Understanding disturbance, vegetation density, seed banks and pollination for the conservation of *Protea curvata*.

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by

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DEDICATION

This thesis is dedicated to my late grandparents, Bonisile and Rose Xaba who exemplified living a purposeful life with discipline, dignity and grace

Abstract

Background Aims: *Protea curvata* (Proteaceae) is a threatened species endemic to Mpumalanga, South Africa. Previous records of the species showed discrepancies in location data and information on population demographics was sparse. At the time of the study (2018), the last IUCN assessment of *P. curvata* was 20 years ago and one subpopulation of the species was reported to have a low number of mature individuals. In 2017, a census of another subpopulation indicated that the mature individuals were the most abundant cohort and there was no recruitment of *P. curvata* seedlings. Thus, there was uncertainty regarding how population size, population demographics and threats faced by the species may have changed over the last 20 years. The study aimed to update the IUCN status of *P. curvata* and to assess factors relating to *P. curvata* recruitment, namely the breeding biology of the species and the woody species composition on *P. curvata* sites.

Methods: A census was conducted on *P. curvata* sites. Census data and site composition was compared between sites with contrasting management. Soil samples were collected from six sites. Pollination modes in *P. curvata* were investigated by manually pollinating inflorescences that were covered at bud phase to exclude animal pollinators. Five treatments were tested: autogamous selfing, tree geitonogamous selfing, inflorescences geitonogamous selfing, natural outcrossing and pollen supplemented outcrossing. Seeds from each treatment were weighed and tested for viability using TTZ staining. Animal pollinators were observed in the field and through camera traps positioned in front of non-covered inflorescences.

Key Results: Five subpopulations were identified. *P. curvata* recruitment was low in two subpopulations and absent in three subpopulations. When comparing the site with the lowest recruitment (*Site A*) to the site with the highest recruitment (*Site C*), woody cover and tree density was higher on the site with low recruitment – indicating bush encroachment. This was attributed primarily to long intervals between fires at *Site A*. *Site A* showed a concerning net decline in subpopulation size (3% loss per year). Despite having more *P. curvata* juveniles, *Subpopulation C* showed a similar rate of decline (2% loss per year). The species was identified as endangered; with severe hailstorms and delayed, intense fires posing the major threats. Hailstorms compounded fire-induced damage on *P. curvata* bark. Flowering declined significantly after a hailstorm. ($\bar{x}_{pre\text{-}hail}$ = 10.94 inflorescences per tree, $\bar{x}_{post\text{-}hail}$ = 4.28 inflorescences per tree, p-value = 0.0031). During pollination experiments, *Protea curvata* was able to self-pollinate. However, seed mass and viability were significantly higher in outcrossed treatments. Viability in naturally outcrossed treatments ($\bar{x}$=42.7%) was similar to pollen-supplemented outcrossed treatments ($\bar{x}$=32.1%), but significantly higher than treatments of autogamous selfing ($\bar{x}$=21.95%), geitonogamous selfing within inflorescences ($\bar{x}$=27.1%) and geitonogamous selfing within trees ($\bar{x}$=26.2%) [C.I= 95%; d.f.= 4, 95; Fcalc= 4.5; p = 0.0021]. Colletid bees and Apid bees contributed to *P. curvata* pollination via geitonogamy and early removal of self-pollen from stigmas. Birds were the most frequent visitors of *P. curvata* and were the most effective pollinators due to their prominent role in natural outcrossing. Bats infrequently visited inflorescences but may possibly complement outcrossing since they make contact with stigmas and do not spend their visit time restricted to one inflorescence or tree. *P. curvata* was non-serotinous and possessed a short-lived, soil seed bank. Across all sites, the average viability of seeds in the soil was well below that of freshly collected seeds from pollination experiments (4–9% for soil seed bank; 22.0–42.6% for fresh seeds). Seed bank size was surprisingly low in subpopulations with high flowering.

Conclusions: Reducing seed loss after release from the canopy appeared to be more important than high flowering for maintaining a large seed bank. Therefore mild, frequent fires will be essential for reducing bush encroachment, making way for seed deposition and encouraging seedling survival.

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I ____Precious Gugulethu Babalwa Mabuza____________ (Student number: ____888236_______)

am a student registered for the degree of __PhD in Animal, Plant and Environmental Science____ in

the academic year _2023_.

I hereby declare the following:

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Precious Gugulethu Babalwa Mabuza

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Papers submitted:

"Fire and Ice: The response of a savanna *Protea* to infrequent burning and severe hailstorms" (Chapter 2)
Journal: Annals of Botany
Manuscript Number: SAJB-D-23-00793

"Evidence for a short-term soil seed bank in *Protea curvata*" (Chapter 4)
Journal: South African Journal of Botany
Manuscript Number: 23329

Introductory Chapter

Protea curvata N.E. Br. is a flowering tree endemic to Mpumalanga, South Africa. It was first recorded in 1890 on a hillside near Barberton. It remained a rare sighting for the next six decades until the original site was relocated in 1956 (Galpin, 1890; Beard, 1956). A second locality was found in 1998 and the species was included on the IUCN Red List under vulnerable status (IUCN Red List, 1998; Hilton-Taylor, 1998). In 2006, one subpopulation was included in the Barberton Nature Reserve (Williamson and Balkwill, 2006; Mpumalanga Tourism and Parks Agency [MTPA], 2012). Despite its vulnerable status, little was known about *Protea curvata* beyond basic occurrence data. Population estimates varied widely, primarily due to uncertainty about the number of subpopulations and estimates made from small sample sizes. Unprotected subpopulations faced the threat of mining and quarrying interests in the area (Protea Atlas, 2008; Rebelo *et al.,* 2020). The subpopulation within the protected area was failing to recruit new seedlings (Mabuza, 2017). Consequently, it became necessary to assess all *P. curvata* subpopulations and identify any ecological and management factors that influence *P. curvata* recruitment.

In a previous study, birds were found to be frequent visitors of *P. curvata* and were identified to species level. Insect visits were also observed, however the ability to discern what type of insects were visiting and whether they made contact with the reproductive whorls was hindered by camera settings at the time (Mabuza, 2017). Therefore further study was required to determine the potential role of insects in *P. curvata* pollination. *Protea curvata* is biogeographically unique. Most *Protea* species are limited to the Cape Floristic Region (CFR) and fynbos biome (Protea Atlas Project, 2002; Protea Atlas, 2008, Valente *et al.,* 2010). Other *Protea* species, such as *P. caffra*, occur in a wider geographic range that includes the CFR. They thrive on quartzitic (calcium-rich) soils (Rebelo *et al*., 2006). *Protea curvata*, on the other hand, is exclusive to serpentine (calcium-poor) soil in the savannas of Mpumalanga (Williamson & Balkwill, 2006). This affords an interesting opportunity to investigate how well such a biodiversity novelty fits into the trends that are associated with its fellow genus members or other savanna species.

Therefore, the aim of this study was to assess factors impacting *Protea curvata* recruitment at the population level (flowering, breeding system, seed retention) and the ecosystem level (disturbances, pollinators, woody species competition). This required meeting the following objectives:

- Undertaking a census of the six extant populations of *Protea curvata*, giving special attention to demographics related to plant reproduction (Chapter 1).
- Determining the effects of disturbances (herbivory, hail, fire) on bark and re-sprouting shoots (Chapter 2).
- Comparing woody species composition on *P. curvata* sites and assessing the potential role of woody species in competing with *P. curvata* individuals or suppressing *P. curvata* recruitment (Chapter 3).
- Examining the type of seed bank in *P. curvata* (Chapter 4).
- Comparing seed viability in *P. curvata* plants that were outcrossed, self-pollinated and autogamously self-pollinated (Chapter 5).
- Identifying potential insect pollinators of *P. curvata* (Chapter 5).

All of the above were synthesized to develop a management plan, which is given in the concluding chapter (Chapter 6).

Currently, published occurrence data are outdated and sometimes conflicting, which can be misleading for conservation efforts. According to the IUCN, only two very small subpopulations exist (Hilton-Taylor, 1998); whereas the Protea Atlas (2008) describes *P. curvata* as having a "healthy large population". However, there were at least four known localities by 2006 and their health had yet to be determined (Williamson & Balkwill, 2006).

The issue of wide-ranging population estimates was addressed by censusing all known subpopulations and adjusting transect sampling based on the size of each subpopulation. This ensured that an ample proportion of each subpopulation was sampled. Similarly, woody species composition was measured around *Protea* trees that were included during transect sampling. There were stark differences between some subpopulations, which raised the question of how vegetation composition of different sites has influenced *Protea curvata* demographics. Although invasive plants were listed as one of the threats to *P.*

curvata, there were no published vegetation surveys of *P. curvata* sites (Rebelo *et al*., 2020). More generally, the threat of bush encroachment challenges various savanna sites (Ward, 2005). Therefore, the third chapter focused on determining the extent of bush encroachment and alien invasion on *P. curvata* sites, and on identifying which species constituted this woody species ingression.

Excessive growth of woody species in savannas can be facilitated by fire suppression or increases in atmospheric CO_2 (Walker *et al.,* 2004; Ward, 2005; Kgope *et al.,* 2010; Bond & Midgley, 2012; O' Connor *et al*., 2014). Fire frequency also relates to fire intensity, thus influencing the destructive and regenerative effects of fire (Pickett and White, 1985; Turner *et al.,* 1989; Huston, 2003). The second chapter of the thesis investigates the impact of fire and herbivory on *P. curvata.* The chapter also investigated the impact of hail (an often overlooked disturbance in African savannas) on *P. curvata* flowering and survival. The interaction of hail, herbivory and fire management was investigated in order to identify management practices that could improve disturbance resilience in *P. curvata*.

In addition to developing a conservation approach for *P. curvata*, the study will contribute to theoretical knowledge. This includes theoretical debates on self-compatibility in Proteaceae and how it relates to pollination or post-fire adapations (Lamont, 1985). For instance Carpenter and Recher (1979) hypothesized that post-fire resprouters are often self-incompatible and favour outcrossing. They suggest that annual seed production is low among these outcrossing plants. This is based on the premise that most of the resources that could be allocated to sexual reproduction (including setting outcrossed or selfed seeds) are used for resprouting instead. Other hypotheses view pollinator scarcity as the driver of self-compatibilty (Baker, 1955; Lloyd, 1965; Inoue *et al*., 1996). The ability of *P. curvata* as a post-fire resprouter or reseeder is examined in the second and fourth chapter of the thesis.

Through analysis of pollinator availability and seed viability in different modes of pollination, the fifth chapter addresses some of the hypotheses regarding self-pollination and outcrossing. A preliminary study sampled multiple flowers for each mode of pollination (6-120 flowers). Despite sampling multiple flowers, a total of only five inflorescences was used to test four modes of pollination (autogamous selfing, within-inflorescence geitonogamy, within-tree geitonogamy and outcrossing). The preliminary study indicated possible self-compatibility in *P. curvata*. Three of the six autogamously selfed flowers set seed. One of the seeds was viable (Mabuza, 2017). Improved understanding of the breeding system of *P. curvata* thus required more robust sampling, wherein sample size would be large enough in terms of both trees and inflorescences.

Prior to this study, it was unclear whether *Protea curvata* can retain seeds in the canopy or underground. Other studies in the woodlands and savannas of Australia and South Africa, and in North American forests have found some form of seed retention in several species, especially those of the Proteaceae family (Lamont *et al.,* 1991). Overtime, canopy seed storage (serotiny) has become associated with the Proteaceae and fire-prone environments (Bond, 1985). Even so, not all tree species in such environments are serotinous. Furthermore, the longevity of stored seeds and mechanism of canopy seed release varies between taxa (Givnish, 1981; Muir & Lotan, 1985; Bond, 1985). Therefore, this study sought to examine seed release and seed storage in *Protea curvata*. In the fourth chapter, possible mechanisms of seed storage in *P. curvata* were investigated through pre-fire and post-fire observations of the canopy and collection of seeds from the soil.

Each of the chapters will serve as a paper for publication. Therefore, the first mention of a plant name in each chapter is followed by the authority. The concluding chapter includes a summary of major findings from preceding chapters and management plan that can be disseminated to site managers.

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Chapter 1. Population demographics and conservation status of an endemic *Protea* in South Africa

Introduction

Protea curvata N.E. Br. is a flowering tree, typically between 2 and 6 m tall, with narrow, grey-green, cuneate leaves and dark pink floral bracts. Its light pink flowers cluster to form an upward-facing inflorescence (Beard, 1958; Rebelo *et al*., 2005; Rebelo, 1991). The first formal record of the species was by Galpin (1890), who collected specimens in Barberton and in Caledonian, a site north of Barberton (Beard, 1958). The exact co-ordinates of the localities are unknown due to Galpin's specimens containing vague or confusing labels. For instance, specimens collected in Barberton were labelled "Kaap River Valley, 2500 – 3000 ft)". Some specimens were labelled with the same collection number but different locality data, making difficult to ascertain where they were collected. In 1956, Dr. P. D. Hamilton found *Protea curvata* in the Caledonian locality. His specimen was collected from a hill formed by a talcose, rocky outcrop rising from the north bank of the Suidkaap River [Figure 1]. The species was found among bushveld species, including *Pavetta edentula* Sond., *Combretum transvaalense* Schinz, *Combretum apiculatum* Sond. and *Bolusanthus speciosus* (Bolus) Harms (Beard, 1958).

In previous assessments, the species was classified as vulnerable (Hilton-Taylor, 1998; Rebelo *et al*., 2020). At the time of its first IUCN assessment, only two *P. curvata* subpopulations were known. Both were cited as being severely fragmented and having a low number of trees (Hilton-Taylor, 1998). By 2006, four subpopulations were known (Williamson and Balkwill, 2006), yet they were not listed in the more recent assessment (Rebelo *et al.,* 2020). Nevertheless, the species is still classified as vulnerable due to its limited area of occupancy and a low number of mature individuals (Rebelo *et al*., 2020, Williamson and Balkwill, 2006). Population estimates were made from one subpopulation (*Protea Atlas*, 2008), and it was cited as having a low number of mature individuals (Hilton-Taylor, 1998). It remained unknown whether the other subpopulations had similar demographics or if any of them have successfully been recruiting over the years.

P. curvata subpopulations are fragmented as a result of granite, shale and slate soils surrounding the serpentine soils on which the species thrives (Williamson & Balkwill, 2006; Roberts & Proctor, 1992). Several implications arise from fragmentation. For instance, fragmentation can hinder access to mates and reduce pollinator diversity on a site (due to a loss of habitat resources), as well as discourage inter-patch movement of pollinators on a site (Stouffer & Bierregaard, 1995; Stouffer *et al*., 2006; Boscolo & Metzger, 2011).

Documented threats to the species include a Sasol pipeline in the Barberton Greenstone Belt, as well as the collection of flowerheads for ornamental use (Williamson & Balkwill, 2006). Although not listed in earlier assessments (Hilton-Taylor, 1998; Rebelo *et al*., 2020), fire regimes pose a major threat. One of the *Protea curvata* subpopulations is under the management of the Mpumalanga Parks Board and the remainder are on privately owned land (Mpumalanga Tourism and Parks Agency [MTPA], 2012). Without effective management of fire on the sites, the resilience and regeneration of *Protea curvata* become compromised (de Bruno Austin, pers. comm., 2018). The repercussions may be harsher when coupled with drought (Protea Atlas, 2008). Considering how rare *Protea curvata* is, it becomes crucial to examine the population dynamics and associated factors that will be relevant in mitigating these threats.

Population density in conjunction with height and size class distributions can influence how a population is impacted by fire (Lamont *et al.,* 1991). With regards to population density, the *Protea Atlas* (2008) found most of the population to be variably distributed. Only a small portion (6%) of the recorded individuals were evenly distributed. Therefore, uncontrolled fires could exacerbate patchiness in *Protea curvata* populations. Tree height and size is of interest since younger trees tend to be less fire tolerant than mature trees. This is primarily due to younger trees having less developed bark or being too short to escape most fires (Crawley, 1990; Wilson & Witkowski, 2003; Dantas & Pausas, 2013). Therefore, a population comprising trees in various life stages will be more resilient to intense fires than a newly established population comprising mostly young trees. Height class distribution also modifies the impact of fire among mature trees, particularly in species where flowering or seed release is stimulated by fire (Cowling & Lamont, 1985). For instance, in some serotinous pine species, short trees can release their seeds in response to a wide range of fire behaviour; whereas taller trees can only release their seeds when fires are slow moving, with high fuel consumption (Johnson & Gutsell, 1993).

Size class distributions of living trees can provide insight on recruitment in a population by documenting the juveniles present in the population. If certain size classes are absent in the living trees that are sampled, yet present in the dead trees, it can point to a recent event being the cause of the particular class' absence. In other words, one can peer into the recent history of the population, rather than simply considering the living individuals at the time of the study. As such, this study intended to monitor *Protea curvata* population dynamics over three consecutive years (i.e., 2017, 2018 and 2019). Similarly, the presence of inflorescences and infructescence receptacles from current and previous seasons will provide valuable insight into how the population's flowering is changing over time. Flowering is important for the attraction of pollinators and sexual reproduction in plants (Lazaro *et al*., 2008; Schmitt *et al.,* 1987).

A previous study on one of the *Protea curvata* populations found that there were no living trees with heights between 0 and 3 m, and no dead *P. curvata* trees in the 0 – 2 m height range (Mabuza, 2017). This absence of both living and dead small trees indicates that a deficiency in recruitment and seedling establishment is a point of concern in the species. Moreover, recruitment in the population may be limited by flowering, since over a third of the sampled living trees had no inflorescences from the year of the study –despite being sampled during the flowering season (Mabuza, 2017).

The key aims of this study were, i) to establish a population estimate for *Protea curvata* and to identify sites wherein low flowering, low recruitment and the absence or presence of management practices could contribute to population size; ii) to provide an updated assessment of the species' threatened status based on IUCN criteria.

Methods

Study site and species

Protea curvata is a plant found growing on serpentine soils in the lowveld of Mpumalanga, South Africa (Protea Atlas, 2008). The area is characterized by savanna vegetation that is denser in high rainfall sites (Botha *et al*., 2004). The area receives summer rains and has a mean annual rainfall of 715 mm (South African Sugarcane Research Institute, 2017). Trees were sampled from six sites [Figure 1]. Sites were designated based on the aggregation of *Protea* trees on a single mountain and/or fenced area. All mountains with the exception of *Site B1* and *Site B2* were separated by a distance of over 1km and local or informal roads. Sites were assessed and then grouped into locations and subpopulations as per the IUCN definitions [Appendix]. As such, sites that were likely to share a threatening event were grouped as a single location. Sites that showed no signs of recent, successful demographic exchange were classified as separate subpopulations. Detailed definitions for the IUCN use of the terms "location" and "subpopulation" are included in the appendix.

"*Site A*" is within Phase 2 of the Barberton Nature Reserve (-25.611°S, 31.004°E) in Mpumalanga. This is a 5400 ha area that includes pieces of State land and it is managed by neighbouring conservation organisations (Mpumalanga Tourism and Parks Agency [MTPA], 2012). When reviewing locality descriptions in previous records of the species, it appears "Site A" was not detected (Onderstal, 1979; Protea Atlas Project, 2002). At Mundt's Concession, about 9 km southeast of "*Site A*", "*Site B1*" and "*Site B2*" are found. "*Site C*", "*Site D"* and "*Site E"* occur further southeast. "*Site D*" is situated near Dixie Farm in Claremont Vale, while "*Site C*" and "*Site E*" are in Clarendon Vale [Figure 1.1]. A summary of recent disturbances and censuses conducted at *Site A* and *Site C* is given in Figure 2. No data were available regarding the fire history of the *Site B1*, *Site B2, Site D*, and *Site E*.

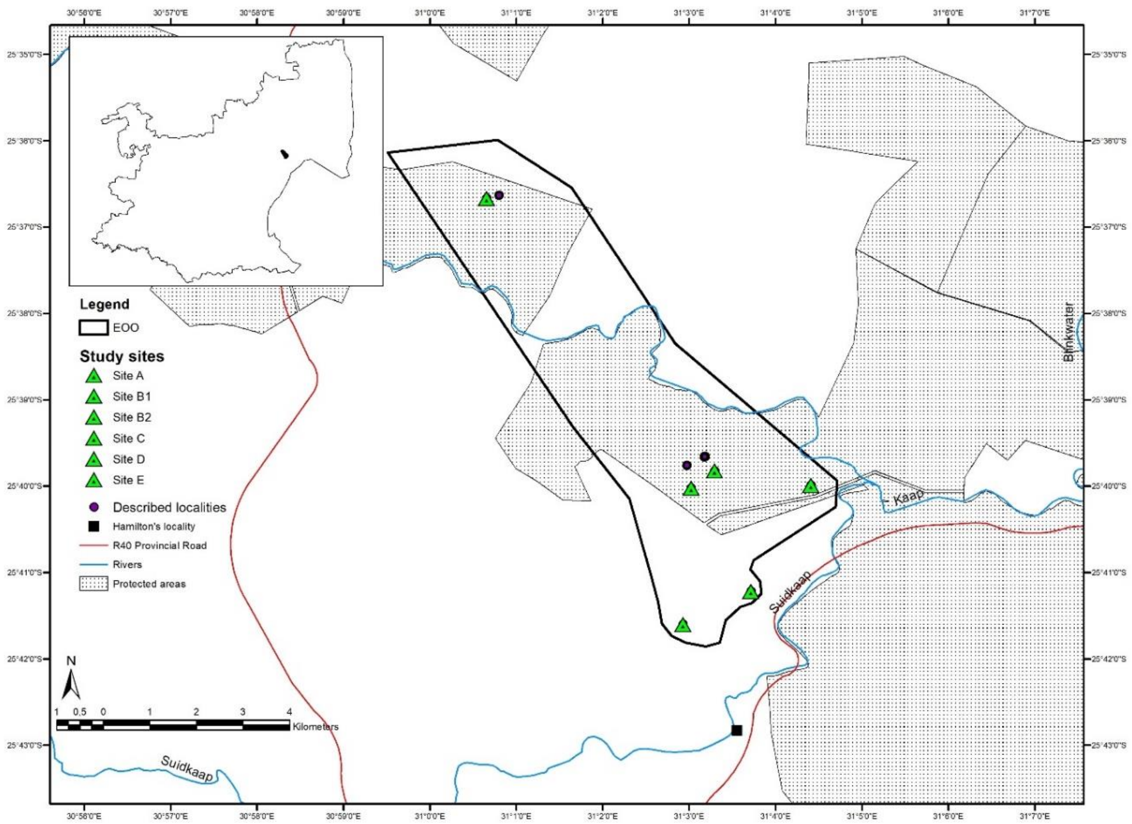


Figure 1.1: Map indicating *Protea curvata* localities in Barberton (Mpumalanga, South Africa).

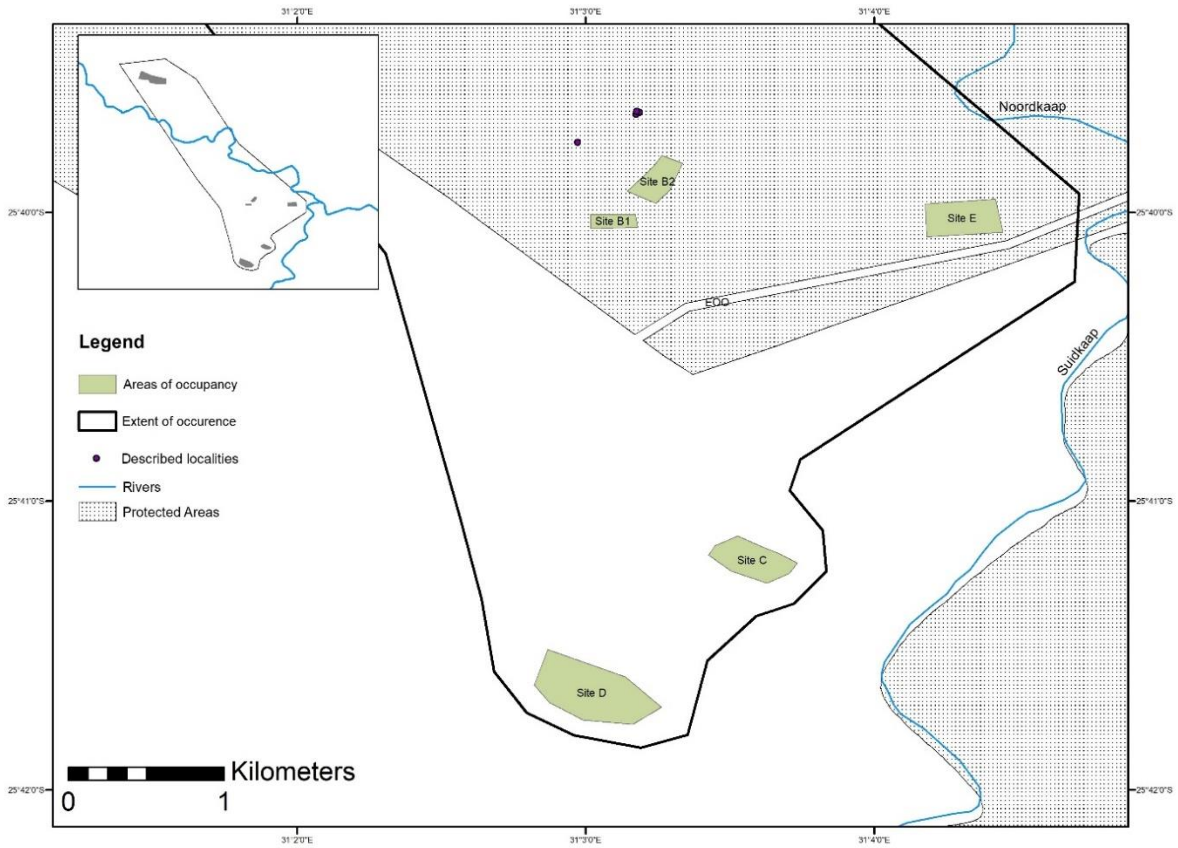


Figure 1.2: Map indicating *Protea curvata* localities in Barberton (Mpumalanga, South Africa).

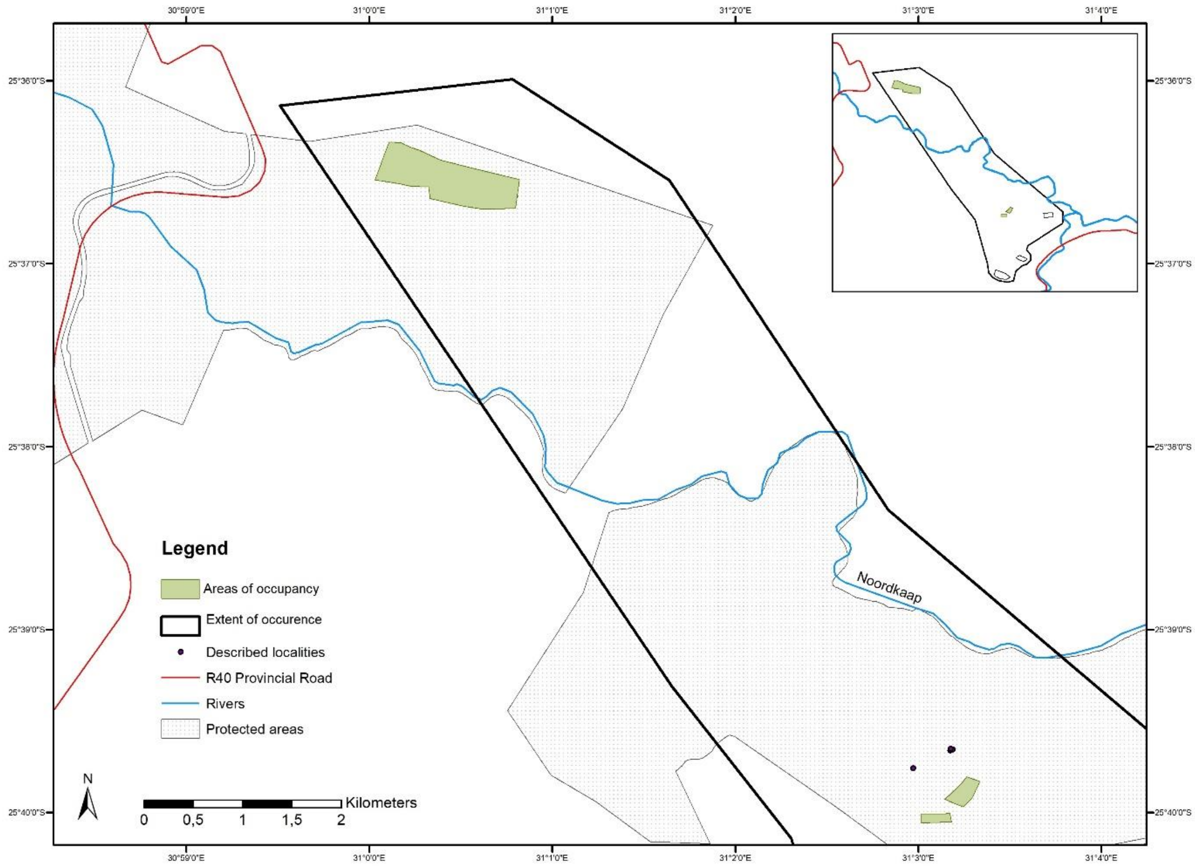


Figure 1.3: Map indicating *Protea curvata* localities in Barberton (Mpumalanga, South Africa).

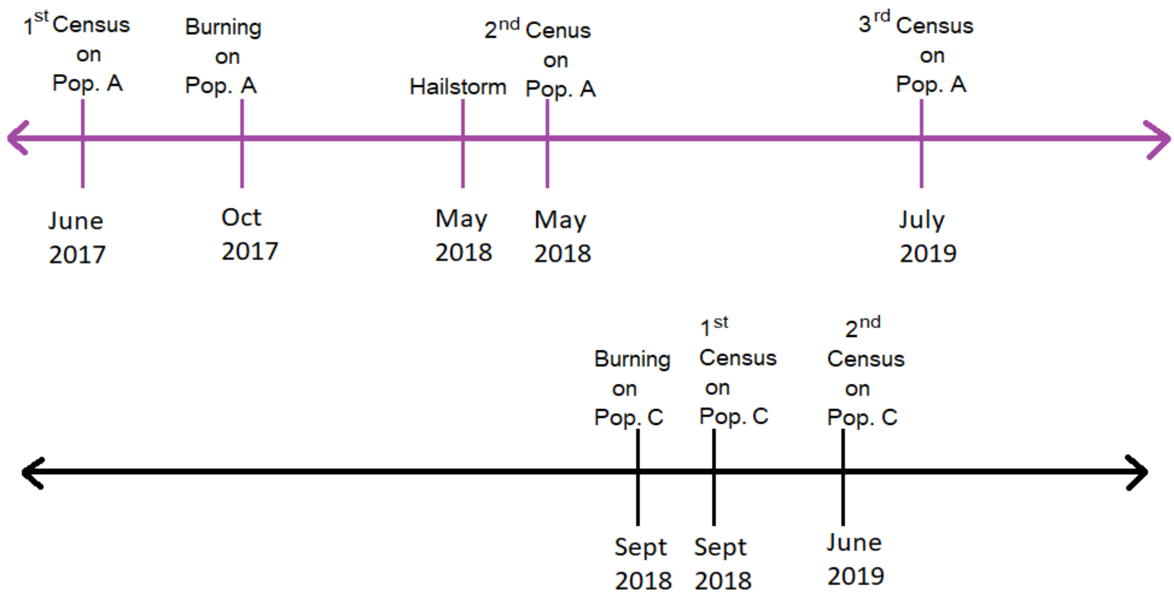


Figure 2: Timeline of recent disturbances and sampling undertaken on two *Protea curvata* subpopulations (*Site A* and *Site C*)

Figure 3: (i) *Protea curvata* tree (ii) a *Protea curvata* inflorescence bud. Barberton Nature Reserve, June 2018

Figure 4: (i) *Protea curvata* open inflorescence (ii) Old inflorescences. Barberton Nature Reserve, June 2018.

Data Collection

All six known sites (*A*, *B1, B2, C, D* and *E*) were sampled between 27 May 2018 and 8 September 2018. The area of each site was divided into equidistant transects with the aim of sampling at least 100 trees. Each transect as well as 5 m on either side of the transect was sampled [Figure 5]. The distance between transects was selected based on shape and size of the site to ensure that a large enough sample size was obtained. On *Site A, Site B2* and *Site E,* transects were 100 m apart. Transects were 20 m apart on *Site C* and *Site B1*. *Site D* had the smallest area, thus all *P. curvata* trees on the site were sampled without using transects. For each sampled tree on all sites, the following was recorded:

- Stem circumference (to the nearest cm, measured at 30 cm from the base)
- Tree height (to the nearest 0.1 m)
- Number of flowering (open) inflorescences from this season
- Number of old receptacles from one year ago (i.e., with involucral bracts)
- Number of old receptacles older than a year (i.e., without involucral bracts)
- Number of inflorescences and inflorescence buds
- GPS location

Receptacles were classified as old (i.e., from a flowering season prior to the census) if they contained no florets, had dehisced their fruit and had dry, discoloured involucral bracts. Receptacles that had dehisced both their fruit and involucral bracts were considered to be the oldest (i.e., at least 2 years old). In 2017, the variables listed above were recorded for *Site A* and sampled trees were marked with copper tags. Reserve managers applied a controlled burn on the site. In 2018, the site experienced a major hailstorm and was resampled. Trees were sampled again in 2019, allowing a comparison of demographics before and after these disturbances.

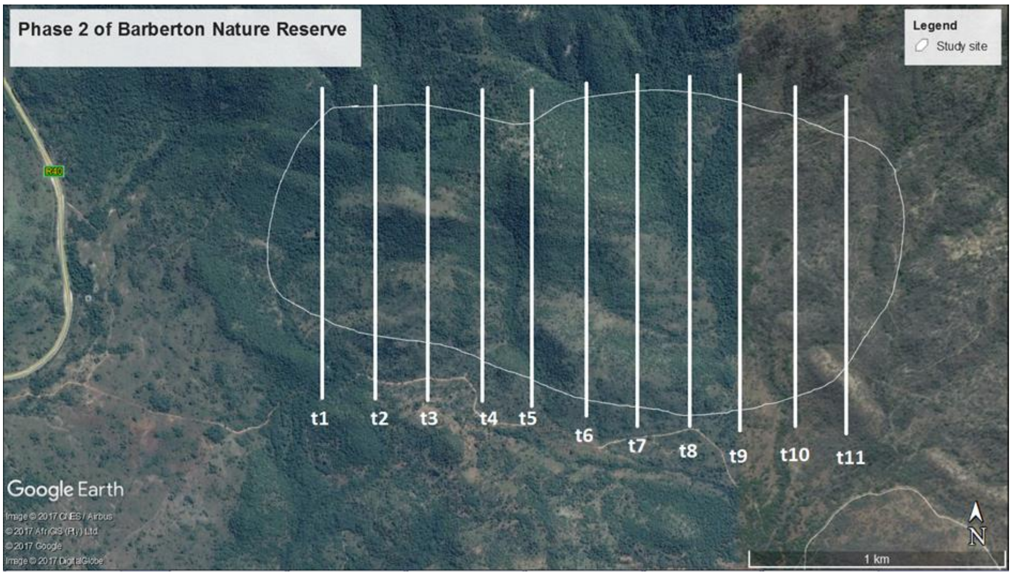


Figure 5: Google Earth image showing extent of subpopulation at *Site A* overlaid with equidistant transects (Phase 2 of Barberton Nature Reserve)

Statistical Methods

Size (above-ground biomass in kg) was calculated for each tree using stem circumference measurements and a standard equation for broad-leafed savannas trees.

The standard equation for an average savanna tree (Nickless *et al*., 2011) was:

$$ln\ (m) = -3.47 + 2.83\ x\ ln(d)$$

Where *m* is tree mass in kg and *d* is stem diameter in cm.

Recruiting populations typically have a higher frequency of small, short trees and fewer large, tall trees (Butt *et al.,* 1994), thus forming a Poisson distribution, i.e., $P(X=r) = \frac{e^{-\mu}\ x\ \mu^{r}}{r!}$. One of the conditions of a Poisson distribution is that the mean and the variance of a dataset are both equal to µ. As such, these were the parameters used to assess how well a Poisson distribution (inverse J-shaped curve) fits the height class and size class data. Equality of the mean and variance was assumed if the mean to variance ratio was within the range 0.8 – 1.2 (Rees, 2001; Mendenhall, 2006). Height class distribution and size class distribution were then graphed. This was done to visualize populat7ion structure, giving special attention to whether it resembled the inverse J-shaped curve typically expected in a recruiting population.

To assess if the number of buds and inflorescences had a relationship with tree height, stem circumference and the number of receptacles form previous seasons, correlation tests were performed separately for each site. Shapiro-Wilk normality tests were performed for each of the following variables: number of buds and inflorescences, number of old receptacles, tree height and stem circumference. Based on the Shapiro-Wilk normality tests, each site had one or more variables that were nonparametric. Therefore, the relationship was measured using a Spearman correlation test.

Mature plants are primarily characterized by the development of reproductive organs and the ability to form seed (Gatsuk *et al.,* 1980). In Proteaceae, this reproductive capability is often concomitant with at least one of the following: plant age (Dupee & Goodwin, 1989; Le Maitre, 1992), post-fire age (Caddy & Gross, 2006; Auld *et al.,* 2007), plant height (Auld *et al.,* 2007) and stem diameter (Abbott, 1985). These can be useful metrics of plant maturity, particularly when assessments are made outside of flowering season or during periods of low flowering for a plant population. Relying on plant age or post-fire age as a metric of plant maturity presents a few challenges. Post-fire age requires knowledge of each site's fire history and this was unfortunately unavailable for some of the *P. curvata* sites (*Site B, Site D* and *Site E*). With regards to plant age, one might not always have the opportunity to observe a population from its establishment in order to calculate the average age of first flowering. As an alternative, growth rings can be used to estimate tree age (Fritts, 1976). This requires felling, which would reduce population size; or tree coring trees, which can introduce health risks to the cored trees (Hart & Wago, 1965, Gao *et al*., 2017) Given *P. curvata*'s threatened status, a less destructive measure of maturity is preferable.

Additionally, the average age of first flowering may vary between subpopulations where maturation is delayed by resource availability or disturbances (Carlson & Holsinger, 2012). A study of *Leucospermum* and *Protea* species found that there was intra-specific variation in the age of plants that flowered and that reproductive maturity in each species was more closely related to plant height than to age (Auld *et al.,* 2007). The longer juvenile periods corresponded with species on drier slopes, indicating the importance of growth rates.

As such, height class was assessed relative to flowering in order to determine which *P. curvata* individuals were mature. Tree measurements were sorted into height classes with intervals of 0.5 m. Individuals which had inflorescence buds or inflorescences in flower at the time of the assessment were considered as having flowered at least once in their lifetime. Individuals which had old receptacles (i.e., flowered in previous seasons) in addition to inflorescences were considered to have flowered in at least two seasons. Height classes which comprised more than 50% of individuals that had flowered at least twice were considered to be mature.

Tree density for each of the three sites was calculated as follows:

Area sampled = (area sampled per transect) x number of transects

= (10 m x transect length) x number of transects

Tree density = Number of trees sampled ÷ Area sampled

The area of each site was obtained in Google Earth using the vertices of the outermost transects i.e., sampled transects on the edge wherein no *P. curvata* trees were found.

The subpopulation size was calculated as:

Number of trees in subpopulation = Tree density x Area of the site

Subpopulation size by number of mature individuals was calculated as follows:

*Number of mature individuals = d*A *p*

where *d* is an estimate of population density, *A* is an estimate of area, and *p* is an estimate of the proportion of individuals that are mature. The area of occurrence of each subpopulation was selected as the *A* value. Total population size was calculated by adding the number of mature individuals from all subpopulations.

Tree mortality was estimated for *Subpopulation A* (which was sampled in 2017, 2018 and 2019) and *Subpopulation C* (which was sampled in 2018 and 2019). Dead trees typically had brown, withered leaves. Estimation of trees that died a year before the census was done by counting dead trees that had old receptacles with bracts. Dead trees with old receptacles that had shed their bracts gave an estimate of tree death 2 years prior to the study. Trees with no remnants of old receptacles were used to estimated tree death that occurred at least 3 years before the census. Trees that had no remnants of old receptacles or leaves were considered to have died more than 3 years before the census. Living trees that were censused and died in following sample years were counted. Recruitment during sampling years was also recorded. These were used to estimate changes in subpopulation size for the two sites.

Results

Site A

Population distribution

Stem circumference of *P. curvata* trees on *Site A* in 2018 did not significantly differ from the previous year ($mean_{2017}$ = 85 cm, $mean_{2018}$ = 81 cm, t = 1.08, d.f. = 201, p-value = 0.2797, C.I. = 95%). Tree height between 2017 and 2018 also showed no significant difference ($mean_{2017}$ = 5.1 m, $mean_{2018}$ = 5.2 m, t = -0.61, d.f. = 197, p-value = 0.5404, C.I. = 95%). The number of buds and inflorescences in 2018 was significantly lower than in 2017 ($mean_{2017}$ = 10.9, $mean_{2018}$ = 4.3, t = 3.00, d.f. = 152.89, p-value = 0.0031). The number of old receptacles was also significantly lower in 2018 than in 2017 ($mean_{2017}$ = 39.0, $mean_{2018}$ = 0.82, t = 9.25, d.f. = 102, p-value < 0.0001, C.I. = 95%).

In both living and dead trees, height classes did not fit a Poisson distribution ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 1.8; $\frac{mean\text{DEAD}}{variance\text{DEAD}}$ = 1.5). Living trees formed an inverse U-shaped curve and occurred most frequently in the 5–6 m height class. Living trees were absent in the 0–1 m and <1–2 m height classes. Similarly, there were no trees between < 2 m in height among the dead trees that were sampled. Notably, dead trees in the < 5–6 m and <6–7 m size classes were absent in 2017 [Figure 6.1]. However, in 2018, two dead trees occurred in the <5–6 m size class. The two trees were tagged while alive in 2017 and were dead once resampled in 2018. Three other trees also died between the two sampling periods, with one of them in the 7–8 m height class. The remaining two were between 3 and 4 m tall – a height class below the average height of dead trees and living trees in the subpopulation ($mean_{dead}$ = 4.2 m, $mean_{living}$ = 5.5 m) [Figure 6.2].

Tree biomass did not have a Poisson distribution in living trees ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 0.0033). The distribution of living trees somewhat resembled an inverse J-curve. However, the lowest size class (0–250 kg) occurred less frequently than the second lowest size class (< 250–500 kg). The < 2250–2500 kg size class, which was empty in 2017, had two trees in 2018 [Figure 6.2].

Biomass in dead trees did not form a Poisson distribution or any typical curve ($\frac{mean\text{DEAD}}{variance\text{DEAD}}$ = 0.0044). Interestingly, none of the dead trees sampled in 2017 were between 0 and 200 kg. However, in 2018, six of the resampled dead trees occupied the 0–200 kg size class. The trees died before reaching the median or average size of trees in the subpopulation ($median_{living}$ = 328.90 kg, $mean_{living}$ = 380.50 kg)

Flowering

The number of buds and inflorescences showed a moderate, positive correlation to tree height (Spearman rs = 0.49, C.I. = 95%, S = 48881, p-value <0.0001). When outliers were removed, the correlation between buds and inflorescences and tree hight remained moderately positive (Spearman rs = 0.52, C.I. = 95%, S = 38132, p-value <0.0001). The number of buds and inflorescences were weakly, positively correlated with stem circumference (Spearman rs = 0.24, C.I. = 95%, S = 72596, p-value = 0.0301). The correlation remained weak when outliers were excluded (Spearman rs = 0.29, C.I. = 95%, S = 56280, p-value = 0.0105) [Figure 6.3]. The number of receptacles from previous seasons had a weak, positive correlation to the number of buds and inflorescences (Spearman rs = 0.22, C.I. = 95%, S = 74513, p-value = 0.0477. When outliers were excluded, the correlation was weaker and no longer significant (Spearman rs = 0.19, C.I. = 95%, S = 64069 p-value = 0.0960) [Figure 6.3].

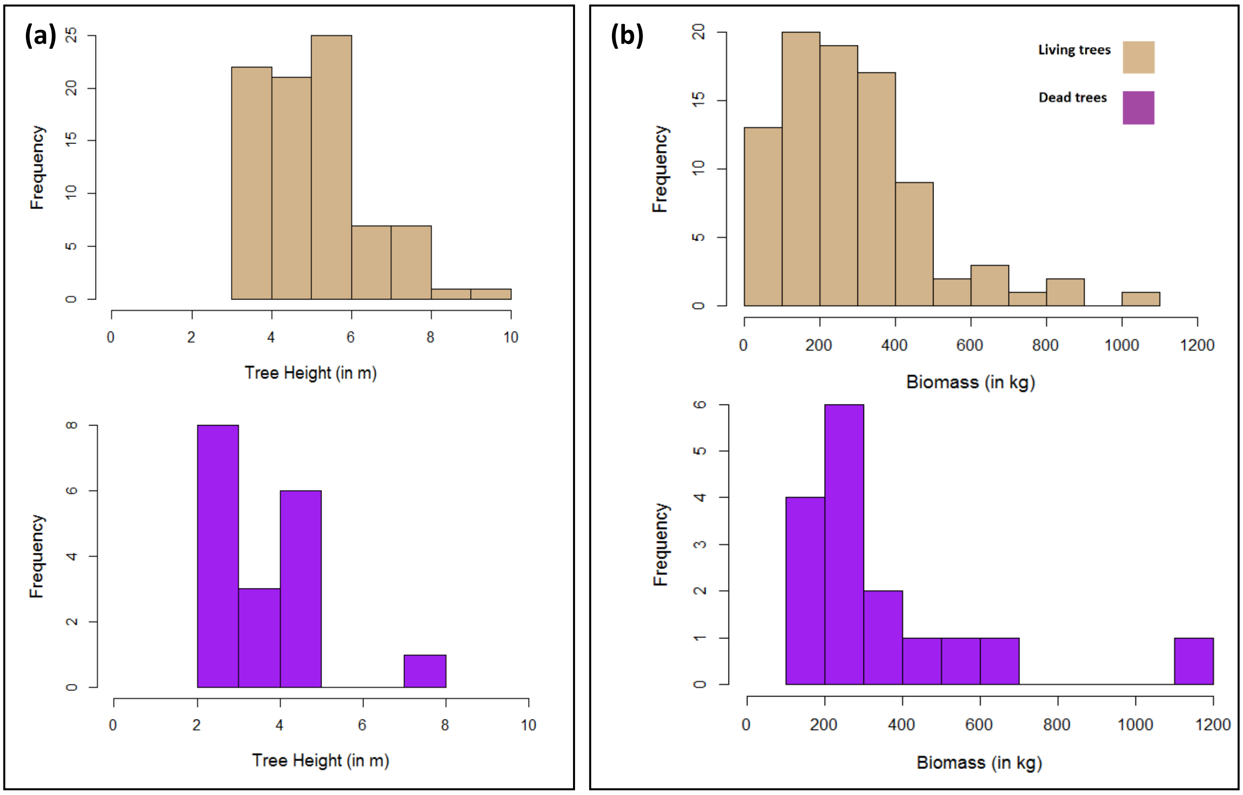


Figure 6.1: (a) Height class distribution in living and dead *Protea curvata* trees at *Site A* (Barberton Nature Reserve) in 2017.
(b) Size class distribution in living and dead *Protea curvata* trees at *Site A* (Barberton Nature Reserve) in 2017.

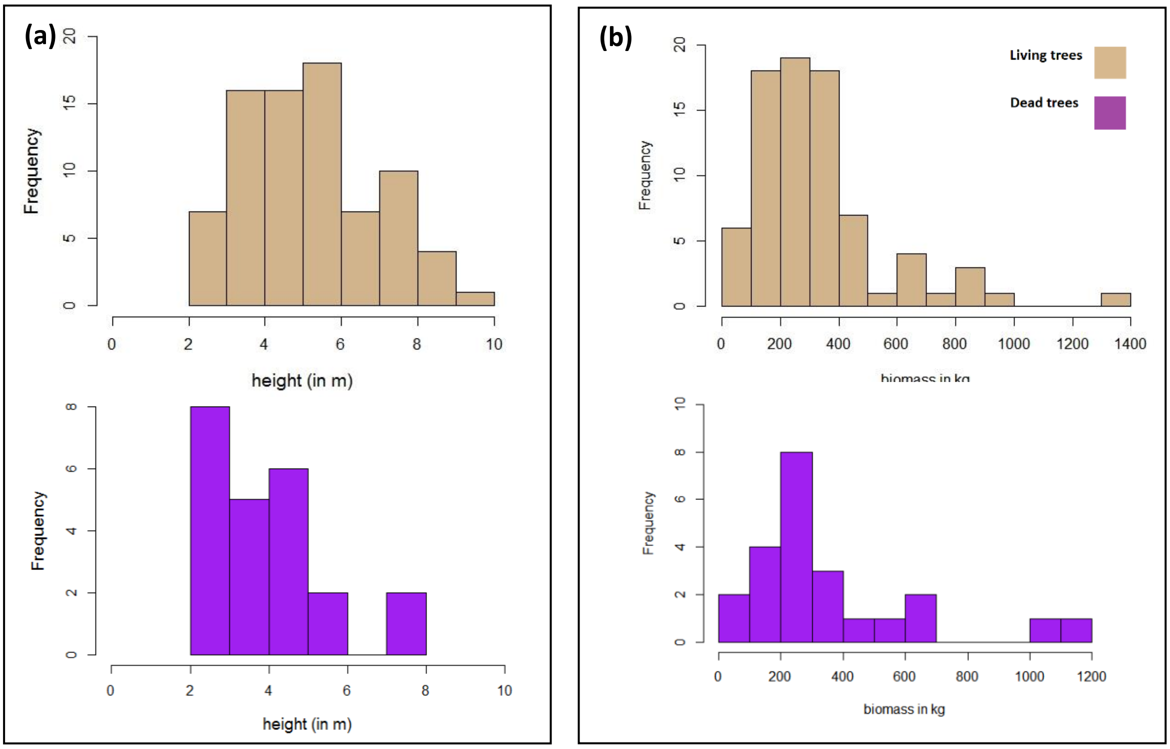


Figure 6.2: (a) Height class distribution in living and dead *Protea curvata* trees at *Site A* (Barberton Nature Reserve, Mpumalanga, South Africa) in 2018.
(b) Size class distribution in living and dead *Protea curvata* trees at *Site A* (Barberton Nature Reserve, Mpumalanga, South Africa) in 2018.

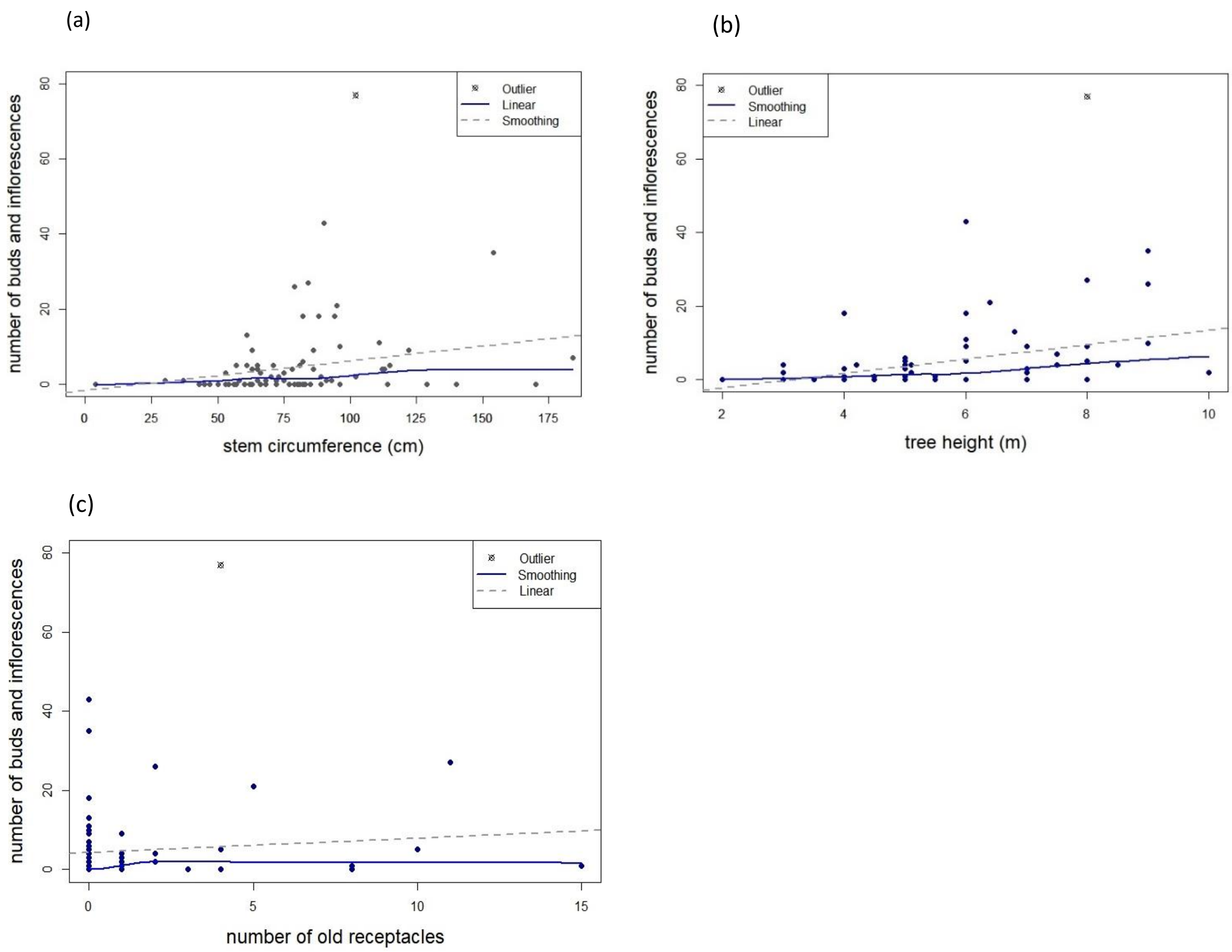


Figure 6.3: Relationship between *P. curvata* inflorescence buds and inflorescences and (a) stem circumference, (b) tree height, (c) number old receptacles at *Site A* (Barberton Nature Reserve, Mpumalanga, South Africa) in 2018.

Trendline and LOESS (Locally estimated scatterplot smoothing) regression curve plotted for data excluding outliers.

Site B1

Population distribution

The height of living trees on *Site B1* did not follow a Poisson distribution ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 2.1). An inverse U-shaped curve can be seen in the height class distribution of living trees. The site had no *P. curvata* trees between 0 and 2 m in height. Only one tree was present in the largest size class (<550–600 kg). Despite most of the trees occurring in the smallest size class, tree biomass did not form an inverse J-curve ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 0.012).

Height among dead trees was irregularly distributed ($\frac{mean\mathrm{DEAD}}{variance\mathrm{DEAD}}$ =1.5). There were gaps in the 0–2 m, <4–5 m and <6–7 m classes. On the other hand, the biomass of dead trees resembled an inverse J-curve, but with a notable gap in the <200–250 kg size class and no trees between 300 kg and 650 kg ($\frac{mean\mathrm{DEAD}}{variance\mathrm{DEAD}}$ =. 0.0040) [Figure 6.4].

<u>Flowering</u>

The number of buds and inflorescences in 2018 showed a weak, positive correlation to tree height (Spearman rs = 0.45, C.I. = 95%, S = 30133, p-value = 0.0001). The number of buds and inflorescences were also weakly, positively correlated with stem circumference (Spearman rs = 0.37, C.I. = 95%, S = 34273, p-value = 0.0016). Number of old receptacles showed a weak, positive correlation to the number of buds and inflorescences (Spearman rs = 0.45, C.I. = 95%, S =30460, p-value = 0.0001) [Figure 6.5].

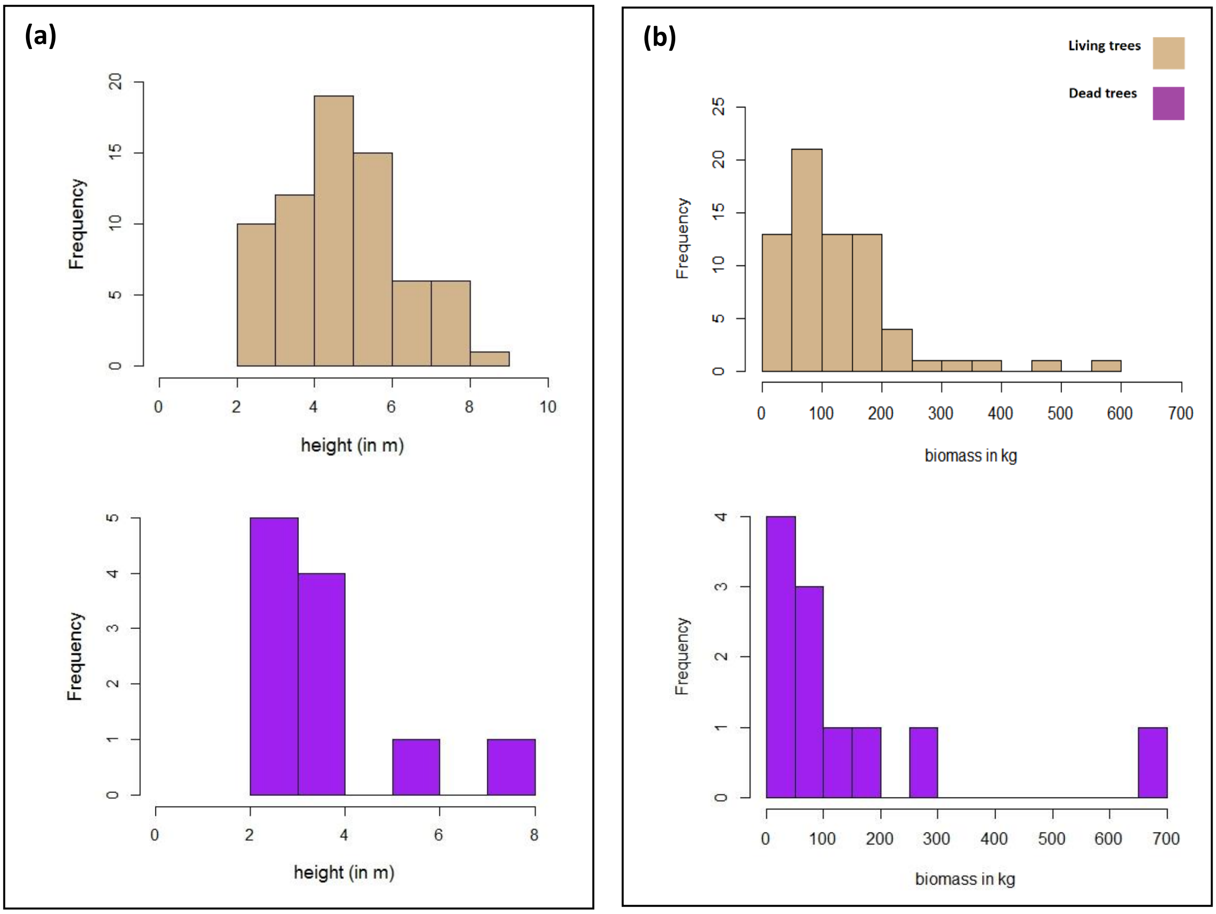


Figure 6.4: (a) Height class distribution in living and dead *Protea curvata* trees at *Site B1* (Mundt's Concession, Mpumalanga) in 2018

(b) Size class distribution in living and dead *Protea curvata* trees *Site B1* (Mundt's Concession, Mpumalanga) in 2018

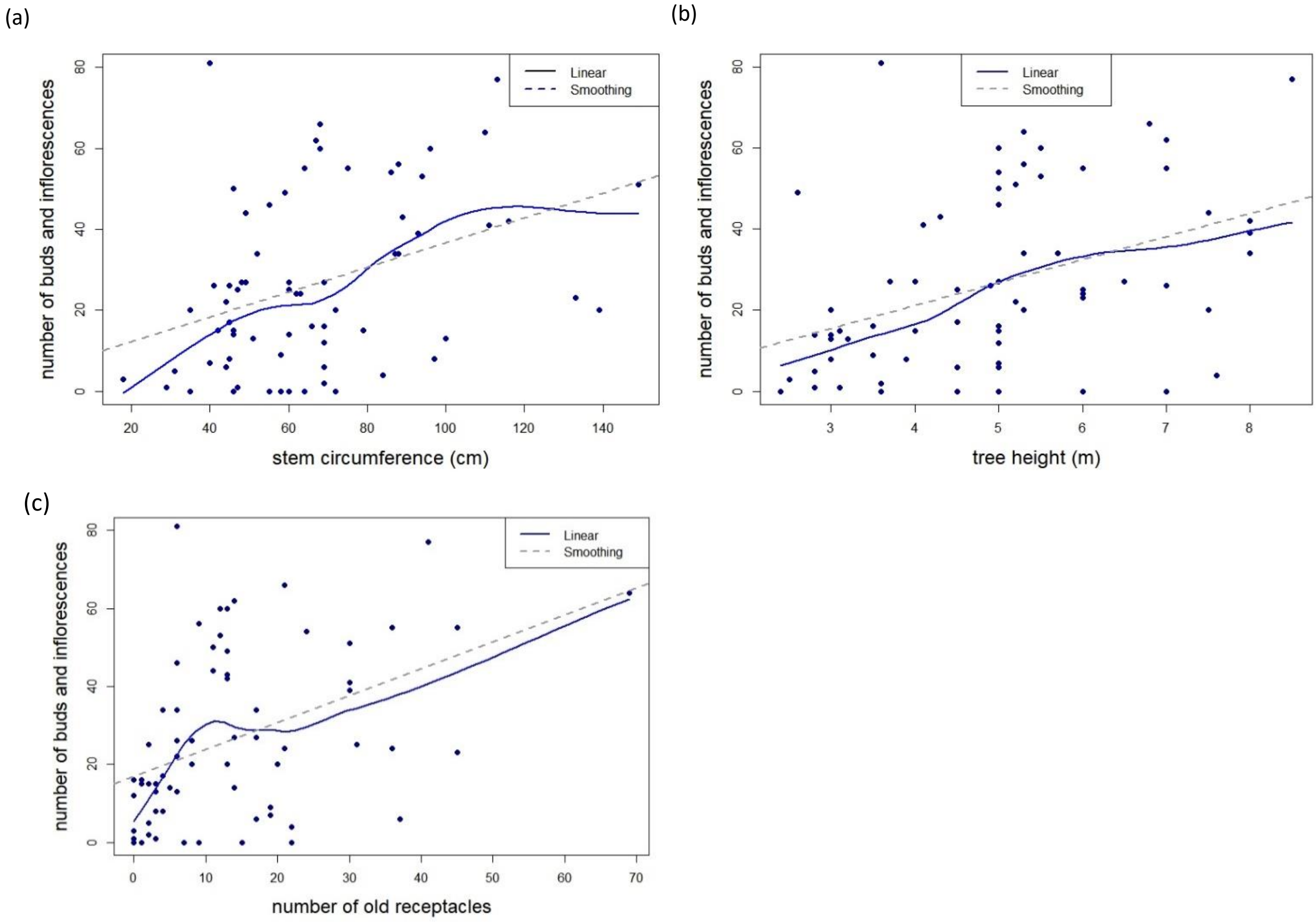


Figure 6.5: Relationship between *P. curvata* inflorescence buds and inflorescences and (a) stem circumference, (b) tree height, (c) number of old receptacles at *Site B1* (Mundt's Concession, Mpumalanga, South Africa) in 2018.

Trendline and LOESS (Locally estimated scatterplot smoothing) regression curve plotted for data excluding outliers.

Site B2

Population distribution

Tree height among living trees did not show a Poisson distribution ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 1.8). There were no living trees in 0–2 m height class. Whereas *Subpopulations A, B1* and *C* had a wider height range of dead trees, *Subpopulation B2* only had dead trees between 3 and 5 m. It is worth noting that trees in the subpopulation can grow beyond that height, as living trees were as tall as 8 m [Figure 6.6a]. The average height of living and dead trees also indicates the persistence of larger trees ($mean_{living}$ = 4.7 m; $mean_{dead}$ =3.7 m).

Biomass did not show a Poisson distribution in living trees and dead trees ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 0.0053; $\frac{mean\text{DEAD}}{variance\text{DEAD}}$ = 0.0085). Much like the other subpopulations, a majority of *Subpopulation B2*'s living trees were between 0 and 200 kg (with 19 trees occurring in the 0–100 kg size class). However, *Subpopulation B2*'s living trees were far less frequent in subsequent size classes. For example, *Subpopulation A'*s 100–200 kg, 200–300 kg, 300–400 kg and 400–500 kg size classes had 20, 19, 18 and 9 trees, respectively. On the other hand, *Subpopulation B2* had 9, 2, 3 and 2 trees in the same respective size classes. This steep decrease in frequency suggests that *Subpopulation B2* had a disproportionately lower number of old/large trees. Furthermore, no dead trees larger than 400 kg were found [Figure 6.6b]. Living trees in *Subpopulation B1* and *Subpopulation B2* showed no significant difference in height (t = 0.866, d.f. = 76.3, p-value = 0.3894 C.I. = 95%, $mean_{B1}$= 4.96 m; $mean_{B2}$ =4.68 m).

Flowering

In *Subpopulation B2*, the number of inflorescences had a weak, positive correlation with tree height (Spearman rs = 0.32, C.I. = 95%, S = 6475, p-value = 0.0490). However, there was no correlation between number of inflorescences and stem circumference (Spearman rs = -0.07, C.I. = 95%, S = 10570, p-value = 0.6726) [Figure 6.7]. Similarly, the number of inflorescences in 2018 was not significantly correlated with the number of old receptacles from 2017 (Spearman rs = 0.18, C.I. = 95%, S =8062, p-value = 0.2624). Flowering in *Subpopulation B1* and in Subpopulation *B2* was similar ($mean_{B1}$ = 27.20 buds and inflorescences per tree, $mean_{B2}$ = 27.79 buds and inflorescences per tree (t = -0.117, d.f. = 71.5, p-value = 0.9075).

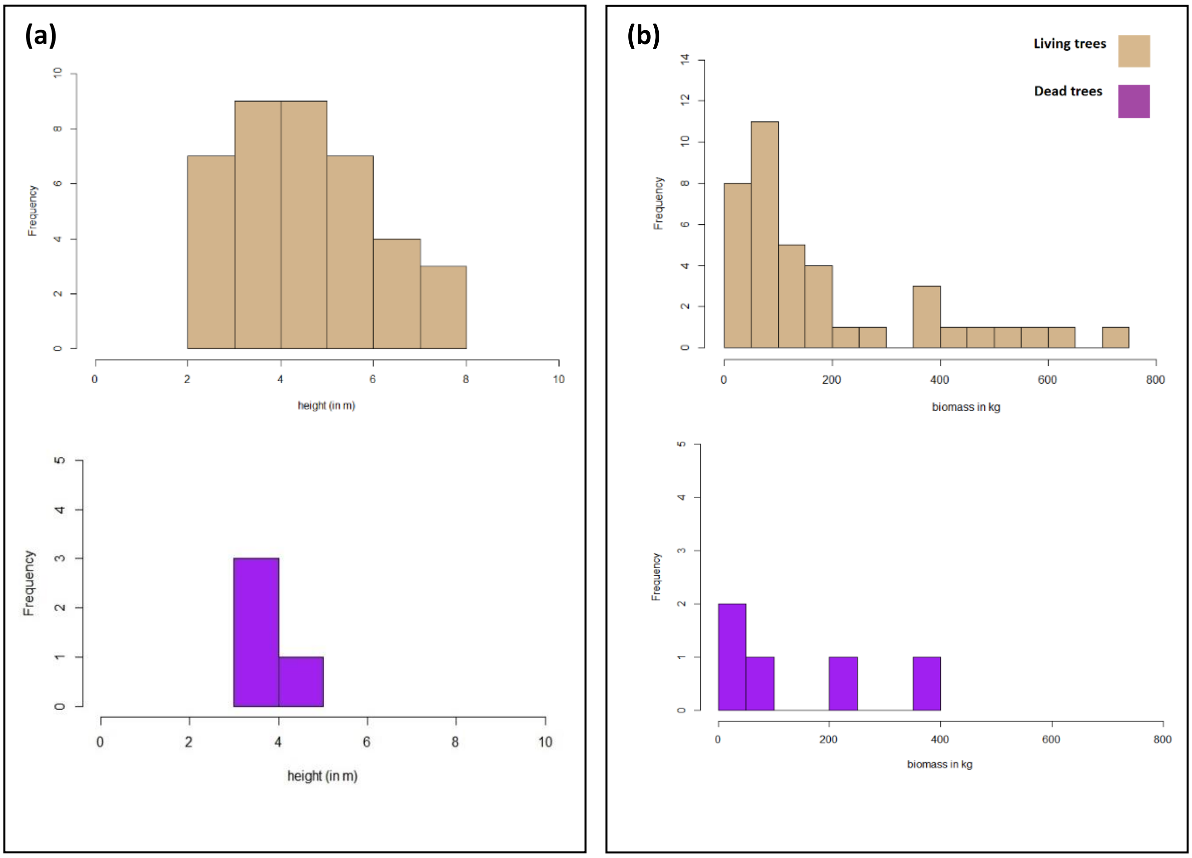


Figure 6.6: (a) Height class distribution in living and dead *Protea curvata* trees at *Site B2* (Mundt's Concession, Mpumalanga) in 2018.
(b) Size class distribution in living and dead *Protea curvata* trees at *Site B2* (Mundt's Concession, Mpumalanga) in 2018.

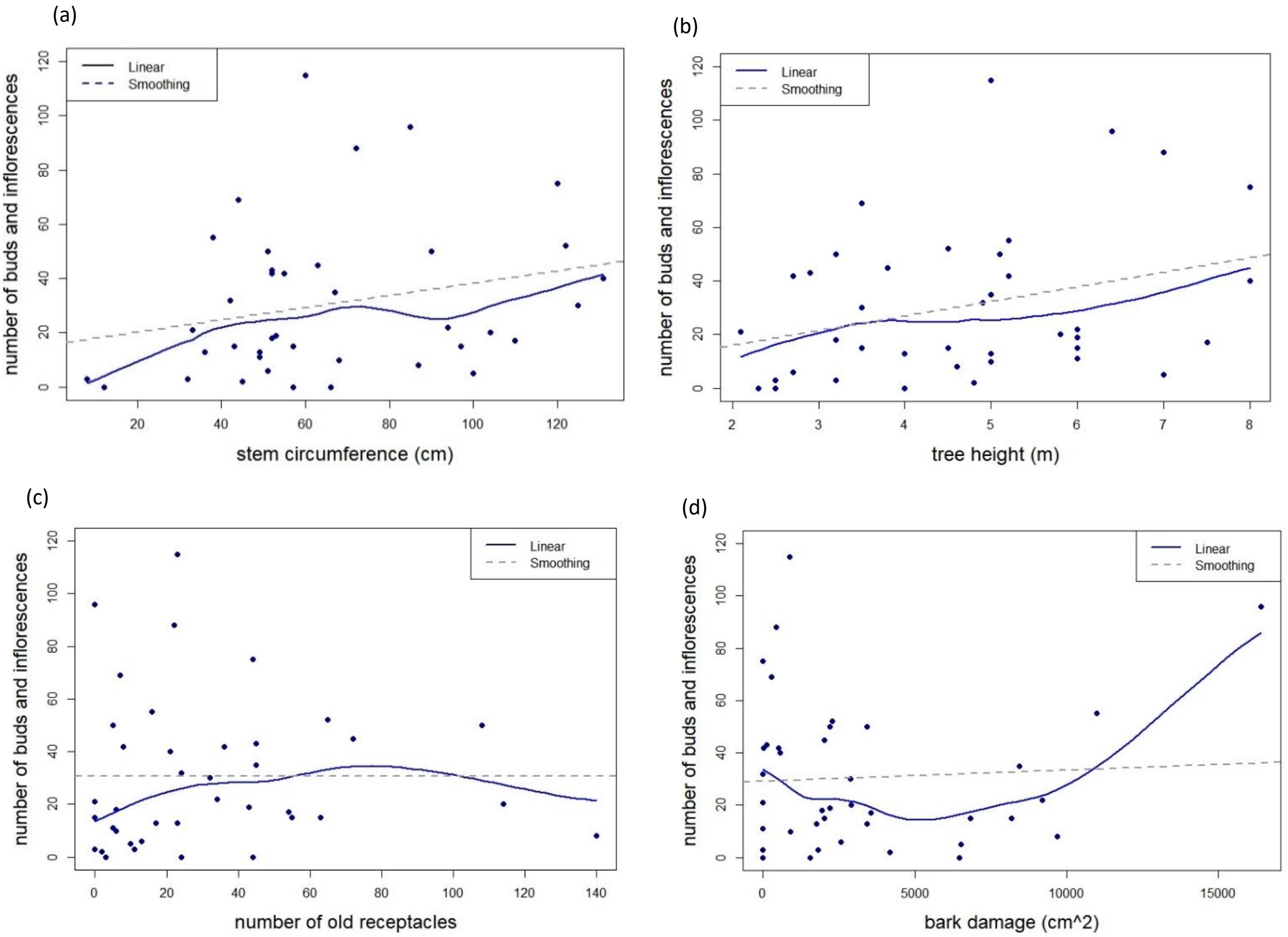


Figure 6.7: Relationship between *P. curvata* inflorescence buds and inflorescences and (a) stem circumference, (b) tree height, (c) number of old receptacles at *Site B2* (Mundt's Concession, Mpumalanga, South Africa) in 2018.

Trendline and LOESS (Locally estimated scatterplot smoothing) regression curve plotted for data excluding outliers

Site C

Population distribution

Neither dead nor living trees in the subpopulation showed a Poisson distribution for height ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 1.3; $\frac{mean\text{DEAD}}{variance\text{DEAD}}$ = 1.5). Living and dead trees were found most frequently in the <2–3 m height class. Like *Subpopulations A* and *B1, Subpopulation C* had no dead trees in the lowest height class (0–1 m). Exceptionally, Subpopulation *C* had living trees in the 0–1 m height class [Figure 6.8].

The biomass of living trees had an irregular distribution with several gaps ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 0.0015). A majority of the subpopulation occupied the 0–250 kg size class. Dead trees did not form a definite curve either ($\frac{mean\text{DEAD}}{variance\text{DEAD}}$ = 0.011). Most dead trees were from the smaller size classes. Size classes above 400 kg had no dead trees whatsoever, despite evidence of (living) trees above 400 kg being found in the subpopulation [Figure 6.8].

Flowering

The number of inflorescences in *Subpopulation C* had a strong, positive correlation to height (Spearman rs = 0.68, C.I. = 95%, S = 173020, p-value <0.0001). Inflorescences were positively correlated with stem circumference (Spearman rs = 0.52, C.I. = 95%, S = 257740, p-value <0.0001). The number of old receptacles showed a strong, positive correlation to the number of buds and inflorescences (Spearman rs = 0.61, C.I. = 95%, S = 208290, p-value < 0.0001). When outliers were excluded, the correlation between old receptacles and the number of buds and inflorescences remained strong and positive (Spearman rs =0.61, C.I. = 95%, S = 208186, p-value < 0.0001) [Figure 6.9].

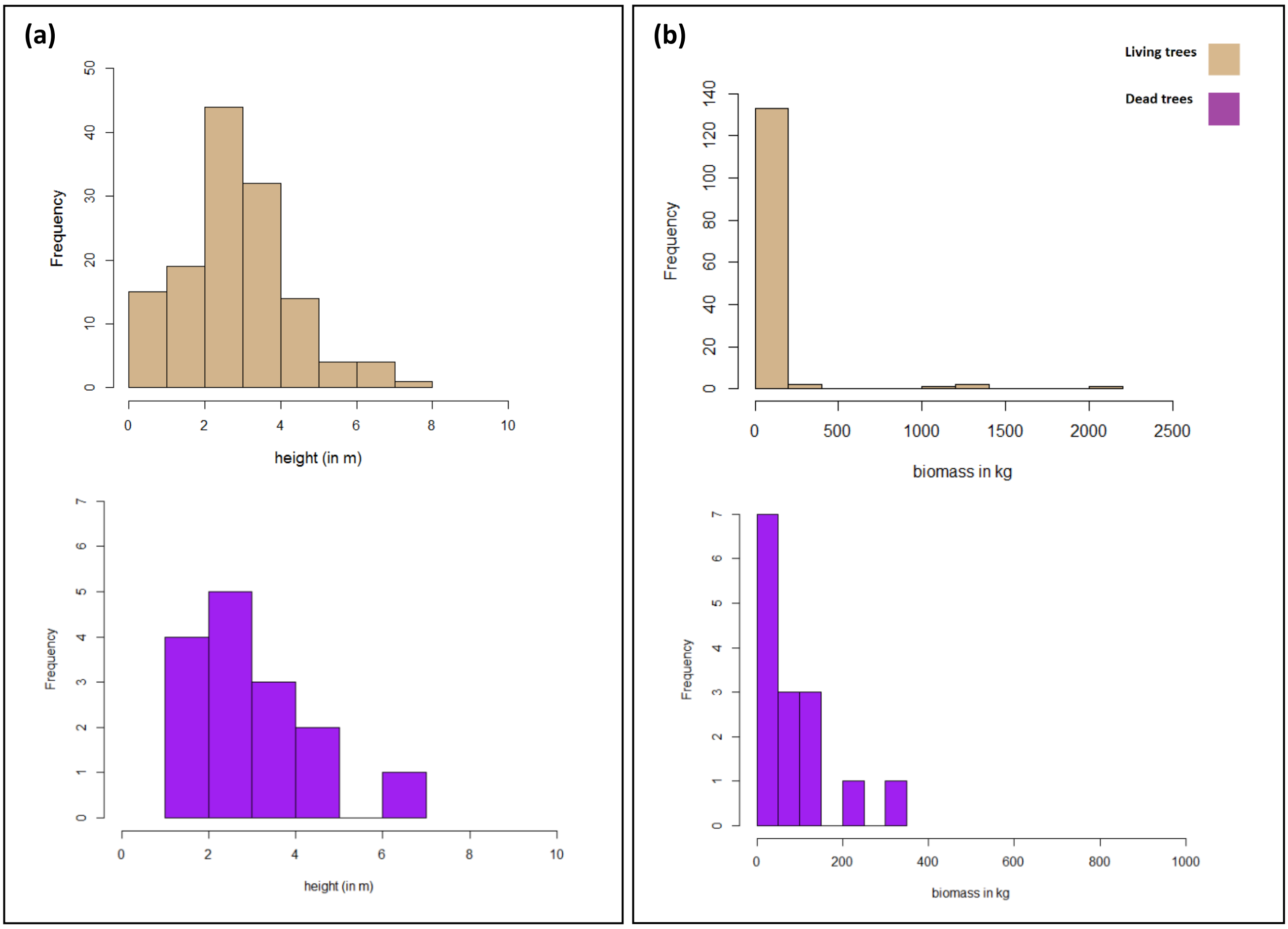
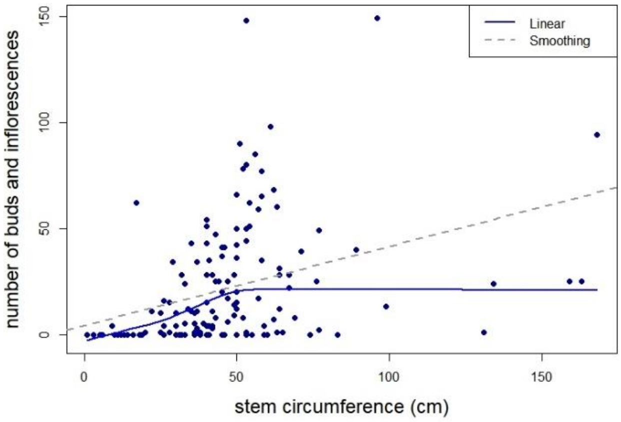


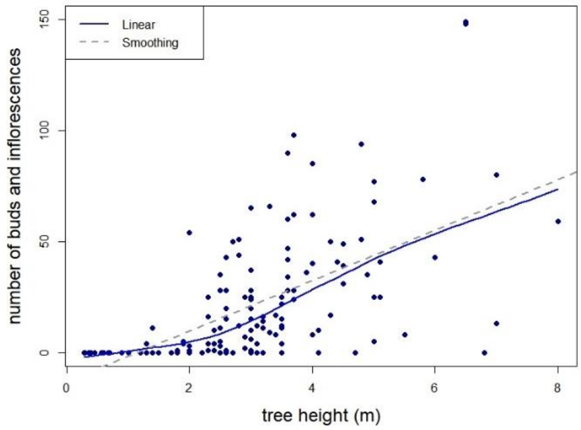
Figure 6.8: (a) Height class distribution in living and dead *Protea curvata* trees at *Site C* (Clarendon Vale, Mpumalanga) in 2018

(b) Size class distribution in living and dead *Protea curvata* trees at *Site C* (Clarendon Vale, Mpumalanga) in 2018.

(a)



(b)



(c)

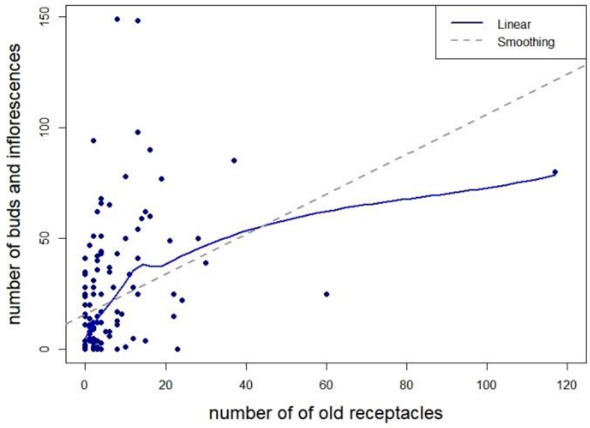


Figure 6.9: Relationship between *P. curvata* inflorescence buds and inflorescences and (a) stem circumference, (b) tree height, (c) number of old receptacles at *Site C* (Clarendon Vale, Mpumalanga) in 2018.

Trendline and LOESS (Locally estimated scatterplot smoothing) regression curve plotted for data excluding outliers.

Site D

Population distribution

Subpopulation D's height classes did not follow a Poisson distribution ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 1.23). There were no trees in the lowest height class (0–1 m) and only two trees in the 1–2 m size class. Like *Subpopulation A*, *Subpopulation D* had trees as tall as 10 m. However, trees were absent in the 8–9 m height class. The 3–4 m height class had the highest number of living trees. However, dead trees occurred equally in the 1–2 m and 3–4 m height classes [Figure

6.10]. This, along with gaps occurring in the lower height classes, gave *Subpopulation D*'s dead trees an irregular distribution ($\frac{mean\mathrm{LIVING}}{variance\mathrm{LIVING}}$ = 1.6).

Although biomass among living trees did not show a Poisson distribution ($\frac{mean\mathrm{LIVING}}{variance\mathrm{LIVING}}$ = 0.01), the lowest size class had the most trees. No living trees occurred in the 250–350 kg and 450–550 kg size classes. *Subpopulation D's* dead trees did not fit a Poisson distribution ($\frac{mean\mathrm{DEAD}}{variance\mathrm{DEAD}}$ = 0.035).

The 100–150 kg size class had the highest number of dead trees. There was only one dead tree under 100 kg and none larger than 200 kg. When comparing the maximum values of height and biomass across subpopulations, *Subpopulation D* has the smallest dead trees.

Flowering

Height had a strong, positive correlation with the number of buds and inflorescences (Spearman rs = 0.68, C.I. = 95%, S = 4549.5, p-value < 0.0001). Stem circumference was positively correlated with number of buds and inflorescences (Spearman rs = 0.52, C.I. = 95%, S = 6821, p-value = 0.0003). The number of old receptacles had a weak, positive correlation with the number of buds and inflorescences (Spearman rs = 0.47, C.I. = 95%, S = 7493.4, p-value = 0.0012).

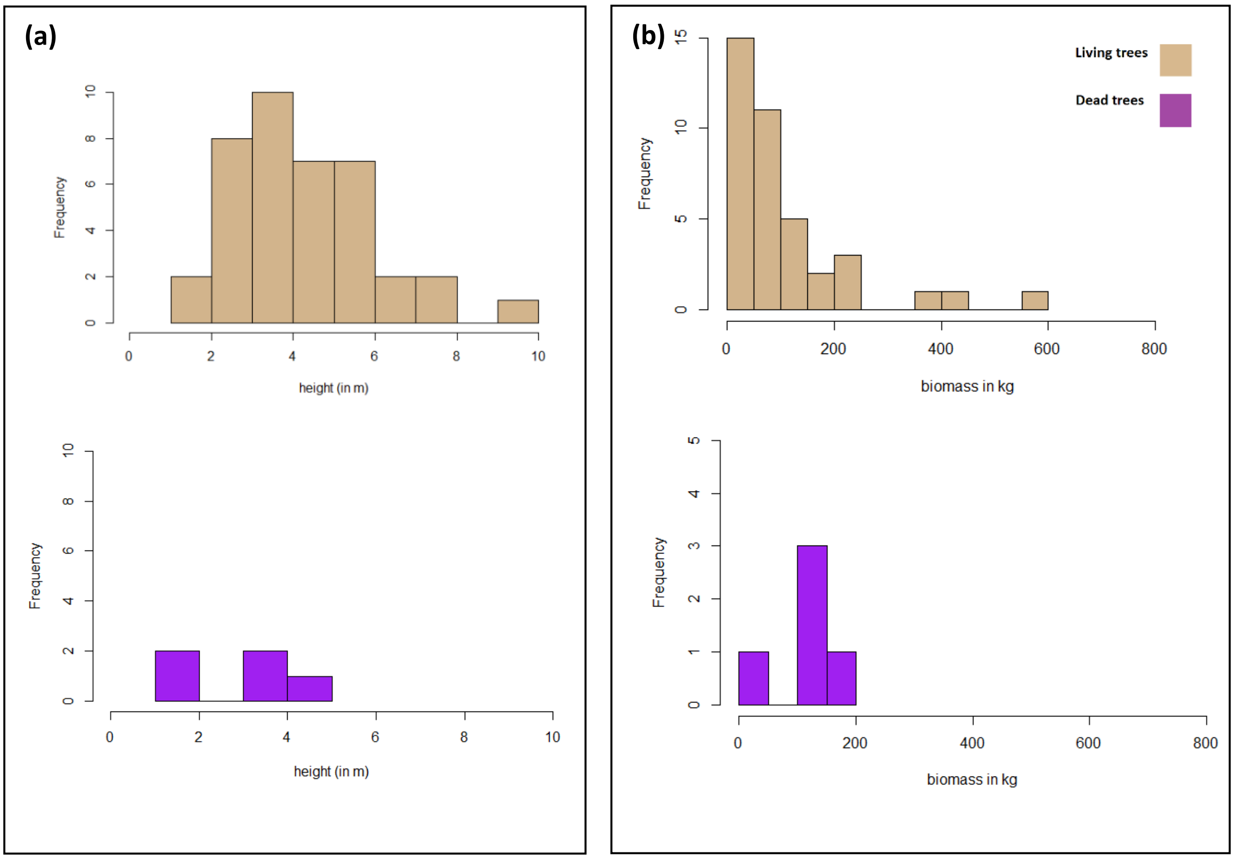


Figure 6.10: (a) Height class distribution in living and dead *Protea curvata* trees at *Site D* (Claremont Vale, Mpumalanga) in 2018.
(b) Size class distribution in living and dead *Protea curvata* trees at *Site D* (Claremont Vale, Mpumalanga) in 2018.

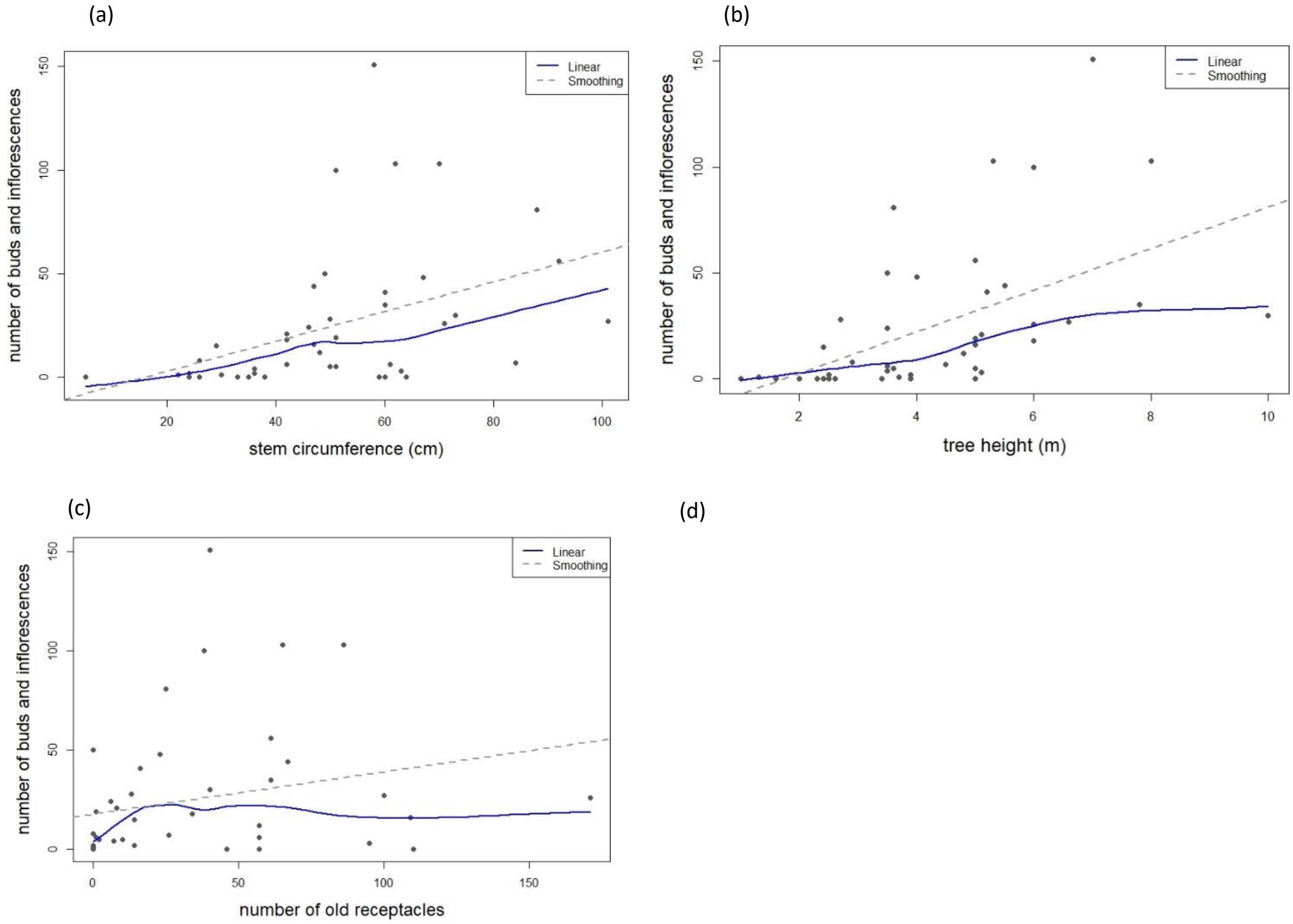


Figure 6.11: Relationship between *P. curvata* inflorescence buds and inflorescences and (a) stem circumference, (b) tree height, (c) number of old receptacles at *Site D* (Claremont Vale, Mpumalanga) in 2018.

Trendline and LOESS (Locally estimated scatterplot smoothing) regression curve plotted for data excluding outliers.

Site E

Population distribution

In *Subpopulation E* height did not fit a Poisson distribution ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 1.3; $\frac{mean\text{DEAD}}{variance\text{DEAD}}$ = 3.5). Living trees were present in the first two size classes but had low frequency. The most frequently occurring trees were in the 4 – 5 m height class. Tree height had a maximum of 7.6 m, with only one tree present in the 7 – 8 m size class. The 0 – 1 m size class was empty for dead trees. The 1 – 2 m, 2 – 3 m and 3 – 4 m height classes had two trees, three trees and two trees respectively. There were no dead trees taller than 4 m, despite taller trees occurring in the living category [Figure 6.12].

Although living biomass did not fit a Poisson distribution ($\frac{mean\text{LIVING}}{variance\text{LIVING}}$ = 0.0064), it had the closest resemblance. Unlike the other subpopulations, biomass distribution in *Subpopulation E* had no gaps and the first two size classes showed the highest frequency (i.e., 0 – 100 kg and 100 – 200 kg). The low frequency in one of the intermediate size classes (300 – 400 kg) is what distinguished the subpopulation's biomass distribution from a Poisson distribution. Dead tree biomass strayed further from a Poisson distribution, having two gaps in the intermediate size classes. The size classes in which most dead trees occurred also had a high frequency in living trees. Dead trees smaller than 300 kg were most common [Figure 6.12].

Flowering

Subpopulation E's flowering had a strong positive correlation with tree height (Spearman rs = 0.69, C.I. = 95%, S = 8120.3, p-value <0.0001) [Figure 6.13]. Flowering was not correlated with stem circumference (Spearman rs = 0.04, C.I. = 95%, S = 19961, p-value <0.08156). The number of old receptacles in the subpopulation had a strong, positive correlation with the number of buds and inflorescences (Spearman rs = 0.72, C.I. = 95%, S = 7315, p-value <0.0001).

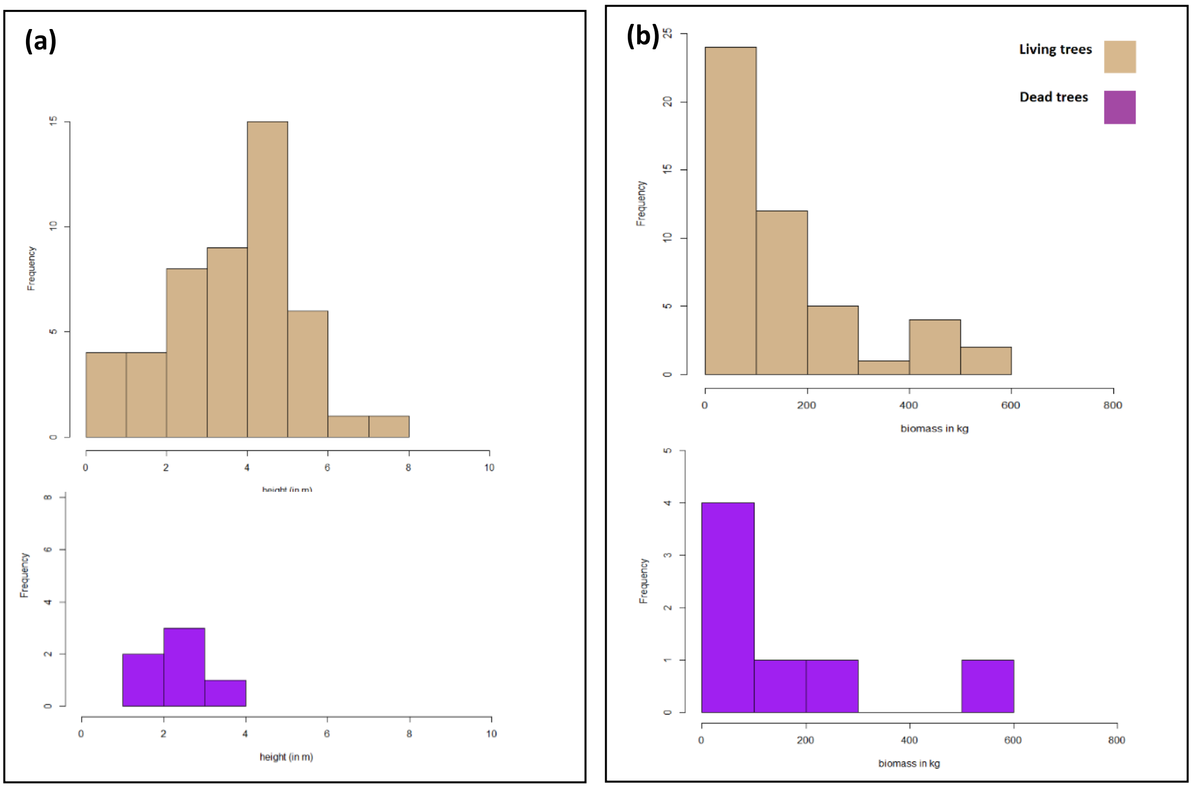


Figure 6.12: (a) Height class distribution in living and dead *Protea curvata* trees at *Site E* (Clarendon Vale, Mpumalanga, South Africa).

(b) Size class distribution in living and dead *Protea curvata* trees at *Site E* (Clarendon Vale, Mpumalanga, South Africa).

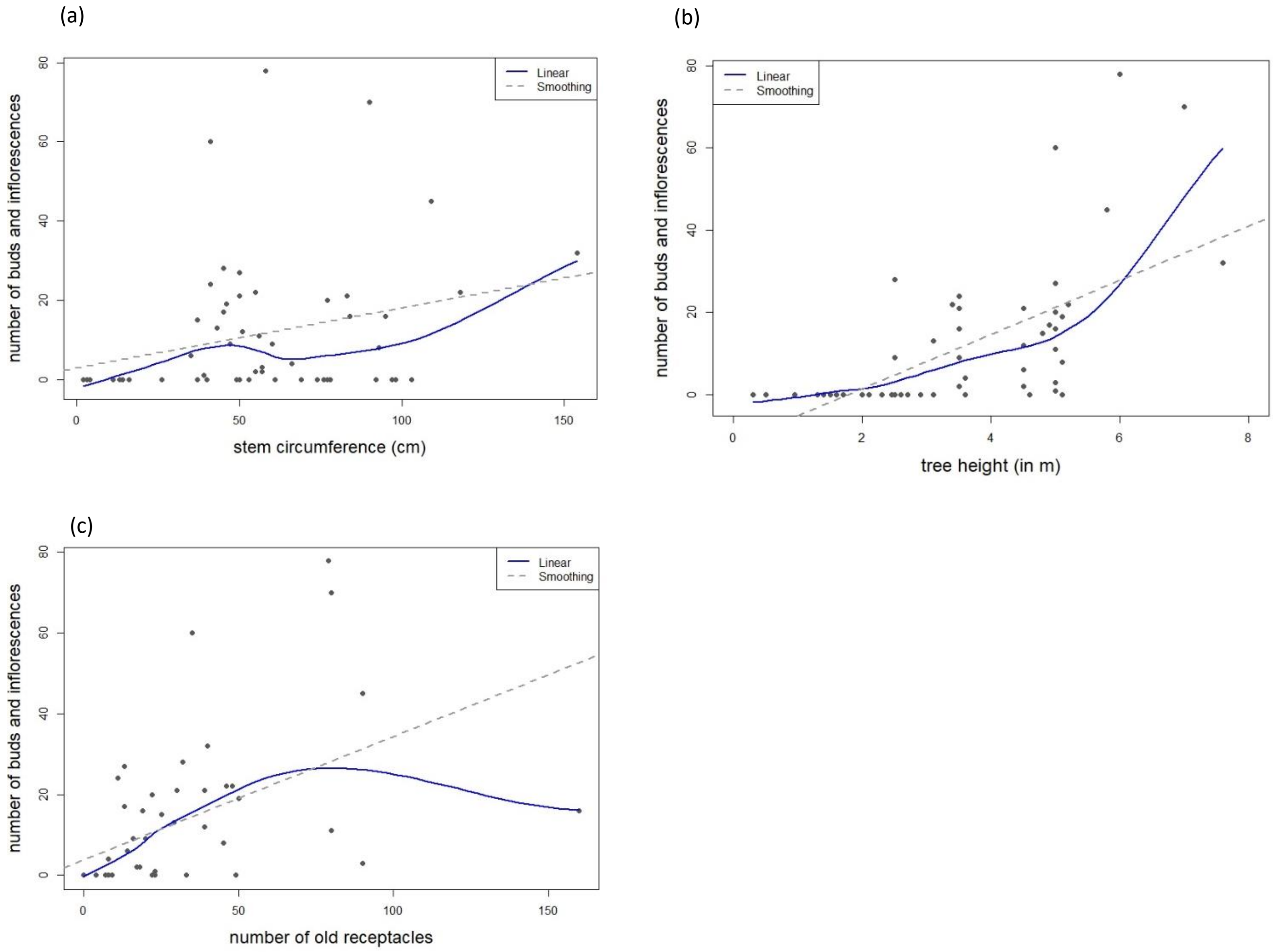


Figure 6.13: Relationship between *P. curvata* inflorescence buds and inflorescences and (a) stem circumference, (b) tree height, (c) number of old receptacles at *Site E* (Clarendon Vale, Mpumalanga, South Africa).

IUCN Assessment

Prior to this study, there have been three published Red List assessments of *P. curvata* (IUCN Red List, 1998; Hilton-Taylor, 1998; Rebelo *et al*. 2020). These are summarized in Figure 7. Two assessments applied IUCN Criterion D (Hilton-Taylor, 1998, Rebelo *et al.*, 2020). Although the criteria used in the earliest assessment are not explicitly noted, there is mention of a “continuing decline in area, extent and/or quality of habitat”. This forms part of Criterion B2b in the latest version of IUCN guidelines (IUCN Standards and Petitions Committee, 2022).

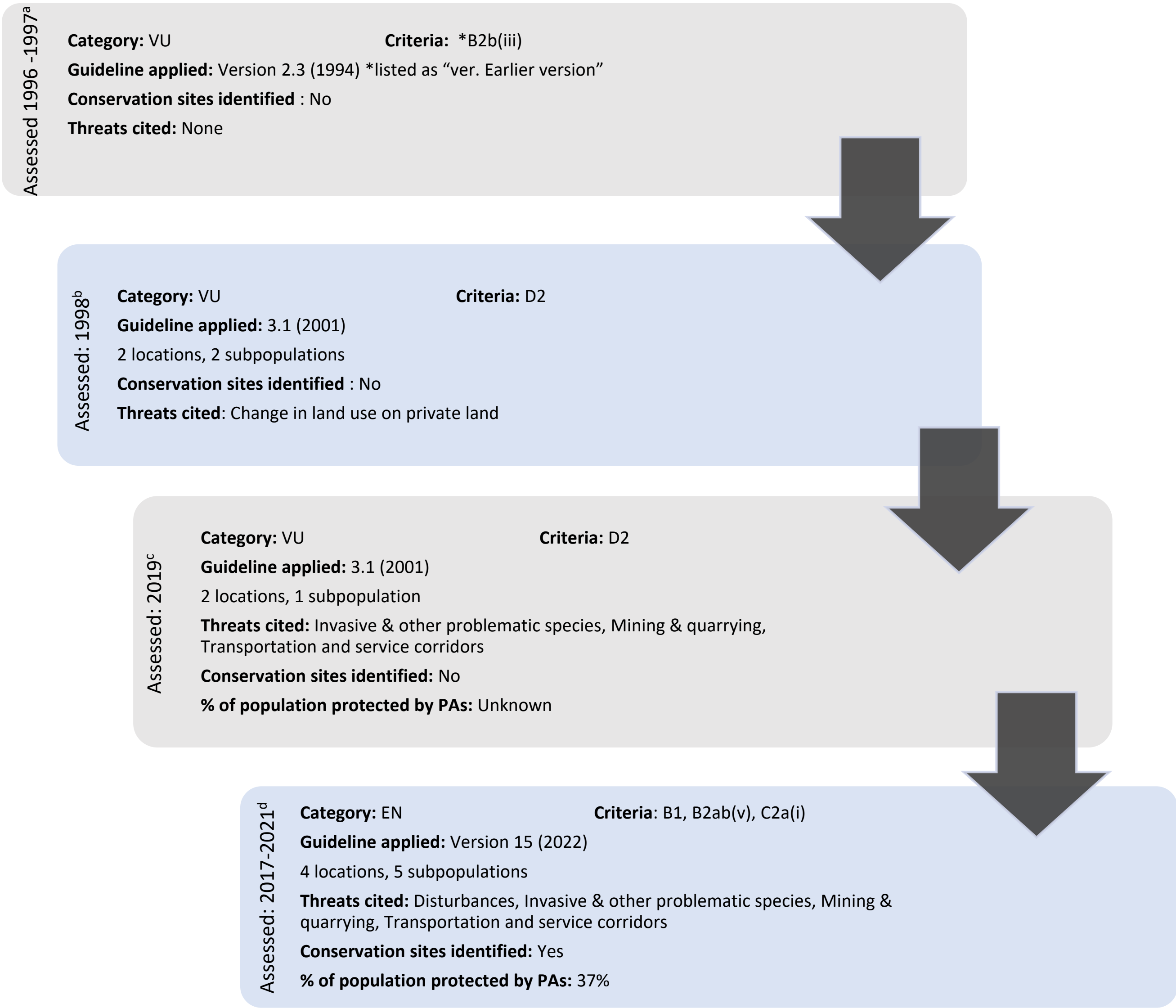


Figure 7: Flow chart showing history of assessments of *P. curvata*

Summary of findings from IUCN Red List, 1998[a]; Hilton-Taylor, 1998[b], Rebelo *et al.*, 2020[c] and Mabuza, 2023[d].

PAs = Protected Areas.VU = Vulnerable. EN= Endangered.

The timeframe of this study did not allow for *P. curvata* to be assessed using IUCN Criterion A or E. While it was possible to estimate population size, there were no data available to estimate population size reduction over the past three generations or the probability of extinction within the next few generations. Therefore, Criteria B, C and D were used in the assessment. An overview of the conditions explored is given in Table 1.1. For the final categorization, some criteria were given precedence based on (i) IUCN guidelines on when it is most appropriate to apply certain criteria (ii) the level of certainty of available information – e.g., observations and estimates were prioritized over inferences and (iii) contextual information gleaned from the demographics and site history of each subpopulation.

With regards to geographic range, *P. curvata* fits two IUCN categories. The area of occupancy was 0.8868 km^2 and the extent of occurrence was 32.3 km^2; thus meeting the prerequisite for the Critically Endangered category. IUCN guidelines define location as "geographically or ecologically distinct area in which a single threatening event can rapidly affect all individuals of the taxon present". For taxa facing many threats, the guidelines recommend basing locations on the threat with the highest likelihood and consequence in terms reducing population size [Detailed definitions included in Appendix]. In the case of *P. curvata*, *Site A* contains the highest percentage of the population [Table 1.3] and occurs within a protected area [Figure 1], thus reducing the likelihood of mining and quarrying activity within the area. Fire management appears to be the threat of greatest consequence since *Subpopulation A* (the largest subpopulation) is not successfully recruiting under the current fire regime [Figure 6.1, Figure 6.2]. The second largest *Subpopulation C* had a few recruits [Figure 6.8], but not enough to counter reduction in subpopulation size [Figure 9].

The remainder of the population (i.e., *subpopulations B, D* and *E*) is found on sites that are not officially protected or in portions of protected areas where access is not heavily restricted. At the time of sampling human settlement could be seen just as few metres from *Site D*. The settlement comprised two houses and a small animal enclosure with chickens and cows. Cars were occasionally observed driving on the dirt roads near *Site D*. At *Site B1* and *Site B2*, cattle grazing and wood harvesting (of non-*Protea* trees) was occasionally observed. Harvesting, farming and human activity was not detected at *Site E*. However, the site is not protected [Figure 1]. These sites are therefore also threatened by fire since they are found on sites that are not under official management, making them vulnerable to unprescribed fires and/or fire suppression. With fire as the location-defining threat, *Subpopulations A, C, D* and *E* are classified as separate locations due the differences in fire management as well as roads and fencing between the sites that serve as potential fire breaks. *Subpopulation B1* and *Subpopulation B2* were on different mountains (~1 km away from each other [Figure 1]), but no persistent fire breaks were identified between the two sites. *Protea curvata* can therefore be considered as having five locations, thus meeting the condition for the Endangered category [Table 1.1]. *Protea curvata* showed maturity at heights above 2.5 m [Figure 8]. At Site A, *P. curvata* height showed no significant increase between 2017 and 2018. Death of mature trees was noted in all censused sites (Figure 6.1, Figure 6.2, Figure 6.4 Figure 6.6, Figure 6.8, Figure 6.10, Figure 6.12]. Mature individuals were also estimated to show a continuing decline [Figure 9]. *Protea curvata* therefore lies in the Critically Endangered–Endangered range based on Criterion B1 and B2 [Table 1.1].

The next criteria explored were small population sizes and decline. The population estimate for all subpopulations combined was 1580–1653 mature trees. The lower estimate excludes trees shorter than 2 m and the higher estimate only excludes trees shorter than 1 m. When including juveniles, the population size is estimated to be 1729 trees. Both estimates are less than 2500 trees, making *P. curvata* a candidate for the Endangered category. The species was then assessed using Criterion C2, which focuses on the size of the largest subpopulation (IUCN Standards and Petitions Committee, 2022). Based on the number of mature trees, *Subpopulation A* was the largest subpopulation. The number of mature individuals in the subpopulation was less than 1000, qualifying for the Vulnerable category. As a result, *P. curvata* is in the Vulnerable category based on Criterion C2a (i).

Criterion C2a(ii) concerns the percent of mature individuals in a subpopulation. This was calculated for each subpopulation. However, IUCN guidelines deem it most appropriate to use this criterion when all or almost all the individuals of the species occur within the same subpopulation. This was not the case for *P. curvata* as individuals were most abundant in *Subpopulation A* and *Subpopulation C*, then variably spread among the rest [Table 1.3]. Criterion C2a(ii) was excluded from the final categorization.

IUCN guidelines advise the use of Criterion D in very small, restricted populations where the total number of mature individuals meets the threshold for placement in the threatened category (i.e., less than 1000 mature individuals in an area of occupancy that does not exceed 20 km^2). This should be coupled with vulnerability to human impact or stochastic events that can critically endanger the species or lead to extinction within two generations. *P. curvata* is subject to such threats and has an area of occupancy less than 20 km^2.

However, the population size of *P. curvata* can be better assessed using the thresholds for small populations (<250; <2500 and <10 000 mature individuals in Criterion C) rather than thresholds for very small populations (<50; <250; <1000 mature individuals in Criterion D). Therefore, Criterion D was omitted. The final listing is thus EN B1ab(v) + 2ab(v)

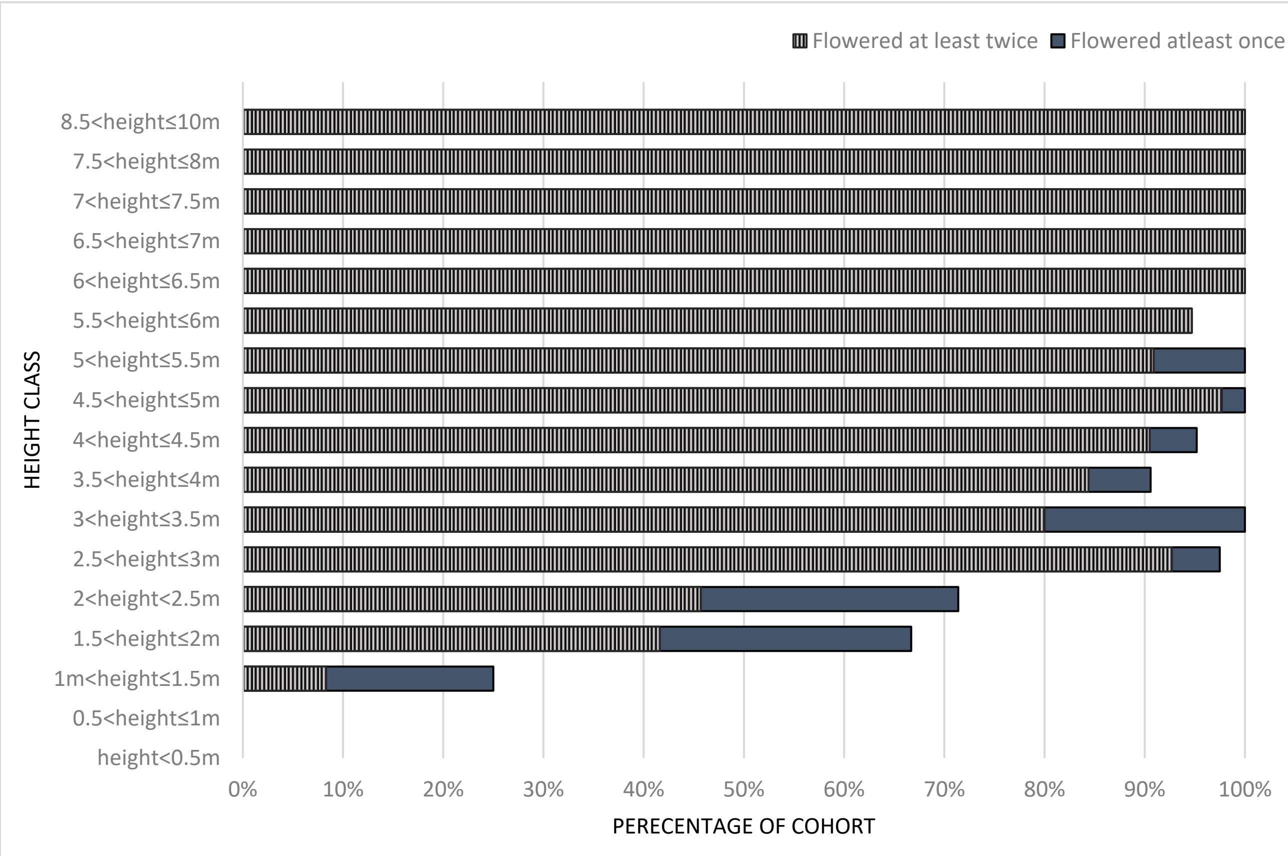


Figure 8: Percentage of *Protea curvata* individuals in each height class that had flowered by 2019.

N= 328 *Protea curvata* plants sampled from six sites in Mpumalanga, South Africa.

Table 1.1: Overview of IUCN Criteria met by *P. curvata* during 2017-2019 Assessment.

A. Population size reduction. Population reduction (measured over the longer of 10 years or 3 generations) based on any of A1 to A4	Critically Endangered	Endangered	Vulnerable
A1	≥ 90%	≥ 70%	≥ 50%
A2, A3 & A4	≥ 80%	≥ 50%	≥ 30%

A1 Population reduction observed, estimated, inferred, or suspected in the past where the causes of the reduction are clearly reversible AND understood AND have ceased.

A2 Population reduction observed, estimated, inferred, or suspected in the past where the causes of reduction may not have ceased OR may not be understood OR may not be reversible.

A3 Population reduction projected, inferred or suspected to be met in the future (up to a maximum of 100 years) *[(a) cannot be used for A3]*.

A4 An observed, estimated, inferred, projected or suspected population reduction where the time period must include both the past and the future (up to a max. of 100 years in future), and where the causes of reduction may not have ceased OR may not be understood OR may not be reversible.

based on any of the following:

- **(a)** direct observation *[except A3]*
- **(b)** an index of abundance appropriate to the taxon
- **(c)** a decline in area of occupancy (AOO), extent of occurrence (EOO) and/or habitat quality
- **(d)** actual or potential levels of exploitation
- **(e)** effects of introduced taxa, hybridization, pathogens, pollutants, competitors or parasites.

B. Geographic range in the form of either B1 (extent of occurrence) AND/OR B2 (area of occupancy)	Critically Endangered	Endangered	Vulnerable
B1. Extent of occurrence (EOO)	< 100 km²	< 5,000 km²	< 20,000 km²
B2. Area of occupancy (AOO)	< 10 km²	< 500 km²	< 2,000 km²
AND at least 2 of the following 3 conditions:			
(a) Severely fragmented **OR** Number of locations	= 1	≤ 5	≤ 10
(b) Continuing decline observed, estimated, inferred or projected in any of: **(i)** extent of occurrence; **(ii)** area of occupancy; **(iii)** area, extent and/or quality of habitat; **(iv)** number of locations or subpopulations; **(v)** number of mature individuals			
(c) Extreme fluctuations in any of: **(i)** extent of occurrence; **(ii)** area of occupancy; **(iii)** number of locations or subpopulations; **(iv)** number of mature individuals			

C. Small population size and decline	Critically Endangered	Endangered	Vulnerable
Number of mature individuals	< 250	< 2,500	< 10,000
AND at least one of C1 or C2			
C1. An observed, estimated or projected continuing decline of at least (up to a max. of 100 years in future):	25% in 3 years or 1 generation (whichever is longer)	20% in 5 years or 2 generations (whichever is longer)	10% in 10 years or 3 generations (whichever is longer)
C2. An observed, estimated, projected or inferred continuing decline AND at least 1 of the following 3 conditions:			
(a) **(i)** Number of mature individuals in each subpopulation	≤ 50	≤ 250	≤ 1,000
(ii) % of mature individuals in one subpopulation =	90–100%	95–100%	100%
(b) Extreme fluctuations in the number of mature individuals			

D. Very small or restricted population	Critically Endangered	Endangered	Vulnerable
D. Number of mature individuals	< 50	< 250	**D1.** < 1,000
D2. *Only applies to the VU category* Restricted area of occupancy or number of locations with a plausible future threat that could drive the taxon to CR or EX in a very short time.	-	-	* **D2.** typically: AOO < 20 km² or number of locations ≤ 5

E. Quantitative Analysis	Critically Endangered	Endangered	Vulnerable
Indicating the probability of extinction in the wild to be:	≥ 50% in 10 years or 3 generations, whichever is longer (100 years max.)	≥ 20% in 20 years or 5 generations, whichever is longer (100 years max.)	≥ 10% in 100 years

Summary sheet extracted from *Guidelines for Using the IUCN Red List Categories and Criteria. Version 15.1* (IUCN Standards and Petitions Committee, 2022). Criteria applicable to *P. curvata* are circled in blue. Use of this summary sheet requires full understanding of the *IUCN Red List Categories and Criteria* and *Guidelines for Using the IUCN Red List Categories and Criteria*. Please refer to both documents for explanations of terms and concepts used here.

Thresholds met during the current assessment are circled or underlined in blue.

*Thresholds assessed in previous assessments are indicated with an asterisk and grey circle.

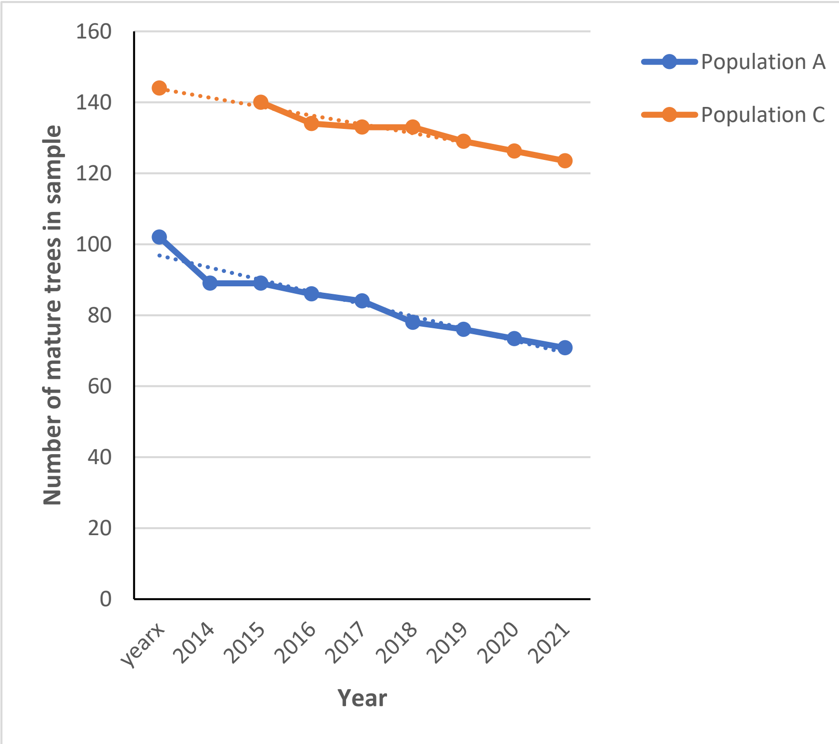


Figure 9: Net decline in number of mature individuals in two *Protea curvata* subpopulations

*Points at 2020 and 2021 are projections based on previously sampled years (where rate of decline was 2.6 trees per year in *Subpopulation A* and 2.75 trees per year in *Subpopulation C*)
*Points prior to 2017 are inferred based on presence of old receptacles from previous seasons found on dead trees in the sample
* First point denoted "yearx" due to uncertainty of how long ago trees without old receptacles and leaves.

Table 1.2: Criterion C– Number of mature individuals in each subpopulation

Criterion C	Mature trees in population:	Thresholds met:	
	1734	EN (< 2500)	VU (< 10 000)

Criterion C2a (i)	**Subpopulation**	**mature trees**	**Category**
	A	639	VU (≤1000)
	B1 + B2	269	VU (≤1000)
	C	477	VU (≤1000)
	D	44	CR (< 250)
	E	305	VU (≤1000)

VU C2ai

Table 1.3: Criterion C2a(ii) – Percent of mature individuals in one subpopulation

Subpopulation:	Estimated number of trees in subpopulation	Estimated number of mature, living trees in subpopulation	% Mature trees of the population	Threshold met
A	639	526	43%	None
B1 +B2	184 + 85	159 + 77	18%	None
C	477	429	21%	None
D	43	39	3%	None
E	305	271	15%	None

Discussion

Population distribution

For *Site A* and *Site C,* there is information from more than one census as well as site management history to provide context to the data. From *Site A* we can gain insight on the short-term effects of disturbance on *Protea curvata*. After the first census in 2017, the site experienced disturbances in the form of a controlled burn. A day before the second census, the site was hit by a severe hailstorm*.* While the hailstorm caused substantial defoliation, the hailstorm and fire had minimal impacts on tree size (Chapter 3). There was only a slight, increase in the height of *P. curvata* trees on *Site A* between 2017 and 2018 (i.e., not statistically significant. The decrease in stem circumference was also statistically insignificant and was possibly due to sampling error i.e., the stem measurements taken slightly higher up the base during a season where bush encroachment encumbers measuring closer to the base. Only two trees grew considerably since 2017; as evinced by the presence of trees in the formerly empty <2250–25000 kg size class.

Site C also experienced a recent disturbance – a fire two days prior to the 2018 census. However, *Site A* and *Site C* are managed differently. While *Site A* is burnt on a four-year cycle (de Bruno Austin, pers. comm., 2018), *Site C* has a history of more frequent burns (Meyer, pers. comm., 2019). The area has attracted herders over the years, who burn the site yearly in the hopes that it would exterminate ticks and encourage regrowth of "sweetgrass" palatable for their livestock. From 2001 to 2009, these yearly burns took place without the authorization of the property owner. Therefore, the area experienced both frequent fires and grazing. When a new owner purchased the site in 2009, more effort was made to avoid uncontrolled grazing and fires, to little avail. Neighbouring herders often managed to enter the site and cross firebreaks prepared before the fire season (Meyer, pers. comm., 2019). *Site A* is well-fenced, making unauthorized burns and grazing less common. This stark difference in management practices is reflected in population demographics.

On *Site A,* the absence of living *P. curvata* trees in the 0–1 m and <1– 2 m height classes in 2017 as well as 2018 indicates that there is no recruitment. The absence of dead trees between 0 and 2 m during both years confirms that low recruitment is not a recent phenomenon in the subpopulation. If it were, one would be able find at least one dead or living tree under 2 m; considering that recruiting populations typically have their smallest size as the most frequently occurring (Condit *et al*., 1998; Miller 1998). Instead, the 5–6 m size class of *Subpopulation A* occurred most frequently. It is not alarming that three of the trees which died between the 2017 and 2018 sampling periods were taller than 5 m. Adult *P. curvata* trees are typically 6–8 m tall (Rebelo, 1991; Mabuza 2017). Taking heed of *Subpopulation A's* lack of juvenile trees in conjunction with its average height of 5.22 m, it is fair to consider trees above 6 m as "older trees". Therefore, the death of the three trees (in the 5–6 m and 7– 8 m classes) may be attributed to senescence.

Subpopulation C showed a very different size class distribution. Unlike *Subpopulation A*, it had trees in the smallest height class (0–1 m) and young trees (<2–3 m) made up the greatest proportion of living trees. A few young, dead trees were also present in the subpopulation. Therefore, recruitment and seedling establishment had been taking place prior to the year of the census.

Both grazing and short intervals between fire events establish a feedback mechanism known to shape savanna ecosystems (van Langevelde *et al.,* 2003). Through this mechanism, herbivory and frequent fire prevent grass biomass from building up at *Site C.* This minimizes the fuel load, thereby ensuring that fires are not intense enough to kill *P. curvata* saplings. *Site A*, on the other hand, is more prone to intense fires. When left unchecked by fire or herbivory, grasses can become overgrown. Such moribund grass is characterized by being less green and less palatable to herbivores as well as having inactive above-ground material.

Despite having grown extensively, the herbaceous layer can no longer effectively outcompete woody species, thus eventually dying out (Trollope, 1980). In the absence of both fire and heavy grazing, it can take as little as three years for grass to reach a moribund state (Scott, 1971; Trollope, 1980; Adie *et al*., 2011). Therefore, long intervals between burns can also encourage bush encroachment.

On pre-fire sampling periods, *Bidens* species (blackjacks) and other thornbushes were notably widespread on *Site A*. *Site C* only had thornbushes predominantly at the lower end of the slope, where *Protea* trees were absent. Bush encroachment in South African savannas is typically extensive on infrequently burnt sites and where browsers and mega-herbivores are absent (De Klerk, 2004; Ward, 2005; O' Connor *et al*, 2014). Since fire management is the major difference between the two subpopulations, it is likely a primary driver of the spread of unwanted bushes at *Site A.* Undesirably, the spread of these bushes fills up the space in which *P. curvata* seedlings could establish.

Seedlings are not the only cohort affected by bush encroachment. With more shrubs and bushes present, the carrying capacity of an ecosystem can decline due to higher water use (Stafford *et al*., 2017). Depending on *Protea curvata's* water-use strategy, the effects may be more detrimental to adult or juvenile trees. For example, *Protea susannae* E. Phillips seedlings transition from a conservative water-use, juvenile stage to a high water-use in the adult phase (Richards *et al*., 1995). Contrastingly, *Protea compacta* R.Br. starts out with high water consumption and later uses a more conservative water-use strategy in the adult stage (Richards *et al*., 1995).

Subpopulation D was similar to *Subpopulation A* in having no recent recruitment. *Subpopulation A* and *Subpopulation D* had the tallest trees (9–10 m). However, *Subpopulation A's* trees had a far larger mass range. This, along with *Subpopulation A's* most frequent height class being the 5–6 m adult trees, suggests that *Subpopulation A* has the highest adult resilience. On the other hand, the height among *Subpopulation D's* trees is mostly due to younger, rapidly coppicing trees rather than older trees. This is highlighted by *Subpopulation D* having the lowest value for maximum biomass and having the highest frequency in the 0–250 kg size class; while having no trees in the smallest height class. All the dead trees in *Subpopulation D* were well below the maximum mass and height of living trees. Evidently, the subpopulation is losing juvenile trees despite showing low recruitment. A study on *Protea caffra* Meisn. showed a similar phenomenon (Adie *et al*., 2011). Where there were fires of great intensity and height, recruitment was suppressed, and population demographics were skewed towards older trees. Younger trees persisted by coppicing. The stems of trees on such sites were severely damaged or lost during fires. Rapid coppicing may also be a response to heavy browsing (Scoging & Macanda, 2005). Although browsing and fire were observed among other subpopulations of *P. curvata* (Chapter 2), the browsing pressure and history of fires experienced by *Subpopulation D* are unknown.

The dead trees in *Subpopulation E* were also mostly juveniles. Fortunately, *Subpopulation E* is a recruiting subpopulation. It had trees in the smallest height class (0–1 m) just as *Subpopulation C* did. However, it did not form the J-curve that characterizes highly recruiting populations. *Subpopulation E*'s most prevalent demographic comprises trees 4–5 m in height, rather than the 2–3 m trees that made up the bulk of *Subpopulation C*. This suggests that either *Protea curvata* recruitment occurs slightly less frequently on *Site E* or that young trees thrive better on *Site C*. Nevertheless, young trees with rapid vertical growth were common in *Subpopulation E.* Much like *Subpopulation D*, *Subpopulation E* had many trees in the smallest size class (0–200 kg) and the taller height classes.

For the most part, *P. curvata* trees on *Site B1* and *Site B2* showed similar patterns in demographics. Both sites showed no signs of recent recruitment. They both showed similar gaps between certain size classes and were made up of trees less than 750 kg in biomass. Height class distribution was also similar and maximum height class varied by 1 m (8 – 9 m for Site B1 and 7 – 8 m for *Site B2*). Similarly, dead trees from *Site B1* were larger than those in *Site B2*. *Site B1* and *Site B2* are on two different hills approximately 200 m apart. Considering the short distance and minor differences between them, they can be treated as one subpopulation. This has implications for how Red List criteria are applied, as discussed in the IUCN Assessment.

Flowering

Flowering had a positive relationship with height and stem circumference. This is not unique to *P. curvata*. Flowering is typically absent in seedlings and only begins to increase in years of reproductive maturity (Hoffman, 2006; See Appendix, Definitions). However, the tree size and flowering relationship was weaker in *Subpopulation A* than in *Subpopulation C.* A possible reason for this is the low recruitment in *Subpopulation A*. In the case of *Subpopulation A*, information on flowering could only be derived from the disproportionately high number of large trees.

New inflorescence buds typically develop at the growth points where the previous season's inflorescences have been dropped. Trees may also develop new branches where buds can later form (Gerber *et al*., 2001). Therefore, during a prolific flowering season, one would expect the number of new inflorescences present on the tree to be much higher than the number of old receptacles. For most *P. curvata* subpopulations, this was the case as there was a positive correlation between the number of old and new inflorescences. Trees on *Site B2* were the exception – showing no correlation between the number of old and new inflorescences. This suggests that flowering during the sample period was not high, relative to the previous season. Although the reasons for this are unclear, we can rule out the loss of a reproductive cohort. Dead trees were between 3 and 5 m tall (i.e., mature) in *Subpopulation B2* and accounted for 9% of the subpopulation. *Site B1* had dead trees between 2 and 8 m which accounted for 13.7% of the subpopulation. Despite losing a slightly higher percentage of mature trees than *Site B2*, *Site B1* maintained a high number of inflorescences relative to the previous season. Furthermore, the number of inflorescences on *Site B1* was similar to inflorescences on *Site B2*. Therefore, other factors affecting *Site B2* likely lead to the number of old receptacles having such a weak relationship with inflorescence number (e.g., shedding of old receptacles occurring early spontaneously or due to a minor disturbance). Overall, this highlights that number of old receptacles can only be used as an estimate of previous flowering, rather than a proxy for measuring flowering over multiple years. Nevertheless, where historical data are unavailable, infructescence numbers (in conjunction with data from the current season) are useful in comparing different populations. For example, an inverse relationship between infructescence and inflorescence number might indicate a major disturbance or a period of serotiny if most of the receptacles contain seeds.

IUCN Assessment

For the assessment, version 15 of the IUCN guidelines was used (IUCN Standards and Petitions Committee, 2022). The most recently published assessment of the *P. curvata* cited version 3.1 as a guideline. Although there are no major differences between version 3.2 and version 15 in thresholds for each category, version 15 provides more detailed definitions of applicable terms as well as recommendations on what equations to use and how to appropriately set each parameter.

The geographic range of *P. curvata* in this study and previous assessments was within the range for Critically Endangered taxa. However, this study found *P. curvata* to have more subpopulations and locations than previously recorded. These are within a small extent of occurrence, yet far enough from each other to be impacted or shielded from certain threats. For example, *Subpopulation A* is located within a Nature Reserve and is less likely to be impacted by mining and quarrying. Conversely, *Subpopulation A* was protected from the more frequent fires experienced by other subpopulations, which likely reduced seedling establishment.

The estimated and projected decline in the number of mature *P. curvata* individuals is due to a lack of recruitment and vulnerability to disturbance. *Subpopulation A* and *Subpopulation C* had similar rates of decline despite the presence of younger trees (recruitment) in *Subpopulation C*.

Number of locations meets the threshold of the Endangered category. Based on location descriptions in previous records (Onderstal, 1979; Protea Atlas Project, 2002) and the locations sampled in this study (Figure 1), it appears *Subpopulation C* and *Subpopulation D* were originally counted as one subpopulation. The subpopulation described as being adjacent to that one is likely *Subpopulation E*. *Subpopulation C* and *Subpopulation D* are a short distance from each other (~2 km) and may have been one population which became fragmented over time, or there simply was not enough information to describe them as separate subpopulations at the time of the first record. We now know that they are on different hills, have different population demographics, and are subject to different levels of competition and disturbance. For instance, *Subpopulation C* is closer to a mine, is on a steeper hill and appeared more open vegetation-wise during the sampling period.

Population size in the previous estimate was 775 – 10 000. The maximum estimate of 10 000 is an overestimate and likely arose from not accounting for the lower density and/or absence of the species in areas within the extent of occurrence. It is also possible that by not sampling more subpopulations, the density value was skewed, thus affecting the population estimate. Furthermore, if the proportion of mature individuals in the sampled subpopulations is 1, it will lead to higher estimates than when adding up the estimates of populations that have different proportion values or when an average of proportion values from multiple populations is used to estimate population size. The matter of how each assessor defines a mature individual will also affect how the proportion value is set. While this assessment used height class relative to flowering as an indicator of which individuals were capable of reproduction, it may be appropriate for future assessors to use adjusted calculations that reflect reproduction limitations such as low density or pollinator scarcity.

Other factors affected the minimum population estimate. The minimum estimate in the previous assessment (775 mature trees) was lower than the one calculated in this assessment. This assessment included populations not surveyed in the earlier assessment – notably S*ubpopulation A*, which accounts for over a third of the individuals of the species. Therefore, even with large values for density and proportion of mature individuals, the lower end of the estimate was too low when this population was not accounted for.

Based on Criterion B, the status ranges from Critically Endangered to Endangered. Based on Criterion C, the status ranges from Endangered to Vulnerable. It is proposed that the Vulnerable status of *P. curvata* be updated to Endangered status based on "non-genuine change". The threats identified in the recent assessment (Rebelo *et al.,* 2020) remain and, due to the overlapping time frame of that assessment and this study, it is unlikely that there were any major threat changes. However, because this study applies the most recent guidelines and synthesizes information from multiple subpopulations, there are some changes in categorization. Population demographics within each site are considered in conjunction with disturbance history and site management. This further informs our interpretation of the species' vulnerability. For instance, reduction in the size of *Subpopulation A* is unlikely to be a part of a healthy fluctuation and more likely to be a concerning decline. Old dead trees were found in the first sample, more trees died during the sampling period and there was a marked absence of young trees in both dead and living trees that were sampled. This indicates poor recruitment of *P. curvata* seedlings while trees in older cohorts continue to die.

Conclusion

In this study, a previously unlisted threat (hail) was identified, which had serious negative impacts on *Subpopulation A* (Chapter 2). The threat of hail and fire damage in most *P. curvata* populations being coupled with a lack of recruitment warrants considering the species to be at a higher risk than previously interpreted. Even among recruiting subpopulations, population demographics were skewed towards older trees. With the exception of the hail damaged site, subpopulations appeared to be floriferous were able to undergo the maturation of inflorescence buds into infructescences. Therefore, factors such as seed set, seed deposition in the soil and seedling establishment may be more influential limitations on *P. curvata* recruitment than flowering. Conservation efforts should therefore focus on improving recruitment improving the establishment of seedings. Additional assessments of management practices and outcomes will be beneficial in elucidating which conditions favour the long-term survival of young *P. curvata* plants to avoid the species being largely represented by senescing trees.

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Chapter 2. Fire and Ice: The response of a savanna *Protea* to infrequent burning and severe hailstorms

Introduction

A disturbance can be defined as “a relatively discrete event in time that disrupts the ecosystem, community or population structure and changes resource pools, substrate availability or physical environment” (White & Pickett, 1985). It may be natural or anthropogenic in origin (White & Pickett, 1985). Throughout the world’s land ecosystems, disturbances (e.g., fire, drought, pests and pathogens) exert a substantial impact on ecosystem dynamics and species resilience (Pickett and White, 1985). It is apparent that disturbances can also have varying impacts based on how they co-occur or interact with other disturbances (Buma & Wessman, 2011; Harvey *et al*., 2014; Gower *et al.*, 2015). In the case of *Protea curvata*, key disturbances that have been identified are drought, hail and fire (Protea Atlas, 2008; Mabuza, pers. observ.).

Two types of disturbance interactions have been identified. The first is “linked disturbance” in which one disturbance affects the behaviour or impact of the next disturbance. For instance, elephants in Kruger National Park are known to alter impacts of fire and *vice versa* (Shannon *et al.,* 2011). The elephants debarked and knocked down more trees on previously burned sites. Due to their post-fire resprout and leaf flush, these sites attracted more elephants, thus they experienced severe elephant disturbance. Subsequent fires resulted in higher mortality among debarked trees.

“Compound disturbance” is when two or more disturbances have an outcome that is greater than the additive effect of each disturbance. The ecosystem responds differently than if the disturbances had occurred independently (Paine *et al*. 1998; Buma & Wessman, 2011, Simard *et al.*, 2011). A good example of compound disturbance can be found in the response of savanna pines after Hurricane Andrew (Platt *et al.,* 2002). The fire regimes preceding the hurricane were important in eliciting contrasting responses to the hurricane.

Pine trees in areas that were burnt during the dry season had a 55% lower probability of withstanding the hurricane and a 94% lower probability of surviving the following 30 months (compared to unburnt areas). Pine tree survival was not reduced on wet season burned sites, but was much higher than on dry season-burned sites and surprisingly higher than on unburned sites (Platt *et al.,* 2002). This re-iterates the assertion that preceding disturbances can change both the magnitude and direction of ecosystem dynamics. Moreover, these changes are contingent on the order of the disturbances and type of disturbances interacting with one another.

In the case of *Protea curvata*, fire, hail and herbivory are interacting disturbances. *Protea curvata* is endemic to Mpumalanga, which has higher rainfall and more frequent hail occurrences relative to other provinces in South Africa [Figure 1].

Despite being simple to diagnose, the impact of hailstorms on trees tends to confound or elude the attention of its observers. This is because it is often noticed by loggers or researchers years after the initial damage and the date of the causative storm is estimated (Riley, 1953; Linzon, 1962). Studies on hail in savannas (where *Protea curvata* is typically found) focus on grasses, crops and/or defoliation (De Beer, 1983; Iledun, 2017). Impacts of hail on tree health are relatively well-studied in forests. Its immediate effects are noticeable in snapped branches, discoloured leaves, rapid defoliation, litter deposition, and bruised or stripped bark (Gower and Fontaine, 2015; Houston, 1999). Hail accompanied by strong winds can alter crown shape. Depending on the direction of wind-driven hail, one side of tree crowns can be thinned by hail. Lesions can also be prevalent on one side of a tree based on the direction of the storm (Riley, 1953).

Beyond mechanical damage, hail can also make trees more vulnerable to fungi and insects (Laut *et al*., 1966). A study of hail impact on forest trees found that all tree species on the site were similarly affected by hail (Linzon, 1962). Over the course of four years, hail-stricken trees had a slightly slower growth rate than trees on undisturbed plots. Since the growth was only slightly stunted by hail, it was proposed that the trees could recover after five years. However, *Protea curvata* can experience more than one disturbance within five years.

For example, one of the sites (*Site A)* was disturbed by fire and a severe hailstorm in a time frame of less than a year [Figure 2].

Fire is a ubiquitous disturbance on savannas. On some sites it may occur more frequently and on other sites it may be linked with hailstorms. For instance, in an Australian shrubland, hail-impacted and fire-impacted trees experienced greater scorch heights than trees that were only disturbed by fire. This was attributed to the canopy biomass that is littered during hail and its contribution to fuel load in the long run (Gower and Fontaine, 2015).

Even on sites where fire is not associated with hail, it is consequential. While compound disturbances can deplete resprout quantity and quality (Gower and Fontaine, 2015), fire alone can have a positive impact. In obligate-resprouters fire stimulates shoot development. Fire can eradicate non-resprouters on a site, thus availing more resources for the remaining resprouting species (Pausas and Keeley, 2017). The variably disturbed populations will shine a light on how single disturbances and interacting disturbances impact *P. curvata* flowering.

Quantifying herbivory on resprouting *P. curvata* shoots will also be informative. Herbivores are generally an important factor to consider in the regeneration of any plant species. Herbivores exert influence on flowering, seedling survival, shoot growth (including resprout), functional leaf area, as well as stand structure and community structure (Gill 1992; Ammer 1996; Danell *et al*., 2003; Didion *et al.,* 2009). This is through foraging, debarking and trampling (Gill, 1992; Skarpe and Hester, 2008; Shannon *et al*., 2011).

A study of two *Banksia* (Proteaceae) species highlighted the importance of herbivory (Witkowski & Lamont, 1997). Overall, seedling survival was lower on experimental plots that were within an open farm than on plots surrounded by longstanding vegetation. Furthermore, both *Banksia* species' seedlings survived longer (on the vegetated area) when herbivores were excluded. This emphasizes a need for herbivores to be limited on certain sites and/or have more than one plant resource (unlike on the open farm).

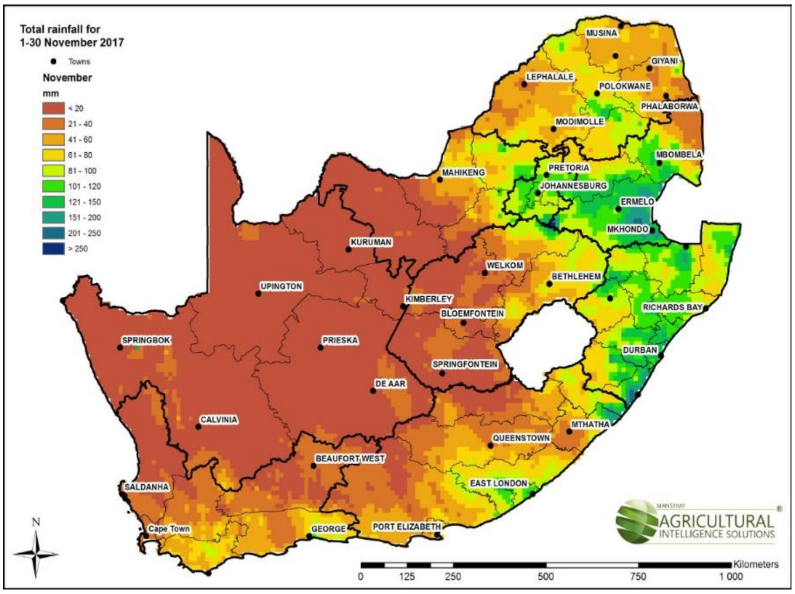

Figure 1.1: Observed total rainfall (mm) in South Africa during November 2017

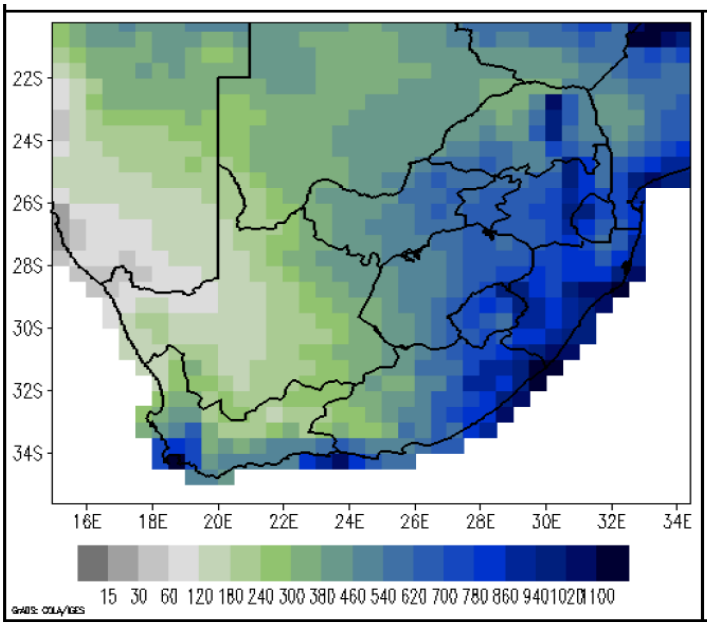

Figure 1.2: Average observed annual total rainfall (mm.year-1) from the Global Precipitation Climatology Centre (GPCC) for the period 1976–2005

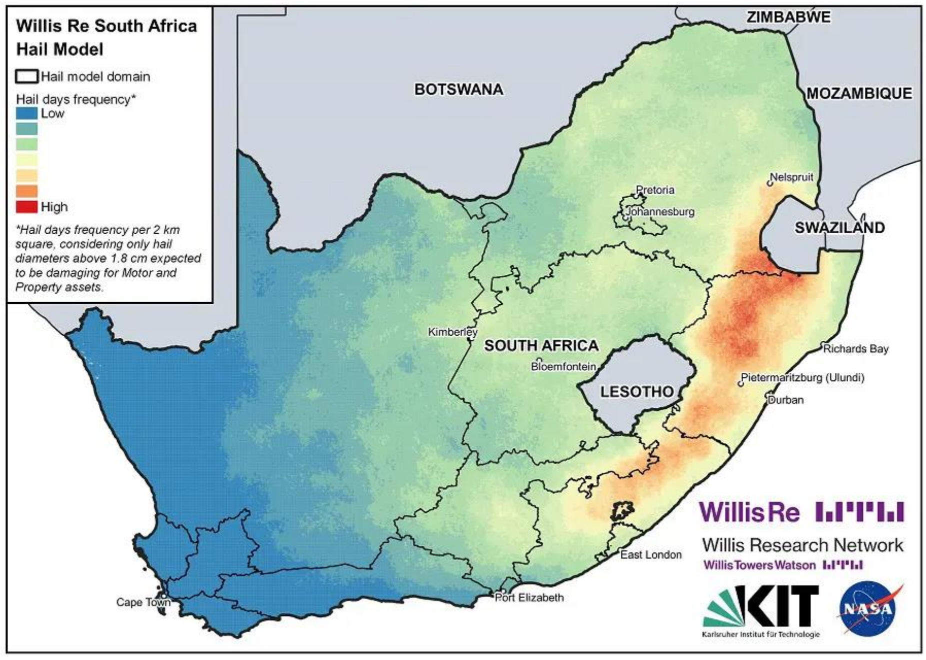

Figure 1.3: Map of the relative hailstorm frequency over South Africa (Wills Re, 2021)

Data derived using SMASH team satellite datasets including overshooting cloud top detections from a 14-year database (2005–2019) of 15-minute resolution Meteosat Second Generation infrared brightness temperatures, TRMM (Tropical Rainfall Measuring Mission) and GPM (Global Precipitation Measurement) hailstorm detections, as well as ERA5 reanalysis data.

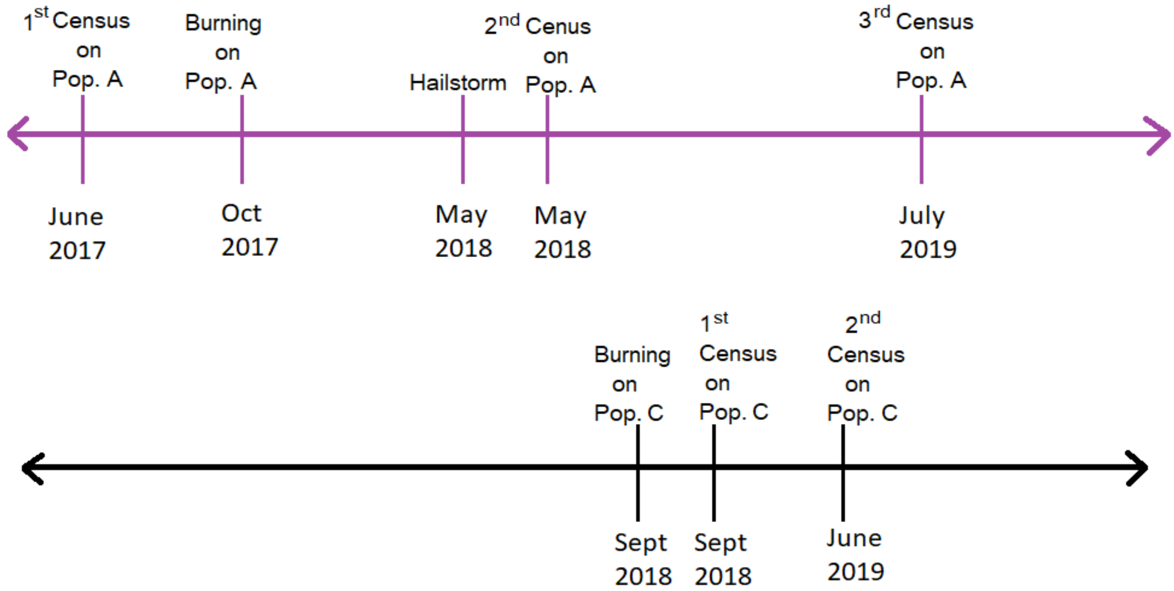

Figure 2: Timeline of recent disturbances and sampling undertaken on two *Protea curvata* subpopulations (*Site A* in Barberton Nature Reserve and *Site C* in Clarendon Vale, Mpumalanga)

Resprouting is one of the strategies used to recover from disturbance and is especially advantageous in fire-prone habitats (Pausas *et al*., 2016). *Protea curvata* exhibits epicormic resprouting [Figure 5], which is widespread in savannas. It is distinguished from basal resprouting in that new shoots emerge from the trunk or from upper branches; rather than emerging from the base or belowground structures of the tree, such as lignotubers and woody rhizomes (Burrows*, et al*. 2010).

While post-fire obligate-seeders have to regenerate much of their population using seed banks, epicormic resprouters have the advantage of retaining the tree structure after a fire. In other words, epicormic resprouters can regain balance of woody matter and carbon much faster than post-fire obligate-seeders. This has positive implications for the functioning of the population and the ecosystem as a whole (Pausas & Keeley, 2017). It is for this reason that the presence of resprout and damage to resprout (caused by herbivores and other disturbances) is so relevant when examining *Protea curvata.*

Excessive defoliation caused by herbivory or disturbances can result in less flowering. This is because leaves are fundamental for producing photosynthates that are directed to flowering. Leaves can also act as signalling tissue for the timing of flowering and anthesis (Gerber *et al*., 2001a; Gerber *et al*., 2002; Smart, 2005). Therefore, herbivory and disturbances can encumber a plant's fecundity or capacity for pollination and reproduction (Michaud, 1990; Heidel *et al*., 2011)

Insect larvae are known to reduce seed mass (Michaud, 1991). Even post-germination, plants remain more susceptible to mortality when affected by herbivores during their early life stages. This is because the impact of herbivores is more pronounced in individuals with a lower biomass and a higher ratio of nutrient rich to woody plant material (Bach, 1993; Sangita *et al*. 1986; Fenner *et al*., 1999). Furthermore, being short, small and lacking mechanical defences (such as multiple branching to make nutrient rich material less accessible to herbivores) makes seedlings vulnerable to more potential herbivores (Boege and Marquis 2005).

Another aspect that is closely entwined with herbivory is stand structure. Both aspects can affect one another. Selective herbivory (e.g., on seeds, seedlings or resprouting shoots) and its intensity can alter the density and demographics of a tree population (Gill, 1992; Ammer, 1996; Danell *et al*. 2003; Didion *et al.,* 2009). Conversely, the density and demographics of a tree population can influence the feeding habits of herbivores. Herbivores may forage more on a certain site depending on the quality of food sources available as well as the protection from predators that can be provided by dense vegetation cover (Kikuzawa 1988; Caccia *et al*., 2009; Nopp-Mayr *et al*., 2015). As seen with elephants on burnt sites of the Kruger National Park, disturbances can also alter stand structure and facilitate foraging (Shannon *et al*., 2011).

The study therefore aimed to:

- Compare damage to flowering, shoots and bark in *Protea curvata* sites with frequent (2 years) fires and infrequent fire (4 years).
- Investigate the presence of fire and herbivory on *P. curvata* shoots.
- Assess the impacts of a hailstorm on flowering and bark in one *P. curvata* subpopulation.

Methods

Study site and species

Protea curvata is a plant growing on serpentine soils in the lowveld of Mpumalanga, South Africa (Protea Atlas, 2008). The area is characterized by savanna vegetation that is denser in high rainfall sites (Botha *et al*., 2004). The province receives summer rains and has a mean annual rainfall of 715 mm (South African Sugarcane Research Institute, 2023). "*Site A*" is within Phase 2 of the Barberton Nature Reserve (-25.611276°, 31.003775°) in Mpumalanga. This is a 5400 ha area that includes pieces of State land and it is managed by neighbouring private conservation organisations (Mpumalanga Tourism and Parks Agency [MTPA], 2012). At Mundt's Concession, about 9 km southeast of "*Site A*", "*Site B*" is found. "*Site C*", "*Site D* and "*Site E"* occur further southeast. These are situated near Dixie Farm and Clarendon Vale. [Figure 3].

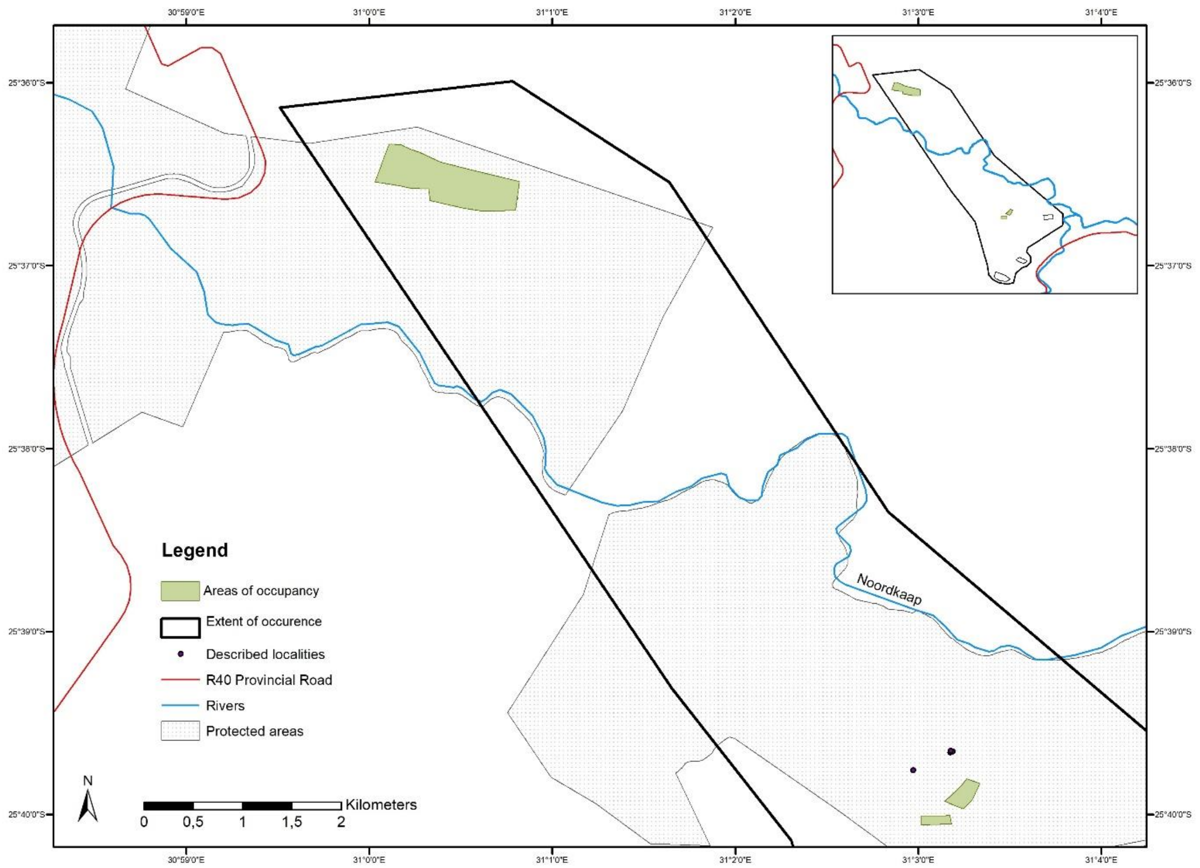

Figure 3: Map indicating *Protea curvata* localities in Barberton (Mpumalanga, South Africa).

Climate and disturbance history

Climate data were obtained from the South African Weather Service (SAWS). Kruger International Airport Station records frost and snow occurrence measured at 08:00, 14:00 and 20:00 daily. The two stations also record a total of rainfall by month. Daily rainfall records were obtained from Barberton Prison Station (-25.7830 31.0500, 852 m) as well as hail records dating back to 1914. To supplement these data, news articles were used to enumerate hailstorm occurrences which may have not been captured by the weather stations (www.businessinsider.co.za, lowvelder.co.za). A variety of standards to classify hailstorms were encountered. The National Weather Service of America defines hailstorms as severe when hailstones are 25 mm in diameter or larger (Cecil and Blankenship,). Although no explicit definition was available from the South African Weather Service, one South African study defined hailstorms as severe when made up of hailstones 35 mm or larger in diameter. South African Weather Service data included notes on the damage caused by each storm or rough estimations of hailstone size (e.g. "golf ball sized stones", "maize fields destroyed"). For the purpose of our study hailstones larger than 25 mm were considered severe. Where hailstone measurements were unavailable, the hailstorms described as having caused extensive injury, property damage or crop loss were also regarded as severe.

The management of Site A is characterized fires applied approximately every four years (de Bruno Austin, pers. comm., 2020). Contrastingly, *Site C* experienced annual fires applied from 2001 to 2009 by local livestock herders. From 2009 onwards, the property owner attempted to limit these unauthorized fires, with little success. Thus, fires occurred irregularly between 2009 and 2018, albeit on a shorter cycle than four years (Meyer, pers. comm., 2019).

Flowering, bark and shoot damage

P. curvata begins budding in April. Flowering can be observed from June to October (*Protea Atlas*, 2008). *Site* C was burnt towards the end of the flowering season in 2018 (2–3 September). It was then sampled a day after burning and again in the following season [Figure 2]. This allowed enough time for shoot development. The effect of fire and herbivory on resprouting shoots was examined.

Parts of *Site A* were burnt in 2017 after initial sampling. Then in 2018, before the second sample, *Site A* experienced a hailstorm. Observations on damaged bark were made. The number of damaged stems per total stems on a tree were counted. The length and width of the damage was measured and the direction it was facing was recorded. The same measurements were done on the remaining subpopulations to compare them to the sequentially disturbed *Site A*.

Hail occurring at *Site A* made it difficult to ascertain whether resprout was absent as a result of hail or if most of the trees simply did not resprout that year. For this reason, resprout damage was only recorded at the other sites. Trees with new and damaged shoots were examined. The number of new shoots was noted. Any damage observed on these shoots was classified as a burn, bite mark or insect hole [Figure 5]. Together with the previous year's data, the data recorded during this study will allow us to ascertain patterns in the population and response to disturbance.

To ascertain how common bark damage was in each population, its presence among trees was noted. The number of damaged trees per number of individuals in a population was then expressed as a percentage. To estimate how commonly bark damage occurred in a population, the percentage of damaged main stems was noted for each tree. This data was then plotted (boxplot) to compare percentage of damaged main stems between all the populations.

A Spearman rank test was performed for each population to check if bark damage was correlated with tree size. Based on which direction the bark damage was facing, aspect was classified into 1 of 8 sectors [Figure 4]. When recording bark damage in the field, it became apparent that some trees were slanted to the point where most some of the main stems laid almost horizontally. For such trees, measurements were taken of upward and downward facing damage. Using boxplot and Kruskal-Wallis test, the amount of damage (in cm^2) facing each aspect was compared. This was followed by a Kruskal-Wallis post-hoc test for multiple pair-wise comparisons (kruskalmc in RStudio).

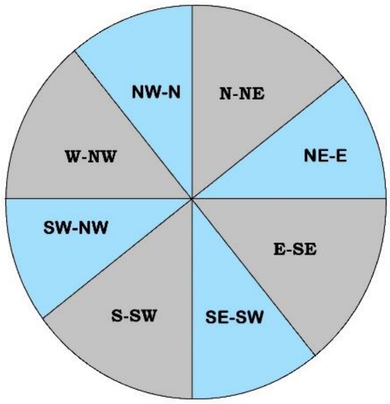

Figure 4: Sectors (aspects) of bark damage

The proportions of i) burnt shoots, ii) bitten shoots and iii) insect-damaged shoots per total number of damaged shoots was recorded for each tree. This was then represented as a boxplot to compare the impact of fire and herbivory on new shoots.

Resprout data were available for all subpopulations, except for *Site A*. Due to a hailstorm occurring in 2018 on *Site A*, many of the inflorescences and leaves were stripped from the trees. This made it hard to distinguish which trees had any resprouting shoots and whether they were grazed on before the hail.

A Spearman rank test was used to check for correlation between inflorescence buds and inflorescences from this season and the following demographics: (i) stem circumference, (ii) tree height, (iii) number of old inflorescences (iv) area of bark damage. Spearman correlation co-efficients less than ± 0.5 were interpreted as a weak correlation and co-efficients greater than ± 0.5 were considered a strong correlation.

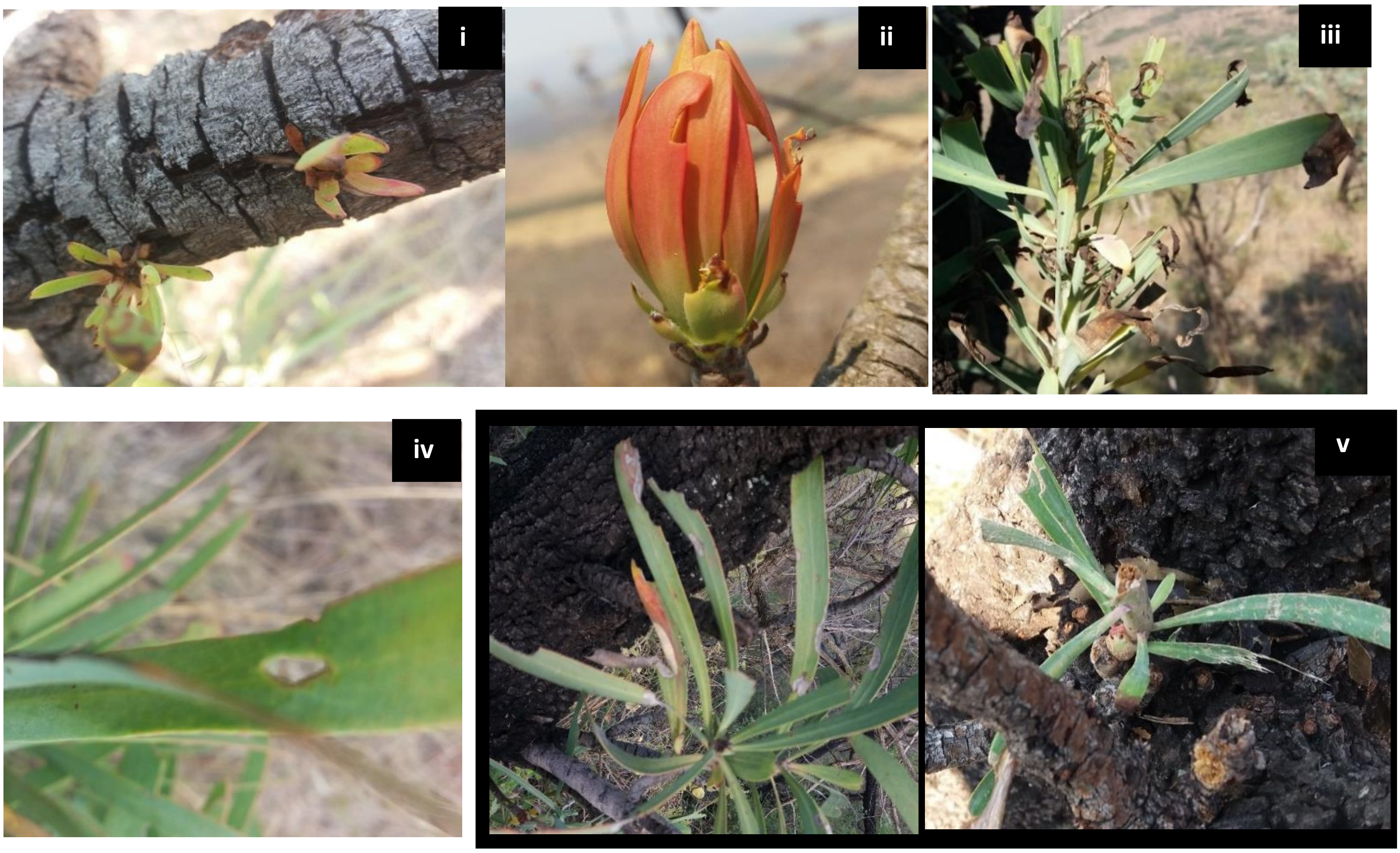

Figure 5: (i) New shoots sprouting *Protea curvata* bark (ii) red leaves distinguishing resprouting shoots, (iii) burnt leaves ,(iv) leaves with insect holes and (v) bitten leaves.

Results

Climate and disturbance history

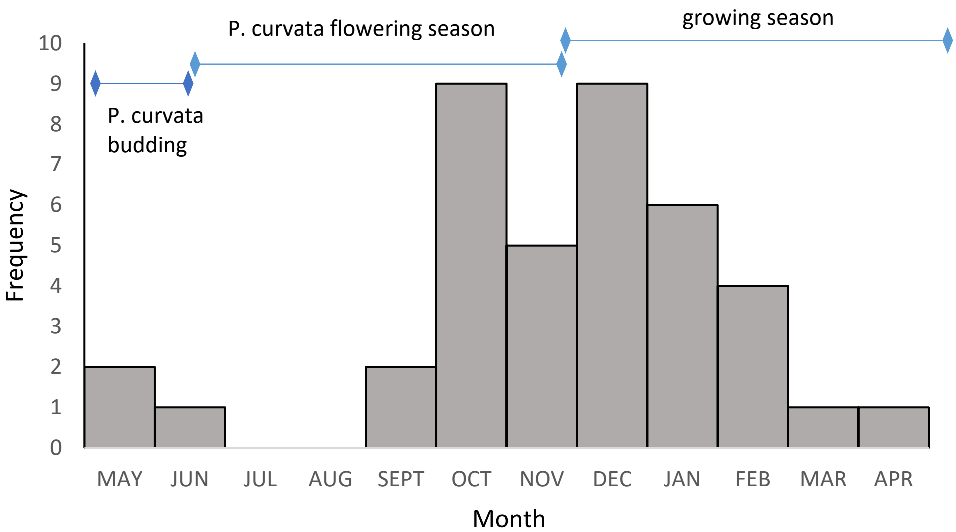

Figure 6: Hailstorm frequency by month for Mpumalanga (1914-2020)

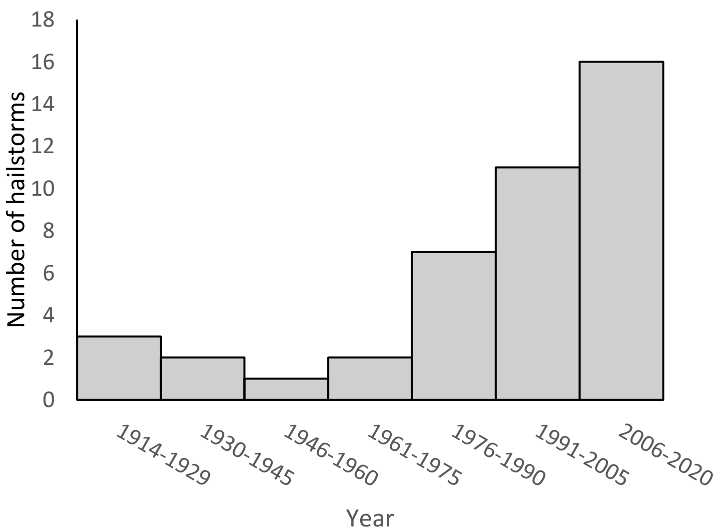

Figure 7: Hailstorm frequency in Mpumalanga Province (1914-2020)

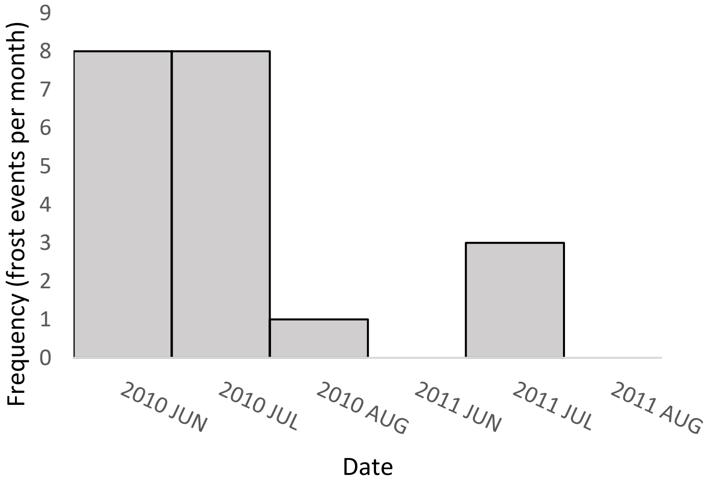

Figure 8: Frost occurence between 2008 and 2020 at Kruger Mpumalanga International Airport weather station

Generally, hailstorms have become more frequent in Mpumalanga over the last 106 years (Figure 7). Most of the province's hailstorms occur in October and December. Hailstorms in May and June stand out since they coincide with *P. curvata's* flowering season (Figure 6). The June hailstorms only begin to appear 77 years into the 106-year record. The year 2018 was first time in 104 years that Mpumalanga experienced a hailstorm in May and this time it was more widespread. The last May hailstorm in 1914 affected one area whereas this one impacted at least five. Based on Barberton weather station daily rainfall data, the hail on May 27, 2018 accounted for nearly a third (27.4%) of that month's precipitation/rainfall and had golf ball sized hailstones. Over the past 5 years, seven severe hailstorms are known to have occurred in Mpumalanga. Three of these events were during *P. curvata's* flowering season, with the recent storm occurring the earliest (during budding stage in 2018).

Although frosting was observed in 2020 at Barberton (where *P. curvata* grows), it was not noted by the SAWS since the Barberton weather station only records rainfall (de Bruno Austin, pers. comm, 2020). The Kruger International Airport Weather Station recorded 20 frost events between 2008 and 2020. All 20 coincided with *P. curvata* flowering season and occurred in the years 2010 and 2011 (Figure 8). Additionally, Barberton Nature Reserve noted frosting towards the end of July 2020 with temperatures between -8°C and -2°C. No snow was recorded either through field observations or weather station data during the 2008–2020 period.

Flowering

For *Subpopulation A*, the number of buds & inflorescences in 2018 was significantly lower than in 2017 ($\mathbf{mean}_{2017}$ = 10.94, $\mathbf{mean}_{2018}$ = 4.28, t = 3.0033, d.f. = 152.89, p-value = 0.0031). It remained significantly low in 2019 compared to the 2017 census. In fact, flowering was at its lowest in 2019 (i.e., a year after the hail disturbance), averaging 2.30 flowers or buds per tree. The number of old receptacles in 2018 did not differ significantly from old receptacles in 2019 ($\mathbf{mean}_{2018}$ = 0.82 $\mathbf{mean}_{2019}$ = 0,90, t = -0.22013, d.f. = 200.87, p-value = 0.826).

In *Subpopulation C*, the number of buds & inflorescences was also significantly lower in 2019 ($\mathbf{mean}_{2018}$ =23.54 $\mathbf{mean}_{2019}$ = 8.28; t = 5.3772, d.f. = 180.44, p-value < 0.001). Conversely, old receptacles (with and without bracts) were higher in 2019 than in 2018 ($\mathbf{mean}_{2018}$ = 5.91; $\mathbf{mean}_{2019}$ = 20.10; t = -4.5188, d.f. = 164.98, p-value < 0.0001). When counting only those with bracts, there were still more old receptacles in the year 2019 than in 2018 (**mean**$_{2018}$ = 3.18750; **mean**$_{2019}$ = 16.50781; $t = -5.3944$, d.f. = 145.9, p-value <0.0001).

During post-fire field observations, burn damage was mostly observed on the lower stems and leaves. In rare cases where inflorescences were burnt, the flowers were singed off and burnt fruits remained attached to the receptacle. During post-hail observations, hail knocked off leaves as well as entire inflorescences (i.e., bracts and seeds included).

Figure 9: Infructescence after fire with bracts and seed remaining on the pedicel (left). Inflorescences removed from pedicles by hail (right).

Damaged Shoots

Site A could not be analysed for resprout and herbivory as a result of defoliation from hail. It is important to note that a single shoot could have none, one or more than one type of damage on it. On average, damaged leaves in *Subpopulation C* had a significantly lower proportion of insect holes than burn marks or large bite marks (Kruskal- Wallis X^2 = 11.759, d.f. = 2, p-value = 0.002796, $\bar{x}_{\text{insect}}$= 21.33%, $\bar{x}_{\text{burn}}$= 38.67%,$\bar{x}_{\text{bite}}$= 49.79%). Insect holes were an uncommon cause of leaf damage (Q1=median=minimum=0%) and where they were present, they often constituted a small portion of the leaf damage. Insect holes made up 50% or more of the damage in only a quarter of damaged leaves (Q3= 50%).

On the other hand, where bite marks were present, they were more likely to account for a majority of the damage. In half of the bitten shoots, bite marks constituted more than 50% of the resprout damage (median=50%) [Figure 10]. Bite marks were found in 43% of trees with resprout damage, whereas burn marks and insect holes were found in 35% and 22% of damaged trees, respectively.

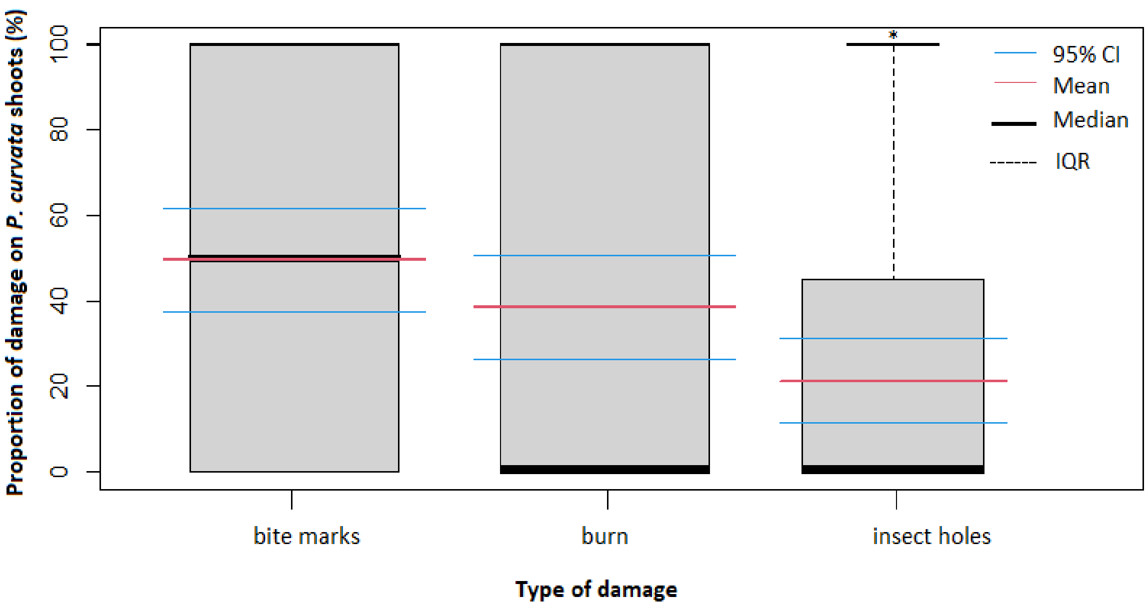

Figure 10: Herbivory and fire damage on resprouting *Protea curvata* shoots (*Site. C)*

Mean and 95% confidence interval given for each type of damage.

*Damage accounted for by insects was significantly lower than damage from fire or bite marks

Damaged Bark

Bark damage was most prevalent at *Site A.* A majority (79.4%) of the trees in *Subpopulation A* had damage on all their stems. The remainder (i.e., trees with damage to only some or none of their stems) were outliers [Figure 11, Table 1]. Similarly, bark damage was prevalent in trees at *Site B1, B2* and *E*. Their lower quartiles had 0–50% damaged main-stems. Therefore, 75% of the trees in these subpopulations had more than half of their stems damaged.

Sites C and *D* had the fewest trees with bark damage [Table 1]. *Site C* and *D* showed similar patterns in terms of damaged main-stems. Within these two sites, it was more common to find trees with a lower proportion of damaged main-stems compared to the other sites. This is indicated by their low median values and lower quartile values, in comparison to the other four subpopulations [Figure 5].

Site A (meanA = 88.97%) showed a significantly higher occurrence of damaged stems than *Site C* (meanC = 62.16%) and *Site D* (meanC = 56.82%, Kruskal-Wallis χ^2 = 34.776, d.f. = 5, p-value < 0.001). Occurrence of bark damage was also the most variable in *Site C* (SD_A = 24.01%, SD_{B1} = 34.92%, SD_C = 41.94%, SD_D = 46.49%, SD_E = 39.75%) [Figure 11].

Subpopulation A showed no significant difference in the area of bark damage between different aspects (Kruskal-Wallis χ^2 = 10.15, d.f. = 7, p-value = 0.1802). In *Site B1*, bark damage extending all around the stem was significantly larger than north-facing bark damage. It was also significantly larger than damage in the northeast to east aspect (Kruskal-Wallis χ^2 = 27.506, d.f. = 9, p-value = 0.0012). The remaining aspects showed no significant difference in area of damage. Within *Site B2* there was no significant difference between bark damage at different aspects (Kruskal-Wallis χ^2 = 13.054, d.f. = 7, p-value = 0.0708).

Trees on *Site C,* like trees on *Site B1*, had a significantly greater amount of damage all around the stem ($mean_{all\ around}$ = 3212.48 cm^2) than in the northeast to east aspect ($mean_{NE\_E}$ = 1448.3 cm^2, (Kruskal-Wallis χ^2 = 22.158, d.f. = 6, p-value = 0.001133). Damage extending all around the stem was also significantly greater than west to northwest facing damage (mean = 1116.77 cm^2) [Figure 8].

In *Subpopulation D*, there was no significant difference between bark damage at different aspects (Kruskal-Wallis χ^2 = 13.072, d.f. = 9, p-value = 0.1594). In *Subpopulation E,* damage facing west to northwest was significantly higher than damage facing north to northeast as well as south to southwest damage ($mean_{W\_NW}$ =3627.333 cm^2; $mean_{N\_NE}$ = 769.76 cm^2; $mean_{S\_SW}$ = 249.33 cm^2). Damage facing west to northwest was also significantly higher than damage in the northwest to north aspect ($mean_{NW\_N}$ = 1385.25 cm^2; Kruskal-Wallis χ^2 = 30.341, d.f. = 9, p-value < 0.0001).

Bark damage was further analysed relative to stem circumference. In *Subpopulation A*, stem circumference and area of bark damage showed no correlation (Spearman rs = 0.00, C.I. = 95%, S = 176520, p-value = 0.9853). This was also the case with trees on *Site B1* (Spearman rs = 0.01, C.I. = 95%, S =84507, p-value = 0.9332). Within *Site B2,* stem circumference and bark damage had a weak, positive correlation (Spearman rs = 0.36, C.I. = 95%, S = 8457, p-value = 0.01724). *Subpopulation C* showed a strong, positive correlation between stem circumference and bark damage (Spearman rs = 0.62, C.I. = 95%, S = 207360, p-value < 0.0001). *Subpopulation D* had a weak, positive correlation between stem circumference and damaged area (Spearman rs = 0.34, C.I. = 95%, S = 9328.1, p-value = 0.0228). *Subpopulation E* had a strong, positive correlation between stem circumference and damaged area Spearman rs = 0.69, C.I. = 95%, S = 8435.6, p-value < 0.0001) [Figure 14].

Table 1: Prevalence of bark damage on *Protea curvata* trees at different sites.

	Trees with stem damage (%)	Trees with damage to all stems (%)
Site A	98	79.4
Site B1	91	67.5
Site B2	76.7	58.1
Site C	62	49.3
Site D	63.6	50
Site E	81.5	68.5
Average:	**83.7**	**65.4**

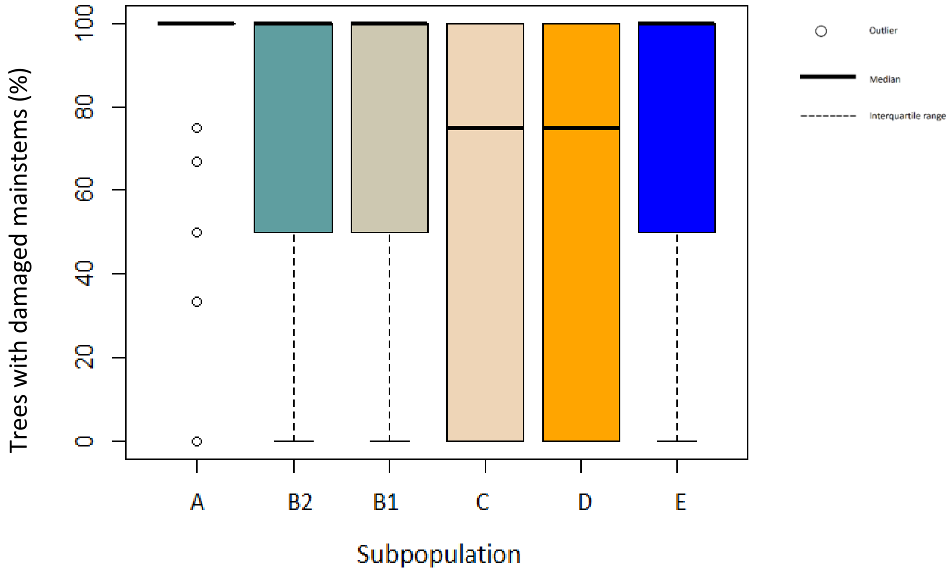

Figure 11: Proportion of trees with damaged mainstems in *Protea curvata* subpopulations

Presence of bark damage recorded in 2018 in trees at Barberton Nature Reserve *(Site A), Mundt's Concession (Site B1* and *Site B2),* Claremont Vale *(Site D) and* Clarendon Vale *(Site C* and *Site E)* in Mpumalanga, South Africa

Figure 12: 12.1) Burns and peeling of *P curvata* bark.

12.2) Hail damage to *P. curvata* outer bark.

12.3) General bark damage and hail damage to inner bark of *P. curvata.*

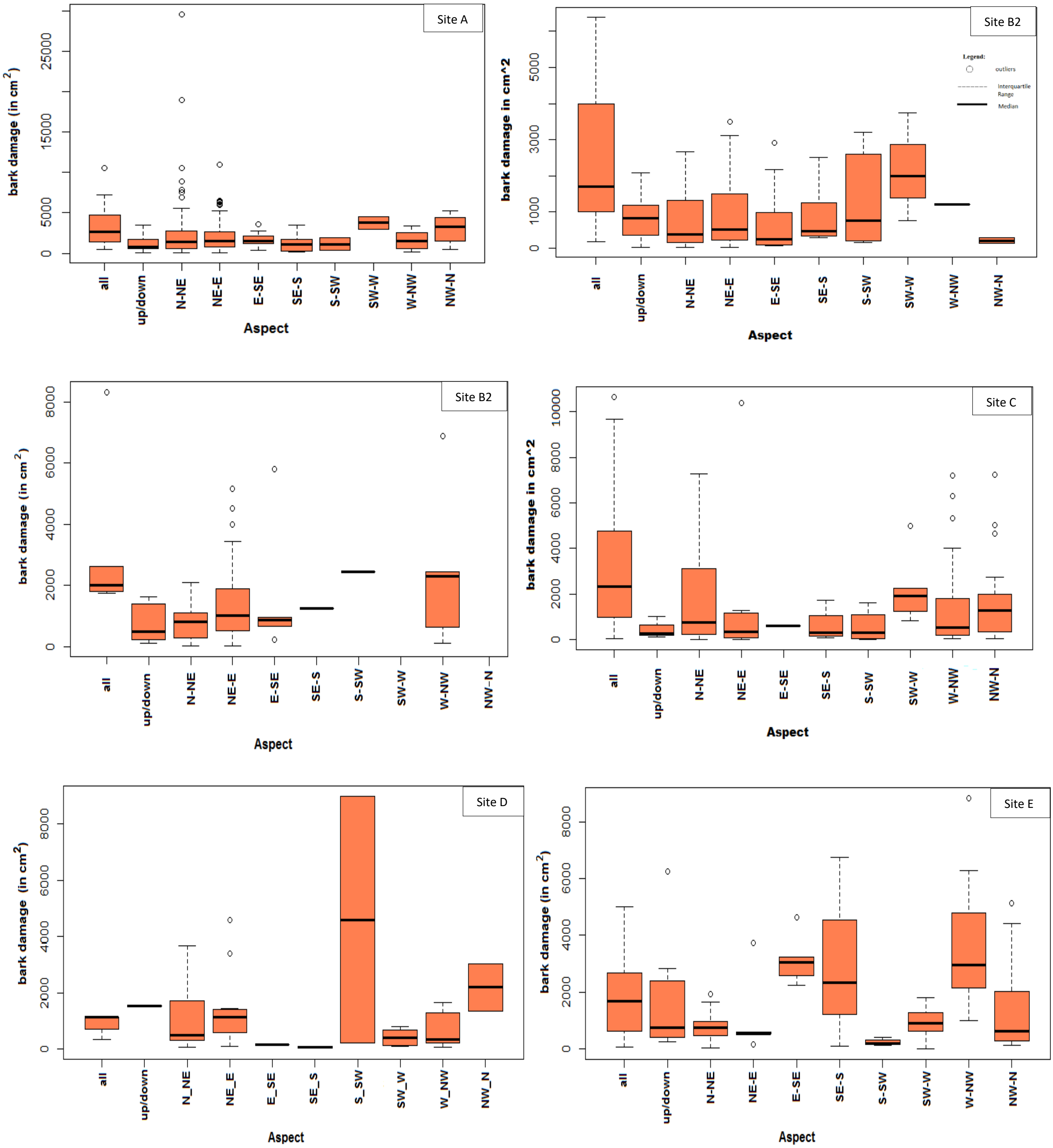

Figure 13: Area of bark damage facing different aspects in *P. curvata* trees sampled from six sites in Mpumalanga, South Africa (*Site A*, *B1, B2, C, D* and *E*)

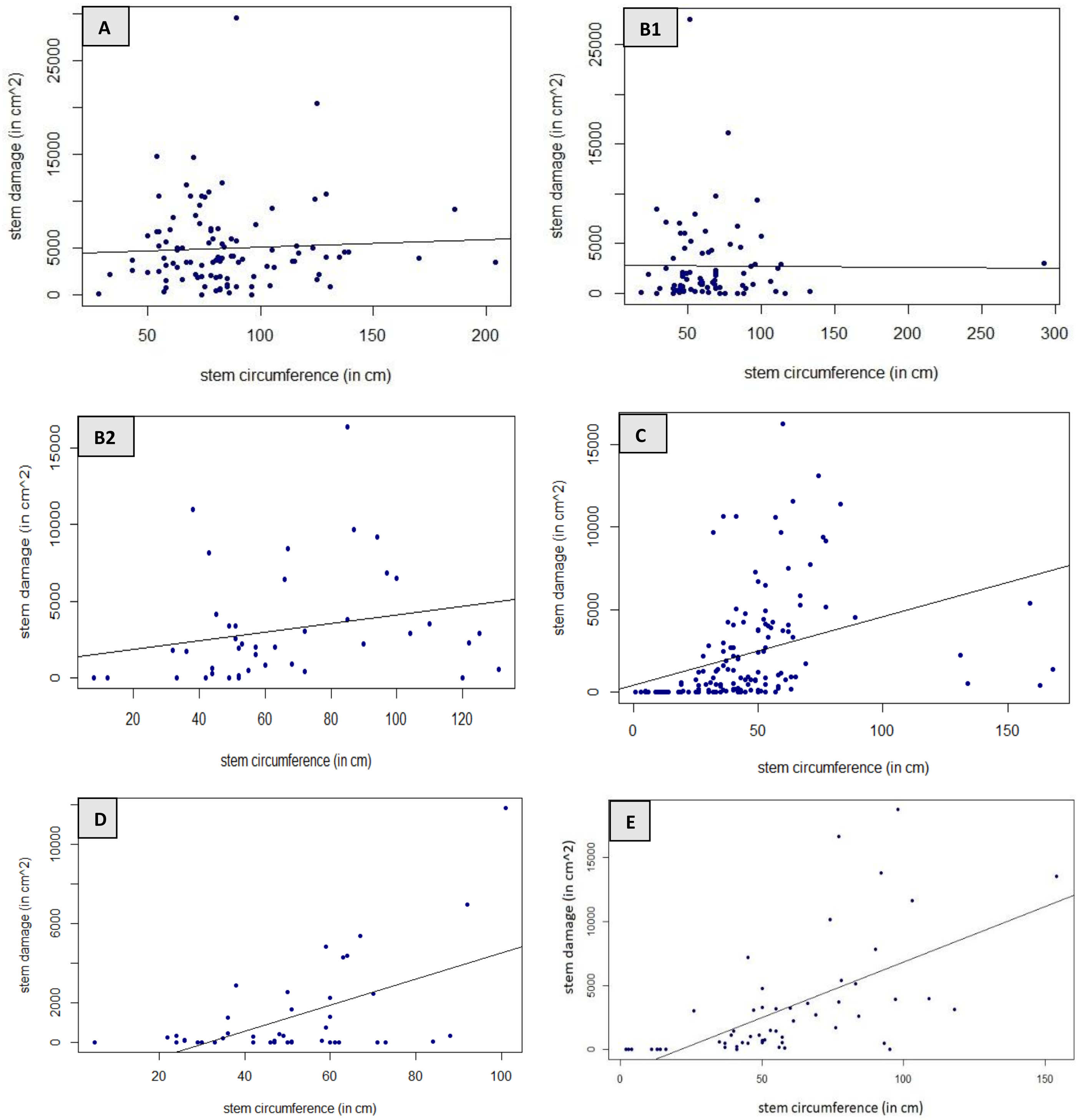

Figure 14: Relationship between stem circumference and area of bark damage in *P. curvata* trees from six sites in Mpumalanga, South Africa (*Site A*, *B1, B2, C, D* and *E*)

Discussion

Flowering and disturbance history

Flowering in *Subpopulation C* was lower in 2019, compared to 2018. This was possibly due to different sampling times (Figure B). Sampling in 2018 was done later in the flowering season when most trees had well-developed, noticeable inflorescences. Sampling in 2019 was done in the budding season. Both *Subpopulation A* and *C* were sampled a day after a disturbance (fire and hail respectively), yet *Subpopulation A* had far less buds and flowers. Flowering in *Subpopulation A* (which has been experiencing infrequent, intense fires) was already low prior to the hailstorm and became significantly lower afterwards. While infructescences appear to be able to survive some fires, more were removed by hail. Therefore, the sharp decline in *Subpopulation A'*s flowering is a result of compound disturbance.

Hailstorms are especially detrimental when storms occur early in a flowering season. Hail-stricken plants experience a reproductive loss as viable seeds can no longer be produced in that season. Furthermore, this means that the resources allocated to developing their flowers go to waste since pollination and seed development cannot occur. Much of the nutrients lost via dropped leaves and un-pollinated flowers form mulch around the trees. Despite still being in proximity to the *Protea* trees, the resorption of resources into the plant is delayed/inhibited by at least two factors. The first factor is the time it takes for the surface mulch to decay into available soil nutrients. Secondly, competition for nutrients remains a challenge. Huffman and Werner (2000) found that bush chopping in a pine savanna produced mulch which occupied the ground space for the entire season. Two growing seasons later, the mulch had decayed and the plants that began to cover the area were mainly grasses and herbaceous plants. In a post-flood study of *Breonadia salicina* (Vahl), flowering had still not recovered five years after the disturbance (Dowson, 2009).

The effect of hail on a subsequent growth flush can be analogous to that of pruning. Pruned *Protea* trees must allocate resources to three processes during a growth flush: the growth of existing leaves, the extension of preformed internodes and the initiation of new floral or vegetative appendages in the apical meristem. Although the timing of these processes can vary, *Protea* species generally produce floral primordia of the next flowering season before the fully differentiated vegetative parts for the upcoming season can start growing. For *Protea "Carnival' (P. compacta* R. Br. × *P. nerifolia R. Br.)* inflorescence primordia for the flowering season are initiated within 29 days of pruning. Over the next 21 days, differentiation occurs until primordia of floral appendages have been formed. This can occur during the extension of preformed shoots (i.e., shoots of the previous season) or during budbreak in the new season (Gerber *et al*., 2001b)

While the presence of inflorescence primordia can be microscopically detected in the tree soon after pruning, the time between initiation and development can vary between species. *Banksia coccinea* trees, for example, initiated most of their inflorescence primordia a month after peak flowering. The initials then took 12 months to fully develop. In *Banksia menziesii* R.Br., the earliest initiation of inflorescence primordia occurred 4 months after peak flowering; with most primordia being formed 5 months after flowering. Inflorescence primordia develop into inflorescences over the course of 8 months. Vegetative primordia can also be detected microscopically during this inflorescence development phase. These develop in the seasons preceding the flowering season (Fuss & Sedgely, 1990).

It becomes clear that the timing of a hailstorm and how it coincides with the initiation and development of inflorescences can impact the vegetative-reproductive balance in *P. curvata.* In the examples above, inflorescence initiation for the next year took place at least a month after peak flowering, whereas *P. curvata* had yet to reach peak flowering when there was a hailstorm. The inflorescences lost during the hailstorm translate into a loss of meristematic tissue and potential inflorescence initials. This is highlighted by the persistently low numbers in flowering a year after the hailstorm.

Another factor to consider is the growth form of *P. curvata* and where points of breakage occur during hailstorms. Proteaceae with axillary inflorescences – e.g., *Protea cordata* Thunb.*, P. decurrens* E. Phillips *and P. subulifolia (Knight*) Rouke – can develop inflorescences in the axils of old stems. Contrastingly, *P. curvata* trees have terminal inflorescences and can only increase their inflorescence number by increasing branching. Plants that are injured at the flowerhead will likely take a shorter time to recuperate flowering than those knocked off at their branches. In *Banksia* trees where cockatoos broke off only the inflorescence of the tree, resprouting was more likely (Witkowski *et al.,* 1994). *Banksia* trees that were harvested by cutting the stem showed less resprouting. This is because trees are more equipped to resprout when the available leaves and apical growth points are kept intact. The continued decline in flowering after a hailstorm is possibly a vegetative growth response to avail more branches for terminal inflorescences. Even in preformed shoots, secondary growth is likely required to gain enough conducting tissue and strength for supporting large inflorescences (Bond & Midgley, 1988; Le Maitre & Midgley, 1991). Resampling of the *P. curvata* subpopulation at *Site A* would help reveal if the decline in flowering noted in 2018 and 2019 continues further and what time is necessary for flowering to return to the levels noted in 2017. Cultivation of *P. curvata* for pruning experiments may help elucidate how resource allocation and resprouting change in response to defoliation and inflorescence removal.

The erratic occurrence of hailstorms in the region is likely due to climate change. The IPCC cites extreme precipitation as one of the global concerns regarding climate change (Seneviratne *et al,* 2012). On a provincial scale, our study shows that hailstorms are least frequent between March and August. On a continental scale, severe hailstorms (those with hailstones > 2.5 cm) had a slightly different pattern. They were least frequent between the months of May and October in south-eastern Africa (Cecil and Blankenship, 2011). Nevertheless, small and severe hailstorms were rare in early flowering season (May and June) at both scales.

The hailstorm of May 2018 proved to be ill-timed for *Protea curvata* and other flora in Barberton Nature Reserve. *Annona senegalensis* Pers. for example, flowers between April and June, and *Faurea rochetiana* (A. Rich.) Chiov. ex Pic.Serm flowers between March and September (Jones, 1994). Mpumalanga’s agriculture industry also suffered, with one farm reporting a loss of approximately 30 000 cartons worth of citrus (SAWS, 2020). Regionally, as hailstorms become more frequent and coincide with the flowering season (Figure 2), *P. curvata* and other winter-flowering trees will have less time to recover between disturbances.

Special attention should be given to hailstorms on *P. curvata* sites. Relying on the weather station records can lead to hailstorms being under reported. Some of the hailstorms present in news reports were absent in the weather station records. Some storms were recorded as being limited to a certain town but appeared to affect a larger area when reviewing news reports. Cecil and Blankenship (2012) noticed that their AMSR-E satellite data indicated a larger number of severe hailstorms than reported in the literature. They suggested that either some storms are not detected by weather stations or that satellite data may overestimate hailstorms by detecting a cluster of small hailstones which melt before reaching the surface. Therefore, it is important to use a variety of sources when examining hail history and to measure it directly where possible.

Based on Kruger International Airport Weather Station records, frosting is neither a common nor increasingly severe problem. Frost incidents all occurred in the winter months of 2010 and 2011 and have become less frequent. However, all frost events occurred early in the flowering season and potentially impacted *P. curvata* phenology. High temperatures and long photoperiods are required to initiate flowering in some members of Proteaceae (Fuss and Sedgely, 1990). Some *Protea* species exhibit both delayed development and less flowering overall after exposure to cold temperatures (Dupee & Goodwin, 1990). Others show accelerated flower development at low temperatures (Storey, 1985). In some species, prolonged exposure is a prerequisite to flowering, rather than an accelerant (Bastow *et al.*, 2004). Although the Kruger International Airport Weather Station did not record frost between 2018 and 2020, site managers recall at least one frost event in Barberton Nature Reserve within in those years (de Bruno Austin, pers. comm, 2020). The decline in *P. curvata* flowering was possibly intensified by these temperature extremes.

Damaged Shoots

Unlike Riley's (1953) study where hail was important in directionally "sheering" the tree canopy, there was no noticeable change in *Protea curvata's* general crown shape. Although it lost some leaves, *P. curvata* kept more of its foliage compared to other trees on the site which appeared bare after the hailstorm. These included *Vachellia, Dichrostachys* and *Searsia* species. The only comparably leafy trees on the site were *Protea curvata* and *Faurea* *rochetiana* – both of which are of the Proteaceae family. Therefore, the tough leathery leaves of *P. curvata* aid endurance during hail.

Laut and Elliott (1965) observed hail damage in a Canadian (Manitoba) forest. A year after a hailstorm, damaged jack pine trees still had lesions and had retained their dead leaves which had turned reddish orange. In our study, lesions were present both immediately after and a year after the hailstorm. However, none of the sampled trees had retained the previous year's leaves. Instead, *Site C* and *Site B2* had two trees which retained dead leaves. In some cases, the entire crown was brown. This is likely due to disease since there was no recent hail disturbance on those sites and no co-ordinated dropping of the leaves to suggest senescence.

The impact of fire and herbivory on leaves was more definitively assessed. Evidently, bite marks are the most common type of damage among resprouting *P. curvata* shoots – accounting for most of the damage in new shoots. Insect herbivory is a less significant pressure since insect holes only accounted for a small proportion of damage in the *P. curvata* trees. Herbivory poses a resource loss to trees. Resprouting tissue typically acquires carbon from current photosynthesis or carbon reserves within the plant. If herbivory is excessive, reserves can be depleted, and photosynthetic capacity reduced as leaves are lost to herbivory (Tiffin, 2000). Therefore, browsing should be limited immediately after fire. Trees will need time to restore carbon reserves in order for their resprouting ability to be effective in mitigating disturbance (Paula & Ojeda, 2011; Schierenbeck *et al.,* 1994 Iwasa & Kubo, 1997)

The livestock herded in the area over the years as well as the goats observed during the field study of *Subpopulation C* have likely browsed on *P. curvata*. Goats tend to browse on young shoots, twigs or leaves. As mixed-feeding opportunists, they can feed on a variety of woody or forb species and even include more grasses in their diet depending on seasonal variation and availability (Lu, 1988; Jordaan 1998; Basha & Nsahlai, 2012). Goats can thus provide a dual service by feeding on woody species that may be in competition with *Protea curvata* and by limiting grass dominance.

The herbivore community on *Site A* is more diverse. While, livestock is excluded, several species of antelope are present. The portion of Barberton Nature Reserve in which *P. curvata* occurs (i.e., Phase 2) should be exposed to mixed feeders as well as grazers. Where browsers are included, they should predominantly feed on shorter shrubs and bushes. This will place less pressure on *P. curvata* and more feeding pressure on competing trees and shrubs in the Barberton Nature Reserve.

Damaged Bark

While the tough leaves help *P. curvata* shoots endure hail, the tree shape makes its bark more prone to hail damage. *P. curvata* trees typically have a broad, open shape or vase shape. This exposes more points of contact on the branches in comparison to trees with a conical or pyramid shape.

Bark damage is present in all *P. curvata* subpopulations. A possible cause of bark damage is the sun. Direct sun on tree bark can cause cambium temperatures to be too high and lead to sunburn injury. Injury may include the formation of calluses that are susceptible to further damage or open wounds that are susceptible to pathogens (Roppolo & Miller 2001). Alternatively, sunscald injury may occur on cold days with low cloud cover where rapid temperature changes within cambium tissue can cause cell rupture (Huberman 1943; Roppolo and Miller 2001). The dead bark gradually sloughs off the tree, leaving it exposed to secondary damage (Litzow & Pellet, 1983). In sun-damaged trees, one would expect aspects of the tree that received direct solar radiation for longer durations or at the hottest time of the day to have significantly greater bark damage (Derby & Gates, 1966; Sheppard *et al*., 2016). *Site E* is the only site where this appeared to be the case. Damage facing west to northwest was the highest. This would be where trees experience brief, yet intense heat during sunset (Rohli & Vega, 2011; Bonan, 2016). Intensity appeared to be more impactful than the duration of exposure to the sun, since west to northwest damage was even higher than damage facing north to northeast.

Five of the six sites either had similar sized sections of bark damage facing different aspects or most of the damage spanning all around the tree. Furthermore, leaf wilting was not observed in aspects with prolonged or intense exposure to sunlight. This indicates that sunlight is not the sole cause of bark damage; other factors appear to contribute to *P. curvata* bark damage.

In *Subpopulation A* (where the area of bark damage in different aspects did not significantly differ and occurred indiscriminately in different directions), hail is a clear contributor. There was conspicuous, newly formed damage on the trees a day after a hailstorm [Figure 12]. The hail stones had left scattered marks, rather than uniform, unidirectional damage on each tree. In some trees, damage was superficial; whereas other trees had their inner bark exposed. Damage also occurred on upward facing stems.

Bark damage was most prominent in *Subpopulation A* (Table 1). *Subpopulation A* was made up of larger older trees, whereas other subpopulations included younger, small trees (Chapter 1). Therefore, bark damage in the subpopulation might be considered a result of a larger trees having more surface area that can potentially be damaged as well as being exposed to more damage over time by virtue of their age. The results testing correlation between stem circumference and area of bark damage partially support this hypothesis. Subpopulations made up of only large trees showed no correlation between stem circumference and damage. Subpopulations where size and age differences were present showed a correlation between stem circumference and area of bark damage. However, the frequency of bark damage (i.e., number of trees and number of stems per tree in which damage was present) in subpopulations with different demographics did not entirely support this hypothesis. With regard to *P. curvata* recruitment (and therefore abundance of small, young trees), sites could be ranked as follows: 1) *Site C*, 2) *Site E*, 3) *Site D,* 4) *Site B2,* 5) *Site B1,* 6) *Site A.* Regarding lowest percentage of damaged trees and lowest percentage of trees that incurred damage on all stems sites ranked as follows: 1) *Site C*, 2) *Site D*, 3) *Site B2*, 4) *Site E*, 5) *Site B1*, 6) *Site A.* This suggests there were contributing factors, besides tree age, that lead to bark damage being the highest in *Subpopulation A across all measures* (mean area of damage, percentage of trees with bark damage, percentage of trees that showed damage in all their stems). Both the intensity of fires and the short interval between the fire and hailstorm on the site likely compounded bark damage. While mild fires leave scorch marks on the outer bark, intense fires can burn into vascular tissue and leave indentations in tree bark (Smith & Sutherland, 2001). Woundwood tissue develops at the periphery of the indentation to initiate wound closure. The process of wound closure can take years. If interrupted (by the impact of hail in this case), wounded areas can expand. Similarly, bark killed during fire can take years to slough off the tree and the impact of hail may serve to accelerate peeling (Smith & Sutherland, 2001). Furthermore, new points of damage are created by hail, thus having a cumulative effect.

Another possibility is that intense fires exert physiological effects on stem tissue which may make tree bark more vulnerable to hail damage. Increased internal temperature during fires can alter sap surface tension and lead to cavitation in xylem tissue. This reduces the ability of the tree to conduct water to distal parts of the tree. As a result, bark may become more brittle and thus prone to chipping off upon hailstone impact (Tyree *et al.*, 1994; Rood *et al*., 2000; Sperry *et al.,* 2002 Michaletz *et al.,* 2012).

If the widespread bark damage observed in *Subpopulation A* is a case of cumulative damage on fire-induced wounds, then larger wounds will be prevalent in positions that match scorch height – i.e., near base of stems for surface fires or in upper parts of stem for ground and crown fires (Wagner, 1973; Alexander & Cruz, 2012). The wounds can also be most prominent on sides facing charred stumps or woody debris that served as a fuel source for recent fires (Smith & Sutherland, 2001). If it is a result of overall brittle bark due to poor xylem conductivity, one could expect the large wounds to be more diffuse across the surface area of the stem. Bark damage in *P. curvata* is likely occurring through the latter mechanism. Although the exact height at which large wounds occurred was not measured, we know that they occurred indiscriminately at different aspects (Figure 13). Even upward facing damage was not very different in size when compared to bark damage facing other directions. Further exploration of bark health, wound size, wound position and how they respond to different fire intensities might be useful in determining fire regimes that make *P. curvata* bark less vulnerable to hail damage.

In *Subpopulation C* damage was slightly less scattered. Starting points of fires are alternated every year. The landowner recalls fires being set alight from the bottom and dying out or being intentionally put out by him towards the steeper mountainside of the site (Meyer, pers. comm., 2019). Similarly, when sampling was done a day after a fire, the areas higher up the mountain were spared from fire. With parts of the site (particularly the bottom of the slope) being burnt more often, it is plausible that those trees would incur more damage. This may account for why damage extending all around the stem was significantly greater than damage facing northeast-east and west-northwest. In other words, where trees are damaged in multiple directions (from sunlight, insects, hail etc), fire can burn off the undamaged patches to form one wound facing multiple aspects or burn a separate area of bark, that may also become vulnerable to secondary damage. Fire-disturbed trees may also show differences in secondary growth and bark strength. In studies of oak and pine trees this was visible in cross sections of stems that either had thinner annual rings or discontinuous annual rings in the years after fire (Jordan, 1966). Varying fire application might be advantageous in creating heterogeneity within *Site C* (Smit *et al.,* 2016). Frequently burnt portions of the site (e.g., bottom of the slope) can have more space for seedling establishment and have a higher number of young trees present (van Langevelde *et al.*, 2003). Trees that experience fewer fires will have bark that is damaged less often, with more time to recover bark thickness and is likely to be more resilient to future disturbances (Catry *et al.,* 2012). *Subpopulation C* is therefore a prime example of fires as a linked disturbances – i.e., the direction of bark damage after a fire appears to be influenced by the frequency of previous disturbances. Disturbances in *Subpopulation C* affect one another, but unlike in *Subpopulation A*, they do not compound each other. This is evinced in *Subpopulation C* (along with *Subpopulation D*) having the fewest trees and percentage of stems with bark damage.

For most sites, the area of damaged bark positively correlated with tree size. The correlation is probably a factor of age since older trees are more likely to have experienced damage over several years. This is further supported by the fact that *Site C* and *Site E* had the strongest correlation between bark damage and tree size, and they were the only subpopulations to have juvenile trees below 1 m in height. In other words, the relationship between bark damage and tree size is more pronounced in subpopulations with a variety of juvenile and adult trees. *Site A* and *Site B1* were the only sites in which stem circumference and area of bark damage showed no correlation. *Site A* mostly had old trees, thus lacking the variety in tree sizes that might highlight any correlation between tree size and bark damage. We can infer that the large area of bark damage observed in *Subpopulation A* is ubiquitous in trees of varying size within the adult cohort (wherein height ranged from 2 m to 10 m).

A study of open savannas in Brazil found fire frequency to primarily affect nutrient availability in the soil and had a less pronounced effect on fire behaviour in comparison to climatic conditions. Climatic conditions at the time of the fire as well as conditions over a longer period before the onset of the fire had the most impact on the fire intensity, heat released and rate of spread (Pivello *et al.,* 2010). It may therefore be beneficial to burn *Site A* more frequently, but with treatments of varying climatic conditions.

Conclusion

The distinct management of *Site A* and *Site C* highlight how intense fire and hailstorms cause immediate and long-term damage to *P. curvata* stems and flowering. *Subpopulation A* was already at a disadvantage prior to hail damage. It had the most bark damage and lower flowering than *Subpopulation C*, which made it less resilient to subsequent disturbance. Reducing fuel load accumulation through shorter fire intervals is the first step. Although it is known that *Subpopulation C* was more frequently burnt than *Subpopulation A,* the temperature, season, or moisture content under which these burns took place is unknown.

Further experimentation is required to establish the nature of fires that are conducive to *P. curvata* survival. Treatments can be considered conducive to *P. curvata* if they elicit vegetation cover & composition that is more resemblant of Site C *than* Site A. As an initial strategy, a disc pasture metre can be calibrated to the grass load on *Site C* to monitor vegetation cover. Thus far, this study has fire frequency as the key influencer of damage to *P. curvata* stems and flowering. Shorter fire intervals with low intensity may be preferable to prevent the loss of flowerheads and inflorescence initial points. Intense fires that result in multiple *P. curvata* shoots snapping off would further delay a prolific flowering season as the trees would first have to increase branching in order to grow and support terminal inflorescences. Therefore, pre-fire bush clearing may be beneficial in reducing the fuel available for the next fire. It is recommended that fires should be applied at least every two years. This is based on *Site C* being burnt annually at alternate starting points, with fires being put out or dying out along the way.

Hail during flowering season reduces the number of flowerheads available for pollination and/or setting seed. Future studies may compare seed set between subpopulations to determine whether seed set per flowerhead is also reduced by hail damage and how resource allocation changes following loss of flowerheads.

A combination of direct and indirect hail observation is best for making accurate disturbance assessments on *Protea curvata.* It is also advisable that the Barberton Nature Reserve establishes its own record of frosting. Kruger International Airport's weather station is 27 km away from the nearest *P. curvata* site. The station is situated on top of the hill and the *P. curvata* site is in a valley, with a 60 m difference in elevation between the two. Therefore, distance between the sites as well as temperature inversions occurring at the top of the hill could lead to station records missing frost events experienced on *P. curvata* sites. Due to the varying effects of frost on different Proteaceae, it may be helpful to note whether frosting has a positive, negative, or negligible effect on *P. curvata* flowering.

Going forward, savanna and disturbance studies should be more cognisant of hailstorms. Despite being less common than fire or herbivory, severe hailstorms proved to be important in altering in flowering responses, resprouting and tree health. For tree species growing in hail zones, the approaches to fire management should include building resilience to hail damage.

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Chapter 3: Effects of fire management on woody cover and vegetation density in *Protea curvata* sites

Introduction

Savannas thrive on a dynamic equilibrium between grass and woody species. The dynamic equilibrium is important for maintaining species diversity and high carrying capacity (Stafford *et al.*, 2017). Dominance of woody species or grass typically arise due to site management, including the application of fire. Fire promotes a healthy herbaceous layer by eliminating dead sward material. This gives way to new growth, rather than the persistence of dry, dormant above-ground material. In the absence of fire, grasses have less vigour and can be easily outcompeted by aggressively growing woody species (Jordaan, 1998).

Overgrazing, infrequent fires and the exclusion of browsers can lead to an increase in bush encroachment (Ward, 2005). The rise in CO_2 concentrations in the atmosphere also encourages the growth of woody biomass (Kgope *et al.,* 2010; Bond & Midgley, 2012, De Klerk, 2004; Walker *et al.,* 2004). Bush encroachment can alter stand structure as bushes start to occur in higher densities and form thickets or as thorny bushes occur more frequently than other tree and grass species (Roques *et al.* 2001, Sheuyange *et al*., 2005). The site then becomes less accessible to herbivores, further increasing vulnerability to an overgrowth of undesired species. This is particularly concerning when managing protected sites with indigenous species, some of which may be rare or threatened (Lotter *et al*., 2014). The study focused on sites with the Mpumalanga endemic, *Protea curvata*. The sourveld in which the species is found consists of open to closed tree savanna, dense vegetation along drainage lines and open grassy escarpments (MTPA, 2012). Despite parts of the population occurring within a protected area, *P. curvata* remains threatened (Rebelo *et al*., 2020). *Protea curvata* recruitment was markedly low on sites with visibly more closed vegetation (Chapter 1). It is therefore necessary to develop management strategies that address tree abundance on these sites and the effects it may have on *P. curvata* regeneration.

Although several studies demonstrate the importance of preventing overgrazing and bush encroachment in savannas, the appropriate grass to tree ratio and management regime will vary slightly for different sites. The various mechanisms through which trees and grasses interact make it implausible to have a singular grass to tree ratio that ubiquitously denotes a somewhat stable savanna. Some models use resource partitioning to explain the abundance of grasses and trees. For example, niche separation by root depth can facilitate the coexistence of grasses and trees (Walter, 1971). Grass to tree ratio can then be predicted by soil and climatic factors. In wetter climates with soils of low water holding capacity, the deep root system of trees generally gives them competitive advantage over grasses. Where surface soils have low infiltration and the subsoil type is a water retaining clay, one could find fewer trees (Hesla, 1985; Johnson & Tothill, 1985; Knoop & Walker, 1985; Skarpe, 1990a). Other models are based on what can be described as balanced competition. The growth form with a competitive advantage (e.g., tree) initially spreads on the site. The strong competitor is then suppressed by competition with plants of the same growth form, making it unable to eliminate other growth forms (e.g., grasses). This maintains a coexistence of trees and grasses. In this case, grass to tree ratio could be predicted by factors limiting tree growth (Scholes & Archer, 1997). Other models place less emphasis on the stable coexistence of trees and grasses. Tree and grass abundance is instead determined by disturbances which intermittently change the environment to favour one growth form or the other (Sankaran, 2005).

Each of these models contains factors relevant to savannas but does not serve as a universal model of grass-tree interactions in savannas. Moreover, factors such as climate, competition for resources and disturbances can have disparate effects on trees and grasses or be dependent on the type of trees or grasses in question. This can be seen in how C3 and C4 grasses respond differently to seasonal conditions and CO_2 concentrations (Harshini *et al.,* 2014; Kurschner *et al*., 2008; Osborne 2008, Scheiter, *et al.,* 2012) or how trees that are resprouters respond differently to fire than obligate seeders (Benwell, 1998; Knox & Morrison, 2005; Marais *et al*., 2014). Plants of the same growth form may also respond differently based on the timing and spatial patterns of competition, climate or disturbance (Scholes & Archer, 1997). For example, a fire on a stand of older trees might be less damaging than a fire on a site predominantly comprising young or recently debarked trees that have less fire protection (Catry *et al.*, 2012; Pausas, 2015). This illustrates the need to explore the interaction between *P. curvata,* disturbances and tree cover. To better understand competitive pressure faced by *P. curvata*, the study also determined the species and demographics of trees constituting much of the woody cover on each site.

In terms of competitive pressure faced by *P. curvata*, the size of neighbouring trees is important to consider. For one, size impacts plant defense potential. Rohner and Ward (1997) found that younger acacias invested more into spinescence than older trees. More importantly, the size of a tree impacts its resource uptake and competitive edge.

The three classes of resources that plants typically compete for are light, nutrients and water. Trees can be more competitive for light by growing taller and having wider canopies that out-shade their competitors (Craine and Dybzinski, 2013). For nutrient acquisition, below-ground mass is vital. With regards to water use, a tree's competitive ability can be a result of both its physiology and developmental stage. In broadleaf species, for example, water-use efficiency (W_i) increases with tree age (Brienen *et al.,* 2017). In other words, one can expect larger trees to be more efficient at water uptake. In addition to the size of woody species on *P. curvata* sites, the distribution of woody species relative to *Protea curvata* was of interest. This has bearing on competition and seed dispersal (Ribbens *et al.*, 1994).

The interplay between tree species, density and demography has been a topic of ecological interest for decades. In what is now commonly referred to as the "Janzen-Connell hypothesis", the density of seedlings and their proximity to the parent plant is proposed to mediate the proliferation of conspecifics, thus being a key driver of species diversity (Janzen, 1970; Connell, 1971; Comita *et al.,* 2014). The hypothesis was tested in this study by determining how frequently *Protea curvata* trees were surrounded by their own species.

In addition to fire, bush encroachment can be prevented by browsers (Raats *et al.,* 1996; Nopp-Mayr *et al*., 2015). Accessibility to browsers may be limited by the height and defense mechanisms of woody species found on the sites. Physical defenses such as thorns can deter herbivores (Bazely & Myers, 1991; Gowda, 1996). Browsers can also be deterred by plants having toxic or unpalatable leaves (Furstenburg & van Hoven, 1994). Generally, when nutrient or water availability is low in African savannas, one tends to find slow-growing trees that are relatively high in tannins and alkaloids (Wrangham & Waterman, 1981; Owen-Smith 1993). *Protea curvata* grows within a savanna on serpentine soil (Protea Atlas, 2008), which is considered nutrient-poor (Alexander *et al*. 2007; Brooks, 1987). Thus, it is likely to find species in the area with defense or tolerance mechanisms such as heavy metal accumulation and toxic leaves. Evaluating the density of such species can help identify when additional interventions (e.g., bush clearing or chemical treatment) may be necessary for curtailing bush encroachment.

Methods

A census of five *P. curvata* subpopulations was undertaken in 2018. The census included a count of living trees and dead trees, as well as the height, stem circumference and flowering of each tree (Chapter 1). The subpopulations with the biggest differences in population demographics were *Subpopulation A* (occurring within Barberton Nature Reserve) and *Subpopulation C* (on privately owned land approximately 10 km from Barberton Nature Reserve). At the time of the census, *Site C* was noticeably more open. *Site A* was extensively covered in trees and bushes. Gaps between trees and shrubs were covered in moribund grass. This was coupled with absence of *P. curvata* seedlings on the site. Management of the two sites also differed. *Site A* is characterized by less frequent fires. The site is burnt approximately every four years (de Bruno Austin, pers. comm., 2018). Contrastingly, *Site C* experienced annual fires applied from 2001 to 2009 by local livestock herders. From 2009 onwards, the property owner attempted to limit these unauthorized fires, with little success. Thus, fires occurred irregularly between 2009 and 2018, albeit on a shorter cycle than four years (Meyer, pers. comm., 2019).

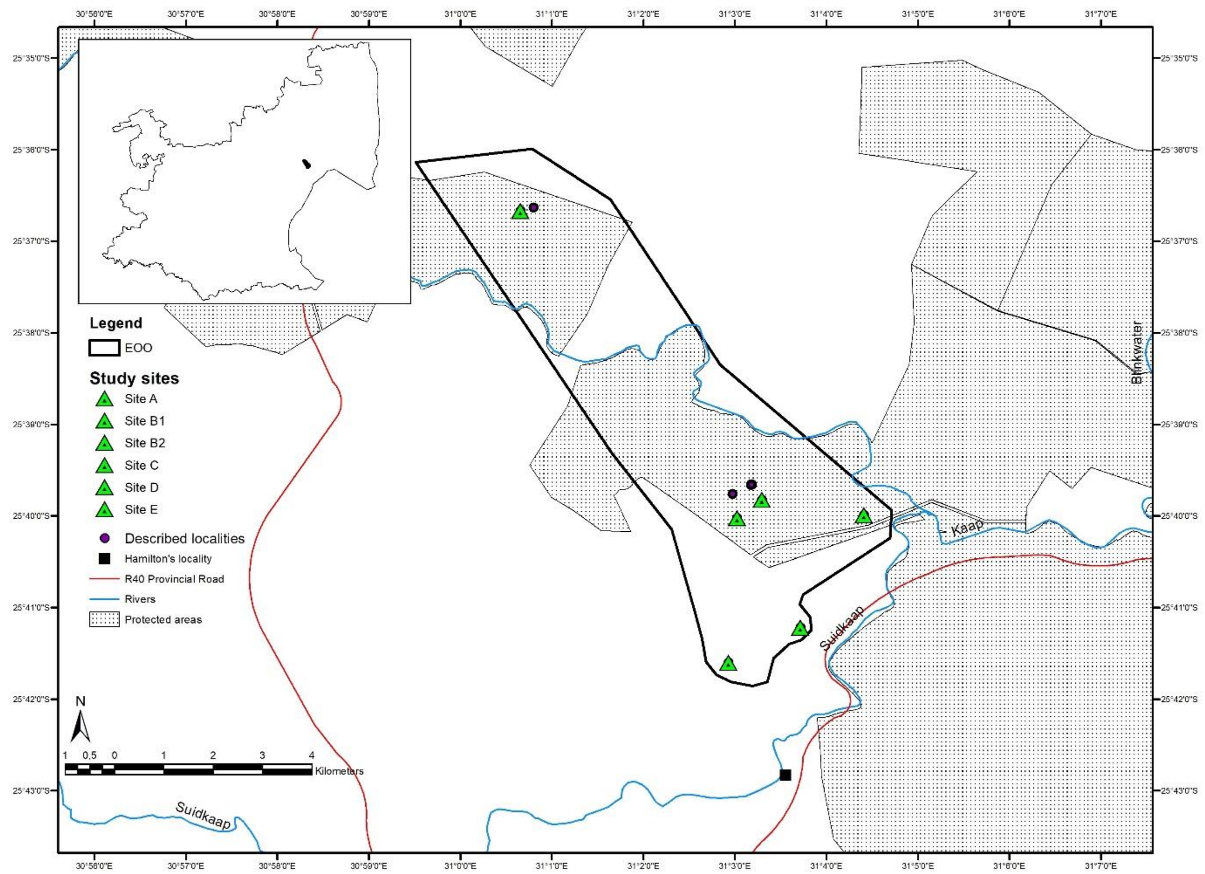

Figure 1: Map indicating Protea curvata localities in Barberton (Mpumalanga South Africa).

Protea curvata trees at *Site A* and *Site C* were censused again in 2019. Tree mortality was estimated for *Subpopulation A* (which was sampled in 2017, 2018 and 2019) and *Subpopulation C* (which was sampled in 2018 and 2019). Dead trees typically had brown, withered leaves. Estimation of trees that died a year before the census was done by counting dead trees that had old receptacles with bracts. Dead trees with old receptacles that had shed their bracts gave an estimate of tree death 2 years prior to the study. Trees with no remnants of old receptacles were used to estimated tree death that occurred at least 3 years before the census. Trees that had no remnants of old receptacles or leaves were considered to have died more than 3 years before the census. Living trees that were censused and died in following sample years were counted. Recruitment during sampling years was also recorded. These were used to estimate changes in subpopulation size for the two sites.

The Point Centred Quarter (PCQ) method was used to determine woody species composition for the two sites. The method was applied at *Site A* and *Site C* upon noting that they have the most distinct management and appeared to have different tree density. PCQ measurements can be used to calculate density, frequency, and cover (Cottam *et al.,* 1953; Morisita, 1954). The method can be carried out by sampling a single transect of at least 100 m or by sampling multiple transects (Mitchell, 2010). Sampling can be randomized by generating 15 two-digit numbers that are at least five units apart. These can used as distances along the transect where sampling will take place.

However, since the aim was to quantify bush encroachment relative to *P. curvata* rather than the entire site, a more specific approach was applied. Instead of sampling one transect, all the *P. curvata* trees that were tagged during initial transect sampling (Chapter 1) were revisited. Each *P. curvata* tree was used as the central sampling point. The area around a *P. curvata* tree was divided into quarters. (denoted N, S, E, W). [Figure 2]. For each tree, the following data were recorded:

(a) quarter in which the tree occurs

(b) distance from the *P. curvata* tree to the closest tree in each quarter (i.e., distance between the two trees' main trunks rounded off to the nearest cm)*

(c) stem circumference near the base.*****

(d) tree height to the nearest 0.1 m

(e) the species of the tree and/or samples of the plant for later identification

***** Distance and stem circumference are typically measured at breast height. The ambiguity of "breast height" leaves room for subjectivity. Brokaw and Thompson (2000) suggest using a standard height of 130 cm. However, it was more useful to measure closer to the base because many of the surrounding bushes were shorter than 130 cm. Additionally, the trunks of *P. curvata* and the other trees branch out into main-stems before 130 cm. It was more practical to measure both stem circumference and distance near the base. Nevertheless, it was important to be consistent about where the stem and distance were measured. For our study, measurements were taken at 30 cm from the base (C_{30}).

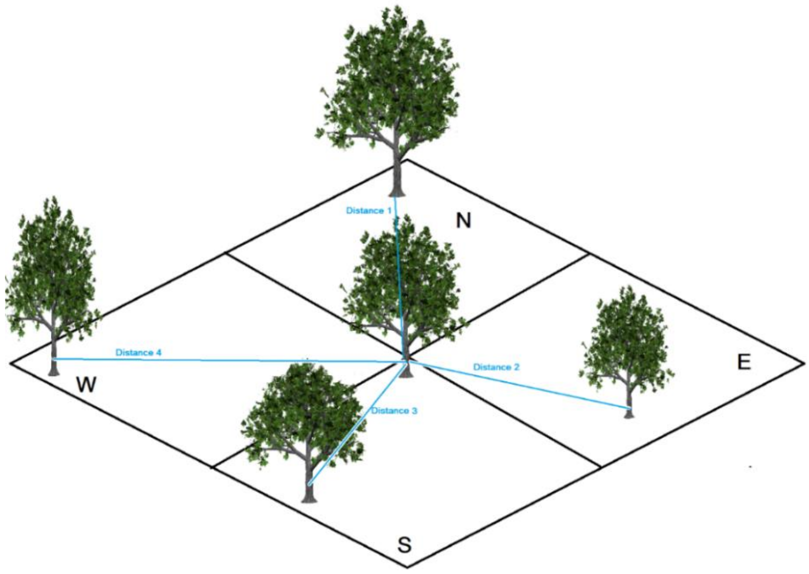

Figure 2: Application of Point Centred Quarter method using a *P. curvata* tree as the centre of a quarter.

Statistical Methods

Measurements from point quarter sampling were used to determine the importance value of each species. Equal weight is given to density, cover and frequency. This is done by representing each of the three values as percentages. Relative density represents the percentage of quarters containing each species. Relative frequency represents the percentage of sample points in which a species occupied at least one of the quarters. Relative density is the percentage cover that a species contributes to the total woody cover on the site. Empirical formulae established by Cottam *et al.* (1953) and Morisita (1954) were used to calculate each value. Firstly, following parameters were calculated:

Σd= sum of distances from all quarters of all sampling points

Qn = total number of quarters sampled

$\bar{r} = \Sigma d \div Qn$ = the mean distance

This was used to calculate the absolute density of all sampled trees as:

$$\lambda = \frac{1}{\bar{r}^2}$$ …………………. (Cottam *et al.,* 1953; Morisita, 1954)

The absolute density of a particular species (λ_k) is the expected number of trees belonging the species per hectare and was calculated as:

$$\lambda_k = \frac{\text{Quarters with species } k}{Qn} \times \lambda$$ ………………… (Mitchell, 2010)

The relative density of each species is the percentage density of a species in relation to the total density of all observed trees. The sum of all the observed species' relative densities should be equal to 100 (plus or minus a small round-off error).

Relative density can be calculated as follows:

$$\text{Relative density of species k} = \frac{\lambda_k}{\lambda} \times 100$$ ………………… (Mitchell, 2010)

Alternatively,

$$\text{Relative density of species k} = \frac{\text{Quarters with species } k}{Qn} \times 100$$

……………… (Cottam *et al.,* 1953)

Both equations were applied to select the output with the smallest round-off error.

The cover or an individual tree is given by its basal area per hectare. The stem circumference measurements of each tree were used to calculate its basal area as follows:

$$\text{Basa Area} = \frac{\text{circumference}^2}{4\pi}$$

In trees with multiple trunks, the area of each trunk was calculated separately then added together. Individuals were then sorted by species to get the mean basal area and total basal area for each species. Absolute cover (in $m^2.ha^{-1}$) for each species was calculated as follows:

$$\text{Absolute cover} = \text{Average basal area of tree from species (in cm}^2\text{)}$$

$$\text{x}$$

$$\text{Absolute density of species (in } \frac{\text{trees}}{\text{ha}}\text{) x } \frac{1\text{ m}^2}{10\,000\text{ cm}^2}$$

………………… (Mitchell, 2010)

Total woody cover for each site was calculated as the sum of absolute cover from all species, i.e., $\text{Site woody cover} = \sum \text{Absolute cover of species}$

Relative cover was calculated as follows:

$$\text{Relative cover}_k = \frac{\text{Absolute cover of species } k}{\text{Total absolute cover of all species}} \text{ x } 100$$

Absolute frequency is the percentage of sampling points (not quarters) at which each species occurs. Absolute frequency and relative frequency were calculated as:

$$\text{Absolute frequency}_K = \frac{\text{No.of sample points with species k}}{\text{Total number of sample points}} \text{ x } 100$$

$$\text{Relative frequency}_K = \frac{\text{Absolute frequency of species k}}{\text{Total frequency of all species}} \text{ x } 100$$

…………………. (Mitchell, 2010)

The importance value of each species was calculated as:

$$\text{Importance value} = \text{Relative density} + \text{Relative cover} + \text{Relative frequency}$$

…………………. (Mitchell, 2010)

Results from the PCQ sampling were also analysed in the context of censuses conducted on the sites. This allowed comparisons of tree demography relative to site composition.

Results

The mean distance between woody species was significantly higher at *Site C* than at *Site A* ($t = -15.3060$, $p<0.001$, d.f. = 678, meanA= 0.61 m, meanC= 1.86 m). For species shared by the two sites, absolute density tended to be much higher on *Site A* than *Site C*. In total, the absolute density of woody species found on *Site A* far exceeded the density of woody species on *Site C* ($\bar{x}_A$= 2671 trees.ha^{-1}, $\bar{x}_C$= 288 trees.ha^{-1}; $t= 3.7089$, $p<0.001$, d.f. = 26.35,). The total woody cover on *Site A* was higher than woody cover on *Site C* (*A*= 22.21 m^2.ha^{-1}; *C*= 5.00 m^2.ha^{-1})

The relative density, cover and frequency determined the importance value of each species. On *Site A* the five species with the highest importance value were *Searsia pentheri* (Zahlbr.) Moffett, *Protea curvata*, *Vachellia davyi* (N.E. Br.) Kyal. & Boatwr., *Berchemia zeyheri* (Sond.) Grubov and *Searsia grandidens* (Harv. ex Engl.) Moffett [Table 1]. The five most important species on *Site C* were *Vachellia davyi*, *Protea curvata, Searsia grandidens, Gymnosporia heterophylla* (Eckl. & Zeyh.) Loes. *and Clutia pulchella* (Table 2). These five most important species were not exclusive to one site. *Site A* and *Site C* showed an overlap of ~75% in species composition The species sampled only on *Site A* were *Combretum hereroense* Schinz*, Combretum zeyheri* Sond*., Bauhina galpinii* N.E. Br., *Heteropyxis natalensis* Harv., *Lantana camara L. and Pearsonia sessilifolia* (Harv.) Dümmer. The species sampled on *Site C* and not *Site A* were *Dichrostachys cinerea* (L.) Wight & Arn., *Ximenia caffra* Sond*., Grewia flavescens* Juss. var. *flavescens, Ozoroa sp. nov.* and *Cussonia spicata* Thunb.

Searsia pentheri was the most dominant species on *Site A*. This was largely due to the high abundance, high relative density and moderate cover of the species. For both sites, *P. curvata* had the second highest importance value, despite occurring relatively infrequently and in low density. On *Site C*, *P. curvata* trees were more frequent, but for both sites, the importance of *P. curvata* was mostly due its high relative cover. During the initial census of *Site C*, juvenile trees were recorded in the subpopulation. However, during quarter sampling on *Site C,* none of the neighbouring *P. curvata* trees were juveniles (i.e., all quarters with *P. curvata* had high basal area). From examining the census data in conjunction with the data from PCQ sampling, only 2 out of 15 juvenile trees had another *Protea* tree as their nearest neighbour.

The height of the five most dominant species was evaluated. Both sites had a mixture of species that grow as tall trees and species that grow as shrubs. At the time of the study, most of the individuals were well below the height of maturity for their respective species. All the dominant species, with the exception of *Searsia grandidens*, had the potential to grow above the fire zone and the height of most browsers on each site. When dominant species were compared, *Site A* tended to have taller trees (Table 3). *Vachellia davyi* and *Gymnosporia heterophylla* were the only dominant species found to be taller on *Site C* than on *Site A.* On *Site C, Vachellia dayvi* showed the highest recruitment and had several trees above 2 m. *P. curvata* had new recruits on *Site C*, although individuals of the 2–3 m cohort were the most abundant. *Searsia grandidens* and *Clutia pulchella* surrounding *P. curvata* were exclusively below 1 m in height and these exceeded the number of *P. curvata* recruits. Among the dominant species on *Site A, Searsia grandidens* showed the highest recruitment. *P. curvata* was the only species that lacked individuals below 1 m – i.e., showed no recruitment (Figure 3). Both sites had a mixture of palatable and unpalatable species in their top five (Table 4).

For plants surrounding *P. curvata* trees on *Site A*, there was a statistically significant relationship between the basal area of each plant and the species it belonged to (Quasi-Poisson GLM: $\chi2 = 90158$; d.f. = 25; $p<0.0001$). Similarly, the basal area and species of plants on *Site C* had a statistically significant relationship (Quasi-Poisson GLM: $\chi2 = 107133$; d.f. = 23; $p = 0.03844$). A model was fitted to determine the impact of surrounding tree species and size on *P. curvata* size. On both sites, the interaction between size and species of neighbouring trees had no significant impact on *P. curvata* size (*Site A:* GLM: $\chi2 = 26967$; d.f. = 23; $p = 0.7582$; *Site C:* GLM: $\chi2 = 148897$; d.f. = 19; $p = 1.00$). On *Site C*, *P. curvata* basal area was higher in sampling points that had *D. cinerea* as one or more of its neighbouring trees. This effect is likely negligible since, overall, the effect of neighbouring tree species in explaining *P. curvata* size was not significant. The basal area of each plant also had no explanatory effect on *P. curvata* size. Tables for analysis of deviance and models are given in the appendix.

Based on the censuses conducted on the two sites, average net decline was 16.3 trees per year in *Subpopulation A* and 8.9 trees per year in *Subpopulation C*. Due to *Subpopulation A* generally having more trees, this represented a 3% annual loss for *Subpopulation A* and 2% for *Subpopulation C.* Seedlings on *Subpopulation C* were able to survive fires between sampling periods.

Table 1: Ranking of species found on *Site A* based on importance value from point centered quarter sampling.

	Species	**Absolute density (trees/ha)**	**Relative density**	**Relative cover**	**Relative frequency**	**Importance Value**
1	*Searsia pentheri* (Zahlbr.) Moffett	480,9	18,00	11,98	16,00	45,98
2	*Protea curvata* N.E. Br.	17,81	0,67	38,81	0,89	40,37
3	*Vachellia davyi* (N.E. Br.) Kyal. & Boatwr.	409,7	15,33	6,65	15,56	37,54
4	*Berchemia zeyheri* (Sond.) Grubov	160,3	6,00	23,27	7,11	36,38
5	*Searsia grandidens* (Harv. ex Engl.) Moffett	329,5	12,33	0,58	11,56	24,47
6	*Zanthoxylum capense* (Thunb.) harv.	106,9	4,00	6,81	3,56	14,37
7	*Senegalia ataxacantha* (DC.) Kyal. & Boatwr.	204,8	7,67	0,14	5,33	13,14
8	*Pearsonia sessilifolia* (Harv.) Dümmer	151,4	5,67	0,07	5,33	11,07
9	*Hippobromus pauciflorus* (L.f.) Radlk	98,0	3,67	0,09	4,44	8,20
10	*Annona senegalensis* Pers.	80,1	3,00	2,03	3,11	8,14
11	*Peltophorum africanum* Sond.	17,8	0,67	5,30	0,89	6,85
12	*Bauhinia galpinii* N.E. Br.	89,1	3,33	0,15	3,11	6,60
13	*Faurea rochetiana* (A. Rich.) Chiov. ex Pic. Serm.	62,3	2,33	1,21	2,67	6,21
14	*Berkheya nivea* N.E. Br.	71,2	2,67	0,02	3,11	5,80
15	*Olea europea* L. subsp. *africana*	62,3	2,33	0,02	2,67	5,02
16	*Euclea natalensis* A. DC.	53,4	2,00	0,93	1,78	4,71
17	*Combretum zeyheri* Sond.	44,5	1,67	0,59	2,22	4,48
18	*Heteropyxis natalensis* Harv.	44,5	1,67	0,15	2,22	4,04
19	*Gymnosporia heterophylla* (Eckl. & Zeyh.) Loes.	35,6	1,33	0,01	1,78	3,12
20	*Combretum molle* (Klotzsch) Engl. & Diels	35,6	1,33	0,38	1,33	3,05
21	*Combretum hereroense* Schinz	26,7	1,00	0,66	1,33	2,99
22	*Clutia pulchella* L.	35,6	1,33	0,00	1,33	2,67
23	*Melhania prostrata* Burch.	26,7	1,00	0,06	1,33	2,39
24	*Lantana camara* L.	17,8	0,67	0,08	0,89	1,63
25	*Maytenus undata* (Thunb.) Blakelock	8,9	0,33	0,00	0,44	0,78
		Total:2672	100	100	100	300

Table 2: Ranking of species found on *Site C* based on importance value from point centered quarter sampling.

	Species	**Absolute density (trees/ha)**	**Relative density**	**Relative cover**	**Relative frequency**	**Importance Value**
1	*Vachellia davyi* (N.E. Br.) Kyal. & Boatwr.	133,30	46,1538	58,2325	31,80534	136,1917
2	*Protea curvata* N. E.Br.	13,89	4,8077	27,6368	6,590296	39,03483
3	*Searsia grandidens* (Harv. ex Engl.) Moffett	36,10	12,5000	0,1488	13,75366	26,4025
4	*Gymnosporia heterophylla* (Eckl. & Zeyh.) Loes.	21,66	7,5000	1,9149	8,596038	18,01095
5	*Clutia pulchella* L.	17,22	5,9615	0,3056	7,449899	13,717
6	*Annona senegalensis* Pers.	10,55	3,6538	3,6312	5,157623	12,44268
7	*Olea europaea* subsp. *africana*	5,00	1,7308	5,0526	2,578811	9,362201
8	*Searsia pentheri* (Zahlbr.) Moffett	9,44	3,2692	0,9519	4,584553	8,805645
9	*Senegalia ataxacantha* (DC.) Kyal. & Boatwr.	7,78	2,6923	0,2780	3,72495	6,695291
10	*Faurea saligna* Harv.	5,00	1,7308	0,4288	2,292277	4,451825
11	*Zanthoxylum capense* (Thunb.) harv.	3,33	1,1538	0,6196	1,719208	3,492655
12	*Berkheya nivea* N.E. Br.	3,89	1,3462	0,0012	1,719208	3,066557
13	*Euclea natalensis* A. DC.	3,33	1,1538	0,0298	1,432673	2,616311
14	*Ximenia caffra* Sond.	2,78	0,9615	0,0587	1,432673	2,452904
15	*Dichrostachys cinerea* (L.) Wight & Arn.	2,22	0,7692	0,6016	0,859604	2,230456
16	*Peltophorum africanum* Sond.	2,22	0,7692	0,0407	1,146138	1,956109
17	*Berchemia zeyheri* (Sond.) Grubov	2,22	0,7692	0,0140	1,146138	1,929327
18	*Ozoroa sp. nov.*	2,22	0,7692	0,0071	1,146138	1,922427
19	*Melhania prostrata* Burch.	2,22	0,7692	0,0013	1,146138	1,916713
20	*Maytenus undata* (Thunb.) Blakelock	1,11	0,3846	0,0001	0,573069	0,957772
21	*Grewia flavescens* var. *flavescens*	1,11	0,3846	0,0227	0,286535	0,693832
22	*Hippobromus pauciflorus* (L.f.) Radlk	1,11	0,3846	0,0002	0,286535	0,671307
23	*Cussonia spicata* Thunb.	0,56	0,1923	0,0218	0,286535	0,500652
24	*Combretum molle* (Klotzsch) Engl. & Diels	1,03 x10^{-5}	0,1923	1,46 x10^{-7}	0,286535	0,286537
		Total: 288	100	100	100	300

Table 3: Comparison of tree height for dominant species on *Site A* and *Site C*

Species	Max Height	Site A: Mean height (m)	Site C: Mean height (m)	p-value	t-value	D.f.
Protea curvata (PCQ)	10.0	5.1	2.4	0.1612	2.7893	1.3722
Protea curvata (census)[3]	10.0	5.5	3.0	<0.001***	10.853	143.49
Vachelia davyi [1]	6.0	0.92	1.03	0.0133*	-1.5165	99.936
Gymnosporia heterophylla[1]	3.0	0.32	0.62	<0.001*	5.6163	40.999
Berchemia zeyheri [2]	10.0	1.09	0.59	0.0240*	2.4729	17.368
Searsia pentheri [1]	6.0	1.11	0.75	0.0062 **	2.8510	54.287
Searsia grandidens[1]	2.0	0.68	0.67	0.8457	0.1957	44.552
Clutia pulchella[1]	6.0	0.46	0.42	0.6910	0.4299	3.7268

Results from Welch's two-tailed t-test in which trees of the same rank compared. Mean obtained from height of trees measured on each site. Maximum height based on typical height of adult trees in each species as per records in literature (Schmidt *et al.,* 2007 [1]; Palgrave, 2005[2]; Mabuza, 2017[3]).” *Protea curvata* (PCQ)” Results from trees found in quarters during PCQ sampling where n= 2 *P. curvata* trees for *Site A* and n=25 *P. curvata* trees for *Site C.*

Table 4: Traits related to feeding suitability of dominant woody species on *P. curvata* sites.

Site A					Site C				
Species	**phenology**	**Leaves**	**Spinescent**	**Palatable(P) or Unpalatable (U)**	**Species**	**phenology**	**Leaves**	**Spinescent**	**Palatable(P) or Unpalatable (U)**
Searsia pentheri[1]	Semi-deciduous	macrophyllus	NO	U	*Vachellia davyi*[2,3]	Deciduous	microphyllus	YES	P
Protea curvata	Evergreen	macrophyllus	NO	P	*Protea curvata*	Evergreen	macrophyllus	NO	P
Vachellia dayvi[2,3]	Deciduous	microphyllus	YES	P	*Searsia grandidens*[4,5]	Deciduous	macrophyllus	NO	U
Berchemia zeyheri[6]	Semi-deciduous	macrophyllus	NO	P	*Gymnospria heterophylla*[7,8]	Evergreen	macrophyllus	YES	P
Searsia grandidens[4,5]	Deciduous	macrophyllus	NO	U	*Clutia pulchella*[8]	Deciduous	macrophyllus	NO	P

(López, A. and Sánchez, 2001[1]; Gordijn *et al*., 2012[2]; Setshogo and Venter, 2003[3]; Mkhize *et al*., 2018[4] Hutchings *et al*., 1996[5]; van Rooyen *et al.* 1986[6]; Da Silva *et al*., 2011[7]; Chinomona *et al*., 2018[8])

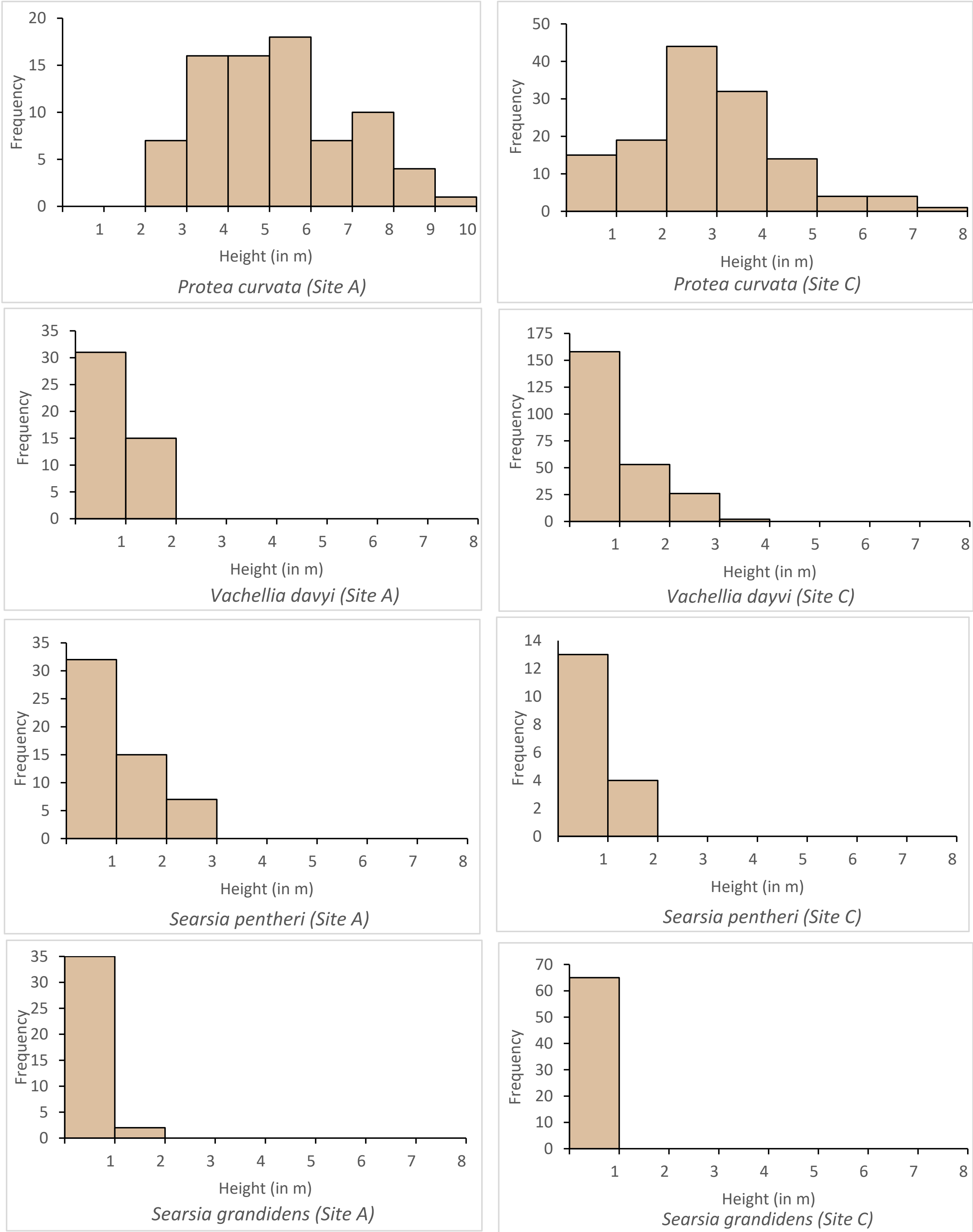

Figure 3.1: Height class distribution of dominant woody species on *Site A* (Barberton Nature Reserve, Mpumalanga) and *Site C* (Clarendon Vale, Mpumalanga)

Height class distribution (HCD) of *P. curvata* obtained from population sampling, while HCD of the remaining species was based on PCQ sampling.

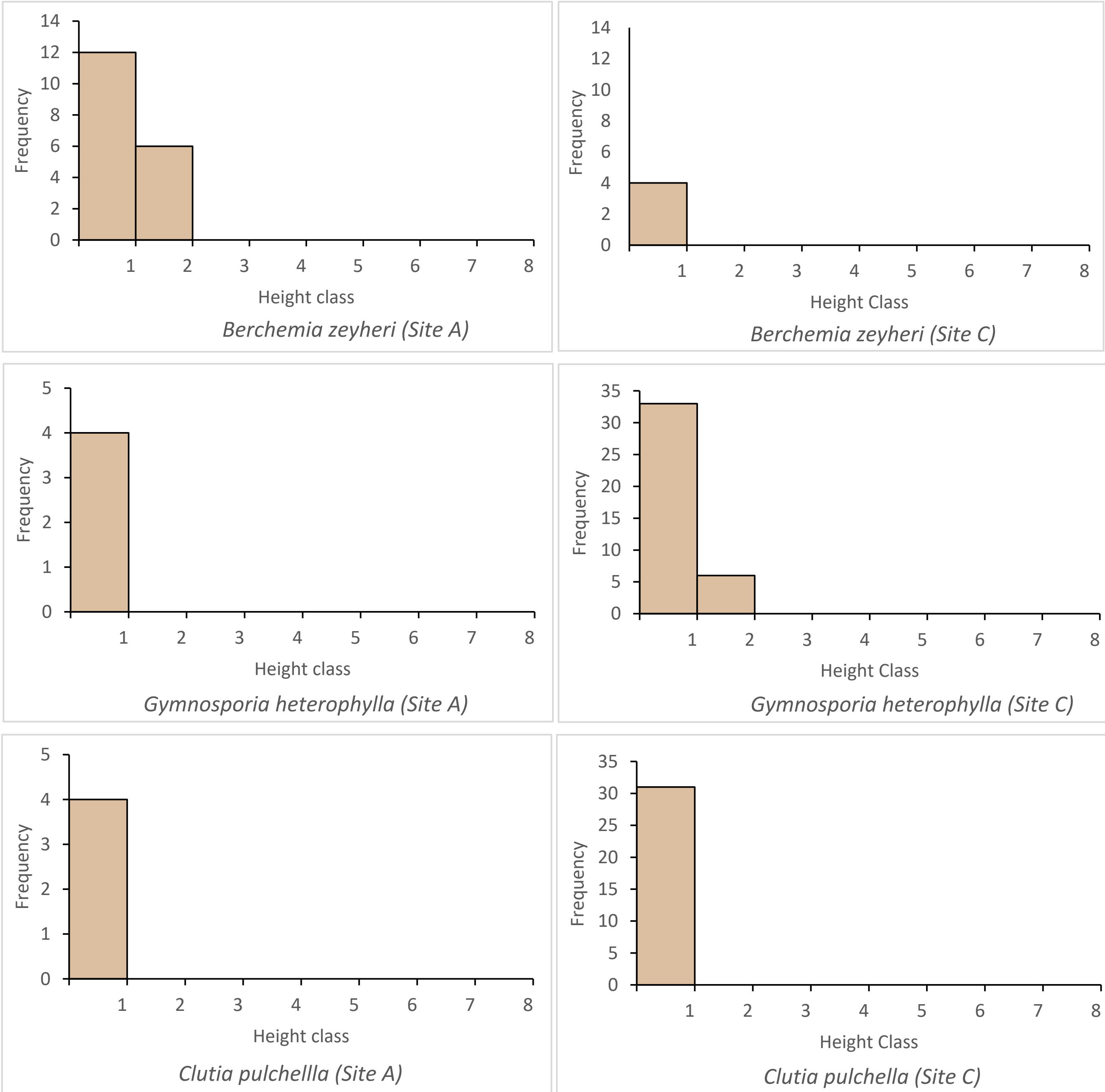

Figure 3.2: Height class distribution of dominant woody species on *Site A* (Barberton Nature Reserve, Mpumalanga) and *Site C* (Clarendon Vale, Mpumalanga)

Height class distribution (HCD) of *P. curvata* obtained from population sampling, while HCD of the remaining species was based on PCQ sampling.

Discussion

Site A had high woody cover and high woody plant density. This is indicative of an unhealthy herbaceous layer that cannot mediate the ingression of woody plants (Trollope, 1980; Skarpe, 1990b; Hoffman & Ashwell, 2001). Contrastingly, *Site C* was characterized by fewer trees and low basal cover. Based on species composition and structure, both sites can be described as mixed bushveld and thornveld. There were trees taller than 6 m, shrubs below 1 m and an abundance of trees with thorns at both sites. Bush encroachment in southern African savannas typically involves the dominance of trees of the *Vachellia, Senegalia*, *Dichrostachys*, *Termanalia*, *Rhigozum* or *Tarchonanthus* genera (Kraaij and Ward, 2006). Both sites had were dominated by *Vachellia davyi,* while *Dichrostachys cinerea* occurred at low density on *Site C.*

The dominance of thorny species on both sites was quite striking. Out of the five most densely occurring species, two were thorny. On *Site A* these were small *Senegalia ataxacantha* bushes (all below 1 m) and *Vachellia davyi* trees ranging between 0.2 and 1.8 m. On *Site C*, *Gymnosporia heterophylla* and *Vachellia davyi* were among the main contributors to tree density*,* with *V. davyi* covering the largest basal area of all species. Thornbushes are detrimental in high densities since they can form thickets that exclude grazers and most browsers from the site (Stafford *et al.,* 2017). For browsers that can access parts of the site, browsing is still limited by tree spinescence. For example, *Senegalia* and *Vachellia* leaves make up a great portion of the kudu diet (Owen-Smith, 1985; Cooper & Owen-Smith, 1986). Even so, bite size and bite rate are reduced by thorns. The kudus also tend to preferentially feed on other palatable trees if the available thornbushes have very small leaves and a high number of prickles (Cooper & Owen-Smith, 1986). Through minimal herbivory, high water use and reduced competitive pressure from a healthy grass layer, thornbushes eventually lower the carrying capacity of a site (Trollope, 1984; Stafford *et al.*, 2017). It is therefore preferable to have non-thorny plants constituting a greater proportion of the woody species cover on *Site A* and *Site C.*

To explore the idea of thornbush control via herbivory, consideration was given to the availability of browsers that feed on microphyllus, thorny species. For *Site A*, these potentially include giraffe and kudu. The average *Vachellia* tree on *Site A* was 0.9 m tall and all *Vachellia* trees on the site were shorter than 2 m. This makes giraffe the least viable option since they primarily feed on *Vachellia/Senegalia* foliage at heights between 2 and 4 m (Pellew, 1983; Birkett, 2002). *Site A* is mountainous with some slopes exceeding 40°, making it inaccessible to giraffe (Bond & Loffell, 2001). Therefore, even a later stage when trees site have grown taller, giraffe are unlikely to be effective browsers on the site. In a study of giraffe, elephant and rhino in a *Vachellia* and *Senegalia* woodland, the impact of these browsers varied with tree height. Annual growth was low for trees shorter than 1 m and trees of the 3 – 5 m class compared to any other class. Growth of trees between 1 and 3 m was significantly lower on sites where the browsers were present, compared to sites where they were excluded. Although growth was limited by feeding for most height classes, a high percentage of trees below 1 m were damaged or killed by black rhinos and elephants (Birkett, 2002). Therefore, despite most *Vachellia* trees on *Site A* being below 1 m, rhinos would not be an advisable introduction. Unlike feeding behaviour which can be selective, trampling can happen to any tree species obstructing a rhino's path. Consequently, desirable tree species (including *P. curvata*) would be vulnerable to damage.

Antelope like impala and kudu appear to be a better option than rhinos. Most microphyllus thornbushes on *Site A* were below 2 m. This makes them appropriate for kudu which typically feed on microphyllus thornbushes below 2 m, and for impala which typically feed on bushes below 1.5 m (Dayton, 1978; Du Toit, 1990). On Site C, *Vachellia davyi* included an abundance of trees below 2 m as well as trees taller than 2 m. Trees taller than 2 m could present a management challenge. In addition to contributing to excessive woody plant density, the reproductively mature trees can recruit seedlings that compete with *P. curvata* seedlings. Additionally, their height allows them to escape mild fires and face low browsing pressure since there are no large herbivores enclosed within *Site C*. Therefore, it will be important to maintain a high fire frequency so that fewer *Vachellia* trees reach the heights at which they can be difficult to manage.

Woody cover in savannas is primarily limited by water availability (Higgins *et al.,* 2007, Stevens *et al.,* 2017). Grasslands that occur adjacent to savannas help illustrate other important determinants of woody cover. Grassland and savanna sites may both be dominated by C4 grasses and have similar fire frequencies, but trees from the savanna site are unable to survive on the adjacent grasslands (Acocks, 1953; Schutz *et al*., 2009). To understand this phenomenon, one study looked at the growth of six species of African *Acacias* (*Vachellia and Senegalia*) on adjacent grassland and savanna sites (Wakeling *et al.,* 2012). Each site consisted of 10 plots of seedlings along an altitudinal gradient. With soil nutrients, water and competitive pressure controlled for, temperature proved to be the most important determinant of woody cover. The upland grassland site was generally colder than the low-lying savanna and showed slower woody seedling growth. The plots in the highest portion of the grassland were prone to frost that led to tree mortality or top-kill. These limitations on tree growth meant that seedlings could not reach maturity in time to escape the fire zone. Notably, day length was the main phenological cue for the *Acacias* at all altitudes. Shoot elongation was highest between November and January, then declined in February, despite temperatures and water availability still being high (Wakeling *et al.,* 2012). With this in mind, bush clearing targeted at *Acacias* seedlings or juveniles on *Protea* sites should be applied before November. This will ensure that unwanted bushes are not tall enough to escape the fires that follow. Effects of frost on seedling survival can be explored by recording frost/temperature and woody seedlings at the upper and lower end of the slope during cold fronts.

Woody species without thorns also contribute to tree density. The most dominant species on *Site A* was *Searsia pentheri*. It had both high relative density and cover on the site. The absolute density of *S. pentheri* was more than 10 times higher on *Site A* than on *Site C*. *Searsia pentheri* and *Searsia grandidens* also had the highest frequency of seedlings on *Site A*, while *P. curvata* had none. Therefore, where there are opportunities for seedlings to establish on *Site A, Searsia* seedlings appear to outperform *P. curvata*. This could be due to germination conditions favouring *Searsia* seeds or factors impacting seedling survival. The demographic of *Searsia* trees currently dominating *Site A* would be accessible to antelope browsers because of their height and lack of spinescence. However, *Searsia* species are typically unpalatable due to the presence of inositol and other toxic compounds in their leaves (Hutchings *et al*., 1996). An introduction of more herbivores on *Site A* would therefore be ineffective in curtailing the dominance of *S. pentheri*. On average, most *S. pentheri* trees on the site are short enough to be impacted by mild fires (Table 3), making fire a more viable option for suppressing the growth of *S. pentheri* trees. In a vegetation study of Ithala Game Reserve, species composition shifted towards an abundance of species that had a low appeal to browsers and relatively high recruitment compared to other trees (Wiseman *et al.,* 2004). It is predicted that in the absence of fire, *Site A* could shift towards being a *Searsia* dominated woodland. This is based on *S. pentheri* and *S. grandidens'* high importance value, high recruitment, and presumably low browsing pressure.

The palatability of *Protea curvata* may also play a role in feeding preferences of herbivores. In this study *P. curvata* was considered palatable (Table 4) based on observations of herbivory in resprouting shoots (Chapter 2). However, it is possible that these shoots were browsed on due to younger leaves lacking the protective waxy layer that older leaves have. Feeding preferences based on leaf age have been found in another *Protea* (Wright & Gilmore). Young leaves of *Protea magnifica* Andrews and *Protea laurifolia* Thunb. had higher nitrogen content than older leaves. In spite of this, the insect herbivores were deterred by trichomes on the young leaves. Older leaves had more tannins, but this was insufficient for deterring herbivores that had digestive adaptations to deal with tannins. In future studies, it may be beneficial to investigate age-related differences in the leaves as well as their efficiency in deterring herbivores.

Lantana camara, which was found on *Site A,* is one of the most invasive alien species in southern Africa (Lowe *et al.,* 2000). The species has been previously recorded on the Reserve. Various action projects have been initiated to reduce and eventually eliminate *L. camara* and other invasive alien species encroaching on the Nature Reserve. These are outlined in an Integrated Management Plan. To suppress the spread of *L. camara* and other invasives, a mixture of physical removal, chemical application and fire removal have been used throughout the reserve (MTPA, 2012). These interventions appear to have been effective for *Site A*, considering that *L. camara* ranked the second lowest in terms of importance due to its low abundance. The two *L. camara* bushes found were presumably young – having main stems smaller than 5 cm in diameter and height below 1 m. This suggests they are recently established bushes and highlights the importance of continuous monitoring of the site. An invasive plant may be absent during one survey but cannot automatically be assumed to be permanently eradicated. For instance, the perennial *L. camara* can germinate from seed or rapidly regrow from the base of cut/burnt stems. It can also slowly re-establish from rooted horizontal stems that lie in moist soil (Swarbick *et al.,* 1995). Thus, the continuous monitoring systems proposed in Barberton Nature Reserve's management plan remain essential. Annual, physical surveys of the area were prioritized, and the long-term goal is to implement remote sensing on a quarterly basis. In the interim, the absolute density of species obtained in this study can serve as a useful reference point for quantifying any changes in the spread of *L. camara* on the site. Another encroaching species which was noted on *Site A* but absent on *Site C* is *Bidens pilosa*. *Bidens pilosa* bushes were especially widespread during the 2017 census of *Subpopulation A*. These visibly declined after application of a fire in late 2017 and other subsequent fires.

Fire and herbivory are well-documented as processes that mediate woody cover on sites exceeding a mean annual precipitation of 650 mm (Sankaran *et al.*, 2005). Mean annual precipitation in the study area is 715 mm. Unlike *Site A*, no herbivores are enclosed on *Site C*. As such, low woody cover and low density of undesirable bushes can mostly be attributed to frequent fires. In comparison to *Site A*, the fire regime on *Site C* has been good for *P. curvata* recruitment. However, *P. curvata* recruitment on *Site C* was still below replacement. It is therefore important to include other management interventions that significantly increase *P. curvata* recruitment and/or reduce *P. curvata* mortality on the site. Due to *Site C* not being in a protected area, fires have been a challenge to control. Some fires occurred without the property owner's consent. Such fires often died out or were deliberately put out by the property owner before they could spread throughout the site (Meyer, pers. comm., 2019). This poses two challenges. Firstly, it is hard to monitor the conditions of uncontrolled burns. Secondly, since different patches are burnt at different times, they may start to develop distinct responses to fire. This makes it even more difficult to apply fire frequency as a blanket estimate of fire intensity. A study on *Protea caffra* Meisn. illustrated how different patch dynamics on a site elicit varying fire conditions under the same fire frequency (Adie *et al*., 2011). On a site burnt every 2 – 3 years, the *Protea* trees on patches with grass fared better than those on patches with bracken fern. Fires on bracken patches burnt longer, had higher char heights and higher temperature maxima. This was a result of bracken fern having less moisture and a structure that lent itself to more intense fires than grasses did (Adie *et al*., 2011). Therefore, the prescribed burns on *Site C* should be applied with the aim of limiting fire intensity on all patches. This could potentially require the area to be partitioned by fire breaks to burn different patches at different times. For example, the site was noticeably more open toward the peak of the mountain. Grass cover was sparse and trees were spaced further apart from each other due to higher rock cover. Such areas could tolerate a dry fire due to low grass fuel and minimal combustion from bushes. At the mid to lower end of the slope, grass cover was less sparse. These patches are likely to have low scorch heights but high fuel loads that incinerate *Protea* seeds and seedlings (Adie *et al.,* 2011). It is advisable that controlled burns be applied on these patches after rain so as to increase plant moisture and reduce fire intensity. The bottom of the slope on *Site C*, had taller grass and clustered bushes. Intense fires on these patches could be detrimental for the adult cohort of *Protea curvata*. Char heights could lead to bark damage, branch breakage and reduced ability to recover from subsequent fires (Canadell & López -Soria 1998; Schutz *et al.* 2009; Adie *et al.,* 2011). Therefore, fires on such patches might need to be preceded by rain and bush clearing.

In addition to fire, herbivory was considered for the management of *Site C*. As a privately owned area, *Site C* lacks the variety of mammalian browsers found on *Site A* (i.e., Barberton Nature Reserve). Currently, *Site C* also lacks the infrastructure to manage the introduction of wildlife. Livestock management is suggested as an alternative. Goats are likely the most practical option, since the site is too steep for most large herbivores. Although goats were frequently observed on the site during tree sampling, the site could benefit from a greater and more controlled addition of this livestock. The controlled application of goat herbivory has shown promising results in the management of savanna bushvelds and thornveld (Raats *et al.,* 1996; Jordaan 1998; Jordaan & Le Roux, 1998). Goats are mixed feeding opportunists that feed on grasses, forbs and shrubs. As such, the extent to which goats control bush or grass dominance varies based on forage availability (McCammon-Feldman *et al.*, 1981; Basha & Nsahlai, 2012). Bush density on *Site C* can be managed by herding goats on the site seasonally rather than throughout the year. The viability of goats as a form of bush control on the site is improved by their ability to feed on *Searsia* leaves, which are typically unpalatable to other browsers (Basha & Nsahlai, 2012; Carrick, 2018; Mkhize *et al.,* 2018).

Although overall woody plant density on *Site C* was lower than on *Site A*, both sites showed similar densities of *Vachellia dayvi* (248,04 trees/ha and 250,63 trees/ha respectively). Moreover, *V. dayvi* was the most dominant species on *Site C*. It showed high recruitment on the site and had a larger height range than the *V. dayvi* population on *Site A*. This increases the potential of thicket formation as older trees are likely to be more well-branched than newly established seedlings.

On *Site A,* the *P. curvata* tree of each sampling point rarely had another *P. curvata* tree as its nearest neighbour (as indicated by the low relative density and frequency). The Janzen-Connell hypothesis was partially applicable to *P. curvata* when assessing the demography of neighbouring trees. *Protea curvata* juveniles were rarely associated with other *Protea.* Moreover, cases of closely occurring *Protea* on *Site C* involved two adult trees or one juvenile and one adult tree. It was rare to find seedlings that had established and survived near each other. This partially confirms the hypothesized limits placed by intraspecific competition on seedling survival. With *Site A*, juvenile trees were absent from the subpopulation, making it even more improbable to find two *Protea* seedlings occurring next to each other. The effect of conspecific tree density on *P. curvata* mortality was less apparent when assessing absolute density. *Site A* and *Site C* had similar mortality rates even though the absolute density of *P. curvata* trees was much higher on *Site C.* Moreover, *Site C* had more seedlings despite there being a higher density of *P. curvata* trees with which to compete. Therefore, conspecific density-dependent mortality appears to only limit the number of *P. curvata* seedlings that can establish close to other *P. curvata* trees. It is not the mechanism preventing *P. curvata* seedling establishment altogether on *Site A*. Rather, *P. curvata* seedlings are likely excluded via fire suppression which has led to high woody cover and density. This is consistent with other studies in which bushes started to invade the area once overgrown grass became moribund (Scott, 1971; Trollope, 1980; Everson *et al*., 2004).

Based on the second part of Janzen-Connell hypothesis, one would expect sites with higher density-dependent mortality to have higher species diversity (Janzen, 1970; Connell, 1971; Comita *et al.,* 2014). Both sites had a similar number of unique species, so there is no indication that density-dependent mortality of a particular species has increased diversity on the sites. However, since the study mainly focussed on woody species closely associated with *Protea curvata,* the species diversity from this study is not an exhaustive record. Overall, the recruitment of *P. curvata* trees and the maintenance of plant diversity on both sites will be dependent keeping both the herbaceous and woody layer competitive. This can be achieved with fire intervals no longer than 2 years, periodic herbivory and bush clearing where necessary.

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Chapter 4: Soil seed banks in *Protea curvata*

Introduction

In fire prone habitats, plants can be categorized into various functional groups based on their life history traits, particularly how they respond to fire. Obligate seeders have plants that are mostly killed by fire, making the species reliant on seeds for recovery on the site. Obligate resprouters are able to survive fires and resprout from vegetative buds after a fire (Le Maitre, 1992). Facultative resprouters can employ both seedling recruitment and resprouting to recover from fire (Benwell, 1998; Knox & Morrison, 2005; Marais *et al*., 2014). Seedling recruitment in obligate seeders and facultative resprouters often involves seed banks. These can be aquatic seed banks, soil seed banks or aerial seed banks (Csontos, 2007).

Protea is a genus within the *Proteaceae* family, with most species occurring in the fynbos of Western Cape and Eastern Cape, South Africa (Rebelo, 2001), where fire has been considered the most important disturbance shaping plant communities (Schwilk *et al*., 1997). For some Proteaceae in the Cape region, aerial seed banks maximise the number of seeds available for post-fire regeneration (Bond, 1985). Serotiny is a life history trait of some woody plants associated with fire-prone habitats wherein the aerial seed bank is formed by seeds in cones, follicles, woody capsules or infructescences within the canopy (de Gouvenain *et al.,* 2019). In weakly serotinous plants, seeds are stored in the canopy for 1–3 years, and in strongly serotinous plants they are stored for 5–30 years (Lamont *et al.,* 1991; Midgley, 2000). Triggers for seed release may include fire, rain, or senescence of the parent plant (Lamont *et al.,* 1991*,* van Oudtshoorn & van Rooyen*,* 1999; Peters *et al.*, 2009). *Protea curvata* N.E. Br is a tree that grows outside the Cape region. It is endemic to Mpumalanga and grows on serpentine mountain slopes with savanna vegetation (Williamson and Balkwill, 2006). Other *Proteas* that are found on the mountains of Mpumalanga include *Protea welwitschii* Engl., *Protea rubropilosa* Beard., *Protea laetans* L.E. Davidson, *Protea comptonii* Beard and *Protea angolensis* var*. divaricata* (Engl. & Gilg) Beard (Rebelo, 2001; Notten, 2012). They are colloquially termed the "mountain sugarbushes" (Protea Atlas, 2008a). Although fire-induced release of seeds has not been demonstrated for mountain sugarbushes (Rebelo, 2001), fire remains an important factor in their population dynamics and responses to fire vary between the species. For example, *Protea laetans* is reliant on fires to clear space for seedlings and prune old branches on mature trees (Notten, 2012; Rebelo *et al.,* 2019). *Protea angolensis* var*. divaricata* resprouts from underground boles after fire; whereas *P. rubropilosa* trees are killed by fire, leaving behind only the seeds (Protea Atlas, 2008a).

Although *P. curvata* has been identified as threatened, the species' post-fire regeneration strategies are not well-defined (Hilton-Taylor, 1998; Rebelo *et al*., 2020). A field guide entry mentions that the *P. curvata* "seedhead shears off when fruits are mature" (Rebelo, 2001), suggesting the absence of serotiny. At the time of the entry, only one subpopulation was known in Claremont Vale, Mpumalanga and *P. curvata* seed ecology had yet to be studied in other sites. An entry on the species in the Protea Atlas (2008a) briefly mentions that *P. curvata* can resprout from underground boles. The entry includes a somewhat misleading use of the term "seed storage". For *Protea curvata,* the description "in seedheads on plant [sic]" was listed under the category of seed storage. Conversely, seed storage for *P. laetans*, *P. rubropilosa* and *P. rupestris* was described as "released to lie on the ground". *Protea*, *Faurea* and certain *Leucadendron* species in savannas are typically non-serotinous (Protea Atlas, 2008c). Therefore, for the savanna species, *Protea curvata*, "seed storage in seedheads on plant" presumably did not refer to serotiny (i.e., the storage of seeds in the canopy for a year or more [Bond, 1985]), and possibly indicated that mature fruit of *P. curvata* remained in the seedhead for longer in comparisons to other mountain sugarbushes. In 2017, a *P. curvata* subpopulation in Barberton Nature Reserve, Mpumalanga was sampled during the flowering season. Receptacles in the canopy held inflorescence buds, open inflorescences or inflorescence bracts. No receptacles containing seeds from the previous year were observed, thus suggesting the absence of serotiny (Mabuza, 2017). Based on the infra-annual release of seed noted in Claremont Vale (Rebelo, 2001) and Barberton Nature Reserve (Mabuza, 2017), as well as the low prevalence of serotiny in savanna species (Williams *et al.,* 1999; Rebelo, 2001; Gashaw & Michelsen, 2002; Protea Atlas, 2008c), *P. curvata* is expected to be non-serotinous. Observations from other sites will be helpful in determining the typical duration of seed storage in the *P. curvata* canopy and whether it fits the description of non-serotinous or weakly serotinous.

Soil seed banking in savannas is well-described for non-Proteaceae, woody species and these seed banks are typically described in terms of burial depth and seed density in the soil (Skoglund, 1992; Williams *et al.,* 2005; Jaganathan, 2018). The various methods used for describing soil seed banks typically investigate at least one of the following traits: (i) seed bank persistence, and (ii) soil seed survival. Seed bank persistence methods measure how long seeds can remain in the soil and/or the proportion of seeds in each soil layer. Soil seed survival methods measure the amount of seeds in the soil that remain germinable after more than a year (Saatkamp *et al*., 2009).

A classification system for describing soil seed banks was developed by Poschlod & Jackel (1993). The system focuses on the seeds that fall from trees ("seed rain") and the dynamics of the seed bank. There are four categories. *Type A* transient seeds are buried in the upper soil layer only and they last for less than one year after seed rain. *Type B* transient seeds remain in the upper soil layer throughout the year, with a marked peak after seed rain and a few seeds being found in the lower soil layer. These can last for up to two years. *Type C* persistent seeds are abundant in the upper soil layer, with some seeds in the lower layer being present the whole year. A marked peak is seen in the upper layer after seed rain, and less distinct peak is seen in the lower layer. These seeds persist for some years to a few decades. For *Type D* persistent seeds, the number of seeds in the lower soil layer is similar to those found in the upper layer all year. Even after seed rain, there is no peak noticeable in both layers. These seeds persist for several decades.

Poschlod & Jackel's (1993) classifications are an improvement from classifications that did not factor in the addition and depth of seeds in the soil over time. Unfortunately, it is very rare to find data that can fit these classifications, primarily because the type of data collection required can be demanding in terms of time and resources (Bakker *et al.,* 1996). For example, relying on direct evidence to determine if a species is *Type C* would require a study period of decades. Alternatively, data would have to be taken from sites where there is certainty regarding how long there has been no input of new seeds. This includes soil under buildings and soils where vegetation has been completely cleared for a known time (Bakker *et al.,* 1996). Similarly, artificial burial experiments can be time-intensive if working with long-lived seeds. Furthermore, artificial burial studies may have an experimental design that obscures the impact of seed predators. Since the impact of seed predators on the seed cannot always be quantified and corrected for, it is possible to draw inaccurate conclusions about seed banks from artificial burial experiments (Bakker *et al*., 1996; Thompson *et al*., 1994). To avoid these constraints, it can be appropriate to use *in situ* sampling and observation of the vertical distribution of seeds in the soil. The correlation between seed age and depth is well established – with older seeds generally being found deeper than newer seeds and persisting longer than seeds that remain on or near the soil surface (Thompson *et al.,* 1997, Bekker *et al.,* 1998). Therefore, the ratio of deeply buried to shallow seeds can be used to estimate seed longevity. If applied correctly, this method can yield results that allow basic comparisons with directly measured seed longevity (Bakker 1989; Bakker *et al.,* 1996). A dichotomous key formulated by Thompson *et al.* (1996) uses this approach and allows seed banks to be classified based on soil samples from one occasion. The key uses the presence or absence of vegetation on a site and the distribution of seeds in soil layers to infer seed persistence.

The classification made after vertical soil sampling is just one step in investigating a species' seed bank. The frequency (density) of seeds found in the soil layers often varies from the longevity and viability of the seeds found in those layers. Seed longevity can be defined as the total time span during which seeds remain viable i.e., the time between the end of seed maturation and the onset of germination (Sano *et al.,* 2016, Smolikova, *et al.,* 2020). Dissimilar results from burial experiments of the same species suggest that edaphic conditions can influence longevity. *Solanum nigrum*, for example, showed 83% viability after 39 years in one study (Toole & Brown, 1946); whereas the same species showed 4% viability after 11 years in another study (Salzmann, 1954). Additionally, the level of disturbance within a site and the depth of seed burial can significantly affect seed longevity (Thompson, *et al.*, 1997; Jaganathan, 2018). This highlights the importance of sampling from multiple sites when attempting to describe the typical seed longevity of *P. curvata*.

Seed viability can be defined as the percentage of seeds with the potential to germinate under suitable conditions and includes dormant seeds. Non-viable seeds are those which would not germinate even under conditions suitable for breaking dormancy or initiating germination (Bradbeer, 1988). Much like seed longevity, seed viability and germination can show intraspecific variation. For example, among seeds of the shrub *Purshia tridentata* (Pursh) DC. that were collected from six different sites, percent germination was 91% for the site with the highest germination and 57% for site with the lowest germination (Meyer & Monsen, 1989). Similarly, in the shrub *Amelanchier alnifolia* (Nutt.) ex M. Roem.*,* percent germination varies between batches collected from different habitats. In one study, *A. alnifolia* seeds were collected from five different locations, and germination of untreated seeds from the five sources ranged between 0% and 44%. A batch of seeds from each habitat was cold-treated to break dormancy. Although germination improved, habitat related differences persisted, with germination ranging between 15% and 85% (Meyer *et al*., 1987). Such differences can occur within a species as a consequence of genetic variation between populations (Fernández-Pascual *et al.,* 2013), clines formed by differences in long-term environmental and climate conditions across collection sites (Zúñiga-Feest, 2015), or different weather (i.e., short-term) conditions at the time of seed maturation (Meyer & Monsen, 1991; Fernández-Pascual *et al.,* 2013).

Evidently, it is possible for seeds in lower soil layers to occur frequently enough to be classified as "long-term persistent", using Thompson et *al*.'s (1996) key; even when most of the seeds have lost their viability. This highlights how Thompson et *al*.'s (1996) key has its shortcomings. A wiser option is to use the key for a preliminary investigation of seed bank persistence. Conclusions regarding the short-term or long-term survival of a seed bank should draw information beyond the dichotomous key. It thus becomes important to evaluate the percentage of viable seeds present in soil samples from *P. curvata* sites.

Protea curvata subpopulations showed low recruitment (Mabuza 2017, Chapter 1) and grow on sites with varying fire management (Chapter 2). *Protea curvata* seeds may therefore encounter various fire intensities or pre-fire and post-fire substrates depending on the timing of fires and seed drop. Given the link between disturbance and the incorporation of seeds into soil depths that can extend seed longevity (Thompson *et al.,* 1997; Jaganathan, 2018), knowledge on whether *P. curvata* seeds can persist for more than a year will be valuable for site management considerations, particularly the timing of fires. Further inquiry regarding the longevity of *P. curvata* seeds after fruit maturation is therefore a key part of understanding how to encourage recruitment within this threatened species. Since *P.*

curvata is not a riparian species, it is unlikely to recruit from an aquatic seed bank. Our study therefore aimed to give a description of *Protea curvata* seed ecology based on seed persistence and seed survival by:

1) Testing if *Protea curvata* seeds are retained in the canopy for at least one year.
2) Testing if *Protea curvata* seeds are deposited and retained in the soil for at least one year.
3) Determining what percentage of seeds are viable amongst *P. curvata* seeds that are at least one year old.

Methods

Study site and species

Protea curvata is a flowering tree found in Legogote Sour Bushveld and Barberton Serpentine Sourveld (Mucina *et al.,* 2005). Trees were sampled from six areas. "*Site A*" is within Phase 2 of the Barberton Nature Reserve (-25.611276° S, 31.003775° E) in Mpumalanga. This is a 5400 ha area that includes pieces of state land and it is managed by neighbouring private conservation organisations (Mpumalanga Tourism and Parks Agency [MTPA], 2012). At Mundt's Concession, about 9 km southeast of "*Site A*", "*Site B1*" and "Site *B2*" are found. "*Site C*", "*Site D*" and "*Site E"* occur further southeast. Studies of "*Site D*" in Claremont Vale formed the basis for the species' description with regards to phenology and seed ecology (Rebelo, 2001).

Protea curvata flowers grow on a flat peduncle, forming a capitulum surrounded by dark pink, involucral bracts. The multiple flowers on the capitulum of *P. curvata* are collectively referred to as an inflorescence (Protea Atlas, 2008d). Inflorescence buds typically form between April and May (*Protea Atlas*, 2008a). Anthesis begins in June and the flowering season ends in October (Rebelo, 2001). Fruit formation takes seven to eight weeks (Hargreaves *et al.,* 2004; Mabuza, 2017). The fruit is an indehiscent achene containing a single seed. It is covered in trichomes (hairs) that form a pappus-like structure (Bond, 1988). Hairs on *P. curvata* fruit are typically reddish-brown throughout (mature fruit) or red near the fruit wall with lightly coloured tips (immature fruit) [Figure 1]. The fruit wall is typically reddish-brown or deep pink. These differ from the fruit hairs of other Proteaceae trees found on the site. Both *Faurea saligna* Harv. And *Faurea rochetiana* (A. Rich.) fruit have white or silvery hairs and light yellow or green fruit. Additionally, the shape of *Protea curvata* fruit is narrow and slender compared to the more spherical fruit of *F. saligna* and *F. rochetiana* (Pooley, 1993). The seeds remain contained in the fruit when released from the canopy and deposited into the soil (pers. observ.).

Data Collection

A census was conducted on six *Protea curvata* sites. The area of each site was divided into equidistant transects. *Protea curvata* trees found on the transect or within 5 m of either side of the transects were tagged and sampled. *Site A* was first sampled in 2017. The site was burnt shortly after the first census and resampled in 2018 and 2019. *Site C* was sampled in 2018 and 2019. *Site B1*, *Site B2*, *Site D* and *Site E* were sampled in 2018 [Figure 2]. All censuses were done during flowering season. Measurements recorded included tree height, number of inflorescences, infructescences and old receptacles. These measurements were used to calculate the number of inflorescences that could contribute seeds at each site.

In 2018, soil samples were collected from each of the six *P. curvata* sites. A count of seeds was made for depths of 0−5 cm, 5−10 cm and 10−15 cm. A depth of 5 cm was considered to be the upper layer as per Thompson *et al*.'s (1996) recommendation. This was also for the sake of comparison; since what is defined as the upper layer in many studies, is typically a minimum of 5 cm (Bakker *et al*., 1996).

Protea curvata occurs on a rocky landscape. It was anticipated that it would be challenging to find spots where soil could be collected beyond depths of 5 cm, particularly if using a random sampling method. Since the priority was to establish if there is any seed bank present, a more targeted sampling method was selected. Samples were collected around floriferous trees to improve the likelihood of finding seeds. Porcupine diggings were observed in the area. This is likely to be where seeds were incorporated deeper in the soil. Therefore, sampling was also focused near such spots. Six *Protea curvata* sites were sampled, with ten soil samples collected from each site.

For each sample a 21 x 30 cm area was marked out on the ground. The area of soil was then dug out. Soil samples were placed in a bag with a piece of paper noting the site, GPS coordinates and layer from which they were collected. In the lab, soil was transferred into beakers to determine the volume of the soil samples. The soil was then sieved for *P. curvata* fruits. The number of seeds found at different depths was determined. The type of seed bank possessed by *Protea curvata* was classified using Thompson *et al.*'s (1996) dichotomous key [Figure 4]. Species that are absent from the soil or only present in the surface soil are classified as transient. Species whose seeds are more frequent in the upper soil layer and present in the lower soil layer are classified as having a short-term persistent seed bank. Species with a relatively equal distribution of seeds between the upper and lower soil layer are classified as long-term persistent. Species can also be classified as persistent regardless of the depths at which the seeds are found, provided that the species has been absent from the site's vegetation for a known amount of time. Those present in the soil but absent from the vegetation for less than 5 years are classified as short-term persistent. Those present in the soil but absent from the vegetation for 5 or more years are classified as long-term persistent. The species is known to have been growing for more than 5 years on at least three of the sites, with flowering being observed at least 5 years prior to the study at *Site A* (de Bruno Austin, pers. comm., 2018), *Site D* (Rebelo, 2001) and *Site E* (Hamilton, 1956; Beard, 1958). Therefore, the key could be used to estimate seed persistence after checking for seeds in the soil.

Seeds were sorted into envelopes based on the site and layer they were collected from. Each seed was weighed. This was followed by a Topographical Tetrazolium Test for viability, as per Moore's (1973) protocol. Keeping in mind the possibility of intraspecific or site-based variation, percentage viability was calculated separately for samples of different sites.

Soil samples were collected in the flowering season of 2018 before fruit formation (1 June – 30 July). Therefore, seeds found in the soil were likely deposited in the soil prior to 2018. Between 5 August and 31 October 2018, soil volume and seed count in each sample was measured. Viability testing of seeds took place between 15 February and 20 April 2019. Any viable seeds found in the samples could thus be presumed to have a longevity of at least 14 months (November 2017 – February 2019).

Breeding system experiments were conducted at Site A to test pollination success (seed set and viability) between different pollination modes. Autogamy and geitonogamy were tested by hand-pollinating inflorescences that were covered with fine mesh enclosures from bud stage. Natural outcrossing and pollen supplemented outcrossing were tested by leaving inflorescences open to pollinators and, in the case of pollen-supplemented outcrossing, hand-pollinating open inflorescences three times (Chapter 5). Viability results from these experiments were compared with viability of the soil seed bank.

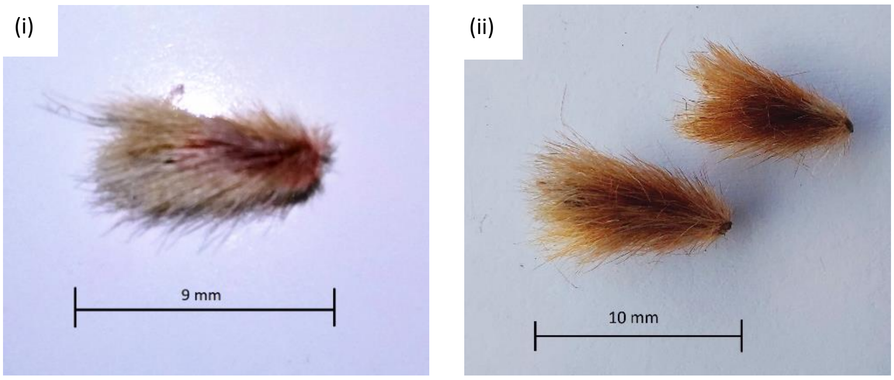

Figure 1: (i) Immature achene collected from *Protea curvata* tree in June 2017 from Barberton Nature Reserve, Mpumalanga, South Africa).

(ii) Mature achene collected in September 2018 from Barberton Nature Reserve, Mpumalanga South Africa).

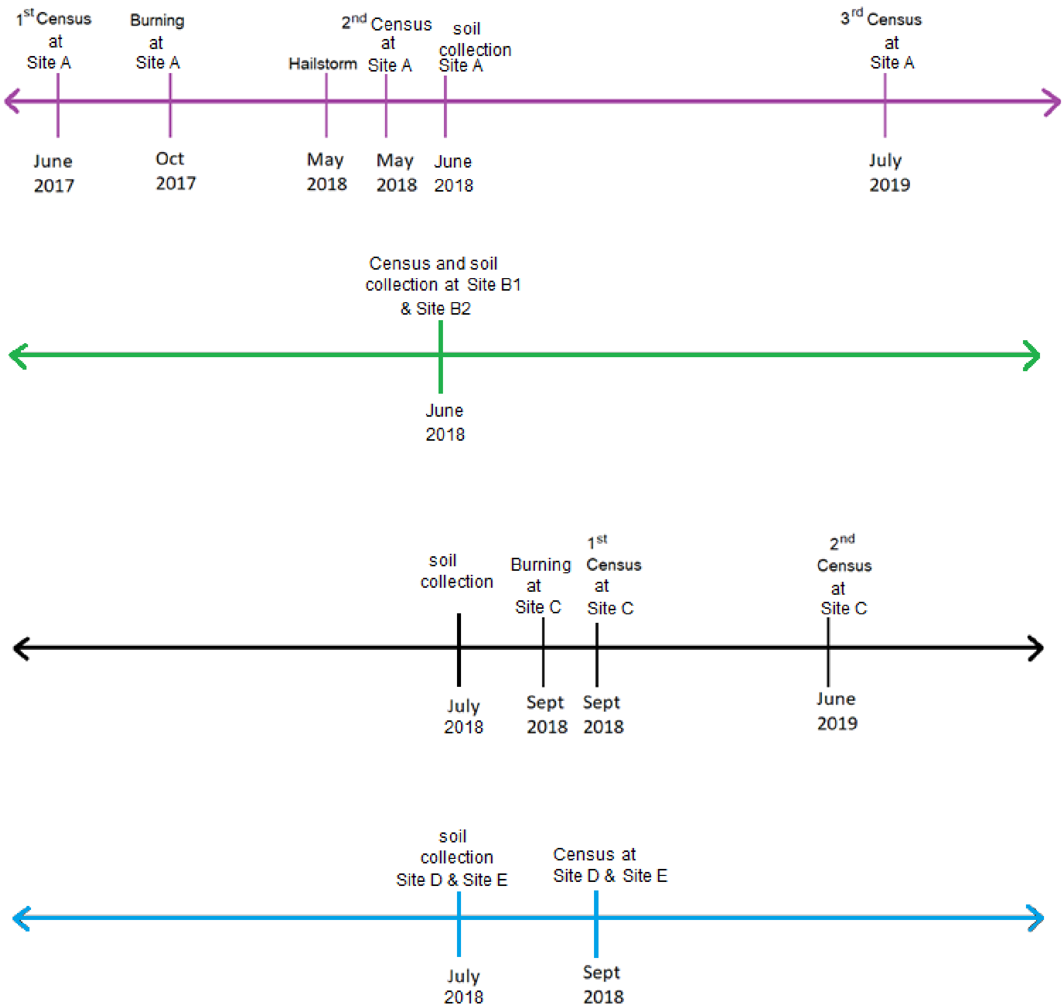

Figure 2: Timeline of recent disturbances and sampling undertaken on *Protea curvata* subpopulations in Mpumalanga, South Africa between 2017 and 2019.

Statistical Methods

Tree density for each of the three sites was calculated as follows:

Area sampled = (area sampled per transect) x *number of transects*

= (10 m x *transect length)* x *number of transects*

Tree density = Number of trees sampled ÷ Area sampled

The subpopulation size was calculated as:

Number of trees in subpopulation = Tree density x Area of the site

Subpopulation size by number of mature individuals was calculated as follows:

*Number of mature individuals = d*A*p;*

where *d* is an estimate of population density, *A* is an estimate of area, and *p* is an estimate of the proportion of individuals that are mature. *Protea curvata* trees were determined to show (reproductive) maturity when 2.5 m or taller (Chapter 1). The area of occurrence of each subpopulation was selected as the *A* value. Total population size was calculated by adding the number of mature individuals from all subpopulations.

Seed input was estimated in terms of total inflorescence number in subpopulation. For *Site A* this was calculated as follows:

$Inflorescences_{2017} = i \times N$

Where i is the average number of inflorescences per tree in 2017 and N is the number of mature individuals on the site.

Seed input for *Sites B1, B2, C, D* and *E* was calculated as follows:

$Inflorescences_{2017} = f \times N$

Where f is the average number of infructescences per tree in the 2018 census and N is the number of mature individuals on the site.

Seed input was also estimated in terms of total inflorescence number per m^2

$Inflorescences\ per\ m^2 = Inflorescences_{2017} \div A;$

where A is the area of occurrence of the subpopulation.

The number of viable *P. curvata* seeds in each site's soil seed bank was estimated by multiplying the percent viability of seeds collected from the site by the mean number of seeds collected per m^2.

For all sites except *Site D*, seed bank size per number of trees in a subpopulation was calculated as:

$$\textit{Viable soil stored seeds per tree} = \frac{(Mean\ number\ of\ viable\ seeds\ per\ m^2\ x\ Area\ of\ site)}{Tree\ density\ x\ Area\ of\ site}$$

All *P. curvata trees* at *Site D* where censused, therefore seed bank size per tree was calculated as:

$$\textit{Viable soil stored seeds per tree} = \frac{(Mean\ number\ of\ viable\ seeds\ per\ m^2\ x\ Area\ of\ site)}{Number\ of\ trees\ in\ subpopulation}$$

Results

A total of 538 seeds was collected from 60 soil samples. A majority of the seeds was found in the upper layer (0 – 5cm), with only 2.6% of the seeds being from lower soil layers [Figure 3] and none of these were viable. Root mass or rocky terrain often prevented sampling into lower layers and only 15 of the 60 soil samples were deeper than 10 cm. When considering only samples that went deeper than 10 cm, the upper layer still contained most of the seeds [Figure 3]. As such, the seed bank can be described as short-term transient based on the dichotomous key developed by Thompson *et al.* (1996) [Figure 4]. Soil samples deeper than 10 cm were most common on *Site B1* (six of the samples > 10 cm deep were found on *Site B1*).

Seed bank size expressed per volume ranged between 1.43 and 13.67 seeds.dm^{-3}, with *Site C* having a significantly larger seed bank than the other sites [Table 1]. Seed bank size expressed as volume was lowest at *Site A* (1.93 seeds.dm^{-3}) and *Site B2* (1.43 seeds.dm^{-3}). Seed bank size in terms of area ranged between 33.67 and 312.65 seeds.m^{-2} for the six sites. *Site C* still had the largest seed bank when expressed by area and both *Site C* and *Site D* had significantly larger seed banks than any other site [Table 2]. *Site A* and *Site B2* had the smallest seed banks when expressed by area (46.49 seeds.m^{-2} and 33.67 seeds.m^{-2} respectively) [Table 4].

Seed viability ranged between an average of 4% and 9.9%, with no significant difference between samples from different sites [Table 3]. During a pollination study (Chapter 5), seed viability was tested in five treatments (autogamous selfing, inflorescence geitonogamy, tree geitonogamy, natural outcrossing and pollen supplemented outcrossing). The viability of freshly harvested seeds from pollination experiments was higher than the viability of the soil seed bank. Naturally outcrossed seeds showed the highest viability (43%) and autonogamy showed the lowest viability (22%) amongst the pollination treatments. When the viability of seeds from soil samples was taken into account, *Site C* continued to have the largest seed bank. Soil from *Site A* and *Site B2* had the fewest viable *P. curvata* seeds [Table 4].

Seed input for *Site A* in 2017 (i.e., a year before soil sampling) was estimated using census data from 2017. Seed input for the remainder of the sites was based on the number of old receptacles and reproductively mature trees present at the time of the 2018 census to get a conservative estimate of the previous year's flowering [Table 5]. *Site C*, which had the largest seed bank [Table 1; Table 2] had relatively low seed input at subpopulation level. Although *Site D* had the second largest seed bank [Table 1; Table 2], it had the lowest seed input at subpopulation level. Trees on *Site D* were highly floriferous, with both average number of inflorescence and old receptacles per tree being the highest among the sites [Table 5]. However, the subpopulation comprised very few trees. *Site D* also had the lowest seed input when expressed as the average number of inflorescences per unit area [Table 5]. *Site A and Site E* had the highest seed input at subpopulation level. For *Site A*, this appeared to largely be a result of the population having the highest number of mature individuals. *Site E* had a lower number of mature individuals but very floriferous trees [Table 5]. Seed input (number of inflorescences) per unit area was highest at *Site B1.* All sites a had a greater number of seeds in the soil than the number of trees in the subpopulation. *Site D* had the greatest number of seeds available for replenishing the subpopulation (Table 6).

Across all sites, receptacles from previous seasons showed no retention of flowers, fruits, or seed. This includes *Site A* which was censused for three years and *Site C* which was censused for two years. A year after flowering, only the old inflorescence bracts remained. During flowering seasons, infructescences were able to release fruit without fire cues [Figure 6]. This happened consistently across sites. Regardless of their differences in fire frequency, all sites flowered by June and dropped most of their fruit by November of the same year. Notably, inflorescences on *Site C* that were mildly impacted by fire had their perianths singed off but were able to retain most of their fruits (achenes) during the fire. Burnt infructescences showed no signs of breakage or seed release [Figure 5]. *Protea curvata* is therefore non-serotinous. Trees on *Site A* and *Site C* that were sampled before and after fire were able to survive fires and sprouted new shoots from the bark [Figure 7].

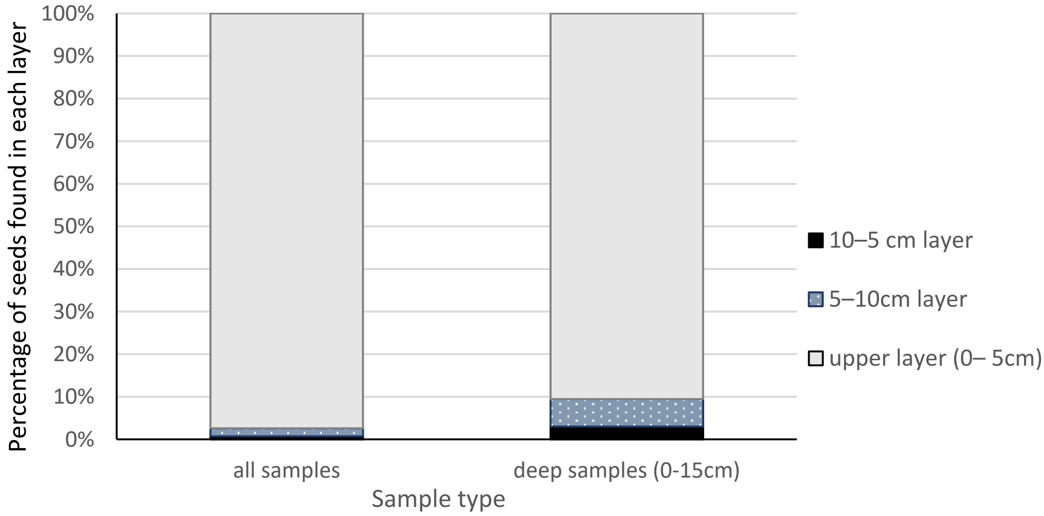

Figure 3: Distribution of *P. curvata* seeds in soil.

Seeds found at depths less than 5 cm beneath the surface constitute the upper layer and those at depths between 5 and 15 cm beneath the surface constitute the lower layers. "All samples" indicates distribution of seeds for all points sampled from five sites, including where sampling deeper than 10 cm was obstructed (N = 60 soil samples). "Deep samples" indicates distribution of seeds when excluding points where sampling deeper than 10 cm was obstructed.

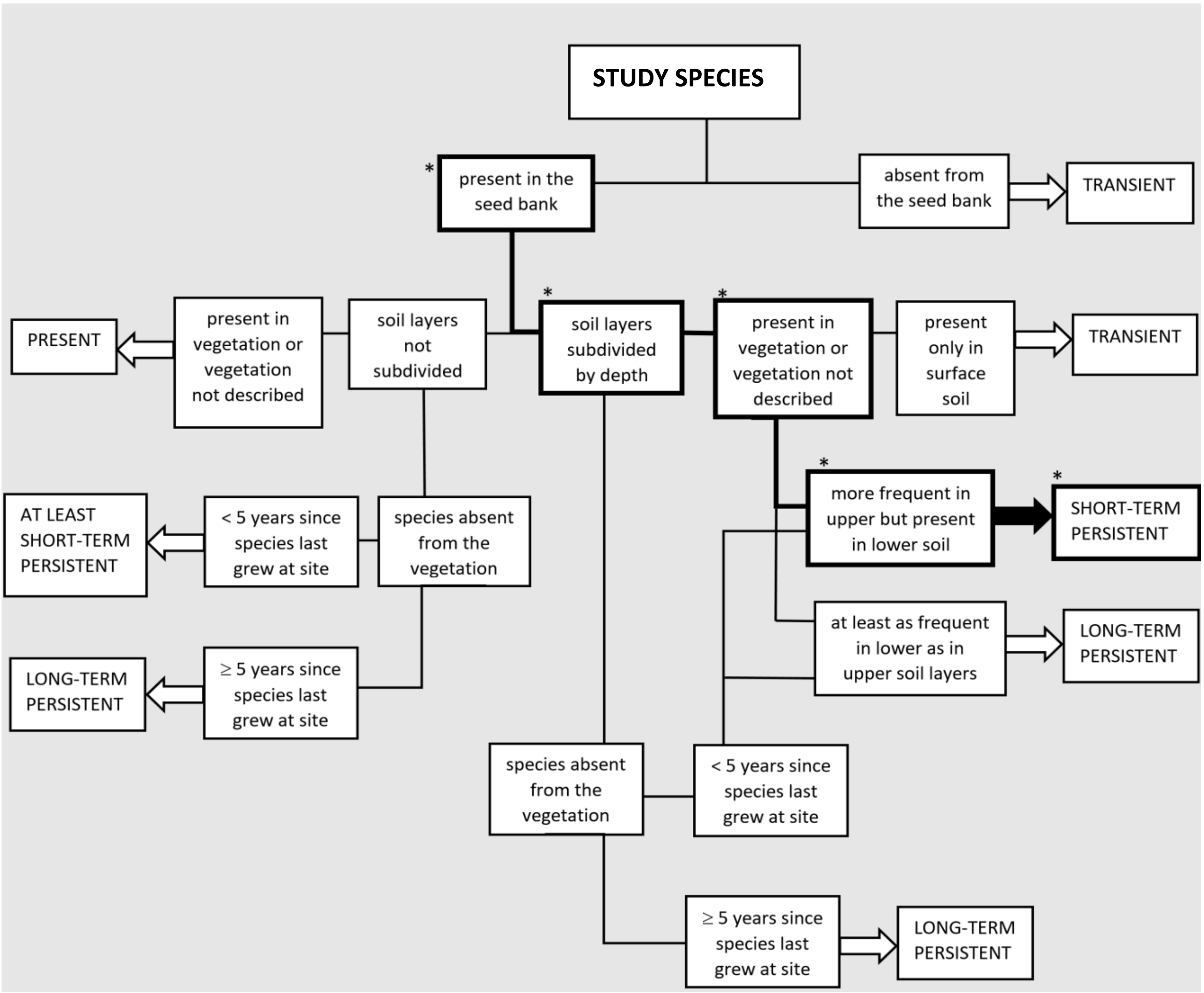

Figure 4: A dichotomous key to the three seed bank types (transient, short-term persistent and long-term persistent) employed in the database of Thompson *et al.* (1996).

*Traits applicable to *P. curvata* are indicated with an asterisk.

Table 1: Results from the Quasi-Poisson distribution generalized linear model for explaining number of seeds per dm^3 of soil sampled at various collection sites

Source	Estimate	SE	t-value	P value
(Intercept)	0.6586	0.6778	0.972	0.3355
Site B1	0.6229	0.8402	0.741	0.4616
Site B2	-0.2956	1.0377	-0.285	0.7769
Site C	1.9567	0.7241	2.702	0.0092 **
Site D	1.1421	0.7785	1.467	0.1482
Site E	0.6066	0.8426	0.720	0.4746

Soil samples collected early in flowering season of 2018 before fruit formation. Therefore, seeds found in the soil were likely from years prior to 2018. Significance levels indicated by asterisks ($p < 0.05$ *; $p < 0.01$**; $p < 0.001$***).

Table 2: Results from the Quasi-Poisson distribution generalized linear model for explaining number of seeds per m^2 at various collection sites.

Source	Estimate	SE	t-value	P value
(Intercept)	3.8394	0.8439	4.550	<0.0001***
Site B1	0.7758	1.0198	0.761	0.4501
Site B2	-0.3228	1.3021	-0.248	0.8052
Site C	1.9057	0.9045	2.107	0.0398*
Site D	1.8691	0.9066	2.062	0.0441*
Site E	0.3704	1.0972	0.338	0.0737

Soil samples collected early in flowering season of 2018 before fruit formation. Therefore, seeds found in the soil were likely from years prior to 2018. Significance levels indicated by asterisks ($p < 0.05$ *; $p < 0.01$**; $p < 0.001$***).

Table 3: Results from the Quasi-Poisson distribution generalized linear model for explaining seed viability based on collection site with 95% Confidence Interval

Source	Estimate	SE	t-value	P value
(Intercept)	1.3863	0.8096	1.712	0.0926
Site B1	1.0940	0.9354	1.169	0.2473
Site B2	0.2231	1.0862	0.205	0.8380
Site C	0.9086	0.9590	0.947	0.3476
Site D	0.4886	1.0284	0.475	0.6367
Site E	0.7853	0.9769	0.804	0.4250

Table 4: Estimation of viable seeds in the *P. curvata* seed bank

Location	Seed bank size ($seeds.m^{-2}$)	Viable seed bank ($seeds.m^{-2}$)
Site A	46.496 ± 53.68	1.86
Site B1	101.01 ± 137.10	12.06
Site B2	33.67 ± 56.28	1.68
Site C	312.65 ± 373.67	31.01
Site D	301.43 ± 559.296	19.65
Site E	67.34 ± 103.85	5.91

Table 5: Factors related to seed input for the *P. curvata* seed bank at various sites.

	Reproductively mature trees	Inflorescences per tree	Old receptacles per tree	Estimate of inflorescences per subpopulation	Estimate of inflorescences per m^2
		2018	2018	2017	2017
Site A	639	4.28	0.82	*6993	0.0147
Site B1	184	27.20	17	3134	0.4873
Site B2	81	27.79	30.93	2496	0.0513
Site C	423	22.92	5.91	2501	0.0953
Site D	43	28.15	33.18	1426	0.0071
Site E	282	13.18	25.81	7286	0.0695

Census and soil sampling performed in 2018. Collected seeds were likely deposited in the soil during or prior to 2017. Average infructescence number for trees from *subpopulations B1 – E* used to infer flowering during the year 2017 and relative seed input among soil samples. **Subpopulation A* was also sampled in 2017, therefore average inflorescence number from trees sampled in the 2017 census was used to estimate inflorescences in the subpopulation.

Figure 5: *P. curvata* inflorescences after fire at *Site C* (Clarendon Vale, Mpumalanga South Africa), September 2018.

Figure 6: Mature achenes in the canopy of *Protea curvata* tree at *Site A* (Barberton Nature Reserve), August 2018.

Figure 7: New shoots sprouting from *P. curvata* bark at *Site A* (Barberton Nature Reserve), 8 June 2019.

Table 6: Average number of seeds available in soil seed bank per number of *P. curvata* trees in various sites in Mpumalanga, South Africa. Sites ranked in ascending order of seed : tree ratio.

Rank	Location	Viable soil stored seeds per tree
1	*Site B1*	33
2	*Site E*	47
3	*Site B2*	51
4	*Site C*	478
5	*Site A*	662
6	*Site D*	35 727

Discussion

In savannas, soil seed banks studied at the species level often involve African acacias – i.e., *Vachellia* and *Senegalia* species (Skoglund, 1992). In a study of *Acacia nilotica*, *Acacia tortillis* and *Dichrostachys cinerea*, 95% or more of the seeds were found in the 0 – 5cm soil depth (Witkowski & Gramer, 2000). Vertical distribution in the soil of seeds from *P. curvata* (a savanna *Protea*) showed similar trends to these savanna trees. More than 95% of *P. curvata* seeds were found in the 0 – 5 cm layer [Figure 3]. Generally, seeds in lower layers tend to be older than those near the surface (Bakker *et al.,* 1996, Thompson *et al.,* 1997, Bekker *et al.,* 1998). *Protea curvata* seeds present in the lower layers (i.e., older seeds) made a minuscule contribution to the seed bank, both in terms of seed number [Figure 3] and viability. Based on the proportion of seeds found in different layers, *P. curvata* has a short-term persistent seed bank – i.e., it is composed mostly of seeds in the upper layers and a few seeds in the lower layer. Although samples were collected from sites with dissimilar management, the upper layer consistently contained the highest proportion of seeds. Thus, it can be inferred that seed persistence trends observed in this study are typical of the species.

When restricting the term seed bank to only include viable, dormant or readily germinable seeds in the soil (Csontos, 2007); *P. curvata* can be described as having a short-lived seed bank. In *P. curvata,* having many reproductively mature trees at *Site A* [Table 5] did not necessarily lead to a larger seed bank [Table 1; Table 4]. For all sites, viability of the soil seed bank was less than half the viability of seeds collected from the canopy. This was the case despite testing soil samples within a year of collection and irrespective of the pollination treatment that the samples were compared to. In this respect, the seed bank is likely replaced every year, with almost no seeds accumulating in lower layers over many years. This further suggests that *P. curvata* possesses a short-lived seed bank. In terms of the classification system developed by Poschlod & Jackel (1993), *P. curvata* falls under the category of *Type B* seed banks – i.e., has seeds that remain in the upper soil layer throughout the year, with a marked peak after seed rain and a few seeds being found in the lower soil layer. In Proteaceae with long-term persistent seed banks, viability declines less rapidly. *Leucospermum cordifolium*, for instance, maintained 80% viability among soil-stored seeds over the course of four years. Seeds stored in ambient conditions showed similar percent viability in year zero and year one. Viability then started to decline between year one and two. It was only after year three that *L. cordifolium* viability reached less than half of what it was in year zero (Brits *et al.,* 2014). The *P. curvata* seed bank, on the other hand, reached this milestone by year one under similar conditions (i.e., 43% viability in naturally pollinated seeds collected from the canopy, 9.9% viability in one year old seeds from the soil). While seed depth greater than 10 cm was most common amongst samples from *Site B1*, seed viability of these samples did not differ significantly from the other sites' samples. Seeds from the soil of *Site B1* also showed less than half the viability of freshly collected seeds from the canopy. Given this rapid decline in seed viability, *P. curvata* seeds are unlikely to remain viable in the soil for more than 5 years as one might expect of long-term persistent seed banks (Figure 4).

Site C consistently had the largest seed bank (by volume, area and absolute number). This was not accompanied by the highest number of inflorescences for the subpopulation or site area. The seed bank in *Site D* was remarkably large despite having the lowest number of inflorescences available for setting seed. *Site A* had the largest *P. curvata* subpopulation and largest number of inflorescences yet had the second smallest seed bank. Despite *Site B1* having the most inflorescences per unit area, it had a seed bank that was significantly smaller than that of *Site C* or *Site D.* Therefore, flowering and population size were not the greatest determinants of seed bank size in *P. curvata.* This suggests differences in seed set or seed loss over time. Proteaceae can show substantial variance in the number of flowers per inflorescence (Ayre and Whelan, 1989), thus affecting the seed set numbers. Site differences in pollinator availability and pollination success may have also affected seed set. Seed loss can occur prior to seed burial, when seeds are consumed in the canopy or on the surface of the soil (Miller, 1996; Salazar *et al.* 2012). Seed predation may also occur once seeds are buried in the soil. Finally, seedling recruitment between fires can also decrease the seed bank size (Pierce & Cowling, 1991; Auld & Denham, 2001). The small seed bank at *Site A* was not due to high rates of seedling recruitment. *Site A* had no seedlings when sampled a year prior to soil collection as well as between fires. Seed predation is the more likely mechanism of *P. curvata* seed loss. Since *Site A* is enclosed in a nature reserve, it is frequented by herbivores that are absent on other sites, such as kudu, impala, red duiker and mountain reedbuck (Mpumalanga Tourism and Parks Association [MTPA], 2012). Their mixed diet includes seed pods or other fruit and could translate into a higher number of seed predators (Van der Merwe and Marshal, 2012; Makhado *et al*., 2016). Red duiker, for instance, feed on fallen leaves and fruits and could play a role in consuming the seeds released from the canopy (Seufert *et al.,* 2010). However, these were not directly observed feeding on *Protea* seeds. Moreover, *Site B2* (which had the smallest seed bank) does not have all the potential seed predators that are present on *Site A.* If seed bank reduction on *Site B2* is indeed driven by an abundance of seed predators, they are likely to be rodents or shrews – the main predators of various Proteaceae seeds (Le Maitre & Botha, 1994)

The significantly larger seed bank size noted at *Site C* and *Site D* may also be a result of effective seed burial (Thompson, *et al.*, 1997; Jaganathan, 2018). Seed deposition and protection from predators could be improved via removal of the litter layer or with the help of ecosystem engineers (Bond & Slingsby, 1984). Porcupines are a possible candidate; namely *Hystrix africaeaustralis,* which have been documented in the area (Rogan, 2016). During our study, spots that were near porcupine diggings seldom had the rock and root masses that obstructed deep sampling in other spots. In other studies, porcupine diggings formed microhabitats wherein runoff water and nutrients would pool, thus facilitating germination and seedling establishment (Gutterman, 1987).

With regards to seed loss via seed decay, there was not much variation in seed viability at different sites. Patterns of seed bank size remained the same when counting only the seeds that were viable. This indicates that there was no intraspecific variation with regards to seed longevity. The next step would be to explore whether there are any variations in soil and environmental conditions across the sites that could be accelerating seed decomposition rates at *Site A* and *B1* (i.e., at sites with the smallest seed banks).

In addition to possessing a short-term persistent seed bank, *P. curvata* exhibits the potential to regenerate from epicormic resprouting (Pausas & Keeley, 2017) [Figure 7]. It can therefore be considered a facultative resprouter. Adult trees within the species were tall and showed no signs of strong serotiny. By growing as tall as 8 m on most sites (and 10 m on *Site A*), the tree canopies can escape most fires. Even in cases where shorter trees were impacted by fire, they showed no signs of releasing or opening fruit [Figure 5]. For *Protea curvata*, being non-serotinous means that the timing of seed release does not always coincide with vegetation being cleared by fire (Bond, 1985). This makes *Protea curvata* more vulnerable to poor germination and establishment.

Conclusion

The results of our study suggest that *Protea curvata is a* facultative resprouter. The species is non-serotinous but possesses both a short-term persistent seed bank as well as epicormic resprouting that can be used to recover from fire. Based on *P. curvata*'s seed ecology, it is advisable to carry out planned burns before the peak of the rainfall season. Preferably, burning should take place in October. This way there can be enough time for the completion of *P. curvata* flowering and fruit maturation (June–October), the litter layer and competitors can be cleared and *P. curvata* seeds can be incorporated into the soil before receiving rainfall required for germination. During the growing season (January – April), seedlings can then benefit from summer rainfall and limited fire damage in their nascent stages. Given that seed bank size did not vary according to subpopulation size or site flowering, mechanisms of seed loss across *P. curvata* should be investigated in future studies. Efforts to replenish the seed bank or juvenile cohort in the area should focus on fire and granivore management that reduces seed wastage. Further research is required to determine the levels of disturbance and soil conditions that maximize *P. curvata* seed longevity in the soil as well as germination of soil stored seeds.

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Chapter 5: Breeding system and pollination of *Protea curvata*.

Introduction

Protea curvata is a flowering tree, typically between 2 and 5 m tall with narrow, grey-green, cuneate leaves and dark pink floral bracts. Its light pink flowers cluster to form an upward-facing inflorescence with a faint, slightly sweet odour (Rebelo *et al*., 2005; Rebelo, 1991, pers. observ). *P. curvata* therefore fits an ornithophilous pollination syndrome (Protea Atlas, 2008a). In a previous study, birds were seen visiting *P. curvata.* Results from pollinator exclusion experiments showed that seed set and seed mass was significantly low in the treatment where both birds and insect were excluded. Seed set in inflorescences that were accessible to birds and insects was not significantly different to seed set in inflorescences that were accessible to insects only (Mabuza, 2017). Seed set showed less variance in inflorescences open to birds and camera data showed birds interacting with the pollen presenters and stigmas of *P. curvata* flower*.* The 2017 study also noted several bee visits. However, poor lighting and fog in some photographs made it difficult to identify any other insects or their behaviour during the visits. During brief field observations, bees rarely made contact with the stigmas or nectaries of *P. curvata* flowers (Mabuza, 2017). Therefore, although birds were identified as contributors to seed set in *P. curvata*, there was reason to deduce that insects may also have a considerable role in the pollination of *P. curvata*. Therefore, a closer inspection of insect-flower interactions is important for determining which insect visitors act as potential pollinators and which insects act as *P. curvata* “pollen thieves”.

A knowledge gap exists regarding the mode of pollination used by the plant. The species’ propensity to set seeds from self-pollinated or cross-pollinated flowers will have implications on gene flow (MacIntyre & Clegg, 2013). There are three possible modes of pollination in *Protea curvata*. These are outcrossing, geitonogamous self-pollination and autogamous self-pollination. During outcrossing, the pollen of one flower pollinates another flower on a different tree (Maki, 1993). Geitonogamous self-pollination involves the transfer of pollen between flowers of the same tree. The transfer of pollen from the anthers of one flower to the stigma of the same flower is termed autonomous or autogamous selfing (Eckert, 2000).

The implications of reproducing by outcrossing can be either positive or negative, depending on the circumstances of natural selection. Firstly, the expression of unfavourable, recessive genes can be reduced through outcrossing (MacIntyre & Clegg, 2013). Fecundity and seed viability is also often higher in outcrossed plants when compared to inbred plants (Maki, 1993). Similarly, selfing can be either detrimental or beneficial to the population. Favourable traits, which may have otherwise been lost over multiple generations of outcrossing, can remain in the population through autonomous or geitonogamous self-pollination. Autonomous selfing has the added advantage of limiting pollen wastage, since pollen is transferred within a single flower (Wright *et al.,* 2013).

From an evolutionary perspective, it is postulated that self-pollination was selected for due to the advantages it carries at the gene level or the advantages it presents at the population level. At the gene level, a selfing gene has the potential to be carried over to more offspring than an outcrossing gene. For obligate outcrossers there are only two potential modes of transfer. The parent plant can either provide pollen to another plant or be the recipient of pollen from another plant. Self-compatible plants have the additional capability to be recipients of their own pollen. [Figure 1]. Therefore, the selfing gene has a 3:2 transmission advantage over the outcrossing gene (Holsinger, 1991). This is known as the automatic selection hypothesis (Fisher, 1941).

The second hypothesis is termed the reproductive assurance hypothesis. At the population level, selfing allows for reproduction even when pollinators or mates are scarce due to distance or low abundance (Baker, 1955; Lloyd, 1965; Inoue *et al*., 1996). In the automatic selection hypothesis, the ability to outcross forms part of the transmission advantage that self-pollinating plants possess. Contrastingly, the reproductive assurance hypothesis recognises a lack of outcrossing opportunities as a driver of self-pollination. Since *Protea curvata* subpopulations are fragmented (Williamson & Balkwill, 2006; Protea Atlas, 2008b), it is plausible that the species may be self-compatible. The reproductive assurance hypothesis also presumes that scarcity of pollinators drives the predominance of selfing in a population.

A preliminary study on *P. curvata* pollination modes suggested the presence of self-compatibility in one subpopulation. Fifty percent of the autogamously selfed flowers set seed (Mabuza, 2017). However, this was not enough to confidently evaluate pollination modes in *P. curvata* since only one inflorescence per treatment was tested and the viability of the seeds was not tested. Thus, the discovery of self-compatibility in *Protea curvata* is only a small part of the picture. An investigation of the efficiency of *Protea curvata* pollinators in cross-pollination is necessary for ascertaining whether *P. curvata* fits the reproductive assurance hypothesis. Therefore, the aim of this study was to:

i) Test the pollination success of autogamous selfing, geitonogamous selfing and cross-pollination in *Protea curvata*.

ii) Identify insect taxa that visit *P. curvata*

iii) Determine visit frequency, visit duration and visit behaviour of insects to distinguish nectar and pollen thieves from potential pollinators.

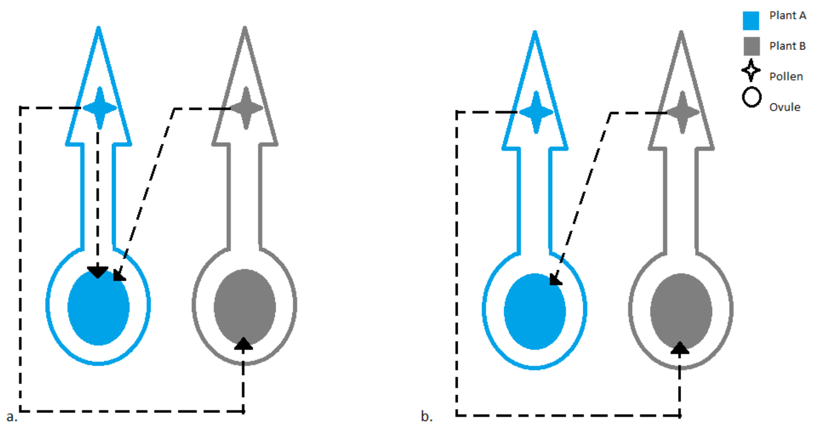

Figure 1: Fertilisation in carriers of (a) Genes promoting selfing and (b) Genes promoting outcrossing (Illustration by Precious Mabuza)

Methods

Study site and species

Protea curvata occurs within the savanna biome of the Mpumalanga lowveld (Protea Atlas, 2008b). Breeding system experiments and camera observations were undertaken on a subpopulation within Phase 2 of the Barberton Nature Reserve (-25.611276° S, 31.003775°E) in Mpumalanga. This is a 5400 ha area that includes pieces of State land and it is managed by neighbouring private conservation organisations (Mpumalanga Tourism and Parks Agency [MTPA], 2012). Due to a recent disturbance, the subpopulation had relatively low flowering compared to previous years (Chapter 2). However, it was the only fenced subpopulation, thus making it preferable for placing cameras and equipment for experiments with low risk of theft and minimal anthropogenic interference. Field observations were undertaken on a subpopulation at Mundt's Concession in Mpumalanga (-25.665833 ° S, 31.050036°E)

Figure 2: (i) *Protea curvata* tree (ii) *Protea curvata* inflorescence bud (iii) *Protea curvata* open inflorescence (Barberton Nature Reserve, Mpumalanga South Africa)

Data Collection

Twenty *P. curvata* trees were arbitrarily selected. On each tree, three inflorescence buds were enclosed with a cage made of wire frame with fine mesh, while two inflorescences were left uncovered. This excluded pollinators and prevented the mesh bags from brushing against the reproductive parts of the flowers. Once the inflorescence buds opened, breeding systems were tested. Pedicels of selected buds were marked with plasticised tags to keep track of the treatments. Stigma receptivity was determined using Zeisler's (1933) test for enzymatic activity. The presence of bubbles on the stigma after application of a hydrogen peroxide solution indicated receptivity (Kearns & Inouye, 1993) and this typically occurred in flowers that were open for at least two days.

Flowers in "Inflorescence A" were used to test for autogamous self-pollination. This involved swiping pollen from the pollen presenter up to the stigma of the same flower using a toothpick. "Inflorescence B" was used to test geitonogamous self-pollination within an inflorescence. Pollen was transferred between different flowers within the same inflorescence using a toothpick. For geitonogamous selfing within a tree, the pollen from flowers of one inflorescence ("Inflorescence B") was transferred to flowers in another inflorescence ("Inflorescence C") of the same tree. To investigate natural outcrossing, one of the uncovered inflorescences was left unmanipulated, thus remaining accessible to natural pollinators). The other uncovered inflorescence was used to test pollen supplemented outcrossing. Pollen-loaded styles were removed from the flowers of inflorescences that did not belong to the same tree as the test inflorescence. The styles were set in an upright position on small Styrofoam platforms. These were enclosed in plastic containers and carried to the test inflorescence. The pollen-loaded portion of the styles were swiped onto the stigmas of the test inflorescence. These inflorescences were pollinated three times (each visited once a day over the course of three days). This would help to determine whether seed set is resource or pollen limited [Figure 3]. To identify which flowers of the inflorescences were part of the certain treatments, colours were assigned to each treatment. Hand-pollinated flowers were tied with a thread of the colour assigned to their treatment. Flowers were left in the canopy for a minimum of seven weeks, since this was established as the time it takes for *Protea* seeds to develop into a size that is easily detectible by touch (Hargreaves *et al.,* 2004; Mabuza, 2017). Inflorescences were collected and achenes removed from the pollinated flowers. Achenes were counted, weighed and classified as follows:

"*Infertile achenes*" which comprised: i) Achenes that crumbled while being counted and contained no embryo ii) Achenes that were firm to the touch during counting but had no embryo visible once dissected.

"*Filled achenes*" which comprised: Seeds that were firm to the touch when counted and an embryo could be seen in the achene once dissected [Figure 4].

Seed studies of *Protea* often use the term "seed mass" when referring to the mass of the single-seeded achenes (Mustart & Cowling, 1993; Carlson & Holsinger, 2010, Carlson & Holsinger, 2015; Schmid *et al.*, 2015). As such, seed mass was measured by placing individual achenes on a weighing balance. Seed viability across treatments was determined using a Topographical Tetrazolium Test as per Moore's (1973) protocol. In addition to comparing seed viability between different treatments, the relationship between seed viability and seed mass was investigated. Seed masses were grouped into mass classes in intervals of 2.5 mg and the percentage of viable seeds in each class was recorded.

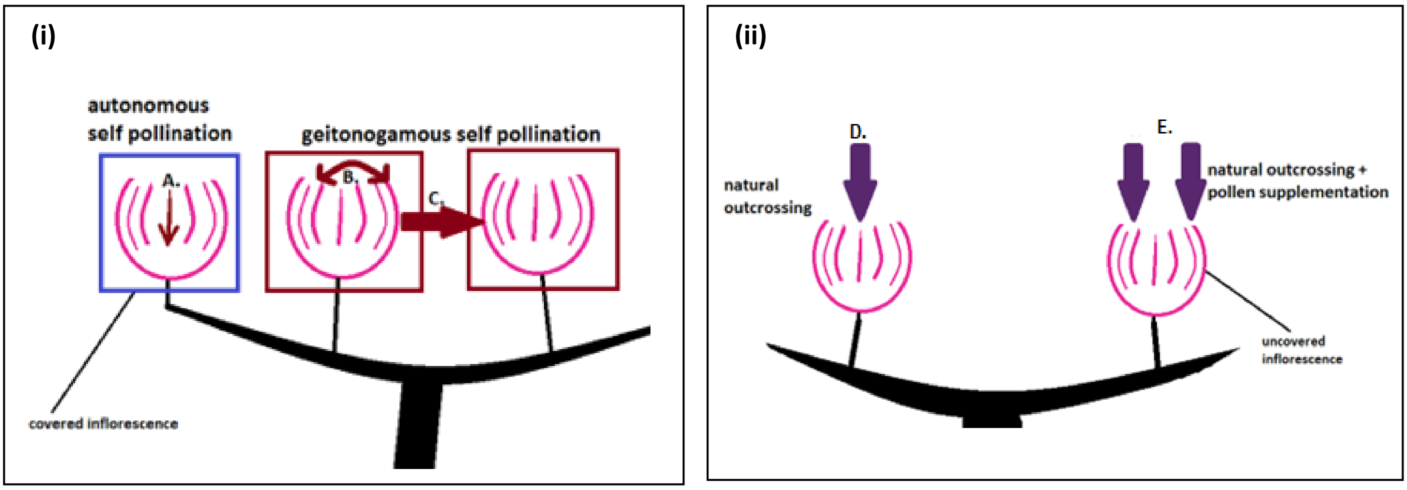

Figure 3: Treatments for breeding system experiments on *Protea curvata* carried out in Barberton Nature Reserve ("*Site A"*), Mpumalanga, South Africa.

(i) Hand-pollinated treatments where natural pollinators are excluded

(ii) Pollination in inflorescences that are accessible to natural pollinators

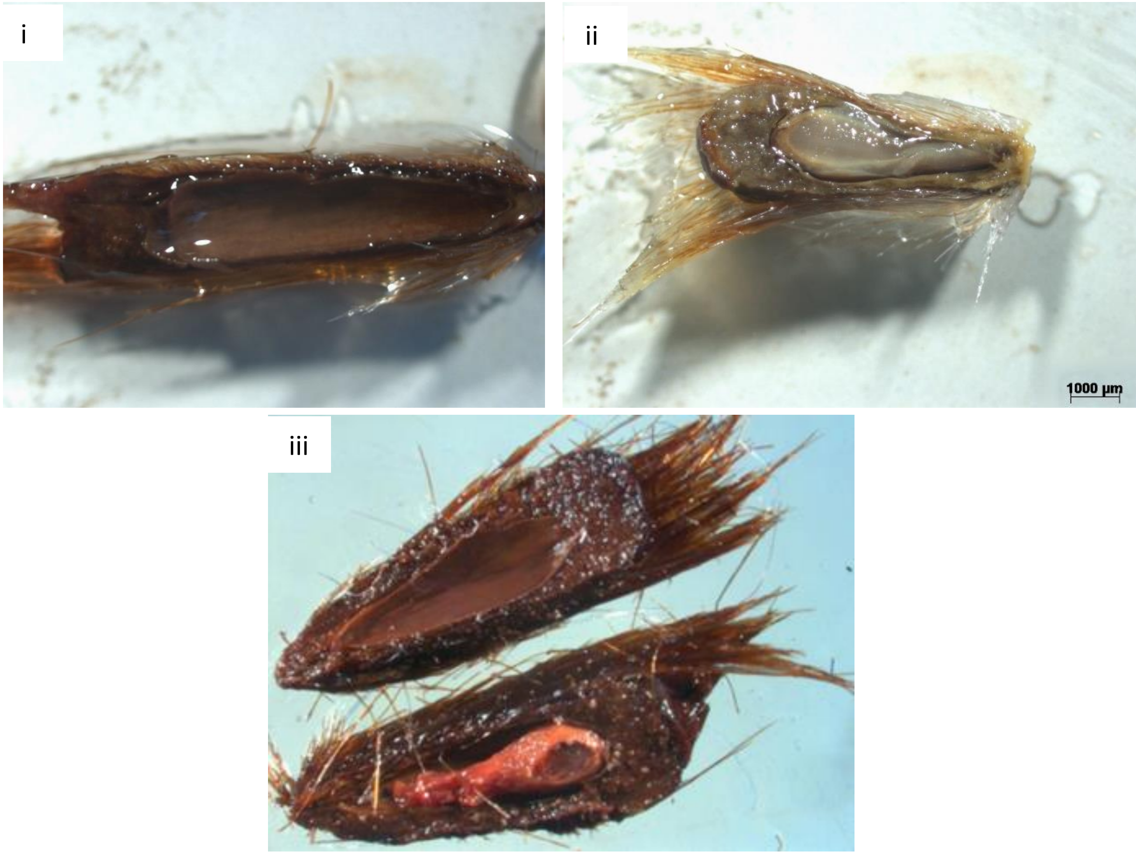

Figure 4: (i) Fruit lacking an embryo was classified as an infertile achene.
(ii) Fruit containing an embryo that did not stain during TTZ tests was classified as non-viable.
(iii) Fruit with an embryo that stained red during TTZ tests was classified as viable.

Both camera and field observations were made with the objective of identifying insects visiting *P. curvata*. Field observations were made at Mundt's concession (12 – 14 June 2018). This entailed standing at one inflorescence and recording the time and duration of insect visits and the taxon of the insect visitor. A different inflorescence from a different tree was observed every 5 minutes. Field observations were then used to determine insect visitation rate and mean visit duration. Taxon identification during the prior study (Mabuza, 2017) was often hindered by unclear camera data. Therefore, insects were collected during field observations and later micrographed for identification. Insects were identified to genus or species level.

Five camera traps were placed around *P. curvata* inflorescences to record insect visits between 30 June and 11 July 2020 in Barberton Nature Reserve. The field scan camera setting was used to automatically take videos at 15-minute intervals and take pictures when the motion sensor was triggered. Insect visits seen on camera were classified based on which part of the *P. curvata* inflorescence the insects made contact with, namely the stigma, the lower part of the style or the tepals and the base of the inflorescence where the nectary is found.

Statistical Methods

Seed viability in each tree was calculated as follows:

% viability = $\frac{\text{No. of filled achenes in treatement with stained embryos}}{\text{Total filled achenes in the treatment}}$ $\text{x } 100$

Seed viability in each tree was calculated as follows:

Seed mass was compared between treatments for filled achenes and for filled achenes containing viable seed.

A one-way ANOVA was performed to compare the effect of pollination treatment on percent of viable *P. curvata* seeds produced. The one-way ANOVA was followed up with Tukey's HSD Test for multiple pair-wise comparisons. The mass of filled achenes (hereafter referred to as "seed mass") was recorded and sorted by tree and by pollination treatment. Similarly, seed mass for viable seeds only was sorted by tree and pollination treatment. Generalized linear models were used to analyse the effect that pollination treatment and the tree on which it was performed had on seed mass. Tukey's HSD test for multiple pair-wise comparisons was used to identify treatments that yielded significantly different seed masses from each other.

For camera data, the number of visits from different animal taxa were counted separately for each camera facing an inflorescence. The run time of each camera was recorded (i.e., total number of hours that camera trap remained switched on the field). Average visit frequency per 24-hour period and mean average visit duration for each taxon was calculated as follows

Average visit frequency = $\frac{\text{No. of visits from the taxon}}{\text{Camera run time (in hours)}}$ $\text{x } 24$

Average visit durationy = $\frac{\sum \text{Visit duration (in seconds)}}{\text{No. of visits from the taxon}}$

A full list of the genera observed in the field is included in the results, however, field data on visit frequency were assessed at family level to facilitate comparison to camera data.

Results

Percent seed set and viability

The one-way ANOVA showed that seed set was statistically different between at least two treatments, (C.I. = 95%; d.f. =4, 95; F_{calc}= 99.65; p < 0.0001). The Tukey HSD post-hoc test found that seed set from natural outcrossing was significantly higher than in all selfing treatments ($mean_{O}$ = 36.2%, C.I. = 95%, p < 0.0001). Outcrossing also showed the widest range in seed set [Figure 5]. Pollen supplemented outcrossing treatments had significantly higher seed set than all selfing treatments ($mean_{PS}$ = 30.9%, C.I. = 95%, p < 0.0001) but did not show a statistically significant difference from seed set in natural outcrossing (C.I. = 95%, p = 0.0535). Although autogamous selfing showed the lowest seed set across all treatments, selfing treatments did not differ significantly from each other with regards to seed set ($mean_{A}$ = 7.6%, $mean_{IG}$ = 10.2%, $mean_{TG}$ = 9.8%, C.I. = 95%, p > 0.50)

There was a statistically significant difference in seed viability between at least two groups (C.I. = 95%; d.f. =4, 95; F_{calc}= 4.5; p = 0.0021). For naturally outcrossed flowers, the percentage of viable seeds was significantly higher than in autogamously self-pollinated flowers (C.I. = 95%; $mean_{O}$ = 42.6%, $mean_{A}$=22.0%, p = 0.0020). Naturally outcrossed flowers also had a significantly higher percentage of viable seeds than geitonogamously self-pollinated flowers (C.I. = 95%; $mean_{IG}$=27.1%, p = 0.0372, $mean_{TG}$ = 26.2%, p = 0.0242). Outcrossing with pollen supplementation showed no significant difference in seed viability when compared with any of the pollination methods (C.I. = 95%; $mean_{PS}$ = 34.0%, p>0.1) [Figure 6].

Seed mass

Seed mass and viability had a positive relationship, with 50% of seeds in the 32.6 mg – 35.0 mg class showing viability. The largest mass class was 42.6 mg – to 44.9 g and showed 90% viability [Figure 7]. Filled achenes from different pollination treatments showed a significant difference in seed mass (GLM: $\chi 2$ = 326.44, d.f. = 4, p < 0.001). Seed mass in naturally outcrossed flowers was significantly larger than seed mass in all other pollination treatments (C.I. = 95%; $mean_{O}$ = 26.3 mg ± 7.1 mg, p<0.0001). Seed mass in autogamous pollination and within-inflorescence geitonogamy showed no significant difference (C.I. = 95%; $mean_A$ = 20.2 mg ± 6.9 mg, $mean_{IG}$ = 20.9 mg ± 6.8 mg, p = 0.1650). Seeds borne of autogamous self-pollination and within-inflorescence geitonogamy had significantly lower mass than seeds borne of within-tree geitonogamy, natural outcrossing and pollen supplemented outcrossing (C.I. = 95%; $mean_{TG}$ = 22.1 mg ± 7.5 mg, $mean_O$= 26.3 g ± 7.1 mg, $mean_{PS}$= 22.6 mg ± 7.1 mg, p<0.0005). Seeds from pollen supplemented flowers and flowers with within-tree geitonogamy had similar mass [Figure 8].

The results of viable seeds showed a significant difference in seed mass between treatments. Natural outcrossing yielded viable seeds with significantly higher mass than all other treatments (C.I. = 95%, p<0.0001, $mean_o$ 28.4 mg ± 6.34 mg). Viable seeds from pollen-supplemented outcrossing (C.I. = 95%, p =0.0154, $mean_{PS}$ =23.7 mg ± 6.19mg) showed significantly higher mass than autogamy (21.8 mg ± 4.83 mg,). Seed mass in pollen-supplemented outcrossing showed no significant difference from within-inflorescence geitonogamy (22.8 mg ± 5.84 mg) or within-tree geitonogamy (23.3 mg ± 6.24 mg) [Figure 9].

The strength of pollination treatment and tree selection as a predictor of seed mass was investigated. Both the pollination treatment as well as the tree on which it was performed had a significant effect on seed mass. The interaction between pollination treatment and tree selection also had a significant effect on seed mass (Table 2).

The model that only includes pollination treatment as a predictor of seed mass suggests that all treatments were significant predictors, with geitonogamy and outcrossing having a significant effect in seeds having a greater mass than seeds of the autogamously selfed treatment. The coefficient estimates in the model also suggests that inflorescence geitonogamy has a smaller effect on seed mass than tree geitonogamy and both types of geitonogamy have a smaller effect than natural or pollen supplemented outcrossing (Table 1). When factoring in tree selection and its interaction with pollination treatment, treatment alone is no longer significant in explaining seed mass among geitonogamously selfed flowers (Table 3). The effects of geitonogamy in yielding higher or lower seed masses than autogamous selfing only becomes significant when significant considered together with the respective tree in which the treatment was performed (Appendix, Table B). An alternative model in which a different reference level for pollination treatment was chosen revealed that, when considered in isolation (without tree selection), autogamous pollination also had a non-significant effect in explaining seed mass (C.I. = 95%, Estimate: -1.8419, $p = 0.4188$, SE = 2.2776, $t = -0.809$). Autogamous and geitonogamous pollination produced significantly low seed mass only when interacting with trees that produced low seed mass across all treatments (Appendix, Table B).

For explaining differences in seed mass of the viable seed cohort, pollination treatment, the tree in which it was performed as well as the interaction between these two variables were significant predictors (Table 4). The model for explaining mass of viable seeds showed similar trends to the model that included non-viable seeds. The effect of geitonogamy (both within inflorescences and within trees) on seed mass was not statistically significant (Table 5). Geitonogamy only became statistically significant in explaining seed mass when it was associated with trees that generally yielded lower seed mass (Table 5). Similarly, autogamous pollination considered without its interaction with tree selection was not a statistically significant predictor of seed mass (C.I. = 95%, Estimate: -0.00433, $p = 0.1080$, SE = 0.00269, $t = -1.609$). Seed mass was better explained by the interaction between the autogamous treatment with certain trees. The outcrossing treatments were significant in explaining seed mass of filled achenes (Table 3) and in explaining seed mass of viable achenes (Table 5). The tendency of outcrossing treatments yielding high seed mass was not largely dependent on the tree in which the treatment was performed. Compared to pollen supplemented outcrossing, natural outcrossing had a slightly larger magnitude of effect on seed mass (Table 5, Table 3).

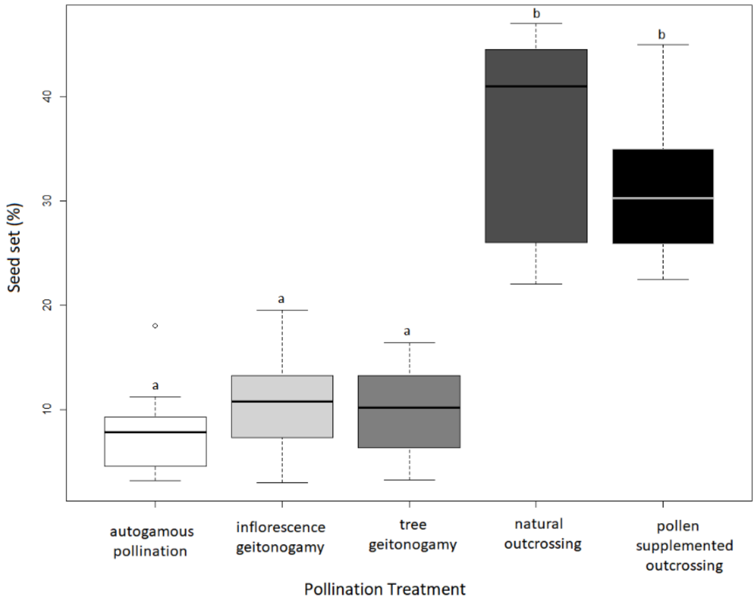

Figure 5: Relationship between pollination treatment and seed set in *Protea curvata*.

Boxes marked with dissimilar letters showed significant difference from each other during multiple (pair-wise) comparisons using Tukey's HSD test. N = 20 trees sampled from Barberton Nature Reserve, Mpumalanga, South Africa. (June – August 2020).

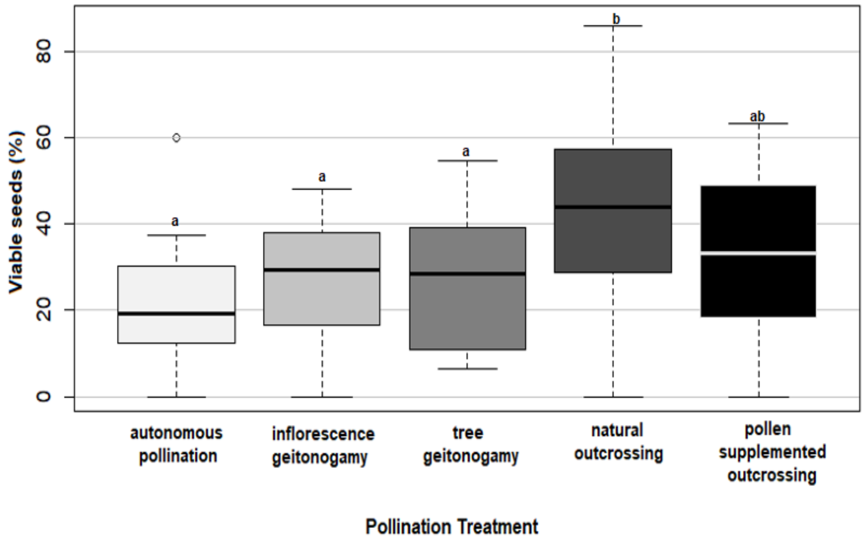

Figure 6: Relationship between pollination treatment and seed viability in filled achenes in *Protea curvata*

"Filled achenes" refers to fruit containing seed. Boxes marked with different letters showed significant difference from each other during multiple (pair-wise) comparisons using Tukey's HSD test (C.I. = 95%). N= 20 trees sampled from Barberton Nature Reserve, Mpumalanga, South Africa (June – August 2020).

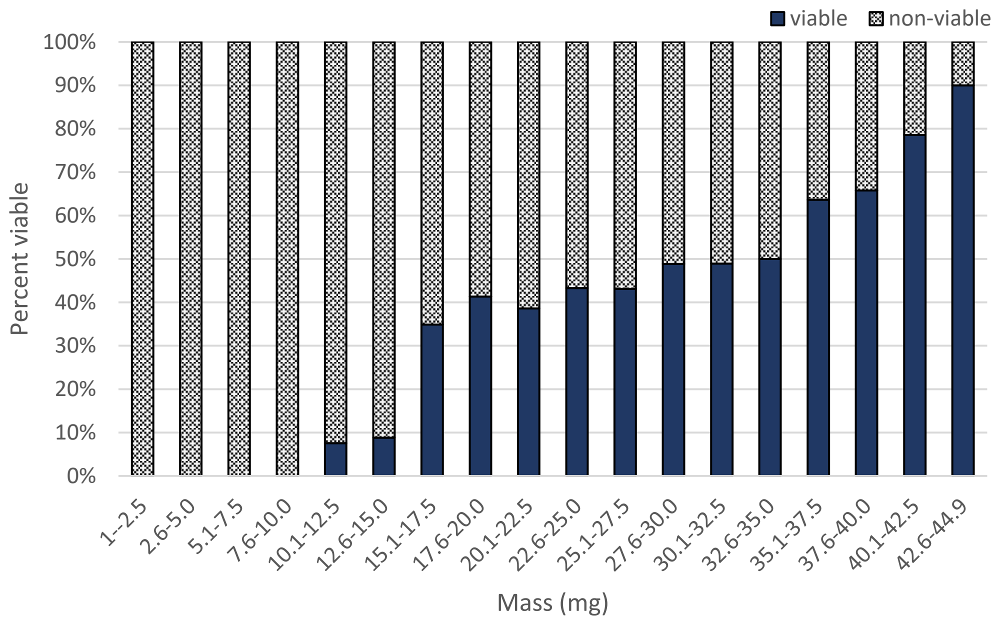

Figure 7: Seed size distributions showing the relationship between seed mass and the percentage of viable seeds per mass class for *Protea curvata*

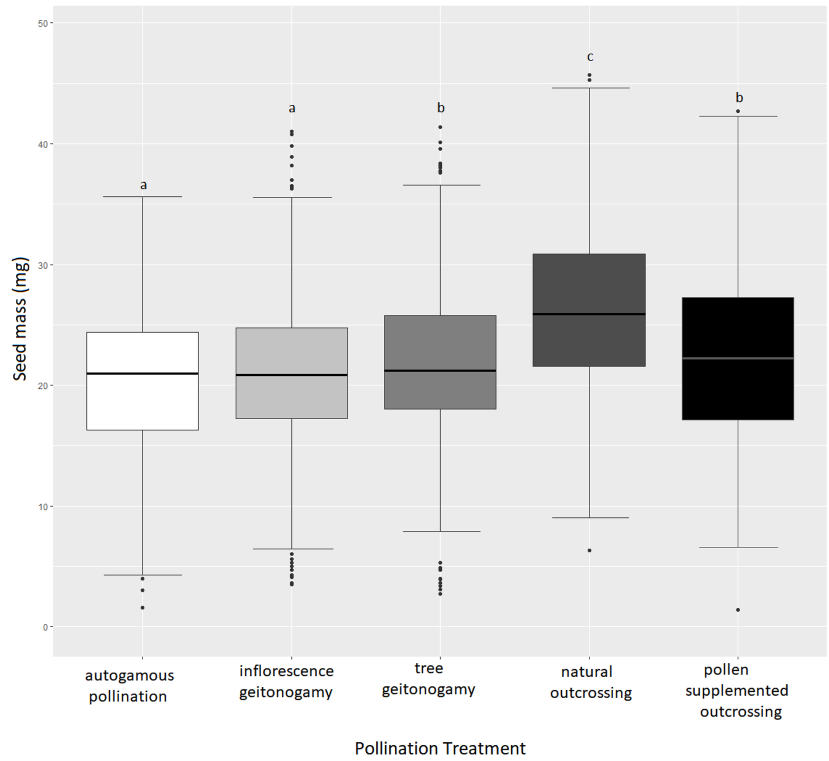

Figure 8: Relationship between pollination treatment and mass of filled achenes in *Protea curvata*. "Filled achenes" refers to fruit containing seed. Boxes marked with dissimilar letters showed significant difference from each other during multiple (pair-wise) comparisons using Tukey's HSD test (C.I. = 95%). N= 20 trees sampled from Barberton Nature Reserve, Mpumalanga, South Africa (June – August 2020).

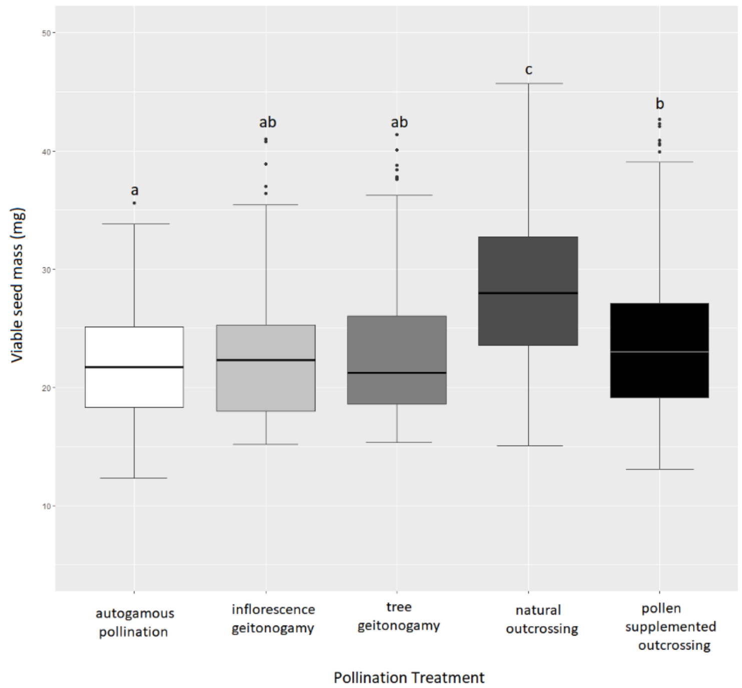

Figure 9: Relationship between pollination treatment and mass of filled achenes with viable seed in *Protea curvata*.

"Filled achenes" refers to fruit which contained seeds and "viable" seed refers to those which had stained embryos during tetrazolium chloride tests. Boxes marked with dissimilar letters showed significant difference from each other during multiple (pair-wise) comparisons using Tukey's HSD test. N = 20 trees sampled from Barberton Nature Reserve, Mpumalanga, South Africa. (June – August 2020).

Table 1: Results from the Gamma distribution generalized linear model for explaining seed mass in *P. curvata* flowers based only on pollination treatment. (C.I. = 95%, link function = inverse)

Source	Estimate	SE	t-value	P value
(Intercept)	49.8204	0.6288	79.227	< 0.0001 ***
Inflorescence geitonogamy (B)	-2.0339	0.9076	-2.241	0.0251 *
Tree geitonogamy (C)	-4.5983	0.8697	-5.287	< 0.0001 ***
Natural outcrossing (D)	-11.7389	0.7810	-15.031	< 0.0001 ***
Pollen suppl. Outcrossing (E)	-5.5368	0.8204	-6.749	< 0.0001 ***

Table 2: Analysis of Deviance Table for pollination treatment and tree interaction in *P. curvata* seed mass.

Source	d.f.	Deviance	Resid.	d.f Resid. Dev	Pr(>Chi)
NULL			2892	352.30	
Treatment	4	25.865	2888	326.44	< 0.0001 ***
Tree	19	95.959	2869	230.48	< 0.0001 ***
Treatment: Tree	76	107.664	2793	122.82	< 0.0001 ***

Table 3: Results from the Gamma distribution model that best explained seed mass in pollinated *P. curvata* flowers based on pollination treatment and tree selection (C.I. = 95%, link function = inverse)

Source	Estimate	SE	t-value	P value
(Intercept)	55.9910	1.4665	38.180	< 0.0001***
Inflorescence geitonogamy (B)	1.8419	2.2776	0.809	0.4187
Tree geitonogamy (C)	4.4886	2.4585	1.826	0.0680
Natural outcrossing (D)	-18.1266	1.8047	-10.044	< 0.0001***
Pollen suppl. outcrossing (E)	-16.2655	1.9044	-8.541	< 0.0001***

Model with pollination treatment, tree selection and treatment:tree selection interaction set as predictor values. Autogamous selfing (Treatment A) and Tree 1 set as the reference levels. Table is truncated to highlight the pollination treatment co-efficients only. The full table of results for the model are given in the appendix wherein the co-efficients of treatment, tree selection and their interaction at each level are shown (i.e., treatment B-E and Tree 2-20)

Table 4: Analysis of Deviance Table for treatment and tree interaction *P. curvata* viable seed mass (C.I. = 95%, link function = inverse).

Source	d.f.	Deviance	Resid.	d.f Resid. Dev	Pr(>Chi)
NULL			948	63.911	
Treatment	4	9.5234	944	54.387	< 0.0001 ***
Tree	19	22.4121	925	31.975	< 0.0001 ***
Treatment: Tree	72	18.1753	853	13.800	< 0.0001 ***

Table 5: Results from the Gamma distribution model that best explained seed mass in viable *P. curvata* seeds based on pollination treatment and tree selection (C.I. = 95%, link function = inverse).

Source	Estimate	SE	t-value	P value
(Intercept)	0.0539	0.001899	28.397	< 0.0001 ***
Inflorescence geitonogamy (B)	0.0046	0.002693	1.609	0.1080
Tree geitonogamy (C)	0.0046	0.003122	1.485	0.1379
Natural outcrossing (D)	-0.0169	0.002127	-7.947	<0.0001 ***
Pollen suppl. outcrossing (E)	-0.0149	0.002414	-6.199	<0.0001 ***

Model with pollination treatment, tree selection and treatment:tree selection interaction set as predictor values. Autogamous selfing (Treatment A) and Tree 1 set as the reference levels. Table is truncated to highlight the pollination treatment co-efficients only.

Insect Pollination

During field observations of 68 inflorescences between 09:00 and 15:00, a total of 458 insects were observed over the course of three days. The most frequent visitors of *P. curvata* were insects in the Apidae and Figitidae families ($\bar{x} = 5.74$ and $\bar{x} = 4.58$ visits per 5-minute period respectively). The number of Apidae visits was significantly higher than visits from all families except Figitidae and Formicidae (Kruskal-Wallis $\chi^2 = 39.085$, d.f. = 8, p-value < 0.05). Conopidae, Calliphoridae and Vespidae had a low average number of visits per 5-minute observation period. Colletidae averaged 1 visit per 5-minute period. The Chrysomelidae and Lepidoptera insect taxa had even fewer visits – each visiting only once over the entire sampling period (i.e., between 09:00 and 15:00 for 3 days) [Figure 10].

During camera observations, pictures and 10 second videos were recorded at 15-minute intervals over the course of 11 days. Camera and field observations showed similar patterns in terms of which insect families frequented *P. curvata* the most. Members of Apidae and Figitidae were the most common visitors. Conopidae, Calliphoridae and other fly families could not be properly distinguished on camera but were still less common than Apidae and Figitidae when grouped at order level (i.e., as Diptera). The cumulative observation period was 991 hours once recordings of inflorescences not in full view were excluded. Inflorescences with longer observation periods did not consistently have a higher number of insect visits. Rather visits were more frequent to inflorescences that were already in flower when first recorded and inflorescence buds that bloomed soon after the initial observation.

Ants (Formicidae) had the longest visit duration (Kruskal-Wallis $\chi^2 = 66.228$, d.f. = 8, p-value < 0.0001). However this did not translate into more pollination. During field observations, ants remained at the base of the inflorescence, presumably for nectar. On camera, ants were frequently situated near the base on the tepals of open flowers (Figure 15) . They were occasionally seen crawling from branches into the base, but were concelaed by bracts once inside the inflorescence. Colletid bees had the second longest visit duration and often made contact with the styles and stigmas of *P. curvata*. Apid bees had far shorter visits but often made contact with the stigmas during field observations. Camera observations showed that Apidae, Colletidae and Figitidae were the insect taxa that made the most contact with the stigmas and the pollen presenters of *P. curvata*.

While camera traps in this study were set up for the purpose of recording insects, visits from other animals were also captured. The most common vertebrate visitors were birds and were mostly the same species as those observed in an earlier study (Mabuza, 2017) [Table 4]. For the first time, bats were seen visiting *P. curvata.* Two bat visits were captured at 01:51 a.m. and 02:15 a.m (Figure 17). Both visits were brief (less than 10 seconds), and the bats appeared to be feeding on nectar. A lizard was seen manoeuvring around the base of a *P. curvata* inflorescence at 12:59 p.m., presumably feeding on nectar or searching for insects within the inflorescence (Figure 17). The visit lasted ~4 minutes.

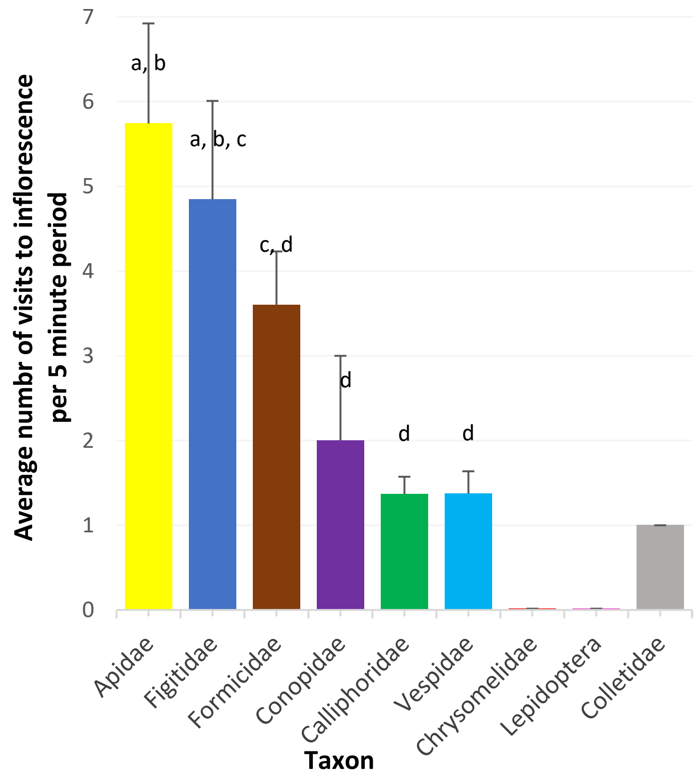

Figure 10: Frequency of insect visits to *P. curvata* inflorescences observed in the field

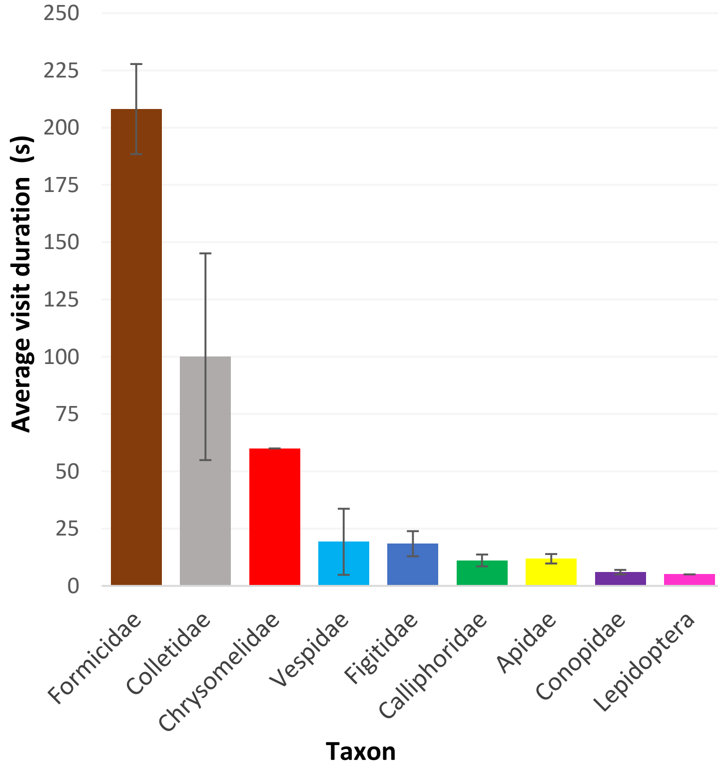

Figure 11 Insect visit duration on *P. curvata* inflorescences observed in the field

Visit frequency and duration were recorded for *Protea curvata* inflorescences at Mundt's Concession ("*Site B1*"), Mpumalanga South Africa. One inflorescence was observed from a different tree every five minutes. N= 68 inflorescences observed for 5 minutes each

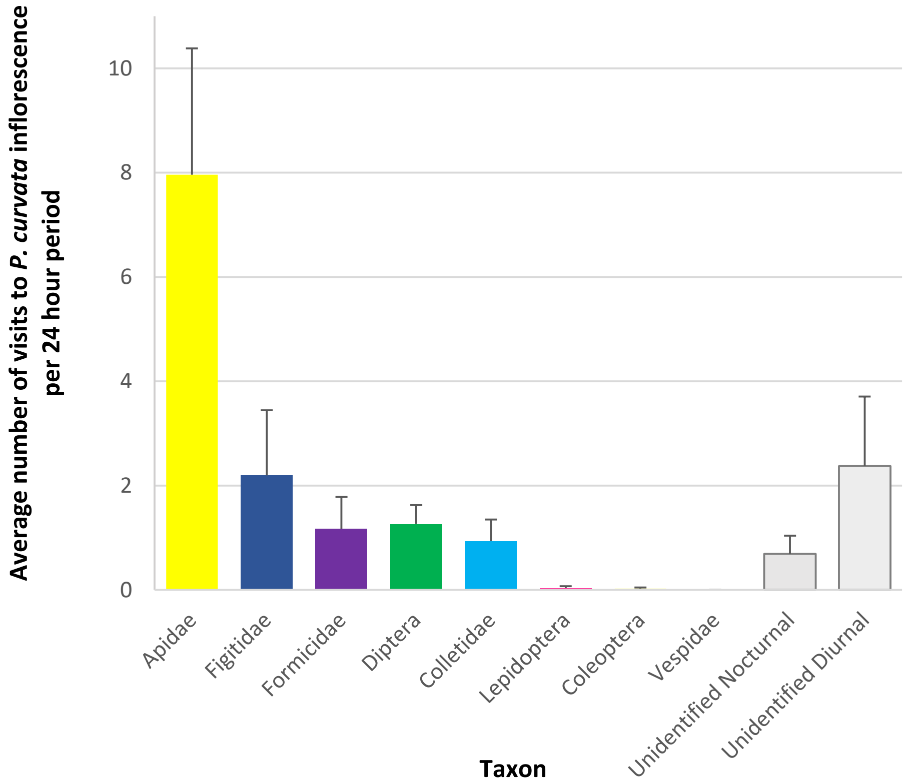

Figure 12: Frequency of insect visits to *Protea curvata* inflorescences observed via camera traps at Barberton Nature Reserve ("*Site A*"), Mpumalanga, South Africa

Insects observed via camera traps over the course of 11 days. N = 5 inflorescences

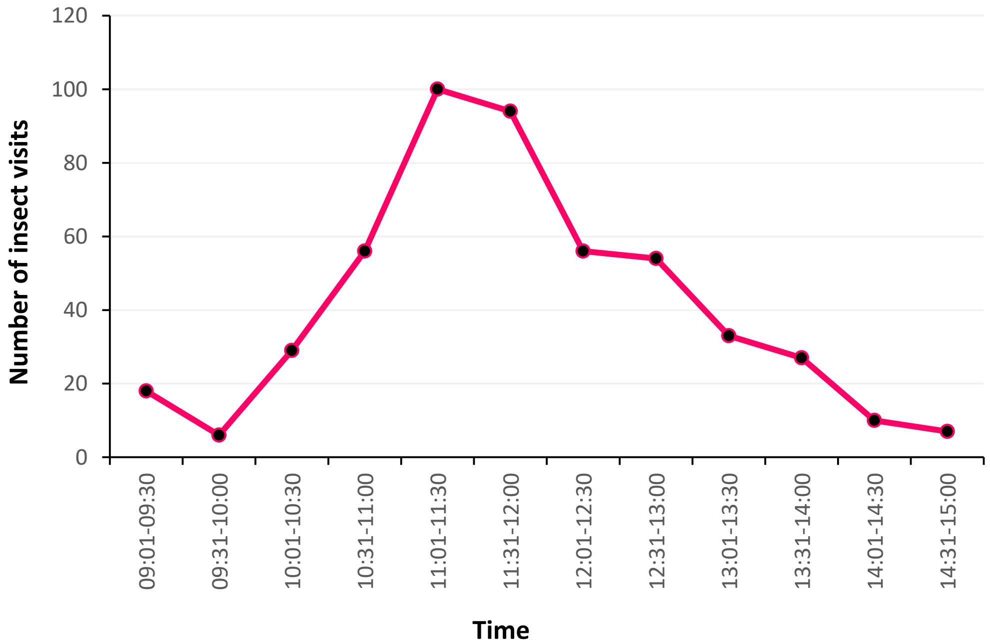

Figure 13: Timing of insect visits on *Protea curvata* inflorescences during field observations at Mundt's Concession ("*Site B1*") in Mpumalanga, South Africa

Field observations between 9:00 and 15:00 over the course of 3 days N= 68 inflorescences observed for 5 minutes each

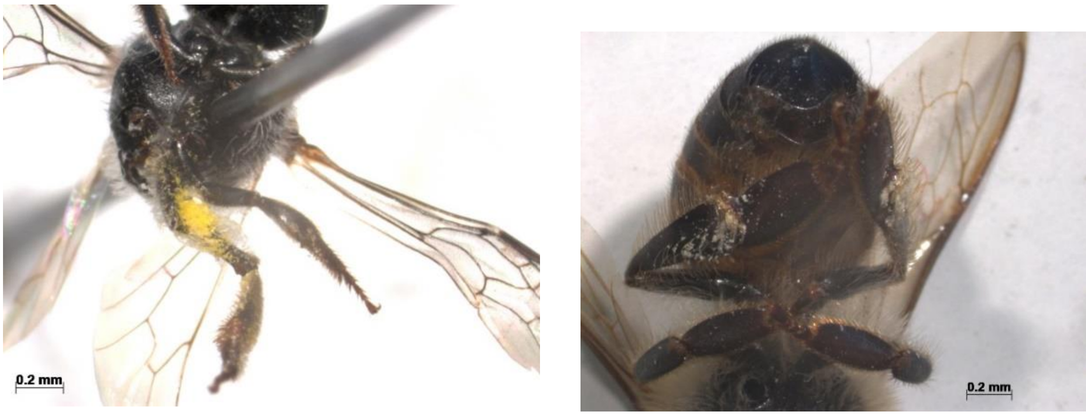

Figure 14: *Hyalinus sp*. and *Apis mellifera* with pollen. Insects were collected during field observations and micrographed under a dissecting microscope.

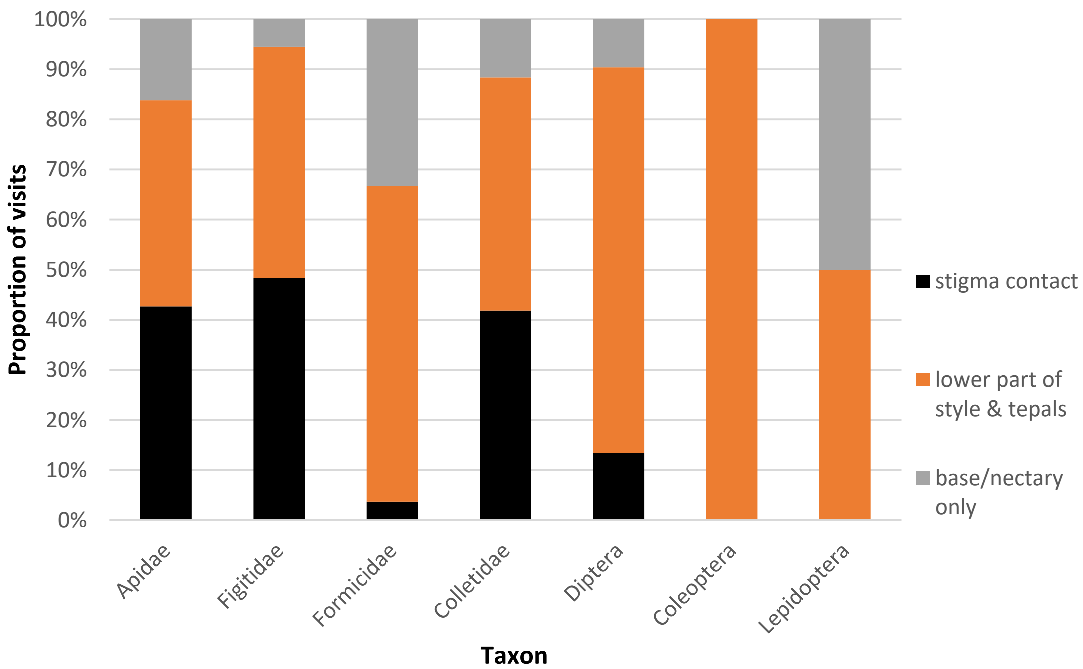

Figure 15: Behaviour observed in insects that visited *P. curvata* inflorescences in Barberton Nature Reserve, Mpumalanga South Africa. Insects observed via camera traps over the course of 11 days. N = 5 inflorescences

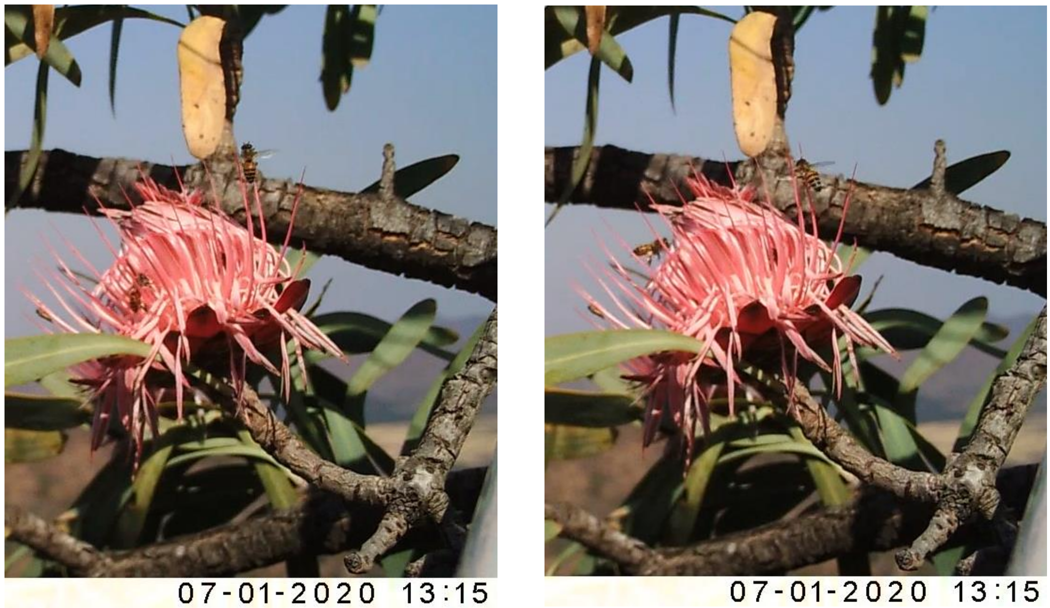

Figure 16: Bees visiting *Protea curvata* inflorescences in Barberton Nature Reserve, Mpumalanga, South Africa.

Figure 17: Bat and lizard visits recorded on *P. curvata* inflorescences in Barberton Nature Reserve, Mpumalanga, South Africa.

Table 4: List of identified insects genera visiting *P. curvata* flowers

Family	**Species and/or Genus**
Apidae	*Apis mellifera*
Apidae	*Xylocopa caffra*
Figitidae	*Pycnostigmus*
Colletidae	*Hyalinus*
Calliphoridae	*Calliphora*
Formicidae	*Polyrachis*
Formicidae	*Camponotus*
Formicidae	*Lepisiota*
Vespidae	*Belonogaster*

Table 5: List of vertebrate visitors to *Protea curvata* observed via camera traps in Barberton Nature Reserve, Mpumalanga, South Africa).

Observed in 2017	**Observed in 2020**
Cape weaver *(Ploceus capensis)*	Cape weaver *(Ploceus capensis)*
Amethyst sunbird *(Chalcomitra ametystina)*	Amethyst sunbird *(Chalcomitra ametystina)*
Cape white-eye *(Zosterops virens)*	Cape white-eye
Scarlet-chested sunbird *(Chalcomitra senegalensis)*	Scarlet-chested sunbird *(Chalcomitra senegalensis)*
Dark-capped Bulbul *(Pycnonotus tricolor)*	Dark-capped bulbul *(Pycnonotus tricolor)*
Rock sparrow *(Petronia petronia)*	Rock sparrow *(Petronia petronia)*
Greater double-collared Sunbird *(Cinnyris afer)*	Greater double-collared Sunbird *(Cinnyris afer)*
Cardinal woodpecker *(Dendropicos fuscescens)*	Fruit Bat (Species not identified)
Square tailed drongo (*Dicrurus ludwigii)*	Lizard (Species not identified)
Fork tailed drongo (*Dicrurus adsimilis*)	

Cameras set up in 2017 captured photos using a motion sensor (Mabuza, 2017). Cameras set up in 2020 captured both photos using a motion sensor and recorded 10 second videos every 15 minutes.

Discussion

Our use of hand-pollination to in autogamous selfing treatments appraises autogamous pollination, but not autonomous self-pollination (also termed "spontaneous autogamy" [Ehlers & Pedersen, 2000]). In other words, the method determines if self-pollinated flowers can set seed but does not necessarily demonstrate how frequently *P. curvata* self-pollinates in the absence of pollinators. A possible method for testing autonomous selfing involves covering inflorescences and leaving them unmanipulated (Lamont, 1985; Hargreaves *et al.,* 2004; Steenhuisen and Johnson, 2011). While it is preferable to do both methods (unmanipulated and hand-pollinated), there was a limited number of inflorescences available for testing. The study was primarily concerned with investigating the ability to set viable seeds from autogamously self-pollinated flowers, since this had yet to be determined for this species. It was also important to ensure the autogamous selfing treatment was similar to the others in terms of pollen load and timing of pollination. This way differences in seed set and viability can be interpreted as a consequence of factors other than pollen load. Furthermore, unmanipulated inflorescences do not necessarily exclude the possibility of geitonogamy occurring (e.g., via wind transferring pollen to adjacent inflorescences). Manual self-pollination was therefore prioritized over unmanipulated inflorescences.

P. curvata, like many *Protea* species, is protandrous. Pollen can develop and be removed from the style by pollinators before the stigma becomes receptive (Carolin, 1961). Our results suggest that in the hand-pollinated autogamous selfing treatment, self-pollen remained viable long enough to pollinate the stigma once it became receptive. Longer periods of pollen viability can have positive implications for protandrous inflorescences. Similar to other *Proteas*, anthesis is centripetal in *P. curvata*, with the outer flowers opening first (Collins & Rebelo, 1987; pers. observ.). If pollen from the outer flowers of the inflorescence remains viable while anther dehiscence begins in the innermost flowers, receptive stigmas of outer flowers can be successfully pollinated by both their own pollen and newly released pollen from inner flowers. In the absence of animal pollinators which remove self-pollen, the pollen from inner and outer flowers could potentially translate into a higher pollen load for geitonogamous selfing within the inflorescence.

If natural pollinators are available, self-pollination may be reduced by a period of protandry, wherein pollinators can pick up pollen from pollen presenters (Carolin, 1961). Cross pollination is then facilitated by long periods of pollen viability which provide pollinators with a wide enough time window to transfer the pollen to another plant. As such, *Protea curvata* likely undergoes facultative self-pollination in natural settings. This is supported by the viability results in which autogamous self-pollination showed some viability (21.95%), while natural outcrossing showed higher viability (42.65%) than other pollination modes. In other words, when natural pollinators were available, *P. curvata* was able to set seed via outcrossing or a combination of outcrossing and selfing. Seed viability among selfed flowers (21.95%) was approximately half that of naturally outcrossed flowers (42.65%). This viability ratio resembles that of seed set in selfed and naturally outcrossed *P. eximia* (Knight) Fourc. flowers – although data on viability ratios was unavailable (Collins & Rebelo, 1987; Horn, 1962). Moreover, the ratio suggests a predominance of xenogamy in *P. curvata*, especially considering the tendency of autonomous selfing to set less seed than hand-facilitated self-pollination (Hargreaves *et al*., 2004; Melidonis & Peter, 2014)

Other *Protea* species which have shown overlap in periods of stigma receptivity and self-pollen viability include *P. simplex* E.Phillips., *P. caffra* Meisn*., P. welwitschii* Engl. and *P. dracomontana* Beard*.* In these species, protandry allowed for a brief period of pollen presentation without self-pollination, but by the time stigmatic grooves opened (~72 hours after anthesis) self-pollen was still viable. Similar trends were noted in *Banksia prionotes*, *Protea repens* (L.) L. 'Sneyd' and *P. eximia* (Salis. ex Knight) Fourc. and *Telopea speciosissima* R.Br. (Collins & Spice, 1986; van der Walt & Littlejohn, 1996a; Offord, 2004) – all of which belong to Proteaceae.

Although TTZ staining is a good method for relative comparisons, it is rarely an absolute predictor of germinability or seedling success. Seed mass can improve predictions on reproductive success. For example, major nutrient content in *Banksia cunninghamii* seeds increased proportionally or more than proportionally with an increase in seed mass. Germination was not greatly affected, but the effects of seed mass on seedling health were pronounced. Seedlings from large seeds were larger and more adept for survival in nutrient deprivation experiments (Vaughton and Ramsey, 2001). In terms of *P. curvata* seed viability, all three types of self-pollination appeared to have similar reproductive success. However, when modelling seed mass, within-tree geitonogamy differed from within-inflorescence geitonogamy and autogamous selfing. The interaction of pollination treatment and tree selection improved the modelling of patterns in seed mass across self-pollination and cross pollination treatments. Moreover, viable seed mass among selfed flowers tended to be significantly low only when associated with trees that generally had a negative impact on seed mass. Therefore, the type of self-pollination alone is not a strong predictor of seed mass. Rather the tree in which it occurs is a stronger predictor. As such, we can deduce that self-pollination's impact on reproductive success is highly dependent on the health or genetics of the tree in which it occurs.

It is unsurprising that seed mass and viability were so similar for autogamous selfing and within-inflorescence geitonogamy since they are considered to effectively be the same at the genetic level (Schoen & Lloyd, 1992). Surprisingly, within-tree geitonogamy had higher seed mass despite being genetically equivalent to autogamous and within-inflorescence selfing (Les, 1988;). One explanation for this is the floral arrangement of *P. curvata.* Flowers are borne on inflorescences. Therefore, flowers within an inflorescence are likely to have similar health and be in similar stages of development compared to flowers on other inflorescences or inflorescence buds. For tree geitonogamy, flowers share genes of the same tree, but resource allocation and developmental stage for each inflorescence may differ (Fuss & Sedgely, 1990; Hoffman, 2006; Smart, 2012). This includes developmental stage and viability of pollen (Carrizo García *et al*., 2017). Self-pollen applied on autogamous and within-inflorescences pollination treatments may have been at similar stages since its application was confined temporally by stigma receptivity. Flowers typically showed stigma receptivity 2 – 3 days after flower opening. On the other hand, pollen for tree geitonogamy could be collected from flowers that had opened less 2 days ago since fresh pollen was readily available in the mesh enclosed inflorescences. Generally, pollen viability gradually declines over time once pollen presentation occurs (Bo and Carrizo García, 2015), Vesprini and Pacini; 2005; Azimi-Motem et al. 2008). Tree geitonogamy introduces greater variation with regards to floret maturation, which affects timing of pollen presentation (Bäurle and

Dean, 2006; Giakountis and Coupland, 2008, Wilson & Zhang, 2009), as well as the duration that pollen grains are exposed, which can affect pollen viability (Hong *et al*., 1999).

Health may also be less aggregated at the tree level than at the inflorescence level. For example, the effects of insect larvae and pathogens are more likely to be pronounced in the inflorescence they occupy before being detectable in other inflorescences on the tree. Effects of pollen thieves in an inflorescence will also vary depending on whether the inflorescence serves as a pollen donor or recipient in a certain pollination event. While flowers within an inflorescence are derived from inflorescence meristem (IM) originating from the shoot apical meristem (SAM) of one stem, inflorescences within the tree originate from the SAM of different stems (Benlloch *et al.*, 2007). Therefore, tree geitonogamy could potentially yield more genetic variety than other types of selfing. This is because mutations occurring in SAM tissue, albeit seldom, could result in slight genetic differences between inflorescences of the same tree (Jack, 2004; Blazquez *et al.*, 2006; Benlloch *et al.*, 2007).

Natural outcrossing proved to be the most successful mode of pollination – even more successful than hand-facilitated outcrossing. This was also the case in a study on *P. humiflora* and the researchers suggested that either the mesh enclosures hindered seed set or the hand-pollination was not effective in placing pollen along the stigmatic groove (Wiens *et al.,* 1983). The former reason likely does not apply to *P. curvata* because neither of our outcrossing treatments were covered. Therefore, as suggested by Wiens *et al.* (1983) the challenge of accurately depositing pollen into the stigmatic slit may have been one of the limiting factors in hand-pollinated treatments. However, given the variation in pollen morphology and size between *Protea* species (van der Walt & Littlejohn, 1996b), it is unclear whether the ease of pollen deposition into stigmatic slits differs significantly between species and how much this would offset pollination success in hand-pollinated flowers. It is also difficult to verify during field experiments whether the pollen being applied has entered the stigmatic slit since this is typically best visible under scanning electron microscopy (Wiens *et al*., 1983). Nevertheless, pollination exclusion and hand-pollinated experiments remain a widely used method for testing pollination modes (). It may thus be of interest for future studies to include a hand-pollinated outcrossing treatment in addition to natural outcrossing and pollen-supplemented hand-pollination. For this study of *P. curvata* pollination, differences in pollen dispersal may explain seed mass in pollen supplemented outcrossing being significantly lower seed mass than natural outcrossing. Although flowers for pollen supplemented treatments were collected from plants that were not in the immediate vicinity of the recipient plant, the variety and distance was limited by how many flowers could be safely carried around in the field (since pollen-loaded styles from different plants were propped on a Styrofoam platform inside a plastic container). This contrasts with natural pollinators such as birds, which can pick up pollen from one inflorescence and fly long distances before depositing pollen onto another inflorescence (Thavornkanlapachai *et al.,* 2018).

Flowering is beneficial for the attraction of pollinators and reproductive capacity of a plant (Schmitt *et al.,* 1987; Goldingay & Whelan, 1993; Lazaro *et al*., 2008; Delnevo *et al.,* 2019). Inaccessible inflorescences can sometimes be more attractive than uncovered inflorescences due to their accumulation of nectar and pollen (Wiens *et al.,* 1983). Many bees would often gather around our enclosed inflorescences while uncovered inflorescences only had a few visitors at a time.

Apidae (bees) and Figitidae (parasitoid wasps) were the main insect families that visited *P. curvata*. They had the highest frequency of visits and interaction with pollen presenters, compared to other insects. Although ants had significantly longer visits, they spent most of their time sheltering themselves in the base of inflorescences and consuming nectar. The short visit duration of bees can be attributed to their frequent movement to other inflorescences in the tree or neighbouring trees (pers. observ). For this reason, it is unlikely that bees contribute genetically diverse pollen to stigmas. Camera observations showed that bees were the earliest visitors of newly opened flowers that had an abundance of self-pollen on stigmas. This is different from insects of the Diptera taxon, for example, which would mostly consume pollen near the bottom of the style and residual pollen on the tepals of older flowers. They act more as pollen thieves. Contrastingly, bees and parasitoid wasps can be considered to be pollen removers that limit autogamous selfing in *P. curvata* by collecting self-pollen prior to stigma receptivity. Colletid bees appear to play a similar role. They were less frequent visitors than Apid bees or parasitoid wasps but spent a similar proportion of their visits making contact with stigmas. Of the insects collected from *P.*

curvata inflorescences, Colletid bees had the most pollen on their bodies, although the composition of the pollen was not determined. During field observations they spent the longest time interacting with flowers within a single inflorescence (and not the base). Based on our observations Colletid bees, Apid bees and parasitoid wasps are likely geitonogamous pollinators of *P. curvata*. Their efficiency as pollinators could be further explored by assessing pollen composition and pollen deposition on *P. curvata* stigmas by these taxa. Birds likely deposit more genetically diverse pollen on *P. curvata* stigmas, leading to successful outcrossing.

Although they were less frequent visitors, bats may play a role in outcrossing. The behaviour of bats in this study resembled the behaviour of bats in a pollination study of the neotropical Proteaceae, *Oreocallis grandiflora* (Cárdenas *et al.,* 2020). The bats hovered in front of inflorescence, making brief contact and quickly flying away. Hummingbirds had a higher visitation rate and contacted more *O. grandiflora* stigmas per visit. However, bats deposited more pollen on stigmas, despite their quick and infrequent visits (Cárdenas *et al.,* 2020). This efficiency of bats in pollen transfer is attributed to their fur being able to carry more pollen than bird feathers (Muchhala & Thomson, 2010). The study of *O. grandiflora* demonstrated an additive effect of birds and bats, whereby pollination success was two times higher when bats and hummingbirds were included than when bats or hummingbirds were the sole pollinators. *O. grandiflora* was able to self-pollinate in the absence of pollen removal. Insects were infrequent visitors of *O. grandiflora* and not considered pollinators (Hazlehurst *et al.,* 2016; Cárdenas *et al*., 2017). Mice were also among the infrequent visitors. Their fur did not contribute much to pollen deposition since they spent most time in the centre and base of inflorescences, managing to avoid contact with stigmas (Cárdenas *et al.,* 2020). This resembles the behaviour of the lizard seen visiting *P. curvata*. Based on our findings, *P. curvata* can be considered as primarily bird pollinated, with insects and bats having a functionally complementary role. Insects seem to be important in removing self-pollen prior to stigma receptivity. Birds and bats aid in carrying genetically diverse pollen, with birds potentially depositing pollen more frequently and bats potentially depositing pollen more abundantly.

Conclusion

The findings of this study point to natural outcrossing as the pollination mode which yields the most viable seeds and highest seed mass. Seed set and seed viability from hand-pollinated outcrossing was similar to natural outcrossing and hand-pollinated had similar viability, but hand-pollinated outcrossing showed lower seed mass. The lower quality of these seeds was likely due to not using a wide range of outcross pollen. Cross pollination by hand may only be adequate as an interim solution while site managers determine ways to support natural pollinators on the site. Conservation plans should therefore include addressing the annual declines noted in the flowering of one of the subpopulation (Chapter 2). To encourage the abundance of natural pollinators of *P. curvata*, site managers may need to consider additional issues not only relating to *P. curvata* flowering. This may involve looking at the foraging and nesting habits of the pollinators identified in this study and assessing how well these can be accommodated within the site. In the same vein, site managers are cautioned against inadvertently compromising pollinators during efforts to address other conservation issues. The role of bees in *P. curvata* pollination, for instance, warrants the avoidance or conservative use of pesticides and herbicides that may negatively impact bees.

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Chapter 6: Concluding Discussion and Management Plan

The findings of this study allowed for comparison to plants of the same family and post-fire regeneration strategy as *Protea curvata* (Chapter 4). Proteaceae which can resprout are hypothesized to not be serotinous due to a lower dependence on seeds for post-fire regeneration (Bond, 1985; Zammit, 1987). This proved to be the case in *Protea curvata* which displayed epicormic resprouting and no serotiny (Chapter 4). Resprouters are also generalized as being self-incompatible and/or showing low seed set and viability after being outcrossed (Carpenter & Recher, 1979; Lamont & Wiens, 2003). Contrastingly, *P. curvata* was self-compatible and had a substantial percentage of viable seeds from outcrossing (Chapter 5).

Both bird and insect pollinators were observed visiting *Protea curvata.* The interaction of these two pollinator types was efficient, as evinced by high pollination success in naturally outcrossed inflorescences. Naturally outcrossed inflorescences yielded higher seed viability than autogamously or geitonogamously selfed flowers. Seed viability was not higher in pollen supplemented plants. *Protea curvata* seed set was therefore not limited by pollinator or pollen availability (Chapter 5). Although results from a small study of pollination modes in *P. curvata* had already suggested the possibility of selfing in *P. curvata* (Mabuza, 2017), the experimental design of this study provided information on the differences in seed quality (mass and viability) between various types of selfing and outcrossing. While differences in the quality of seeds from tree geitonogamy, inflorescence geitonogamy and autogamous selfing were indiscernible in the small study, they were identified in the fifth chapter. This was in part due to large sample sizes. In breeding system experiments, sample sizes that are adequate for publication range from 5–10 plants or 10–20 inflorescences. Each treatment can have 5–300 flowers depending on the treatment i.e., how practical it is to simulate the particular mode of pollination in the field (Steenhuisen and Johnson, 2012, Gross & Caddy, 2006). In this study, pollination modes were tested within 20 trees, each with at least five inflorescences (this excludes the number of trees sourced for outcross pollen).

This served as the first study to sample *P. curvata* in six localities. Moreover, it is the first study to sample a *P. curvata* subpopulation for three consecutive years, thus improving deductions about population growth or decline over time (Chapter 1). Of the six sites in which *P. curvata* was surveyed, the short distance between two sites and the similarities found in the demographics of those *Protea* trees warranted treating them as one subpopulation (Chapter 1). In the future, attempts to mark out either of these two sites (*Site B1* and *B2*) for formal protection should include both sites so as not to further fragment them. Only two subpopulations (*Subpopulation C* and *Subpopulation E*) showed recruitment (Chapter 1). Comparison of woody vegetation on two sites with dissimilar population demographics and site management brought understanding on how woody plant density affects *P. curvata* recruitment (Chapter 3).

Overall, four key intervention points were identified as imperative for *Protea curvata* regeneration. These are (i) to reduce woody plant density and invasive species on sites with *P. curvata*, (ii) to increase flowering and/or seed set in subpopulations affected by hail (iii) to minimize damage to *P. curvata* bark as well as reduce *P. curvata* mortality, (iv) to enrich the soil seed bank by facilitating seed deposition and reducing seed predation.

Protea curvata seedlings were unable to establish on *Site A,* which had a woody plant density of 2672 trees.ha^{-1}. *Protea curvata* seedlings were present on *Site C* where woody plant density was 288 trees.ha^{-1} (Chapter 3). *Protea curvata* recruitment on *Site C* was not enough to offset the mortality rate. Both sites' recruitment rates were below replacement, resulting in a 3% and 2% annual loss for *Subpopulation A* and *Subpopulation C* respectively (Chapter 1). Therefore, both sites are in need of a management strategy that enables the recruitment and long-term survival of *P. curvata* trees. Large trees (over 400 kg in biomass and 6-10 m tall) made up the minority of dead trees *in Subpopulations A, B, C*, and *E*. Most dead trees were relatively young (under 300 kg and shorter than 4 m). This indicated that a large proportion of mortality in *P. curvata* trees was not due to senescence (Chapter 1). All trees showed extensive bark damage. Damage was more prominent in *Subpopulation A,* which experienced more intense fires and a recent hailstorm. Bark damage in *Subpopulation A* and *Subpopulation C* was not restricted to heights of the fire zone (Chapter 2). This suggested that most bark wounds did not form immediately after fire. Instead, internal heat damage to the stem likely resulted in bark being vulnerable to secondary damage (e.g., insect damage), thus forming scattered wounds. Moreover, observations immediately after disturbances revealed the variety of wound appearances as well as the ability of hail to inflict secondary damage on previously burnt or peeled bark. Overtime extensive damage to the tree stems and their xylem tissue can result in tree mortality (Tyree *et al.*, 1994; Rood *et al*., 2000; Sperry *et al.,* 2002 Michaletz *et al.,* 2012). Damage from fire and hail disturbances was attributed as the primary driver of premature mortality in *Protea curvata* (Chapter 2).

Hail resulted in an immediate reduction in flowering by damaging inflorescences. Flowering was lowest a year after the hailstorm (Chapter 2). This appears to be a result of defoliation and branch breakage during hailstorms. Primordia of the next flowering season's inflorescences are lost as a consequence of branches breaking off. Additionally, the shoot structure and conducting tissue required to support inflorescences as large as those of *P. curvata* often requires secondary growth. This likely delays a return to prolific flowering while resources are directed towards shoot growth. (Bond & Midgley, 1988; Le Maitre & Midgley, 1991).

Protea curvata was found to have a short-lived soil seed bank in which the viability of year-old seeds showed less than half the viability of freshly collected seeds from the canopy. Seed bank size was surprisingly not reflective of inflorescence availability in previous seasons. The largest seed bank of the six sites that were studied did not belong to a subpopulation with the most floriferous trees (*Site D*) or to the site with the most inflorescences per unit area (*Site B1*). Curtailing seed predation or decay is therefore important for reducing the wastage of seeds set by floriferous subpopulations (Chapter 4). Both seed bank size and seedling recruitment were highest in the subpopulation with lower woody plant density (Chapter 4, Chapter 1, Chapter 3). This reiterates the importance of bush control in facilitating seed deposition.

The following management plan aims to address the aforementioned issues and provide a measurement standard to evaluate the success of intervention measures. The evaluation system in Table 1 outlines possible management outcomes and scores them based on how favourable they are to *P. curvata* subpopulations. Since none of the sites are research stations, it might not always be possible to have ecological experts conducting long-term research on *P. curvata* and other plants in the area. Therefore, the purpose of this evaluation system is to embed scientific rationale into a scorecard that landowners/site managers can employ during basic vegetation surveys. The scorecard does not require the user to perform extensive statistical calculations, making it more accessible. Guidelines are also provided on how to interpret the final score. Management recommendations and the rationale behind each category of evaluations is discussed below.

Bush encroachment appeared to be the main driver of differences in *P. curvata* recruitment at different sites, and invasive species were not the largest contributors to tree density. At sites where woody vegetation was surveyed, dominant species were a mixture of palatable, unpalatable and spinescent, indigenous species. Within *Site C, V. davyi* showed the highest recruitment, but *P. curvata* seedlings were still able to establish on the site. Furthermore, absolute density of *V. davyi* was similar on *Site A* and *Site C* sites and was among the highest densities observed for all woody species. The basis for limiting the dominance of this species on both sites is to keep the site accessible to browsers rather than allowing dense thickets to form. On *Site A*, *S. pentheri* appeared to provide the highest competition for *P. curvata* seedlings. The density of *S. pentheri* was far higher at *Site A* than at *Site C*. Therefore, a general reduction in *S. pentheri* density could help *Protea* recruitment on *Site A* to resemble the recruitment noted on *Site C.* Likewise, the low recruitment observed in *Subpopulation B* and *Subpopulation D* may be addressed via bush control.

Woody plant density should be controlled primarily using bush clearing in conjunction with fire. Woody plant density was drastically high within *Site A* relative to the woody plant density observed within *Site C*. Consequently, *Site A* and other dense sites with *P. curvata* subpopulations will benefit from extensive bush clearing. *Protea curvata* stems and flowering were negatively impacted by vegetation that increased scorch heights (Chapter 2). Bush clearing will therefore aid in reducing wood density and in limiting the damage cause by subsequent fires. Fire generally shapes savannas (van Langevelde *et al.,* 2003) and fire regimes were the strongest difference in the management of the two sites. For *Site A,* an increase in fire frequency is recommended. Initially, this should involve applying different fire treatments to blocks of land. The woody plant density and cover on each block should be measured before and after fire. Treatments which yield results closest to the woody plant density and woody cover (per hectare) found in our study of *Site C* should be considered conducive for *P. curvata* seedlings. Treatments resulting in high *P. curvata* mortality should be considered unfavourable. The optimal fire treatment will be one which decreases bush encroachment and eradicates invasive species without significantly increasing *P. curvata* mortality. Fire conditions of the optimal treatment should then be repeated at least twice within a 6-year period. Thereafter, at least one transect on the site should be sampled before fire, immediately after fire and a year after to monitor fire effects on *P. curvata*. This will enable monitoring of seedling establishment, number of trees lost over time as well as the demography of trees most affected by the fire.

Since *P. curvata* has experienced a great deal of disturbance over the years (severe hailstorms and intense fires after long fire intervals), it is expected that tree mortality after the shift in fire regimes will initially remain as high as it was before. This is due to cumulative damage and low resilience (Chapter 2), as well as the subpopulation being made up mostly of older trees – thus higher incidence of senescence is expected (Chapter 1). It is recommended that Barberton Nature Reserve establish its own frost record and place weather monitoring devices as close to *Site A* as possible. Site managers will then be able to capture weather events affecting the region as well as those unique to the microclimate of *Site A*'s valleys. Similarly, frost occurrence should be recorded for *Site C*. Continuing the record of *P. curvata* flowering established by our study is highly recommended. At the very least, where resources are limited, sampling of one transect should be conducted to keep track of flowering. For all sites, flowering will be important for pollinator attraction and seed input (Chapter 5). However, it is particularly important that the already low recruitment observed on *Site A* is not exacerbated by a lack of inflorescences able to set seed. Flowering after 2017 was significantly reduced by hail on *Site A* (Chapter 1, Chapter 2). Accordingly, the scoring system took into account pre-hail flowering and how slow recovery can be after extreme disturbances (Dowson, 2009). To estimate what might be in the healthy range for each subpopulation, previous years' flowering was considered, as well as comparisons of flowering in other sites. Population demographics were also considered. *Site A* was made up entirely of adult trees, whereas *Site C* had seedlings. Therefore, slightly lower numbers of flowering in *Site C* could be attributed to the presence of juveniles that have yet to reach reproductive maturity. In light of these demographics and *Site A*'s lack of recruitment, the penalty for low flowering on *Site A* was slightly higher than for *Site C*. Where sites receive a penalty of -1 or -2 in the flowering category for two consecutive years, there should be two interventions. Firstly, pollination should be facilitated manually since attraction of natural pollinators may be reduced by low flowering. Pollinator visitation rates and consequent reproductive success can be influenced by the size of floral displays in the form of number of flowers per inflorescence (Schmid-Hempel & Speiser, 1988), number of flowers per plant (Klinkhamer *et al*., 1989), number of inflorescences per plant (Goldingay & Whelan, 1993) or per area of site (Delnevo *et al.*, 2019). For instance, a study of *Teleopea speciosissima* (Proteaceae) showed a positive relationship between number of bird visits and number of open inflorescences on a tree (Goldingay & Whelan, 1993). Secondly, timing or intensity of subsequent fires should be reduced because low flowering might indicate that terminal points of branches (where inflorescences should form) are being damaged during disturbances. Population demographics were also considered for scoring the presence of seedlings. *Site A* showed an average loss of *16.3* trees annually, whereas *Site C* lost 8.9 trees annually (Chapter 1, Chapter 3).

Fire control should be complemented with herbivory. For *Site A*, antelope browsers will likely be most effective. For *Site C*, which lacks wild vertebrate herbivores, it is advisable to introduce goats at carefully controlled stocking rates. One study tested the effect of fire, browsers and grazers in bush control (Jordaan & Le Roux, 1998). Different combinations of the following conditions were applied: burning, no burning, no grazing, rotational grazing by cattle and continuous or rotational browsing by goats. The combination of fire and continuous grazing by goats was the best for bush control. All burned treatments showed a short-term increase in bush density due to increased seedling establishment. Among the burned treatments, bush density showed a continuous increase when goats where absent or when cattle were stocked on the site (with or without rotational browsing from goats). Regeneration of woody species occurred faster on burned sites with cattle than on those without. This was due to cattle reducing grass competition (Jordaan & Le Roux, 1998).

Overall, fire will be important for suppressing the growth of the woody component on the sites. Goats and/or other browsers will be important in curtailing any new woody seedlings that emerge soon after fire. Although goats were not directly observed feeding on *Protea curvata* leaves, goat stocking rates should be applied with caution; particularly when new *P. curvata* seedlings start to grow on the site. To keep the grass layer competitive, sites ought to have a post-fire recovery period before the re-introduction of grazers.

Based on communication with the landowner of *Site C*, there is no intention of mining or clearing the area for industrial development. The owner is interested in managing the land with great consideration for the native flora. As such, *Site C* can be recognised as a privately owned protected area. The IUCN's 2003 World Parks Congress defined a private protected area (PPA) as "a land parcel of any size that is i) predominantly managed for biodiversity conservation; ii) protected with or without formal government recognition; and iii) is owned or otherwise secured by individuals, communities, corporations or non-governmental organisations." As many as ten categories of PPAs have been identified. These include ecotourism reserves, in which conservation generates income from tourists, and hybrid reserves in which land uses provide or require the conservation of ecological services to foster production – e.g., the protection of pollinators or watersheds to aid farming practices (Langholz and Lassoie 2001; Sims-Castley *et al.*, 2005). A variety of opinions exist on what type of governance or adherence to formal standards constitutes a PPA. From a practical standpoint, it is beneficial to regard the site as a PPA so the owner may be able to share in the knowledge and resources of conservation-driven NGOs, government programmes and civic organisations.

Various studies have shown the importance of privately owned conservation areas in maintaining biodiversity. Chacon (2005) lists several possible motivations for landowners to commit to making their land a PPA. One of the listed motivations applicable to the owner of *Site C* is a "personal understanding of the importance of protecting nature and their potential role as a private landowner". This is considered an intrinsic motivation that often requires an extrinsic incentive in order for the landowner to act on the motivation (Mitchell, 2005). From the other listed motivations, "technical support for sustainable development activities" stands out as being infused with extrinsic incentive and strongly aligned with goals previously expressed by the landowner. Thus far, the landowner's biggest challenges in managing the land have been keeping the area well-fenced and limiting unauthorized access. This has spurred activities such as small-scale mining by locals as well as unauthorized burns and livestock herding (Meyer, pers. comm., 2019). Therefore, provision or subsidization of fencing and security would benefit the landowner, in turn equipping him to conserve the area. It is of personal, public and environmental interest to conserve this area. *Site C* hosts serpentine endemics and biodiversity novelties such as *Ozoroa sp. nov.* and the endangered *P. curvata* which is unique to South Africa. With technical support, the landowner can protect these unique species and explore other opportunities that incentivize conservative stewardship of the land. For example, the owner may explore an ecotourism venture in which the patrons can enjoy activities such as hikes, picnics and birdwatching on the site. The owner may also periodically permit wood harvesting on the site to stave off bush encroachment. For either of these ventures to be successful, technical support is essential so that the owner may be aware of which species can serve as attractions for tourists and need to be restricted from harvesting, and be able to monitor changes in the site over time. Like many PPAs, the site may also have important, yet undocumented contributions towards regional and national biodiversity targets. One solution to this is to regularly conduct comprehensive biodiversity audits (Sims-Castley, 2005). This would also require technical support which the owner can seek from environmental organizations and research institutions.

Having identified the potential of goats to supplement fire management on *Site C*, a suggestion is provided for the implementation of goat herding. The owner could form partnerships with community-integrated conservation programmes such as “Herding 4 Health (H4H)”. The H4H project aims to assist farmers living within or near protected areas to herd their livestock while preventing land degradation and abating issues that arise from livestock interacting with wildlife. This includes reducing the spread of foot and mouth disease and reducing livestock losses incurred from inefficient herding or predation due to poor fencing in protected areas (Peace Parks Foundation, 2020; UP, 2020). The programme brings together local farmers and their herds. Rotational grazing is co-ordinated based on water sources and seasonal vegetation growth. Herders are provided with predator proof bomas that can be assembled at times when the herds are not required to be taken to a distant water source. The bomas are also assembled on degraded patches of land so the dung of livestock can provide fertilization. Participants of the programme select some of their fellow community members to become eco-rangers. These eco-rangers receive training on herding techniques, co-ordinated grazing, animal production, primary animal care, tracking, security and a host of other skills. Herders also receive veterinary assistance in the form of co-ordinated vaccination and disease monitoring programmes. They are also given access to markets in which they can sell their animal products. Involvement in such a project could be beneficial for both the landowner and other locals. The owner gains access to both the fauna and technical support required to manage their land, while the herders receive space to herd as well as the skills and assistance listed above. This is an improvement from the small scale, erratic goat herding previously occurring on the site. With the programme, herders can be incentivized to bring livestock to the area only when eco-rangers and landowners have co-ordinated to do so. Since herd monitoring and administration is largely handled by the programme, the owner can have a better idea of what stocking rate is appropriate for his site without undertaking a lot of personal costs. Other technical support which can be gleaned from the collaboration is vegetation surveys and bush clearing, which some eco-rangers are trained to do (Africa Geographic, 2021).

For both sites, site managers should be cognisant of when they apply certain interventions. It is recommended that bush thinning be undertaken between March and April. Focus should only be on trimming undesirable trees that are situated in the densest part of the site and anticipated to escape the fire zone. Where necessary, smaller trees and shrubs can also be trimmed in if they are clumped in ways that will limit access for herbivores. Fire breaks can be created as early as March. On *Site C,* three types of burns are likely to be appropriate based on the general vegetation structure of certain patches. Very rocky areas with sparse vegetation are prone to land degradation and erosion. As such, it will be appropriate to apply a “cool burn” by burning between April and June. This will remove cured grass material while maintaining a portion of healthy herbaceous and shrub layers (Larson, 1997). Such areas should be burnt first, with the aim of making them less prone to hotter fires later in the season. Should there be any unauthorized anthropogenic fires, unintentional crossovers of fire breaks or unexpected natural fires later in the year, the area's flammability will have been reduced.

Around the same time or slightly later, cool burns can also be applied on patches where *Protea* trees are surrounded mostly by closely packed grass tufts. Although both cool and hot burns can heat similar depths of soil, the heating effect from hot burns lasts longer and can reach higher temperature maxima in the upper soil layer (Auld, 1986). Detrimental effects of this prolonged heating are likely to be more pronounced in herbaceous species than in woody, aerial resprouters since both their buds and resource stores are located closer to the soil surface. Cool burns can therefore be beneficial in burning cured grass material without eradicating the rhizomes, bulbs and corms of the herbaceous layer. This will be important for grass:woody balance. It will also benefit plant diversity by facilitating the survival of serpentine endemics in the area such as *Gladiolus serpenticola* (Rutherford *et al.,* 2006).

A "wet burn" (e.g., burning a few days after mild rainfall) is recommended for patches with a high density of *Protea* and other woody species. The higher moisture levels will serve to mitigate the high char height and high temperatures to which dry branches might lend themselves (Adie *et al*., 2011). Burning these areas between September and October is advisable, since this will coincide with the maturation of *P. curvata* fruits, thus making way for seeds to be deposited in the soil.

Finally, outlying patches where there is high woody plant density but no *Protea* trees can be treated with hot burns. This should preferably be applied between July and October to intercept the period of peak shoot elongation in thicket forming trees such as *Vachellia* and *Senegalia* (Wakeling *et al.,* 2012). Higher stocking rates and/or longer feeding periods for goats can also be implemented shortly after to suppress the growth of undesirable woody seedlings. During the 2-year gap where no fires will be applied, goats can be stocked on the area throughout the year. Goats can feed on leaf litter from deciduous trees, shrubs, grasses and forbs. For example, the goats may consume a dominant woody species during one season but switch to rare grasses and forb species during another season. Food choice can also vary by plant species depending on seasonal availability. Therefore, stocking rates should be adjusted each season based on management goals, feeding preferences of goats and availability of plant species. This further highlights the importance of vegetation surveys in both site and livestock management.

With *P. curvata* being identified as Endangered in our study, diligent management practices will be necessary to lift *P. curvata* out of Threatened status. The intervention measures proposed here (fire management, herbivory and bush control etc.) are estimated to be sufficient for *Protea curvata* subpopulations that score “Fair” to “Good” on our evaluation scale. Application of our recommendations is expected to increase *P. curvata* recruitment and build disturbance resilience. This will be essential for recovering from unexpected disturbances such as severe hailstorms. Disturbances (whether natural or anthropogenic) should be considered extreme if subpopulations reach the “Critical” point on the evaluation scale. In such cases, the fire and herbivory regimes proposed here may need to be paused to allow the species to recover. Scores on the lower end of the “Critical” range might call for a re-assessment of the species’ IUCN status, potentially shifting to “Critically Endangered”. This is particularly true for *Site A*, whose subpopulation accounts for nearly a third of all *P. curvata* trees; making it a prime indicator of the species’ status. Scores on the higher end of the “Excellent” range may also call for IUCN re-assessment to potentially move the species to the Vulnerable or Least Concern category.

Table 1: Evaluation system for scoring outcomes of management practices on *P. curvata* sites

Site A	**Site C**	**Score**
Tree density greater than or equal to 2672 trees/ha	Tree density 15% greater than 288 trees/ha	-2
1-20% reduction from 2672 trees/ha	Tree density 10 % greater than 288 trees/ha	-1
21-40% reduction from 2672 trees/ha	Tree density 5% greater than 288 trees/ha	0
40-60% reduction from 2672 trees/ha	Tree density 288 trees/ha	1
Over 10% increase in *V. davyi* trees taller than 2 m (i.e., height classes likely to escape fire and herbivory zones)	Over 10% increase in *V. davyi* trees taller than 2 m (i.e., height classes likely to escape fire and herbivory zones)	-2
Less than 10 % increase in *V. davyi* trees taller than 2 m	Less than 10 % increase in *V. davyi* trees taller than 2 m	-1
V. davyi size class distribution same as 2019 records.	*V. davyi* size class distribution same as 2019 records.	0
V. davyi size class distribution similar to 2019 records, but with fewer *V. davyi* trees overall	*V. davyi* size class distribution similar to 2019 records, but with fewer *V. davyi* trees overall	1
Increase of 5% or more in *S. pentheri or S. grandidens* density	Increase of 40 % or more in *S. pentheri* or *S. grandidens* density	-2
Less than 5 % increase in *S. pentheri* or *S. grandidens* density	20 – 39 % increase in *S. pentheri* or *S. grandidens* density	-1
0 – 15 % <u>decrease</u> in *S. pentheri or S. grandidens* density	5 – 19 % increase in *S. pentheri* or *S. grandidens* density	0
16 – 30 % <u>decrease</u> *in S. pentheri or S. grandidens density*	Less than 5% increase in *S. pentheri or S. grandidens* density	1
Mortality in trees ≤ 3 m	Mortality in trees ≤ 2 m	-3
Mortality in 3 ≤ 4 m	Mortality in 2 ≤ 5 m	-2
Mortality in 4 ≤ 6 m	Mortality in 5 ≤ 6 m	-1
Mortality in 6 ≤ 10 m	Mortality in 6 ≤ 8 m	0

No mortality	No mortality	2
0 – 15 seedlings within 1 year after fire	0 – 10 seedlings within 1 year after fire	0
16 – 25 *P. curvata* seedlings within 1 year after fire	11 – 20 *P. curvata* seedlings within 1 year after fire	3
26 – 35 *P. curvata* seedlings within 1 year after fire	21 – 30 *P. curvata* seedlings within 1 year after fire	6
> 35 seedlings within 1 year after fire	> 30 seedlings within 1 year after fire	12
Mortality of over 50% of seedlings within a year	Seedlings survived less than a year	0
At least 50% of seedlings survived for over a year	Seedlings survived for a year	2
Seedlings survived 2 years	Seedlings survived 2 years	4
Seedlings survived next fire	Seedlings survived next fire	6
Net loss averaging 2.5 trees per year or more	Net loss averaging 2.5 trees per year or more	-2
Net loss between 2.1 and 2.5 trees per year	Net loss between 2.1 and 2.5 trees per year	-1
Net loss of between 1.0 and 2.0 trees per year	Net loss of between 1.0 and 2.0 trees per year	0
Net loss of less than 1.0 tree per year	Net loss of less than 1.0 tree per year	1
2 or more species of invasive plants	2 or more species of invasive plants	-2
Invasive species with density higher than 17 trees/ha	Invasive species with density higher than 17 trees/ha	-1
Invasive species with density less than 17 trees/ha	Invasive species with density of 17 trees/ha or less	0
No invasive species	No invasive species	1
Average flowering 1–5 inflorescences per tree(Resembling low flowering a year after hail)	------	-2

Average flowering 5–10 inflorescences per tree (Indicative of recovery after hail disturbance)	Average flowering <10 inflorescences per tree (Resembling lowest flowering observed in *subpop. C* in 2019)	-1
Average flowering 10–15 inflorescences per tree, (Resembling pre-hail flowering of *subpop. A*)	Average flowering 20–25 inflorescences per tree (Resembling flowering observed in 2018)	0
Average flowering 15–20 inflorescences per tree (Higher than pre-hail flowering observed in *subpop*. *A*)	Average flowering 25–30 inflorescences per tree (Resembling more floriferous subpopulations)	1
Average flowering 20–30 inflorescences per tree (Resembling more floriferous subpopulations)	----	2
	Total:	

Total score:

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Appendix

Definitions

Inflorescence: the arrangement of flowers on a specialized stalk (peduncle). (Collins English Dictionary, 2012)

A group of flowers growing from a common stem, often in a characteristic arrangement. (American Heritage Dictionary, 2016)

Infructescence: the fruiting stage of an inflorescence (American Heritage Dictionary, 2016) i.e., the arrangement of fruits derived from the ovaries of flowers in an inflorescence.

Receptacle: a. the modified or expanded portion of a plant stem or axis that bears the organs of the florets of a flower head (Random House Kernerman, 2010).

b. the shortened flattened peduncle bearing the florets of the capitulum of composite flowers (Collins English Dictionary, 2014).

c. the area at the stem tip that bears flower organs or groups of flowers (Hollender *et al.,* 2014)

Old receptacles: receptacles on which the florets and fruits of previous seasons were borne but retained no fruits at the time of censusing.

Juvenile: period between germination and age of first flowering during which the plant is not yet sexually mature (Hackett, 2011; Kraaij *et al.* 2013)

Juvenile individuals: structurally simple plants that no longer have cotyledons, though some embryonic structures may still be present. Juvenile plants have features not found in mature plants, such as different form of leaves and of shoot and root systems (Harper & White, 1974).

Seedling: a. a subset of the juvenile stage in which the plant consists of the cotyledons and a pair of simple leaves (Harper & White, 1974).

b. a plant which still has conspicuous cotyledons present near the base of the stem. Cotyledons are the large, flat 'seed-leaves' that are the first to appear when a seed germinates. Note that most (*Protea*) seedlings have young leaves which are often hairy, more curved, smaller, and of a different colour to that of mature plants. (Rebelo, 2008)

Reproductive plants: characterized by the development of sexual organs and by the ability to form seed. In young reproductive individuals, formation of new parts prevails over death of old parts, and is reflected in the balance between living and dead parts, and between actively growing and fully-grown structures (Harper & White, 1974).

Mature plants: show a relative equilibrium in the processes of formation and death of structures. They usually show the maximum yearly increase in biomass and maximum seed productivity. These are the individuals which are at the peak of ontogenetic development, whereas in old plants, death of parts prevails over the formation of new ones and the reproductive activity is diminished, as is the rate of root and shoot formation. (Harper & White, 1974).

Number of mature individuals: the number of individuals known, estimated or inferred to be capable of reproduction. (IUCN Standards and Petitions Committee, 2022).

Additional considerations to be made when estimating this quantity are detailed in the page 26 of the IUCN. Notably, the use of the term "reproduction" when applying the IUCN guidelines means "production of offspring – not just mating or displaying other reproductive behaviour".

Population: The term 'population' is used in a specific sense in the Red List Criteria that is different to its common biological usage. Population is here defined as the total number of individuals of the taxon. For functional reasons, primarily owing to differences between life forms, population size is measured as numbers of mature individuals only. In the case of taxa obligately dependent on other taxa for all or part of their life cycles, biologically appropriate values for the host taxon should be used. (IUCN Standards and Petitions Committee, 2022).

Subpopulation: Subpopulations are defined as geographically or otherwise distinct groups in the population between which there is little demographic or genetic exchange (typically one successful migrant individual or gamete per year or less) [IUCN Standards and Petitions Committee, 2022].

Location: The term ‘location’ defines a geographically or ecologically distinct area in which a single threatening event can rapidly affect all individuals of the taxon present. The size of the location depends on the area covered by the threatening event and may include part of one or many subpopulations. Where a taxon is affected by more than one threatening event, location should be defined by considering the most serious plausible threat. (IUCN,) For example, where the most serious plausible threat is habitat loss due to development, a location is an area where a single development project can rapidly eliminate or severely reduce the population. The time frame should be short (e.g., within a single generation or three years, whichever is longer, but not any longer than is possible to project the threats and their impacts on the species). When there are several threats, locations should be based on the one that has the maximum product of probability and consequence (in terms of percentage reduction in population) [IUCN Standards and Petitions Committee, 2022].

Continuing decline: A continuing decline is a recent, current or projected future decline (which may be smooth, irregular or sporadic) which is liable to continue unless remedial measures are taken. Fluctuations will not normally count as continuing declines, but an observed decline should not be considered as a fluctuation unless there is evidence for this (IUCN Standards and Petitions Committee, 2022).

Extreme fluctuations: Extreme fluctuations can be said to occur in a number of taxa when population size or distribution area varies widely, rapidly and frequently, typically with a variation greater than one order of magnitude (i.e., a tenfold increase or decrease) [IUCN Standards and Petitions Committee, 2022].

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Chapter 3

Table A1: Analysis of deviance table for size and species of trees surrounding *P. curvata* at *Site A*

Source	d.f.	Deviance	Resid.	d.f. Resid. Dev	Pr(>Chi)
NULL			299	423982	
Basal area of neighbouring tree	1	818	298	423164	0.4567
Species of neighbouring tree	25	41704	273	381460	0.2968

Table A2: Results from the Quasi-Poisson distribution generalized linear model for explaining basal area of *P. curvata* trees on *Site A*

Source	Estimate	SE	t-value	P value
(Intercept)	8.481	0.1154	73.491	2 $\times 10^{-16}$
Basal area of neighbouring tree	1.837 $\times 10^{-5}$	9.371 $\times 10^{-5}$	0.196	0.845

Table A3: Analysis of Deviance Table for size and species of trees surrounding *P. curvata* at *Site C*

Source	d.f.	Deviance	Resid.	d.f. Resid. Dev	Pr(>Chi)
NULL			519	1067486	
Basal area of neighbouring tree	1	996	518	1057490	0.08212
Species of neighbouring tree	23	101963	495	955527	0.12702

Table A4: Results from the Quasi-Poisson distribution generalized linear model for explaining basal area of *P. curvata* trees on *Site C*.

Source	Estimate	SE	t-value	P value
(Intercept)	7.8383532	0.3055627	25.652	<0.0001***
Basal area of neighbouring tree	-0.0001336	0.0001203	-1.111	0.2671
Species: *Dichrostachys cinerea*	1.0633191	0.4562590	2.331	0.0202*

Chapters 4 and 5

Tetrazolium staining test for viability

The tetrazolium test is commonly used to assess viability as it allows any biochemically active, respiring tissue to be stained; thus giving a visual indication of seed viability. Moore's (1973) protocol for tetrazolium staining was used as a guideline on how to prepare solutions and stain seeds. According to the protocol, some species require tissues to be exposed prior to staining for the tetrazolium solution to penetrate seed tissues. For this study, the selected method was longitudinal cutting of the fruit. The fruits were very small and hard, making them difficult to work with. Therefore, seeds were soaked in water as recommended by Moore (1973). After a few soaking trials, it was found that soaking for a minimum of 24 hours made the fruit easy to dissect. From then on, all tested fruits were first imbibed in distilled water for 24 hours at 22 ^{o}C. This was followed by longitudinal cutting of the fruit to expose seed tissue. The exposed seeds were then dipped in a sodium hypochlorite solution to kill any bacteria and fungi whose microbial activity might cause misleading staining results. The seeds were then placed in ice trays, to ensure that each individual seed remained separate. Two solutions were prepared by dissolving 9.078 g of KH2PO4 in 1000 ml distilled water (solution 1) and dissolving 9.472 g of Na2HPO4 in distilled 1000 ml water (solution 2). To make a buffer solution, 200 ml of solution 1 was added to 300 ml of solution 2. A pH meter was used to ensure that this solution was within the recommended range. After obtaining a pH of 7.03, 5g of tetrazolium chloride salt was added to the buffer solution, making a 1% tetrazolium chloride solution. This solution was poured over the trays of seeds. The tray was then covered in foil and kept in the dark for 24 hours. Seeds were then viewed under a dissecting microscope to assess viability. An example of the variety of results and how they were classified is given in Figure A.

Figure A: (1) Unstained embryo was classified as non-viable. (2) Empty fruit lacking embryo was classified as non-viable. (3) Seed with red-stained embryo was classified as viable.

Table B: Result from the Gamma distribution model that best explained seed mass in pollinated *P. curvata* flowers (C.I. = 95%)

Source	Estimate	SE	t-value	P value
(Intercept)	55.9910	1.4665	38.180	< 0.0001 ***
Inflorescence geitonogamy (B)	1.8419	2.2776	0.809	0.418747
Tree geitonogamy (C)	4.4886	2.4585	1.826	0.067996
Natural outcrossing (D)	-18.1266	1.8047	-10.044	< 0.0001 ***
Pollen suppl. outcrossing (E)	-16.2655	1.9044	-8.541	< 0.0001 ***
Tree2	1.0447	2.5261	0.414	0.679227
Tree3	-14.1134	2.3035	-6.127	< 0.0001 ***
Tree4	-14.1134	2.3035	-6.127	< 0.0001 ***
Tree5	-28.5339	2.0140	-14.168	< 0.0001 ***
Tree6	-23.6715	2.1620	-10.949	< 0.0001 ***
Tree7	62.3221	4.1730	14.935	< 0.0001 ***
Tree8	-8.8243	2.5312	-3.486	0.000498 ***
Tree9	-14.8068	2.3277	-6.361	< 0.0001 ***
Tree10	-5.2586	2.4452	-2.151	0.031595 *

Tree11	-6.2866	2.6349	-2.386	0.017104 *
Tree12	-13.5271	2.2981	-5.886	< 0.0001 ***
Tree13	8.8944	3.0471	2.919	0.003540 **
Tree14	-13.1711	2.4432	-5.391	< 0.0001 ***
Tree15	-6.6200	2.7015	-2.450	0.014327 *
Tree16	-1.5533	2.8606	-0.543	0.587163
Tree17	-22.0974	2.1632	-10.215	< 0.0001 ***
Tree18	-10.1993	2.3014	-4.432	< 0.0001 ***
Tree19	-13.4674	2.4647	-5.464	< 0.0001 ***
Tree20	-9.3481	2.5817	-3.621	0.000299 ***
B:Tree2	7.6892	3.5458	2.169	0.030204 *
C:Tree2	-3.9822	3.7316	-1.067	0.285997
D:Tree2	-8.3196	2.8918	-2.877	0.004046 **
E:Tree2	1.8788	3.0687	0.612	0.540434
B:Tree3	-4.0990	3.1080	-1.319	0.187331
C:Tree3	-1.1174	3.3849	-0.330	0.741346
D:Tree3	3.4899	2.7393	1.274	0.202761
E:Tree3	6.2807	2.8901	2.173	0.029847 *
B:Tree4	-4.0990	3.1080	-1.319	0.187331
C:Tree4	-1.1174	3.3849	-0.330	0.741346
D:Tree4	3.4899	2.7393	1.274	0.202761
E:Tree4	6.2807	2.8901	2.173	0.029847 *
B:Tree5	7.4968	2.8144	2.664	0.007773 **
C:Tree5	-3.2038	2.9332	-1.092	0.274815
D:Tree5	19.8644	2.4125	8.234	< 0.0001 ***
E:Tree5	35.4324	2.8316	12.513	< 0.0001 ***
B:Tree6	6.4085	3.0433	2.106	0.035314 *
C:Tree6	-1.7717	3.1017	-0.571	0.567911
D:Tree6	19.4733	2.6357	7.388	< 0.0001 ***
E:Tree6	37.3913	3.0496	12.261	< 0.0001 ***
B:Tree7	5.0421	5.9348	0.850	0.395628
C:Tree7	-64.7466	5.0023	-12.943	< 0.0001 ***
D:Tree7	-49.5719	4.6200	-10.730	< 0.0001 ***
E:Tree7	-18.0331	4.9778	-3.623	0.000297 ***
B:Tree8	11.2602	3.3556	3.356	0.000802 ***
C:Tree8	-7.2648	3.6147	-2.010	0.044548 *
D:Tree8	7.2669	2.9455	2.467	0.013681 *
E:Tree8	13.5744	3.0806	4.406	< 0.0001 ***
B:Tree9	13.2574	3.1305	4.235	< 0.0001 ***
C:Tree9	3.8875	3.4289	1.134	0.257007
D:Tree9	23.8117	2.8746	8.284	< 0.0001 ***
E:Tree9	25.6851	2.9480	8.713	< 0.0001 ***
B:Tree10	-2.8439	3.2055	-0.887	0.375054
C:Tree10	8.8651	3.8658	2.293	0.021909 *
D:Tree10	14.8405	3.0125	4.912	< 0.0001 ***
E:Tree10	12.7215	3.0602	4.157	< 0.0001 ***
B:Tree11	52.5577	4.3446	12.097	< 0.0001 ***
C:Tree11	-7.2888	3.7725	-1.932	0.053453
D:Tree11	13.5206	3.1147	4.341	< 0.0001 ***
E:Tree11	26.6703	3.4349	7.764	< 0.0001 ***
B:Tree12	2.1706	3.1614	0.687	0.492392
C:Tree12	1.2613	3.5266	0.358	0.720624

D:Tree12	3.2260	2.7496	1.173	0.240786
E:Tree12	1.6124	2.7587	0.585	0.558929
B:Tree13	-21.1345	3.6613	-5.772	< 0.0001 ***
C:Tree13	-20.7192	4.1806	-4.956	< 0.0001 ***
D:Tree13	-14.6705	3.4150	-4.296	< 0.0001 ***
E:Tree13	-16.8631	3.4457	-4.894	1.04 $x10^{-6}$***
B:Tree14	-4.1122	3.1462	-1.307	0.191311
C:Tree14	0.1841	3.4512	0.053	0.957457
D:Tree14	45.8120	3.4915	13.121	< 0.0001 ***
E:Tree14	7.3286	2.8693	2.554	0.010698 *
B:Tree15	-6.1700	3.4917	-1.767	0.077332
C:Tree15	-6.5135	3.8515	-1.691	0.090916
D:Tree15	13.0123	3.1271	4.161	< 0.0001 ***
E:Tree15	35.2939	3.5192	10.029	< 2 $x10^{-9}$***
B:Tree16	-13.1201	3.4348	-3.820	0.000136 ***
C:Tree16	-4.9905	3.8084	-1.310	0.190173
D:Tree16	13.1065	3.4615	3.786	0.000156 ***
E:Tree16	7.3531	3.3679	2.183	0.029098 *
B:Tree17	34.5158	3.4626	9.968	< 2 $x10^{-16}$***
C:Tree17	-5.9633	3.1338	-1.903	0.057159
D:Tree17	18.8542	2.5546	7.381	2.07 $x10^{-13}$***
E:Tree17	56.7426	3.2456	17.483	< 2 $x10^{-16}$***
B:Tree18	-0.2589	3.0293	-0.085	0.931897
C:Tree18	3.8835	3.8536	1.008	0.313662
D:Tree18	8.5260	2.7923	3.053	0.002284 **
E:Tree18	10.9549	2.8667	3.821	0.000136 ***
B:Tree19	1.4030	3.2887	0.427	0.669689
C:Tree19	-1.3822	3.5271	-0.392	0.695174
D:Tree19	23.0901	3.1752	7.272	4.58 $x10^{-13}$***
E:Tree19	13.0898	3.1348	4.176	3.06 $x10^{-5}$**
B:Tree20	-2.9060	3.3433	-0.869	0.384811
C:Tree20	-7.8298	3.7652	-2.080	0.037660 *
D:Tree20	18.1472	3.3366	5.439	5.83 $x10^{-8}$***
E:Tree20	9.9242	3.1239	3.177	0.001505 **