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The reproductive system of *Campuloclinium macrocephalum* and its implications for biocontrol implementation.

by

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Declaration

I declare that this dissertation is my own, unaided work. It has been submitted in fulfilment of the requirements of a Master of Science at the University of the Witwatersrand. It has not been submitted before for any degree or examination to another university or similar institution.

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Table of contents

Abstract

Invasive species are a threat to biodiversity therefore it is imperative to determine the factors that facilitate the invasion potential of a species. *Campuloclinium macrocephalum* Less. (DC), the 'pompom weed', is an alien invasive species in South Africa and is currently threatening the persistence of the grassland, wetland, and savanna biomes. The species is also significantly contributing to a decline in plant diversity by outcompeting native vegetation in these areas. Various integrated approaches using combinations of chemical, mechanical and biocontrol management programs have been developed to manage the spread of the species in its invaded range, however the species has still been able to persist.

The persistence of the species was hypothesised to be a consequence of the co-occurrence of apomixis and polyploidy, however despite the identification of triploid and tetraploid cytotypes in South African populations of the pompom weed, the reproductive strategy of the species has not yet been determined. The aim of this study was therefore to infer whether populations of *C. macrocephalum* (pompom weed) reproduces via vector-mediated crosses, self-pollination or apomixis (either facultative or obligate) and examine the relationship of the mode of reproduction with ploidy level. Male fertility was also assessed to ascertain if interploidy gene flow was possible. The collated information was then used to infer the potential impact of reproductive strategies and polyploidy on biocontrol. All examined populations were shown to have high mean pollen viability percentages of 90% and 98% with no significant differences in pollen viability amongst the four populations. The high pollen viability percentages were supported by prolific pollen grain germination on the stigmatic surfaces (margins of style at base of style branches) and the sides of the style. This suggested that the pollen grains can fertilize and interploidy mating is likely possible in South African populations of the pompom weed. It is plausible that the high pollen viability is enabling triploids to act as a 'triploid

bridge'. However, the high pollen viability was confounded by the pollen tube analyses revealing that pollen tube growth is being arrested on the stigmatic surface suggesting that overall male fertility is low. The arrested pollen tube growth is typically associated with a 'triploid block'. Nevertheless, the production of viable gametes can reduce the triploid block and facilitate gene flow between populations.

The predominant mode of reproduction was determined by assessing the contribution of insects to pollen transfer, pollinator exclusion experiments, germination trials, pollen tube growth to the ovules and genetic analyses. We found that the African Monarch butterfly (*Danaus chrysippus)* and the honeybee (*Apis mellifera)* contributed most to pollen transfer in comparison to the other insects visiting *C. macrocephalum*. A pollinator exclusion experiment showed that the pompom weed can set seed in the absence of pollinators, albeit at lower quantities than in the open treatments. Nevertheless, germination percentages showed that reproductive success was similar between open and bagged treatments in each population. The Modderfontein population showed lower reproductive success and seedling establishment in comparison to the other populations, presumably due to the severity of the biocontrol infestation on this population. Genetic analyses revealed low genetic variation within and amongst populations. Pollen tube analyses showed no pollen tube growth to the ovules in all samples, which suggests that seed set is independent of fertilization. The lack of pollen tube growth is a strong indicator of obligate autonomous apomixis which is further corroborated by the low genetic differentiation between maternal plants and their respective offspring.

The co-occurrence of apomixis and polyploidy made it difficult to discern which factor contributes more to the invasiveness of the species, however, we hypothesise that autonomous apomixis provides the pompom weed with the competitive advantage to persist in its invaded range. However, further studies on the reproductive strategies of tetraploid cytotypes are needed to confirm this hypothesis. The low genetic variation suggests that all populations

should be equally susceptible to biocontrol agents, however that this may be affected by other factors such as environmental conditions or phenotypic plasticity. Phenotype plasticity refers to a single genotype producing different phenotypes in response to environmental conditions. This could reduce the efficacy of biocontrol agents as they may exhibit differential responses on different phenotypes.

Keywords: Invasive species; Autonomous apomixis; Polyploidy; Male fertility; Biocontrol

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Introductory chapter

Rationale

Plant invasions threaten biodiversity and ecosystem function by outcompeting native vegetation (Pimentel *et al.*, 2001; Hawkes, 2007). Invasive species typically invade agricultural fields and natural areas, reduce productivity and biodiversity respectively, and alter interactions amongst native species (Holzmueller and Jose, 2009). Additionally, the sustainability of native communities is further threatened by the structural, functional, and compositional changes that follow a successful plant invasion (Holzmueller and Jose, 2009).

Campuloclinium macrocephalum (Less) DC., colloquially referred to as the 'pompom weed', is native to South and Central America but is an invasive species in South Africa (Henderson, 2001; Henderson, 2007). Predicted distributions suggest that as the species expands its range, it poses a considerable risk to the conservation of the savanna and grassland biomes (Trethowan *et al*., 2011). The species outcompetes native vegetation thereby causing a significant decline in plant diversity (Aileen, 2005). Despite the use of various mechanical, chemical and biocontrol methods, pompom weed has persisted.

The success of *C. macrocephalum* was hypothesised to be linked to the co-occurrence of polyploidy and apomixis (Gitonga *et al.,* 2015). Polyploid apomictics are formidable invaders (Richards, 2003), however the association between polyploidy and apomixis is rarely considered in biocontrol management plans. Both polyploidy and apomixis have implications for progeny formation and the subsequent genetic diversity within progeny – factors which may facilitate invasion success (Krahulcová and Krahulec, 2021). Triploid and tetraploid cytotypes have been identified in South African populations of *C. macrocephalum* (Gitonga *et al.*, 2022), however there is no clear consensus on what reproductive strategy the species uses.

Research on the reproductive strategies employed by an invasive species reveals important information on their invasive mechanisms and enables us to develop strategies to mitigate the spread and effects of these species (Yan *et al.,* 2016).

Sexual reproductive strategies

In plants, sexual reproduction is mediated by the transfer of pollen from the anthers to the stigma using wind, water, mammals, birds, insects, or gravity (Richards, 1996; Abrol, 2012). Cross-pollination is the transfer of pollen between individuals on different plants, whereas geitonogamy occurs when the pollen transfer is between individual flowers on the same plant and autogamy is pollen transfer within the same flower (Charlesworth, 2006; Abrol, 2012). Autogamy can either be autonomous or vector-mediated. Autonomous autogamy is self-fertilisation in the absence of pollinators whereas vector-mediated autogamy is within flower pollen transfer using biotic vectors (Bowers, 1975; Solís-Montero *et al.,* 2021). When determining the reproductive strategy used by a species, three major aspects should be considered: i) whether there are occurrences of sexual reproduction, ii) whether individuals are bisexual or unisexual, and iii) whether the co-sexual individuals are self-compatible (Charlesworth, 2006).

Co-sexuality is the rule in angiosperms (Charlesworth, 2006). The presence of both sexes within the same flower (hermaphroditism) can complicate reproduction by promoting self-pollination (Harder *et al.*, 2000) as well as interfering with sexual function (Lloyd and Yates 1982; Harder *et al.*, 2000; Dai and Galloway, 2011). The effects of hermaphroditism are mediated by reducing pollen-stigma interference (i.e., one sexual function being obstructed by another) and promoting outcrossing by ensuring that pollen presentation and stigma receptivity occurs non-simultaneously (Ramirez, 2005). This is accomplished by temporally separating sex organs (dichogamy) or spatially separating sex organs (herkogamy) (Lloyd and Webb,

1986; Webb and Lloyd, 1986; Harder *et al.*, 2000). Dichogamy may be protogynous (the dispersal of pollen after stigma receptivity) or protandrous (the maturation and dispersal of pollen before stigma receptivity) (Stout, 1928; Sargent and Otto, 2004). On the other hand, herkogamy includes monoecy, dioecy and gynomonoecy. Monoecy is when staminate flowers and pistillate flowers are separate but occur on the same plant whereas dioecy is when staminate flowers and pistillate flowers occur on different individual plants. Some studies hypothesise that these spatial segregations evolved to reduce self-pollination and thereby prevent inbreeding depression (Harder *et al.*, 2000); however, Barrett (2003) noted that most plants that exhibit herkogamy are already exempted from selfing by physiological mechanisms. Instead, herkogamy may play a more important role in reducing sexual interference between maternal and paternal functions (Fetscher, 2001; Barrett, 2002).

Despite most flowering plants being hermaphroditic and having the potential for self-fertilisation, they usually develop self-incompatibility (SI) systems to enforce outcrossing (Goldberg *et al.*, 2010). SI systems enable plants to recognize their own pollen and subsequently reject it (Goldberg *et al.*, 2010). This rejection can occur on the stigmatic surface (sporophytic incompatibility system) or after the pollen grain has germinated and the pollen tube has penetrated the stigma (gametophytic incompatibility system) (Newbigin *et al.*, 1993). Gametophytic incompatibility is common in Solanaceae, Rosaceae, and Papaveraceae (e.g., Miller and Kostyun, 2011; Del Duca *et al.*, 2019; Bilinski and Kohn, 2012), whereas sporophytic incompatibility is often reported in Brassicaceae, Asteraceae, and Convolvulaceae (e.g., Mable *et al.*, 2003; Hiscock *et al.*, 2003; Hou *et al.*, 2021).

SI has a short-term disadvantage when there is no outcross pollen available and reproduction is hindered (Newbigin *et al.*, 1993), which can lead to the breakdown of SI systems (Newbigin *et al.*, 1993). Transitions from SI to self-compatibility is a common evolutionary shift seen in angiosperms and self-compatibility is a prerequisite for self-fertilization (Stebbins, 1974; Igic

et al., 2008). Self-fertilization allows species to reproduce uniparentally in the absence of pollinators or in unpredictable environments (Pannell and Barett, 1998; Rea and Nasrallah, 2010). While self-fertilization provides reproductive assurance, it is regarded as an evolutionary 'dead-end' (Dobzhansky, 1950; Stebbins, 1957; Igic and Busch, 2013). Selfing taxa have a lower potential for adaptation which may be linked to genetic drift and limited recombination efficiency (Nöel *et al.,* 2017). Due to increased homozygosity, inbreeding depression allows for increased frequencies of recessive traits or mutations to be expressed (Charlesworth, 2006). Selfing taxa are therefore more prone to extinction than outcrossing taxa (Stebbins, 1957; Glémin *et al.,* 2006; Schoen and Busch, 2008).

In contrast, outcrossing taxa have high recombination rates and consequently maintain high effective population sizes; they exhibit better responses to purifying selection than selfing taxa and display high genetic diversity across their geographic distribution (Goldberg *et al.,* 2010). Despite the fitness advantages of outcrossing, outbreeding depression can promote a change from outcrossing to selfing (Charlesworth, 2006). Outbreeding depression typically occurs when there is gene flow between fragmented populations and favourable gene combinations are broken up therefore causing an increase in the frequency of maladapted gene complexes (Charlesworth, 2006).

The reproductive strategies used by a plant species have implications for the ecology, evolution, and genetic diversity within populations of the species (Charlesworth, 2006). It is evident that both outcrossing and selfing have implications for the genetic variation and consequently the evolutionary patterns within populations (Richards, 1996). Many species adopt a 'mixed' reproductive strategy as both outcrossing and selfing confer fitness benefits to the offspring (Richards, 1996). Additionally, many perennial plants may be able to reproduce asexually, and their evolutionary potential may be linked to the association between

outcrossing and selfing or outcrossing and asexual reproduction (Richards, 1996; Bengtsson and Ceplitis, 2000).

Asexual reproductive strategies

Asexual reproduction includes vegetative propagation and apomixis. Vegetative propagation is the formation of progeny by specialized structures, such as bulbs, tubers, and rhizomes, rather than seeds or spores (Richards, 1997; Hojsgaard and Hörandl, 2019). Meiosis and syngamy are circumvented and offspring have the same genetic composition as the maternal plant (Richards, 1997). Clonal populations often occur in environments where sexual reproduction is prevented by either a lack of suitable pollinators or unfavourable ecological conditions (Barrett, 2015).

Apomixis is clonal reproduction through unfertilised seeds (Asker and Jerling, 1992). There are two types of apomixis: sporophytic (or adventitious embryony) or gametophytic apomixis. Sporophytic apomixis occurs when the nucellus or integument of the ovule gives rise to an embryo which develops via mitotic division (Koltunow *et al.*, 1995). Sporophytic apomixis is the most taxonomically widespread apomictic development pathway, however the genetic mechanisms are not as well understood as those of gametophytic apomixis (Hojsgaard and Hörandl, 2015; Hojsgaard and Hörandl, 2019).

Gametophytic apomixis occurs via two distinct mechanisms: diplospory and apospory. Diplospory occurs when a megaspore mother cell produces an unreduced embryo sac by mitosis or modified meiosis. Apospory occurs when somatic cells, often from the nucellus of the ovule, give rise to an unreduced embryo sac (Bertasso-Borges and Coleman, 2005). After the production of unreduced embryo sacs in both mechanisms, meiosis is bypassed, and the unreduced ova (egg-like cells) develop by parthenogenesis – a form of asexual reproduction where the unfertilised egg cells give rise to new individuals (Nogler, 1984). The subsequent

endosperm formation can be pseudogamous or autonomous. In pseudogamy, the polar nuclei are fertilised and only the egg cell develops by parthenogenesis. In autonomy, the polar nuclei and egg cell are independent of fertilisation, and both develop by parthenogenesis (Nogler, 1984).

True obligate apomictic species, that can form seeds exclusively by apomixis, are rare (Asker and Jerling, 1992). Although most reports of obligate apomixis were dismissed as it was believed to be an 'artifact of screening tools' (Asker and Jerling, 1992), some incidences of obligate apomixis have been published (e.g., Connor and Dawson, 1993; Grusz *et al.*, 2021). Sorensen *et al.* (2009) provided compelling evidence for the expression of obligate apomixis in *Corunastylis apostasioides* Fitzg, the common midge orchid. Apomictic characteristics in the orchid species included seed development without fertilisation, periodically closed flowers that still produced mature embryos, subsequent endosperm by diplospory and adventitious embryony, inviable pollen grains, and the expansion of ovaries despite the lack of fertilization (Sorensen *et al.*, 2009). The expression of apomixis was linked to the inability of *C. apostasioides* to produce a citronella scent to attract pollinators (Sorensen *et al.*, 2009).

Most apomictic species maintain a degree of sexuality and are referred to as 'facultative apomictics' (Asker and Jerling, 1992; Richards, 2003). Apomixis and sexuality are thus not mutually exclusive reproductive strategies; therefore, it is difficult to predict their role in evolution and speciation (Hojsgaard *et al.*, 2014). However, the combination of sexual and asexual reproductive pathways within a species is hypothesised to be more beneficial than obligate outcrossing (Cosendai *et al*., 2013). Facultative apomictics benefit from the advantages of both sexual and asexual reproduction; asexual reproduction enables apomictic populations to rapidly colonize novel niches because they are not limited by mate or pollinator availability (Baker, 1965; Baker,1967; Pannell *et al*., 2015), whereas sexual reproduction maintains genetic diversity within the population (Barcaccia *et al.*, 2006).

On the other hand, apomixis and sexual reproductive pathways are mutually exclusive in the ovaries of diplosporous species. This is in contrast to aposporous species where both sexual reproductive pathways and apomixis can co-exist within the same ovary (Asker and Jerling, 1992; Hojsgaard *et al*., 2013). This results in seeds containing either sexually derived embryos or parthenogenetic embryos with variable genotype frequencies (Koltunow *et al.*, 1995; Hojsgaard *et al.*, 2013). The presence of both reproductive pathways within a plant has a direct impact on its genetic contribution to the population's gene pool (Hojsgaard *et al.*, 2013). Offspring derived from facultative aposporous apomixis often have varying levels of genetic diversity (Hojsgaard *et al.*, 2013). This genetic variation, however, was presumed to be significantly lower than in populations with plants that reproduce predominantly through sexual reproductive pathways such as cross-pollination (Asker and Jerling, 1992).

In contrast, apomictic populations have high proportions of heterozygotes across multiple loci, thereby maintaining genetic variation within individuals (Halkett *et al*., 2005), however, due to the lack of recombination, these populations are genotypically depauperate (Peredo,, 2013; Grusz and Pryer, 2015). This is not applicable to all apomictic groups because the maintenance of male meiosis may generate genotypic variation, thereby providing an evolutionary advantage over apomictic groups with no incidences of meiosis (Whitton *et al*., 2008). Additionally, variation within apomictic lineages may arise when asexual individuals continue producing sexually functional male gametes and thereby mate with sexual individuals. This transmission of apomixis genes to sexually producing plants may contribute to the long-term spread of apomixis and generate genotypically diverse populations – even if these events occur infrequently (Adolfsson and Bengtsson, 2007; Whitton *et al.*, 2008).

The co-occurrence of apomixis and polyploidy

Apomixis is often associated with polyploidy (Carman, 1997). The presence of odd ploidy levels, such as triploid and pentaploid, is generally a reliable indicator of apomixis – with most natural apomictic populations being polyploids (Asker and Jerling, 1992; Koltunow and Grossniklaus, 2003). This might be attributed to tetraploid and hexaploid individuals maintaining the capability for selfing, which often results in fixed heterozygosity that can reduce their potential for establishment (Liu *et al.*, 2012). In contrast, in triploid and pentaploid individuals, the transition to apomixis helps overcome female sterility caused by meiotic incompatibilities in uneven ploidy levels (Liu *et al.*, 2012).

Carman (1997) proposed that polyploidy may have triggered apomixis during the Pleistocene due to cycles of glaciation and deglaciation in North America and Eurasia. Most apomictic families such as Asteraceae, Poaceae, and Orchidaceae, have apomictic species that are presumed to have a Pleistocene origin. Repeated deglaciation events resulted in these areas being revegetated by species adapted to cool climates and short growing seasons, with adaptations for precocious meiosis and embryo sac development. The climatic fluctuations would have also caused range shifts that would have resulted in secondary hybrid contact zones between high latitude and low latitude flora which facilitated the formation of polyploid populations with different cytotypes. Gene flow between cytotypes within these zones may have led to asynchronous gene expression in the megasporogenesis and metagametogenesis phases of sexual reproduction (e.g., Polegri *et al*., 2010). This have may resulted in the megasporogenesis phase being skipped, leading to the suppression of sexuality and the expression of apomixis. This was supported by Grimanelli *et al.*, (2003) who found that the frequencies of apomictic phenotypes were influenced by the timing of the steps in developmental pathways from meiosis to post-fertilization.

The relationship between polyploidy and apomixis is confounded by their co-occurrence, making it difficult to discern the causal mechanism of both. Nevertheless, some studies suggest that polyploidization may indirectly contribute to the establishment of apomictic individuals (e.g., Bierzychudek, 1985; Hojsgaard, 2018; Hojsgaard and Hörandl, 2019), while others suggest that apomixis may promote the establishment of polyploid populations (e.g., Karunarathne *et al*., 2018; Kirchheimer *et al*., 2018). Interestingly, despite the lack of knowledge regarding their origins, both polyploidy and apomixis are typically associated with invasion potential (Grusz *et al.*, 2009; Pandit *et al*., 2011; Beck *et al.*, 2011; te Beest *et al*., 2012).

The range expansion in apomictics is often linked to geographical parthenogenesis – a phenomenon where asexual organisms occupy a wider distribution, typically at higher altitudes or in harsher environments, than their sexual relatives (Vandel, 1928; van Dijk, 2003; Hörandl, 2009). This might be accounted for using Baker's Law which states that long distance dispersal typically favours self-fertilising and apomictic species because they are not limited by mate or pollinator availability (Baker, 1955; Stebbins 1957; Baker, 1967). Additionally, only one individual capable of uniparental reproduction is needed to establish an entire population (Baker, 1967). For example, a study conducted on Iridaceae in South Africa, found that species with higher rates of uniparental reproduction were able to naturalize whereas those without the capacity for uniparental reproduction failed to naturalize (van Kleunen and Fischer, 2008).

Much of the polyploid literature debates the invasion potential of polyploids within the context of their ability to occupy new habitats (te Beest *et al.*, 2012; Hahn *et al.*, 2012; Moura *et al.*, 2021). However, the reproductive strategies of polyploids are probably more important when escaping minority cytotype exclusion (MCE; Levin, 1975; Stebbins, 1985). MCE describes a situation where the establishment of newly formed polyploids is limited by mate availability because they are less common in a randomly mating population. Polyploids were presumed to

overcome these limitations by occupying habitats outside their diploid parent populations' ecological range thus accounting for the extensive reports of polyploidy in invasive species (Hollingsworth and Bailey, 2000; Pandit *et al.*, 2011; te Beest *et al.*, 2012; Baudel *et al.*,2018). However, this was challenged by various studies that noted that niche shifts or the broadening of niches does not necessarily contribute to the establishment of polyploid cytotypes (Theodoridis *et al*., 2013; Glennon *et al.*, 2014; Rice *et al.*, 2019). Rather, the success of polyploid lineages may be attributed to the competitive advantages provided by asexual reproduction (e.g., Kirchheimer *et al.*, 2018).

Male fertility, polyploidy and apomixis

Male fertility is an important factor to consider when investigating invasive species. High male fertility could facilitate interploidy gene flow which may generate new beneficial traits in fertile progenies (Alexander, 2020). Assessing the fertility of potential parents may provide insight on cross combinations that may occur within a species (Alexander, 2020). Male fertility in polyploid species has implications for the reproductive strategies used in populations (Atlagić *et al*., 2012) and the subsequent genetic variation within these populations (Whitton *et al.*, 2008).

Ramsey and Schemske (1998) noted that pollen grains of variable ploidy level can facilitate the formation of polyploid or hybrid cytotypes – this may have long term implications for the invasion of novel niches, establishment of populations and an increased distribution of genotypes. For example, interploidy mating between triploids and tetraploids produces polyploid progeny with odd chromosome numbers (Peckert and Chrtek, 2006). This results in the progeny having novel combinations of the parental genes thereby increasing the gene pool in populations which may provide an adaptive advantage in invaded regions (Rotreklová and Krahulcová, 2006; Alexander 2020).

In polyploid agamic complexes (i.e., sexual reproduction is partly or completely replaced by asexual reproduction), high pollen grain viability may be linked to the expression of apomixis (Rotreklová and Krahulcová, 2006). In both facultative apomictics and pseudogamous apomictic species, endosperm development is still dependent on fertilisation; therefore, the production of viable pollen is maintained in most apomictics (Nogler, 1984; Asker and Jerling, 1992). This allows for genes to be transmitted to facultative apomictic and sexual relatives, which enables the formation of genetically diverse clone complexes (Whitton *et al*., 2008; van Dijk, 2009).

The presence of clonal diversity within a population makes biocontrol using a genotype-specific agent challenging (van Dijk, 2003; Chapman *et al.,* 2004). A study conducted on invasive *Chondrilla juncea* L. (skeleton weed) in Australia found that the introduced biocontrol agent, the rust fungus *Puccinia chondrillina*, had different effects on the survival rate of the three clones within the population (Burdon *et al.,* 1981). The frequency of one clone type increased while the other two decreased. Conversely, in the native range of *Chondrilla juncea* in Turkey, the most abundant clones were over-infected (Burdon *et al.,* 1981). Investigating the sources of genetic diversity, such as reproductive strategies and polyploidy, within invasive taxa is therefore important before releasing biocontrol agents.

Campuloclinium macrocephalum

*Campuloclinium macrocephalum (*Asteraceae, Eupatorieae), or 'pompom weed', is endemic to South America – specifically Brazil, Paraguay, Uruguay, Bolivia, Argentina, Central America Farco *et al.,* 2012). The species has medicinal value in these areas and the leaves are often used as a sedative and anti-inflammatory (Vega *et al.,* 2008). *C. macrocephalum* was introduced to South Africa in the 1960s and the earliest population was found in Pretoria (McConnachie *et al.,* 2011). By the 1980s the species had spread rapidly across Gauteng and into Limpopo

(Henderson, 2007). The species then entered an exponential expansion phase and its range increased to include Mpumalanga, Limpopo, and North-West provinces (Henderson, 2007) (Figure 1.1, this thesis).

The pompom weed is a perennial herb that develops from a woody rootstock with perennating buds. The bright pink capitula (Figure 1.2) comprise multiple florets with ovaries that mature into cypselas (asteraceous fruits with a single seed). The cypselas have a persistent pappus (Goodall *et al.*, 2010). Small clusters of capitula on a common peduncle form synflorescences. The plant invests a considerable amount of its resources in underground structures to ensure its survival during winter months (Goodall *et al*., 2010). Moisture availability is the main environmental stressor for the pompom weed as the onset of winter causes the plants to senesce (Goodall *et al.*, 2010). The weed is typically found in areas that receive more than 600 mm of rain per annum, however the amount of rainfall that limits establishment and spread is unknown (Goodall *et al.*,2010).

Campuloclinium macrocephalum typically occupies disturbed sites, such as road margins and old fields and is consequently considered a pioneer species. The species thereafter invades grasslands, savannas, and wetlands (McConnachie *et al*., 2011) and transforms such ecosystems to the extent that the indigenous species are eliminated. The species does this by increasing soil erosion, reducing biodiversity by displacing native species, interfering with the establishment of grass species, and reducing the carrying capacity of invaded areas (Dixon, 2008). Due to the absence of natural enemies in the non-native region/country, this species' distribution was predicted to exponentially increase every year unless a successful biocontrol agent was found (Trethowan *et al*., 2011).

Invasion opportunity into rangelands is predominantly through wind-dispersal; however, establishment is restricted by vegetation quality – specifically sward and basal cover (Goodall

et al., 2010). The vegetation invaded by this weed is usually in poor condition and typically occurs in rangelands affected by unsustainable grazing, poorly managed/abandoned agricultural fields and drained wetlands (Goodall *et al.,* 2010). *Campuloclinium macrocephalum* does not invade undisturbed grasslands (Lake and Leishman, 2004; Goodall *et al.,* 2010). This species reduces the grazing potential of grasslands as it is unpalatable to livestock. This may be due to the glandular trichomes on stems and leaves producing phytotoxic substances (McConnachie *et al*., 2011). The success of pompom weed's spread is not attributed to its allelopathic nature (McConnachie *et al*., 2011), but could be due to its ability to reproduce prolifically and asexually.

The eradication of *C. macrocephalum* has proven to be a difficult endeavour (McConnachie *et al*., 2011). In the past, management plans for the pompom weed have relied on mechanical and chemical methods. The mechanical methods, such as hoeing and ploughing, were unsuccessful as it encouraged regeneration from the xylopodium and the plants subsequently thrived in the disturbed environments (McConnachie *et al*., 2011). Chemical methods, such as herbicides, negatively impacted other vegetation in the vicinity and use of this method is restricted to roadsides (McConnachie *et al*., 2011). Additionally, *C. macrocephalum* does not use vegetative reproduction, however the xylopodium of the woody underground rootstock functions as a storage organ (Farco *et al*., 2012). This may be co-opted for persistence of the plant if the above ground portion dies (Farco *et al*., 2012). Therefore, the pompom weed is resistant to herbicide damage, fire, and drought, resulting in it being a successful invader (Farco *et al*., 2012).

Biological control programmes using *Liothrips tractabilis* Uzel 1978 (thrips) and *Puccina eupatorii* Cummins 1978 (leaf rust pathogen) have produced better results than both the mechanical and chemical methods. The thrips cause foliage deformation thereby reducing growth and biomass accumulation – particularly in younger plants (Ramanand *et al.,* 2016; Ramanand *et al.,* 2017). The leaf rust pathogen causes premature senescence; however, this

stimulates the production of compensatory growth in late autumn (Goodall *et al.,* 2012). Therefore, the leaf rust pathogen is unlikely to cause measurable reductions in pompom weed densities (Goodall *et al.,* 2012).

The success of biocontrol agents on a species may be linked to the type of reproductive system used by a species, polyploidy, and the consequent genetic variation within a species. A previous study conducted on the reproductive strategy of the pompom weed found that the species is capable of uniparental reproduction, however it was unclear if it was autonomous autogamy or apomixis (Kgaboesele, unpubl. data). Gitonga *et al.* (2015) found low genetic variability amongst populations of the pompom weed, but the presence of apomixis could not be confirmed. Gitonga *et al.*, (2022) identified triploid and tetraploid cytotypes of *C. macrocephalum* in South African populations, however the predominant reproductive strategy used by populations of *C. macrocephalum* in South Africa remains unclear.

Aim and objectives

The aim of this investigation was to infer whether populations of *Campuloclinium macrocephalum* (pompom weed) reproduce by vector-mediated crosses, self-pollination or apomixis (either facultative or obligate) and assess the relationship between polyploidy and the mode of reproduction.

The objectives associated with this aim were to:

1) Survey insect visitors to capitula and investigate whether effective vector-mediated cross-pollination may occur in populations of *C. macrocephalum* in Gauteng.
2) Compare pollen size, shape, and viability between selected triploid and tetraploid populations to assess male fertility and infer possible reproductive barriers in populations of different ploidy levels.

3) Assess whether pollen tubes are reaching the ovaries and/or ovules in bagged capitula to determine if autogamy or apomixis is predominantly occurring, and the type of apomixis.

4) Compare genetic diversity within and among triploid and tetraploid populations of *C. macrocephalum* in order to infer whether plants self-fertilise or reproduce via apomixis, and if apomixis is occurring – assess whether it is facultative or obligate.

Dissertation outline

This thesis is divided into four chapters: the current introductory chapter, chapter 2, chapter 3, and a concluding chapter. Due to the structure of the thesis, there may be some repetition in background material presented. The chapters are listed below:

1) An introductory chapter that comprises the rationale and literature review, aim and objectives of the study.

2) A characterisation of male fertility in triploid and tetraploid populations of *C. macrocephalum.*

3) Reproductive biology of *C. macrocephalum* and its applicability in biocontrol management plants.

4) A concluding chapter to synthesise the thesis results and recommendations for future studies

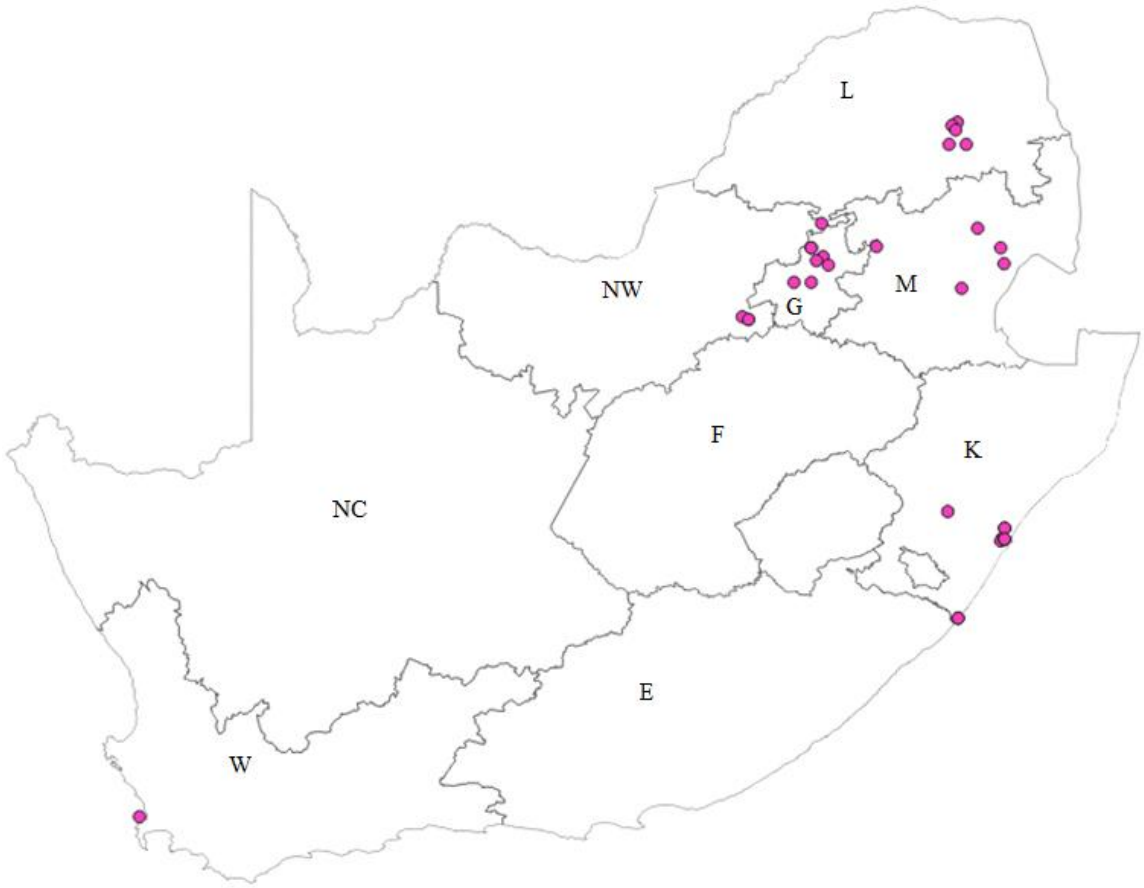

Figure 1.1. The current recorded distribution of *Campuloclinium macrocephalum* in the Gauteng (G), Limpopo (L), Mpumalanga (M), Kwa-Zulu Natal (K) and Western Cape (W) provinces of South Africa. The location data were obtained from the SANBI NewPOSA database (Last updated: 14 October 2021).

Figure 1.2. Dense populations of *Campuloclinium macrocephalum* in Greenstone (Gauteng), characterized by bright pink florets, during the December 2020 flowering period.

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Chapter 2

Male fertility and potential gene flow of triploid and tetraploid populations of *Campuloclinium macrocephalum.*

ABSTRACT

Campuloclinium macrocephalum, commonly referred to as the pompom weed, is an alien invasive species in South Africa. The species is currently threatening the survival of biomes in South Africa by causing a reduction in indigenous vegetation. Without intervention, the species will invade the entire grassland biome and threaten food security. Previous studies hypothesised that a species' invasiveness is linked to ploidy and apomictic reproduction within its invaded range. This study assesses male fertility in two triploid and two tetraploid populations of *C. macrocephalum* in Johannesburg, South Africa to test if gene flow is occurring amongst populations of different ploidy levels. Pollen from triploid and tetraploid populations were stained with aniline blue and acetocarmine; populations exhibited mean pollen viability percentages of 98% and 90% which indicates high male viability. Such viability could enable gene flow among populations and result in the formation of genetically diverse progeny via a 'triploid bridge'. In contrast, fluorescence microscopy and scanning electron microscopy of pollen tubes showed that pollen tube growth is being arrested on the stigmatic margin and on the sides of the style branches, which may be indicative of a triploid block. Previous studies suggest that triploid blocks can be overcome by the production of viable gametes; therefore interploidy mating may be occurring at low frequencies. Interploidy mating may result in the production of offspring that exhibit differential responses to biocontrol agents.

Keywords: Male fertility; pollen viability; polyploidy; interploidy mating; gene flow

INTRODUCTION

Ploidy levels have been shown to influence pollen biology within a species (Johansen and Bothmer, 1994; Katsiotis and Forsberg, 1995; Zlesak, 2008; Knight *et al.*, 2010). Ploidy can often lead to changes in the breeding system strategies used by a plant species therefore a better understanding of male fertility is required (Maia *et al.*, 2015). Pollen grains are responsible for the transfer of male genetic material and directly contribute to the reproductive output in plants (Mert, 2009). Pollen grain characterisation and pollen viability can offer insight into the differences in male fertility among populations with different ploidy levels (Johansen and Bothmer, 1994; Czarnecki *et al.*, 2014).

Pollen grain characterisation includes assessing the morphological variation of pollen grains within a species – particularly pollen grain size and shape. In many genera, pollen size is positively correlated with genome size (Beaulieu *et al*., 2008; Knight *et al.*, 2010) and ploidy level (Katsiotis and Forsberg, 1995; Zlesak, 2008). There is also a trade-off between pollen grain size and the amount of pollen produced (e.g., Vonhof and Harder, 1995). Selfing species often produce fewer pollen grains than outcrossing species, resulting in selfing species having larger pollen grains (Cruden, 2000; Naghiloo and Siahkolaee, 2019). Additional pollen grain characteristics such as apertures, projections on the pollen grain surface, and clumping has implications for the pollination mechanism and consequent reproductive strategy used by a species (Mignot *et al.*, 1994, Nagihloo *et al.*, 2018 and Ackerman, 2000). These factors may influence pollen viability (Till-Bottraud *et al.*, 1994), increase the efficiency of pollinator interactions (Naghiloo *et al.*, 2018) or facilitate pollen dispersal (Ackerman, 2000).

Pollen grain morphology is also influenced by polyploidy (Liu *et al.*, 2003). Tetraploids produce larger pollen grains than both diploids and triploids (Tas and van Dijk, 1999). Larger pollen grains in diploids increase reproductive success as there is a strong relationship between

pollen grain size and seed siring (Cruzan, 1990). Larger pollen grains may have greater resource content (e.g., starch granules or lipids) which increases the probability of an ovule's maturation due to increased metabolic vigour (Cruzan, 1990). Notably, diploids produce pollen grains of uniform size and shape within a species. In contrast, triploids produce pollen of irregular size and shape because individuals exhibit high levels of aneuploidy due to irregular meiosis during microsporogenesis (Aparicio, 1994; Tas and van Dijk, 1999; Mráz *et al*., 2002). The size and shape of pollen grains has implications for its germinability. Diploids often have higher pollen germination rates than both tetraploids and triploids (Liu *et al.,* 2003; Maggi *et al.,* 2008; Ovchinnikov *et al.,* 2017).

Pollen tube growth rates can be affected by ploidy level (Lankinen *et al.,* 2009). Pollen tube growth in diploids is usually faster than in triploids, which may be due to the high proportion of aneuploid pollen grains produced by triploids (Lankinen *et al.,* 2009). An increase in DNA content results in an increase in cell size, nuclear size, and the duration of the cell cycle. This negatively effects pollen tube growth rates as more time is required for pollen tube wall formation (e.g., Soares *et al.,* 2014). Pollen tube growth rate is positively correlated with the fitness of progeny (Walsh and Charlesworth, 1992). A lower pollen tube growth rate may be a pre-zygotic barrier that prevents fertilization from pollen with malfunctioning genomes (Lankinen *et al.,* 2009). The visualisation of pollen germinability and pollen tube growth allows for the identification of pre-zygotic reproductive barriers (Atlagić *et al.,* 2012). These barriers consequently influence the plant breeding strategies that are used to mitigate such incompatibilities (Atlagić *et al.,* 2012).

The reproductive strategy used by a species has implications for the occurrence of interploidy mating (Yamauchi *et al.,* 2004). The conjugation of haploid and diploid pollen produced by diploid and tetraploid individuals, respectively, may result in the formation of triploids, however these triploids typically exhibit lower fitness levels than their progenitors (Burton and

Husband, 2000). This lower fitness can occur via two mechanisms: endosperm collapse due to the unbalanced ratio of maternal and paternal genomes known as the 'triploid block' or the reduced production of gametes due to irregular meiosis (Ramsey and Schemske, 1998). Both of these factors may limit the role triploids play in polyploid evolution by preventing them from reproducing (Thompson and Lumaret, 1992). Conversely, triploids may facilitate the formation of tetraploids via a 'triploid bridge' (Ramsey and Schemske, 1998). Triploid bridges occur when haploid gametes from diploids fuse with unreduced gametes from triploids thereby forming tetraploids (Yahara, 1990). Burton and Husband (2001) suggested that partial viability and/or fertility of triploids may facilitate tetraploid establishment and result in the formation of genetically diverse progeny.

Apomixis, clonal reproduction by seed, is a common reproductive strategy used by polyploids (Ramsey and Schemske, 1998). This has been observed in *Sorbus* L. (Robertson *et al.,* 2010), *Amelanchier* Medik (Burgess *et al.,* 2014) *Boechera* A. Love and D. Love (Luise *et al.,* 2012) and *Potentilla* L. (Dobes *et al.,* 2013) among other genera. Meiosis is often deregulated (the reduction or elimination of certain steps in the meiotic process) in newly formed hybrids, which leads to increase in the production of unreduced gametes (Asker and Jerling, 1992). This often results in individuals favouring apomixis to avoid the formation of lower fitness offspring due to genetic abnormalities (Ramsey and Schemske, 1998). The type of apomixis expressed within a population can thereafter have implications for pollen morphology and viability (Whitton *et al.,* 2008).

Obligate apomictic plants often have low pollen viability or complete pollen sterility (Maia *et al.,* 2015). This is presumably due to individuals occurring in areas with little or no insect visitors (Baker, 1967). In contrast, facultative and pseudogamous apomictic plants have high pollen viability (Maia *et al.,* 2015). Pseudogamous apomictic plants require high pollen

viability for the fertilisation of the polar nuclei. Due to the mixed breeding system used by facultative apomictic plants, high pollen viability is required for sexual reproduction to occur successfully (Maia *et al.*, 2015). Consequently, pollen viability is a reliable indicator of the type of apomixis used by a plant species, once it is established that apomixis occurs (Maia *et al.*, 2015).

Pollen production constitutes a substantial reproductive cost in plants (Meirmans *et al.*, 2006; Mráz, 2009). For example, male-sterile apomictic dandelions produce more flower heads per plant, and consequently more seeds, than pollen producing apomictic dandelion species (Meirmans *et al.*, 2006). Pollen production may be maintained in phylogenetically recent autonomous apomictics because they have not yet accumulated sufficient mutations for male sterility (Smith, 1978). As the frequency of apomixis increases however, pollen transmission become less efficient and results in pollen fertility declining over time (Smith, 1978).

Introduced populations of *Campuloclinium macrocephalum* (Less.) DC. (Asteraceae, Eupatorieae) in South Africa have been shown to comprise triploid and tetraploid individuals with a few aneuploid individuals among them (Gitonga *et al*., 2022). The factors contributing to the invasiveness of this species are not well understood (McConnachie *et al*., 2011; Farco and Dematteis, 2014) however, polyploidy may be enhancing the species' potential for habitat colonisation via a triploid bridge. In a previous study, Farco and Dematteis (2014) conducted a study on the meiotic system and pollen viability (estimated via staining techniques) in *C. macrocephalum* populations in Argentina and Uruguay and found variable meiotic behaviour with irregular chromosome pairing in both triploids and tetraploids. Meiotic indices suggested that only four out of fourteen South American populations were meiotically stable and were therefore normally fertile. The other ten populations had variable pollen viability, with greater pollen viability in triploids than tetraploids. The low viability of male gametes and the expression of apomixis in *C. macrocephalum* polyploids in Argentina and Uruguay was

attributed to meiotic abnormalities and subsequently reduced probabilities of producing viable offspring.

Male fertility (i.e., the ability of pollen grains to sire seeds) in populations of *C. macrocephalum* in South Africa has not yet been assessed. An investigation of male fertility is important because viable pollen could enable gene flow between populations of different ploidy levels (Alexander, 2020). Rapidly evolving populations of *C. macrocephalum* would be difficult to eradicate. Moreover, complete, or partial male sterility could promote reproduction via apomixis, a reproductive mode that allows for the rapid production of large numbers of offspring (Farco and Dematteis, 2014). In South Africa, *C. macrocephalum* is hypothesised to reproduce via apomixis due to the low genetic variation among populations despite the prolific seed production (Gitonga *et al.,* 2015). However, this aspect has not been investigated further. Assessing male fertility could provide information about the breeding system strategies used by *C. macrocephalum*, the potential of interploidy mating and its implications for biocontrol.

The aim of this investigation is to assess male fertility using comparisons of pollen size, shape, and viability between selected triploid and tetraploid populations of *C. macrocephalum.* These data will help me to infer possible reproductive barriers in populations of different ploidy levels. This study will improve our understanding of the reproductive strategies used by *C. macrocephalum* – a factor which may be contributing to the species invasiveness in South Africa. I hypothesize that triploids will exhibit lower male fertility than tetraploids due to irregular meiosis. This should reduce gene flow between triploid and tetraploid cytotypes.

METHOD AND MATERIALS

Study sites

This investigation was conducted on four populations of *C. macrocephalum* in the Gauteng province of South Africa. These populations were in Tembisa (triploid, 26.05 S; 28.16 E),

Midrand (triploid, 26.02 S; 28.13 E), Modderfontein Nature Reserve (tetraploid, 26.09 S; 28.15 E) and Greenstone (tetraploid, 26.12 S; 28.15 E). These populations were selected based on their putative ploidy levels identified by Gitonga *et al.* (2022).

Study species

Campuloclinium macrocephalum, referred to as the pompom weed, is a South American perennial herb that has invaded South African grasslands. The species is conspicuous during its flowering months from December to March. The pompom weed is characterised by its pink flowerheads that are produced in dense clusters. The leaves form a rosette at the base of the plant; however, they decrease in number and size along the length of the stem. The stem and leaves are covered in rough hairs. Adult plants grow to approximately 1.5 m tall. At the end of the flowering season, mature florets each produce a cypsela with tufts of brown hair. The species has a woody rootstock with perennial roots. In summer, new shoots emerge from the rootstock and in autumn they die back down. The rootstock, constituting a xylopodium, enables the species to persist when its aerial parts die.

Pollen viability

Aniline blue stain

After anthesis, capitula from thirty individuals per population were cut and placed in brown paper bags. Five florets from each individual were placed in Eppendorf tubes containing 0.5 ml of 0.1% aniline blue to preserve them for laboratory analyses. Prior to viewing under a microscope, the anthers were dissected out of the florets using a dissecting needle and placed back into the vials. The vials were then vortexed for one minute to resuspend the pollen grains. Thereafter, 40 µL of the aniline blue solution was placed on a microscope slide and a coverslip was placed over it. A 25 mm x 25 mm plastic grid was then placed over the coverslip. The slides were examined under a Zeiss compound light stereomicroscope at 400× and the number

of all the viable and non-viable pollen grains in the 40 µL of the aniline blue solution were counted.

Acetocarmine stain

Pollen viability using the acetocarmine stain was assessed following Farco and Dematteis (2014). This stain was used to confirm the pollen viability results from the aniline blue stain as the aniline blue stain made it difficult to differentiate between viable and non-viable grains. Capitula from 10 individuals per population were harvested and fixed in Carnoy's fixative (6:3:1, 95% ethanol: glacial acetic acid: chloroform) for 2 hours. Thereafter, the capitula were stored in 70% ethanol until further analyses. Prior to staining, all the anthers from three florets per individual were dissected and placed on a microscope slide with a drop of 70% ethanol to prevent the tissue from drying out. The anthers were then squashed to extract the pollen grains and the excess anther tissue was removed from the slide. A drop of acetoglycerol stain was then added to the slide and a coverslip was placed over it. The slides were then examined under Zeiss compound light stereomicroscope at 400x. Dark red stained nuclei were indicative of viable pollen grains while unstained/lightly stained nuclei were indicative of non-viable pollen grains.

The percentage viability/non-viability for both stains was calculated as follows:

$$\text{Percentage viability} = \frac{\text{Number of viable/ non viable pollen grains}}{\text{Total number of pollen grains}} \text{ x } 100$$

After the mean percentage viability was calculated, the distribution of pollen grain viability percentages was tested using a Shapiro Wilk test in R studio (R version 4.0.3) using the fBasics package (Wuertz *et al.*, 2017). The data were found to be non-parametric, therefore a Kruskall-Wallis test was used to test for differences between the pollen viability percentages of the four populations.

Pollen tube analyses

Pollen tube analyses followed the protocol outlined by Kalinganire *et al.,* (2000) for the visualisation of pollen tube growth. Twenty florets from ten individuals per population were removed from the capitula harvested from "open treatments" (i.e., naturally occurring capitula) under a Zeiss Stereo Discovery V12 dissecting microscope. The florets were then fixed in 'Carnoy's' fixative, comprising ethanol:chloroform:acetic acid (6:3:1), for two hours. Thereafter, the florets were transferred to 70% ethanol for storage. The pistils were not softened or cleared using NaOH as the delicate tissue disintegrated during this step. The pistils were rinsed with distilled water and stained with aniline blue in potassium buffer for two hours; longer durations made it difficult to work with the tissue. The florets were then placed on a microscope slide with a drop of 80% glycerol and squashed using a coverslip. The slides were examined under the BX63 OFM fluorescent microscope to observe pollen tube growth.

To confirm the results obtained from fluorescence microscopy, five pistils from three individuals per population were dissected out of florets and placed on carbon tape adhered to aluminium stubs. The pistils were left to dry overnight and then coated with one coat of carbon and one coat of gold/palladium. The stubs were viewed under a Tescan Vega Scanning Electron Microscope. The presence of pollen tube growth was indicative of the viability of the pollen grains.

Pollen grain size and shape

The slides prepared using the aniline blue stain were used to measure pollen grain size and shape. Five florets from thirty individuals were placed in Eppendorf tubes with the aniline blue stain. Thereafter, the tubes were vortexed and 40 µL of the solution was deposited onto a slide and covered with a coverslip. The size of 50 viable pollen grains and all of the non-viable grains (as there were very few non-viable pollen grains) from 10 individuals per population were

measured using the available function on the Axio Imager connected to a Carl Zeiss compound light microscope at 400×. Irregularities in the shape of pollen grains were also noted. Pollen shape was described and compared qualitatively between the putative tetraploid and triploid populations. The distribution of the pollen grain size data was tested using a Shapiro Wilk test on R studio (R version 4.0.3) using the fBasics package (Wuertz *et al.*, 2017). The data were analysed for significant differences in pollen grain size between the populations using a Kruskal-Wallis test followed by a Kruskal-Wallis multiple comparison post hoc test in R studio (R version 4.0.3) using the pgirmess package (Giraudoux *et al.*, 2018). Within population variation of pollen grain sizes was also evaluated (mean ± standard deviation).

Flow cytometry and anther squashes

Flow cytometry was used to confirm the ploidy levels obtained by Gitonga *et al.* (2022) in 2013–2014 for the study populations. Flow cytometry provides estimates of DNA content relative to a known standard from which ploidy level can be inferred. The protocol outlined in Dolezel *et al,* (2007) was followed. Seedlings from three maternal plants per population were grown in a growth chamber (at 25 ºC and 60% humidity on a 16-hour light schedule) until they were large enough to harvest. Leaves from two offspring per maternal plant were used to estimate ploidy. *Zea mays* L. was used as the internal standard. A 1 x 1 cm section of leaf tissue from *Z. mays* was added to a Petri dish containing a 1 x 1 cm section of *C. macrocephalum* leaf tissue. The leaves were simultaneously chopped using a razor in a 500 µl lysis buffer (Otto I). Unhomogenised leaf tissue was removed from the solution by filtering it through 30 µm mesh filters. Thereafter, the solution was incubated for 15 minutes at room temperature. A 1000 µl of Otto II containing DAPI (4', 6-diamidino-2-phenylindole) was added to the solution to stain the cells. This process was repeated for all the samples. The samples were then analysed using a CyFlow^{R} Space flow cytometer at the University of Pretoria. FloMax software (Partec,

Münster, Germany) was used to calculate the mean size of each peak (sample and standard) and calculate the coefficient of variation.

Relative DNA content was calculated as follows:

Relative DNA content = Fluorescence value of the sample/Fluorescence value of the standard

Anther squashes were used to determine chromosome number and to ascertain if irregular meiosis occurs in *C. macrocephalum*. Anther squashes were conducted following Windham *et al*. (2020). Young capitula were harvested from five individuals per population and fixed in Carnoy's fixative (6:3:1 95% ethanol:chloroform:glacial acetic acid) for 24 hours. Thereafter, the fixed material was transferred into 70% ethanol and stored in a freezer at -20 ºC. To prepare the material for squashing, an array of buds was chosen and placed in a Petri dish with 70% ethanol to prevent buds from drying out. The anthers were excised and if they were white and showed normal development, they were selected for the staining process. A drop of diluted acetocarmine stain was placed on one side of a microscope slide. The anthers were transferred to this drop and further isolated from surrounding tissue. A drop of full strength acetocarmine stain was placed on the other side of the microscope slide and the anthers were transferred to it. A dissecting needle was then used to crush the anthers until the sample could not be homogenized anymore. All of the unhomogenized material was then removed and a drop of Hoyer's solution was placed on the slide. The Hoyer's solutions reduced coverslip rebound and partially de-stained the cytoplasm. A coverslip was then lowered onto the droplet and gently tapped to get rid of air bubbles. The slide was then placed in paper towel and squashed. The slide was viewed under a Carl Zeiss compound light stereomicroscope and photographs were taken of the observed stages of meiosis/mitosis on the slide at 400x and/or 1000x.

RESULTS

Pollen viability

There was no significant difference between pollen viability percentages amongst the four populations using the aniline blue stain (H_3 = 7.43; P = 0.06) (Figure 2.1). When stained with aniline blue, the average pollen viability of populations of *C. macrocephalum* was 98%. The acetoglycerol stain, however, revealed an average pollen viability of 90% for the same populations. There was no significant difference between average pollen viability amongst the four populations using the acetoglycerol stain (H_3 = 0.64; P = 0.59, Figure 2.1). The discrepancies between the data obtained from the two stains may be attributed to the difficulty in differentiating between viable and non-viable pollen grains using the aniline blue stain (Figure 2.2).

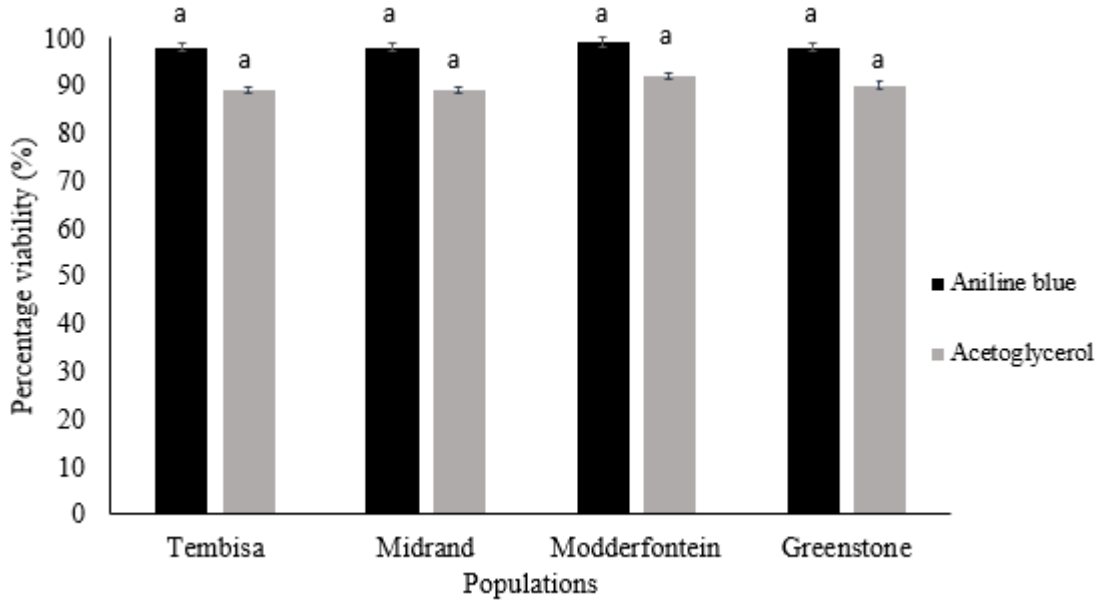

Figure 2.1. Mean pollen viability (%) in four populations of *Campuloclinium macrocephalum* collected across Gauteng, South Africa. Thirty plants per population were assessed using aniline blue stain and ten plants per population were assessed using the acetoglycerol stain. The bars above the column represent standard error. The letters above each bar represent statistical significance between populations for each stain.

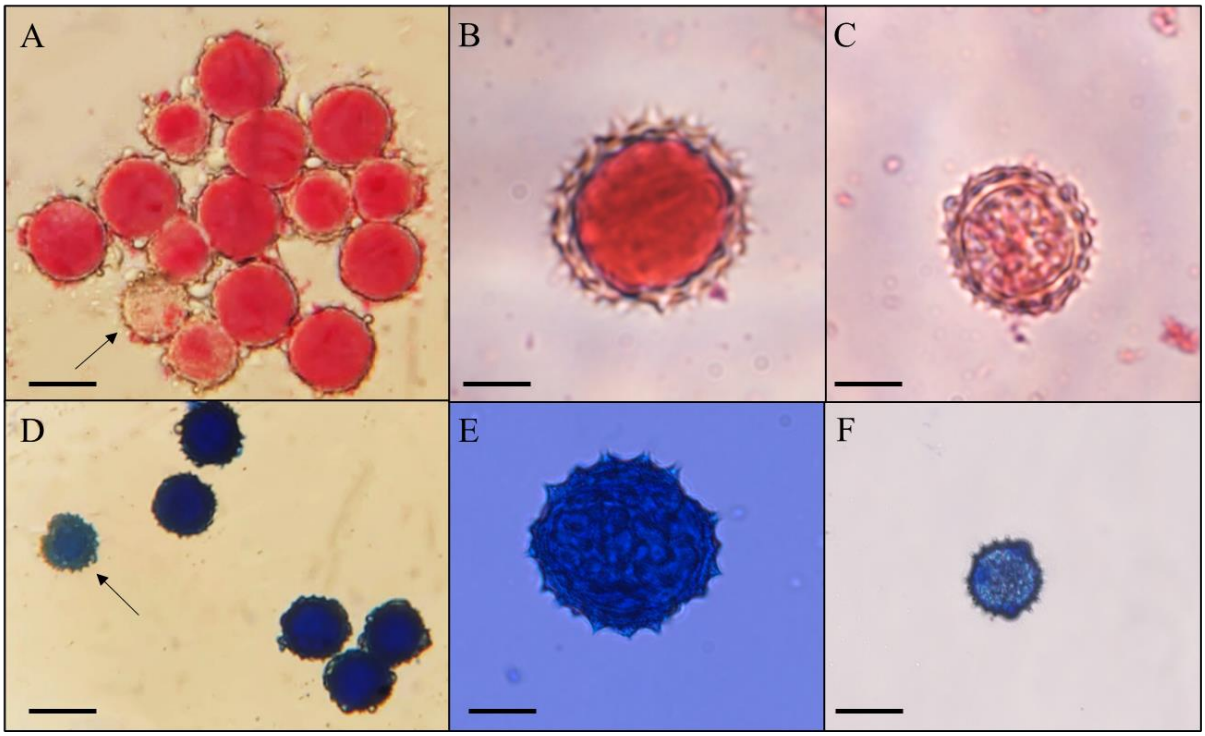

Figure 2.2. Pollen grains of *Campuloclinium macrocephalum* stained for viability assessments using acetocarmine (A, B, C) and acetoglycerol (D, E, F). A: Pollen grain stained by acetoglycerol with an arrow indicating a non-viable pollen grain (scale bar = 24 µm), B: viable pollen grain stained by acetoglycerol (scale bar = 18 µm), C: non-viable pollen grain stained by acetoglycerol (scale bar = 13 µm), D: Pollen grains stained by aniline blue with an arrow indicating a non-viable pollen grain (scale bar = 22 µm), E: viable pollen grain stained by aniline blue (scale bar = 15 µm), F: non-viable grain stained by aniline blue (scale bar = 17 µm).

Pollen tube analyses

Pollen tube analyses were also used as an indicator of male fertility. No pollen tube growth was observed using fluorescence microscopy, however scanning electron microscopy showed pollen tube growth on the outside of the style and on the stigmatic margin (Figure 2.3). The location on the style where the pollen grains adhered matched where the anthers enclose the style before the latter emerges thus suggesting that it is autogamous pollen (Figure 2.4). The presence of pollen tube growth on both sides of the style branches, including near the stigmatic surface, indicates that the species produces viable pollen and therefore supports the pollen viability count data.

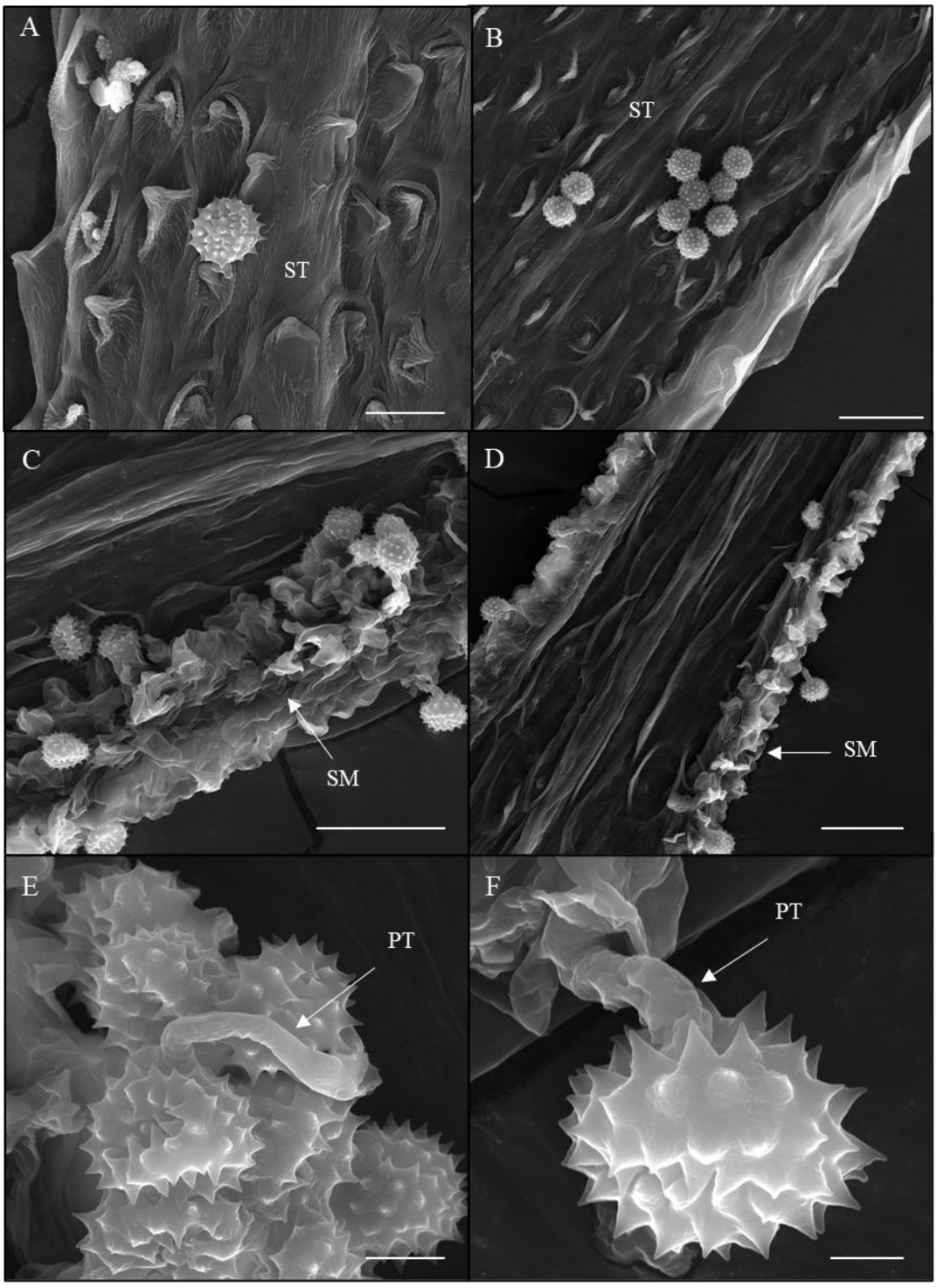

Figure 2.3. Pollen tube growth in *Campuloclinium macrocephalum* observed under a scanning electron microscope. A–B: Pollen grains on the style with no visible pollen tube growth (scale bar in A = 100µm, B = 50 µm), C–D: Pollen germinating on the stigmatic margin (scale bar in C = 20 µm, D = 50 µm) and E–F: Pollen grains with pollen tube growth situated on the outside

of the styles (scale bar in E = 10 µm, F = 5 µm). ST: style, SM: stigmatic margin and PT: pollen tube.

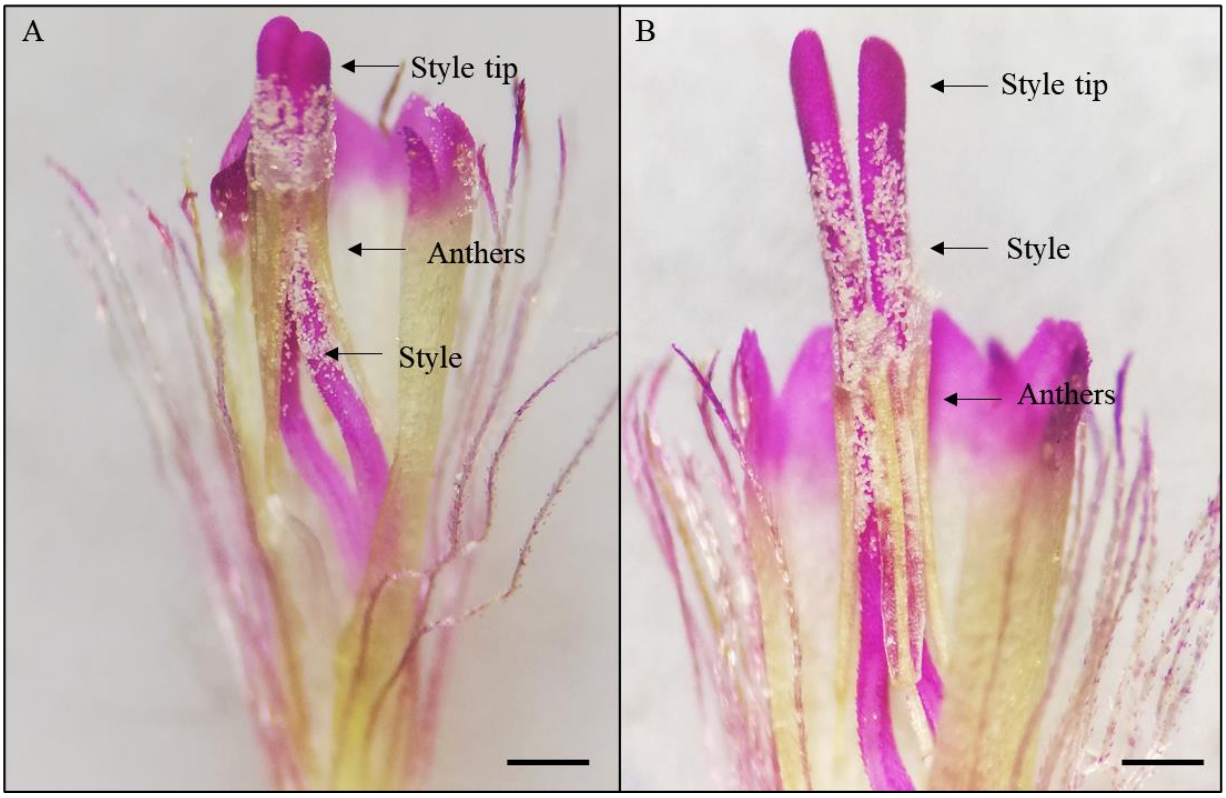

Figure 2.4. The position of the anthers relative to the style in developing florets of *Campuloclinium macrocephalum*. A) A ring of fused anthers surrounding the style branches, with appressed style tips, before it elongates, B) Elongated style branches with separated style tips. Scale bars = 1.7mm.

Pollen grain diameter

There is a significant difference between viable and non-viable pollen grain diameter in all four populations (H_7 = 53.81; P < 0.0001, Table 2.1). Viable pollen grain diameter also differs significantly amongst the four populations (H_3 = 204.9; P< 0.0001, Figure 2.5). There was a significant difference between Tembisa (3x) and Midrand (3x) (P < 0.001), Tembisa and Modderfontein (4x) (P < 0.001), Tembisa and Greenstone (4x) (P < 0.001), and Modderfontein and Midrand (P < 0.001). There was no significant difference between Greenstone and Midrand (P = 0.311), Greenstone and Modderfontein (P = 0.133) (Appendix 2.1).

Table 2.1. Mean pollen grain diameter (± SE) of viable and non-viable pollen grains in four populations of *Campuloclinium macrocephalum.*

Population and putative ploidy level	**Mean diameter of viable pollen grains (±SE)**	**Mean diameter of non-viable pollen grains (±SE)**
Tembisa (3x)	24.05 ± 0.70	21.19 ± 0.82
Midrand (3x)	27.92 ± 0.71	25.58 ± 0.62
Modderfontein (4x)	26.43 ± 0.34	21.62 ± 0.30
Greenstone (4x)	26.98 ± 0.30	23.52 ± 0.41

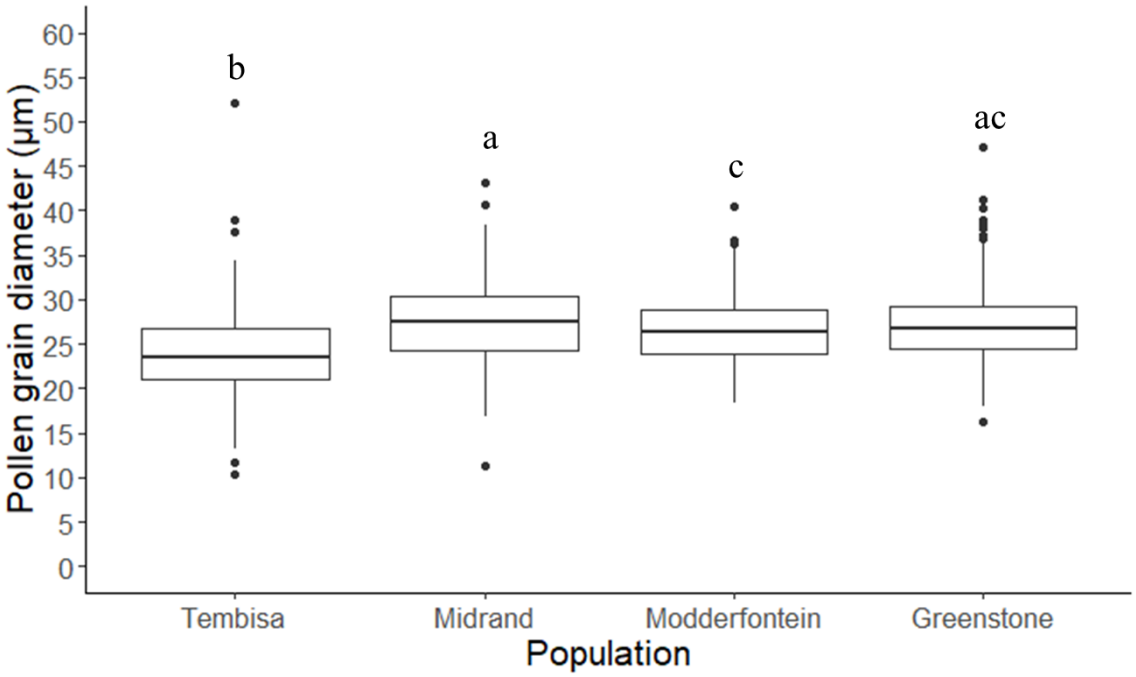

Figure 2.5. Viable pollen grain diameter in four South African populations of *Campuloclinium macrocephalum* using aniline blue. Tembisa and Midrand are triploid populations while Modderfontein and Greenstone are tetraploid populations. The circles represent outliers, the bars represent minimum and maximum values and the box represents variation in the data and the midline represents the mean.

Pollen grain morphology

Pollen grains of *C. macrocephalum* are spherical and echinate (i.e., covered in micro-spines). The spines have a regular arrangement that was consistent in all of the observed pollen grains. Pollen grain tetrads and triads were found in all sampled individuals from each population. The presence of triads is indicative of irregular meiosis occurring in this species. Most of the tetrads and triads were stained as viable by the aniline blue stain, however they were stained as non-viable using the acetoglycerol stain (Figure 2.6). While pollen grain shape was consistent in all four populations, pollen grain size differed (Figure 2.6).

Flow cytometry and anther squashes

From the 24 individuals analysed using flow cytometry, four samples had to be excluded because their coefficient of variation exceeded 5%, which does not provide a reliable estimation of DNA content. The estimated ploidy for the remaining 20 individuals was based on their relative 2C DNA content (Appendix 2.2; Appendix 2.3). Relative 2C DNA content ranged from 2.87 – 3.26, indicating that they were most likely triploid individuals. This was corroborated by anther squashes revealing a chromosome number of approximately 15 (base chromosome number x = 10; Farco *et al.* (2012)). It was challenging to obtain exact counts due to the limitations of the technique (such as obtaining anthers with a high meiotic index) and the available equipment (Figure 2.7). All of the offspring from the Modderfontein and Greenstone populations were identified as triploid despite being harvested from populations previously identified as tetraploid.

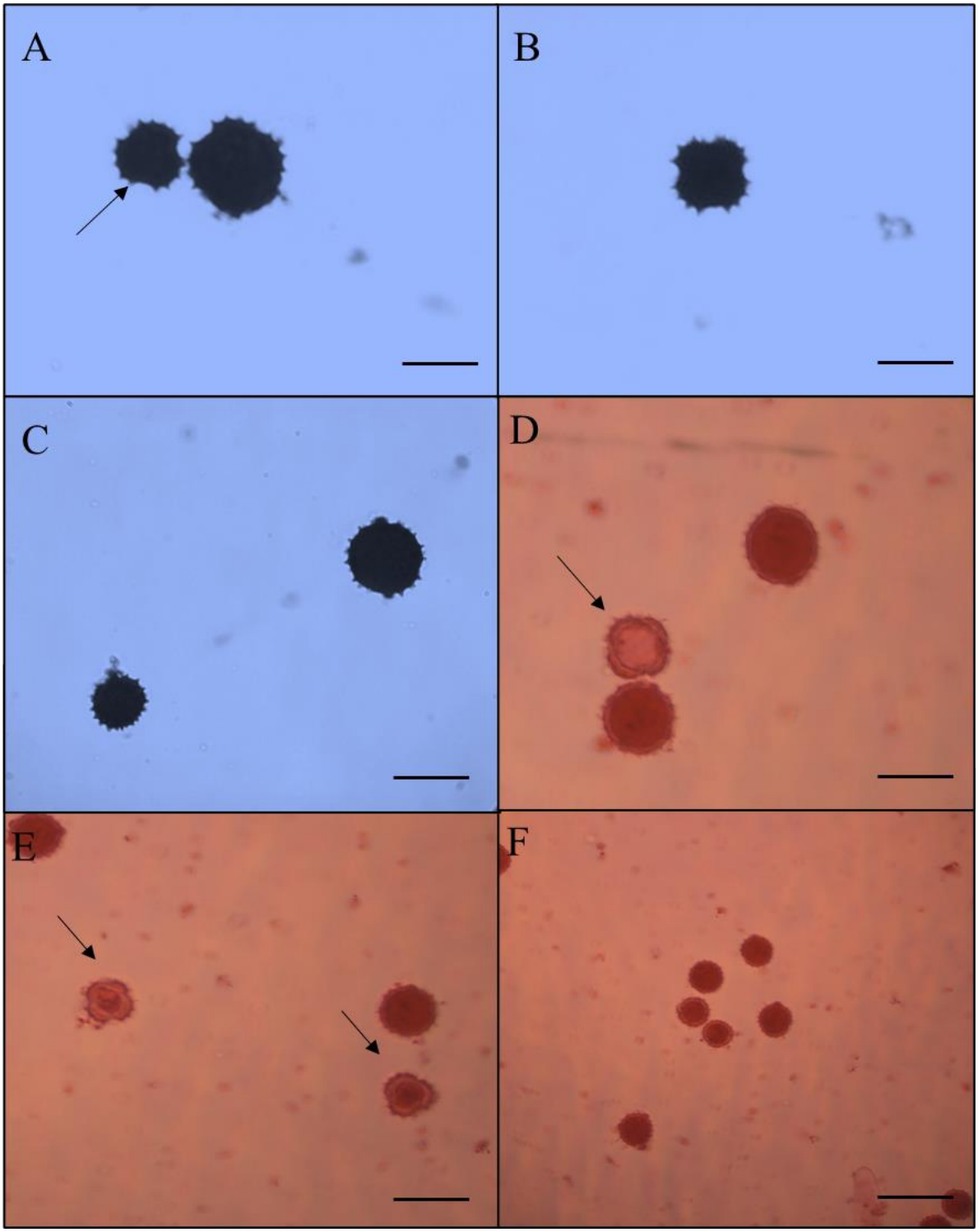

Figure 2.6. Size variation in pollen grains of *Campuloclinium macrocephalum.* A) Viable triad grain indicated by the arrow (scale bar = 28 µm), B) viable tetrad pollen grain (scale bar = 10 µm), C) viable pollen grains with consistent shape (scale bar = 10 µm), D) non-viable tetrad pollen grain (scale bar = 28 µm), E) non-viable triad pollen grains indicated by the arrow (scale bar = 27 µm) and F) viable pollen grains with consistent shape but different sizes (scale bar = 30 µm). Pollen grains in panels A–C were stained by aniline blue while pollen grains in panels D–F were stained by acetocarmine.

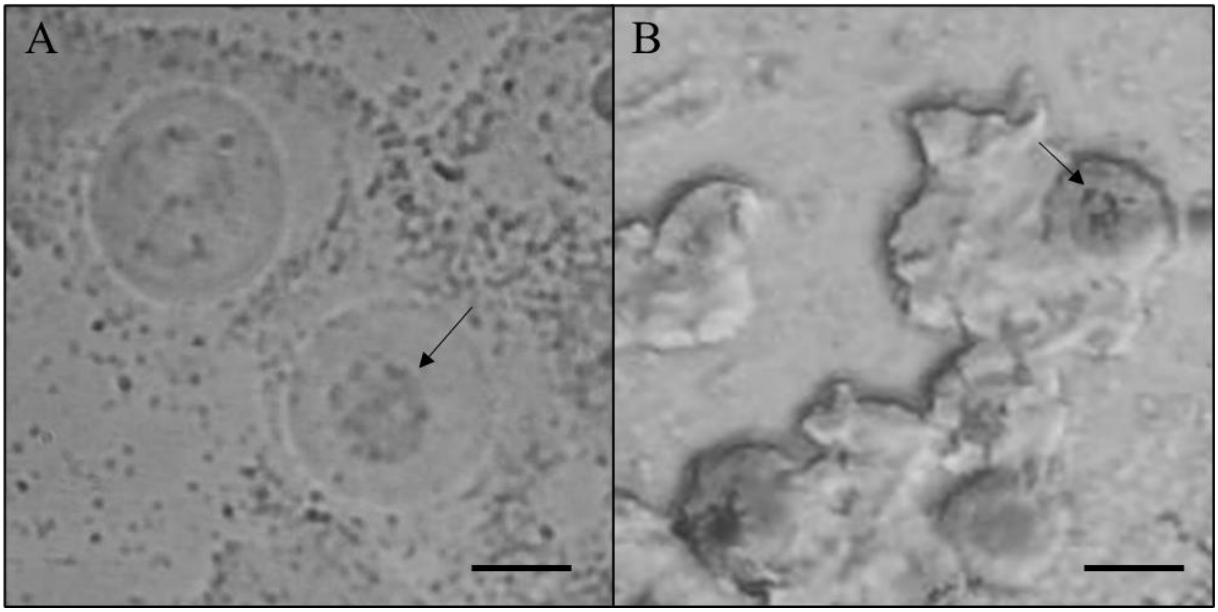

Figure 2.7. Meiotically dividing cells obtained from anther squashes. Both panels show cells in interphase and arrows indicate chromosomes. Scale bars = 18 µm.

DISCUSSION

The purpose of this study was to assess male fertility in South African populations of *Campuloclinium macrocephalum* by estimating pollen viability using staining techniques, pollen tube analyses, and pollen grain characterisation. Additionally, flow cytometry and anther squashes were used to determine the putative ploidy level of offspring formed in each population. Collectively, these data were used to determine the relationship between male fertility and ploidy level thereby enabling inferences on the potential gene flow between populations and its implications for biocontrol management.

The relative fitness of pollen grains is determined by estimating their viability and fertility (Wizenberg *et al.,* 2021). Pollen grain viability, estimated by staining techniques, measures the number of pollen grains that can engage in reproduction under any conditions (Alexander, 1969; Wizenberg *et al.,* 2021) while male fertility is measured by determining the number of pollen grains that can germinate under specific conditions (Heslop-Harrison, 1987; Soares *et al.*, 2008). In contrast, Snow and Lewis (1993) defined male fertility as the proportion of seeds

sired by a given individual. For the purpose of this study, ‘male viability’ describes the pollen grains’ ability to engage in reproduction while ‘male fertility’ refers to whether or not the pollen grains were able to effect fertilization.

High male viability in all examined populations of *C. macrocephalum* may be contributing to gene flow amongst populations. Male viability was high for both staining techniques and this was supported by pollen tube growth on the stigmatic margin and on the side of the styles. The high male viability suggests that the pollen grains could actively fertilise ovules, thereby facilitating gene flow amongst South African populations of *C. macrocephalum*. The production of viable male gametes in populations of *C. macrocephalum* suggests that the species should be reproducing predominantly via sexual reproduction (xenogamy, autogamy, or geitonogamy), facultative apomixis (i.e., the occasional expression of sexuality) or pseudogamy (apomixis with endosperm development dependent on fertilisation).

However, in the analysed triploid individuals, pollen tube growth was arrested on the stigmatic margin and on the outside of the styles, suggesting that overall male fertility is low. This may be indicative of a ‘triploid block’ in South African populations of the pompom weed. Triploid blocks typically prevent endosperm collapse as a consequence of the unbalanced ratio of maternal and paternal genomes (Ramsey and Schemske, 1998). Nevertheless, the presence of viable gametes can still contribute to gene flow albeit at low levels. Burton and Husband (2001) suggested that the production of viable gametes can reduce the reproductive barrier formed by triploid blocks.

The production of viable gametes in triploid populations of *C. macrocephalum* may therefore be contributing to the formation of individuals with diverse karyotypes. Triploids exhibited more pollen grain variation than putative tetraploids. Additionally, the triploid Tembisa population had a smaller mean pollen grain diameter than the triploid Midrand population

despite having the same ploidy level. This could be due to the occurrence of irregular meiosis in triploids, as triploids can produce haploid and diploid gametes (Ramsey and Schemske, 1998, Henry *et al*., 2005) which may have implications for pollen grain size within populations. Alternatively, meiotic instability in triploids can result in chromosome loss (aneuploidy) and a lower nuclear DNA content might cause a reduction in pollen grain size (Conceição *et al.,* 2019).

Due to the high male viability, gene flow between the triploid and tetraploid populations of *C. macrocephalum* could be possible and may have consequences for the genetic variation within the species (Burton and Husband, 2000). Functional gametes produced by triploids, depending on the ploidy level of the gametes, may act as a "triploid bridge" therefore resulting in the production of tetraploid individuals via backcrossing with diploids or mating with other triploids (Husband, 2004; Marciniuk *et al.,* 2010). Moreover, interploidy mating between triploids and tetraploids produces polyploid progeny with odd chromosome numbers (Peckert and Chrtek, 2006). This results in the progeny having novel combinations of the parental genes which increases the genetic variation in populations (Krahulcová and Rotreklová, 2009; Alexander 2020). For instance, Burton and Husband (2001) found that while the fecundity of offspring produced by triploids is low, their gametes can still lead to fertilisation and produce offspring of variable ploidy. Triploids may therefore contribute to polyploid establishment by influencing the rate at which polyploids are formed (Levin, 1975; Husband, 2004). Gene flow between the triploid and tetraploid populations of *C. macrocephalum* could be possible and may have consequences for the invasive potential of the species.

The ploidy data obtained from flow cytometry and anther squashes suggest an increase in the production of triploids in populations previously identified as tetraploid by Gitonga *et al.,* (2022). This may indicate that triploids are expanding their range within Gauteng due to reproduction via apomixis which facilitates rapid range expansion (Baker, 1967) or sexual

reproduction in triploids forming ecologically diverse clones that are increasing the invasion success of the species (e.g., Burdon, 1981). The latter could also account for the low efficacy of current biocontrol agents in South African populations.

Gitonga *et al.,* (2022) hypothesized that polyploidy would not affect the success of biocontrol agents as the natural enemies of *C. macrocephalum* would have co-evolved with polyploids in the native range. This is contradicted by a study conducted on male viability in the species' native range (Argentina and Uruguay), which found that male viability was low within triploid (46.64 – 54.83 %) and tetraploid (3.54 – 45.30 %) populations of *C. macrocephalum* (Farco and Dematteis, 2014). Low male viability would have limited gene flow within these populations and most individuals would have exhibited a similar response to the natural enemies. The present study, however, suggests that biocontrol efficacy in South African populations may decrease due to the high male viability facilitating gene flow amongst populations of different ploidy levels. High genetic variation or unique genetic combinations within these populations may result in biocontrol agents being limited by host specificity and not being able to successfully affect or infect all individuals (Ward *et al.,* 2008).

CONCLUSION

Triploid populations of the pompom weed exhibited low male fertility as pollen tube growth was arrested on the stigmatic margin and on outside of the styles, which was indicative of a triploid block. However, the effects of a triploid block can be reduced by the production of viable gametes. Therefore, the high male viability suggests that gene flow between triploid and tetraploid populations of *Campuloclinium macrocephalum* is still possible. The production of viable male gametes, indicated by high pollen viability and germinability, suggests that may be playing an important role in the formation of tetraploids via a 'triploid bridge'. This would

result in the formation of progeny with unique genetic compositions that may exhibit differential responses to a genotype-specific biocontrol agent.

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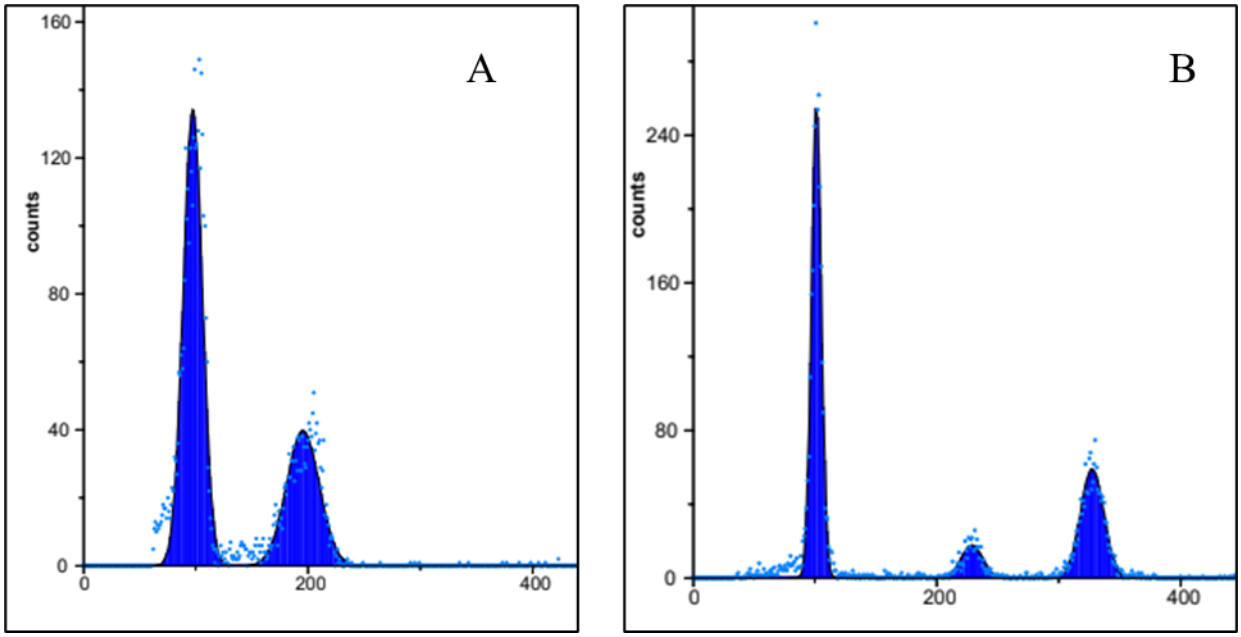
Appendix 2.1. Results from a Kruskall-Wallis multiple comparisons post hoc test comparing viable pollen grain diameter amongst four populations of *Campuloclinium macrocephalum.* Statistically significant values are indicated with an asterisk (*) at $\alpha = 0.05$

	Midrand	Tembisa	Modderfontein
Greenstone	0.311	$p < 0.001$*	0.133
Modderfontein	$p < 0.001$*	$p < 0.001$*	
Tembisa	$p < 0.001$*		

Appendix 2.2. Relative mean fluorescence of sample, relative mean fluorescence of standard, relative 2C DNA content, coefficient of variation (CV) percentage of sample mean, estimated ploidy and 1C DNA content in 24 *Campuloclinium macrocephalum* individuals. The letters correspond to the respective populations, i.e., Tembisa (T), Midrand (DD), Modderfontein (M) and Greenstone (G). The numbers indicate which maternal plant the leaf samples were harvested from.

Individual ID	Relative mean fluorescence of standard	Relative mean fluorescence of sample	CV (%) of sample	Relative 2C DNA content	Estimated ploidy
25T	105.46	336.76	2.90	3.19	3x
25T2	102.14	328.4	2.89	3.22	3x
7T	111.78	349.76	4.04	3.13	3x
7T2	107.51	336.79	3.14	3.13	3x
22T	106.68	356.06	7.81	3.34	–
22T2	94.19	270.38	28.57	2.87	–
7R	106.69	326.68	6.34	3.06	–
7R2	105.44	336.67	2.98	3.19	3x
24R	102.94	329.27	3.45	3.20	3x
24R2	105.37	333.93	2.8	3.17	3x
17R	107.80	327.85	3.87	3.04	3x
17R2	99.91	318.74	3.27	3.19	3x
23M	103.91	337.78	3.91	3.25	3x
23M2	101.82	325.88	2.92	3.20	3x
22M	104.27	335.14	2.91	3.21	3x
22M2	99.74	312.14	3.69	3.13	3x
15M	102.37	330.00	3.6	3.22	3x
15M2	103.24	329.76	2.9	3.19	3x
14DD	107.47	344.44	3.11	3.20	3x
14DD2	104.03	322.89	3.68	3.10	3x
3DD	102.57	325.04	3.16	3.17	3x
3DD2	100.34	327.30	4.14	3.26	3x
11DD	99.29	305.71	9.12	3.08	–
11DD2	114.01	362.97	3.83	3.18	3x



Appendix 2.3. Flow cytometric profiles of A) *Zea mays* (standard) and B) *Zea mays* (standard) and *Campuloclinium macrocephalum* (sample).

CHAPTER 3

The effect of the reproductive strategies of *Campuloclinium macrocephalum* on its invasiveness in South Africa.

ABSTRACT

Campuloclinium macrocephalum (pompom weed) has rapidly invaded South African grasslands and contributed to a significant decline in indigenous vegetation. Predicted distributions show that without intervention, the species will invade a substantial portion of the grassland biome. Previous studies have suggested that the species' success may be linked to its ability to reproduce uniparentally in its invaded range, however the predominant mode of reproduction could not be determined. This study aimed to identify the reproductive strategy employed by *C. macrocephalum* in its invaded range. Thereafter the link between ploidy level and the predominant mode of reproduction was considered to assess the implications of this link for biocontrol management plans. Typically, an invasive species uses either self-fertilization and/or apomixis in its invaded range, however self-fertilization in *C. macrocephalum* might be prevented by dichogamous protandry, a sporophytic incompatibility system operating on the stigmatic surface, and secondary pollen presentation. Instead, the pompom weed appears to reproduce predominantly via gametophytic apomixis with autonomous endosperm development. The maintenance of a sexual pathway, indicated by pollen tube growth on the stigmatic margin, was indicative of gametophytic apomixis while seed set in the absence of fertilization provided evidence for autonomous endosperm development. The co-occurrence of polyploidy and apomixis appears to enhance the species' invasion success in South Africa, but the low genetic differentiation amongst populations and between maternal plants and their offspring suggests that a genotype-specific biocontrol agent should be equally successful on all populations of pompom weed. Nonetheless, in this study,

the reproductive success in only one population was significantly affected by the biocontrol rust fungus and thrips as shown by lower seed set and poor seedling establishment. Additional studies should be conducted to determine what is limiting the success of biocontrol agents.

Keywords: Invasive; Polyploidy; Apomixis; Genetic diversity; Biocontrol efficacy

INTRODUCTION

Invasions by alien plant species have varying effects on biodiversity worldwide (Tilman, 1999; Davis, 2003; Shiferaw *et al.,* 2018; Linders *et al.,* 2019). In recent years, however, more focus has been placed on the negative effects of these alien invasive species on local plant communities and ecosystems (Simberloff *et al*., 2003; Shah *et al.,* 2020). This increased focus includes understanding the mechanisms that enable these species to invade and alter areas outside their native range – particularly their reproductive strategies (Levine *et al.,* 2003). The type of breeding system adopted has implications for genetic diversity and genome evolution within populations of a species (Charlesworth, 2006). The influence of breeding systems on population dynamics makes it an important aspect to consider when investigating biological invasions (Baker, 1995; Ward et al., 2012).

Biological invasions involve the long-distance dispersal, multiplication, and rapid range expansion of species in areas outside of their native range (Barrett *et al.,* 2008). The successful invasion of a species is dependent on the production, dispersal, and genetic composition of propagules – these factors are influenced by the breeding system of populations (Kinlan and Hastings, 2005). Flowering plants exhibit diversity in breeding systems and associated factors such as sexual versus asexual reproductive strategies, the degree of outcrossing and/or selfing, the vectors used for pollen transmission and seed dispersal, as well as the timing of reproduction can all influence invasion dynamics (Barrett *et al.,* 2008).

Baker's Rule predicts that species capable of uniparental reproduction, via mechanisms such as autonomous self-fertilisation and apomixis, are more successful at colonisation than self-incompatible or dioecious species (Baker, 1955; Rambuda and Johnson, 2004). Uniparental reproduction ensures reproduction in the absence of suitable mates or pollinators (Hao *et al.,* 2011). Species that are capable of uniparental reproduction are therefore more likely to re-establish after long distance dispersal to new regions and subsequently invade them (Pannell and Barrett, 1998; Hao *et al.,* 2011). For instance, an assessment of seventeen invasive plant species in South Africa found that all were either self-compatible or apomictic (Rambuda and Johnson, 2004). The findings of Rambuda and Johnson (2004) are consistent with Baker's Rule (Baker, 1955); however, two alternative hypotheses were proposed for the observed patterns: i) uniparental reproduction may be used to alleviate allee effects (i.e., the correlation between mean individual fitness and population size/density) in founding populations and ii) uniparental reproduction compensates for a lack of suitable pollinators in the invaded range. Both hypotheses are not mutually exclusive and suggest that uniparental reproduction in invasive species increases the likelihood of establishment after introduction (Rambuda and Johnson, 2004; Hao *et al.,* 2011).

Selfing occurs more frequently than apomixis (Richards, 1996). Selfing may occur within the same flower without the aid of pollinators (autonomous autogamy), within the same flower with the pollen being transferred from anthers to stigma by biotic vectors (autogamy) or between flowers on the same plant (geitonogamy). Self-fertilisation results in decreased heterozygosity which could potentially lead to inbreeding depression (Lloyd, 1992). Geitonogamy and autogamy are therefore considered to be 'non-adaptive by-products' of outcrossing (Lloyd, 1992) as they provide no advantage to self-compatible species due to their dependency on pollinator visits (Hörandl, 2010). Autonomy, however, provides reproductive assurance in the absence of pollinator visits (Hörandl, 2010).

Apomixis, i.e., clonal reproduction by seed, is common in the Asteraceae, Poaceae and Rosaceae (Noyes 2007; Hörandl, 2010; Hojsgaard *et al*., 2014). This mode of reproduction also provides reproductive assurance when pollinator visits are scarce or absent, yet there is little to no loss of heterozygosity in the offspring because genetic variation of the maternal genotype is maintained by the egg cell (Hörandl, 2010). There are two types of apomixis, gametophytic apomixis and sporophytic apomixis. In gametophytic apomixis, an unreduced embryo sac is either derived from i) a somatic cell of the nucellus (apospory) or ii) an unreduced megaspore mother cell (diplospory). Thereafter, the unreduced egg cell develops without fertilisation into an embryo (Hörandl, 2010). The subsequent endosperm formation may be dependent on fertilisation (pseudogamy) or independent of fertilisation (autonomy). In sporophytic apomixis, the embryo is derived directly from somatic tissue within the ovules (Hörandl, 2010).

Apomixis may be facultative or obligatory and the latter is more frequently encountered in plant lineages (Koltunow and Grossniklaus 2003; Curtis and Grossniklaus 2007). Gametophytic apomixis is not considered to be an independent trait but rather a deregulation of the sexual pathway (Hörandl, 2010). This deregulation and the shifts between the two reproductive modes may be triggered by polyploidy and hybridisation via gene expression repatterning (Grimanelli *et al*., 2001). Both apomixis and self-fertilisation may be linked to polyploidy however the association between self-fertilisation and polyploidy is less pronounced than that of gametophytic apomixis and polyploidy (Ramsey and Schemske 1998). Self-compatibility, a prerequisite for self-fertilisation and pseudogamy, is hypothesised to be common in polyploid taxa for reproductive assurance and/or an expected decrease in inbreeding depression due to their ‘extra’ gene copies (Lande and Schemske, 1985; Miller and Venable, 2000).

Polyploidy is common in invasive species (Hollingsworth and Bailey, 2000; te Beest *et al.*, 2012; Baudel *et al.*,2018). Polyploidization provides an advantage to invasive species by

altering plant physiology, morphology, and phenology in fewer generations than native plants due to genomic changes and increased genetic diversity (Parisod *et al.,* 2010; van de Peer *et al.,* 2017). These shifts in various traits often result in individuals that can exploit new niches, adapt to variable environmental factors, and outcompete native species–thereby enabling them to progress along the introduction–naturalization–invasion continuum (Richardson *et al.,* 2000). However, the persistence of polyploids within their invaded range is typically linked to their ability to reproduce asexually (Kirchheimer *et al.,* 2018).

Campuloclinium macrocephalum (Less.) DC. (Asteraceae, Eupatorieae) is an alien invasive species in South Africa. The species invades grasslands, savannas, and wetlands and transforms such ecosystems to the extent that the indigenous species are eliminated (McConnachie *et al*., 2011). The successful eradication of this species is likely dependent on its ploidy level and reproductive strategies as these factors could affect the efficacy of biocontrol agents (Gitonga *et al.,* 2015). In its native range (Argentina, South America) triploid populations of *C. macrocephalum* have been found to reproduce via facultative apomixis (Farco *et al.,* 2012).

Gitonga *et al.* (2022) identified triploid and tetraploid cytotypes in South African populations of *C. macrocephalum* and hypothesised that the species may be capable of uniparental reproduction. Further, a reproductive study conducted on South African populations of *C. macrocephalum* where groups of capitula were bagged or left open to test for xenogamy and autonomous autogamy/apomixis respectively, showed little difference between the number of cypselas produced in both treatments; however, it was unclear if the cypselas produced in the bagged treatment were produced by autogamy or apomixis (Kgaboesele, unpubl. data). The present study expands on these previous findings to more fully investigate a potential link between ploidy level and the reproductive strategies of *C. macrocephalum.*

The aim of this investigation was to assess the predominant mode of reproduction in South African populations of *C. macrocephalum* (pompom weed) and explore its link to polyploidy.

The objectives associated with this aim are:

1. Survey insect visitors to capitula and investigate whether effective vector-mediated cross-pollination may be occurring in populations in the Gauteng province of South Africa.
2. Assess whether pollen tubes are reaching the ovaries and/or ovules in bagged capitula to test hypotheses of whether autogamy or apomixis is predominantly occurring, and the type of apomixis.
3. Compare genetic diversity within and among putative triploid and tetraploid populations of *C. macrocephalum* to infer whether plants self-fertilise or reproduce via apomixis, and if occurring – whether apomixis is facultative or obligate.

METHOD AND MATERIALS

Study site

Four populations in the Gauteng province were used for this investigation. These populations were located in Tembisa (26.05 S; 28.16 E), Midrand (26.02 S; 28.13 E), Modderfontein (26.09 S; 28.15 E) and Greenstone (26.12 S; 28.15 E). The populations were chosen based on their previously determined ploidy levels using flow cytometry (Gitonga *et al.*, 2022).

Study species

Campuloclinium macrocephalum typically flowers from November to March in South Africa, however the amount of rainfall may limit establishment and spread. The species is characterised by bright pink capitula surrounded by purple bracts. Each capitulum comprises many florets, a feature that enables the species to produce a prolific number of cypselas (fruits derived from an inferior, bicarpellate ovary containing a single seed). These cypselas are easily

wind dispersed allowing the species to establish across a wide range of areas. The leaves are serrate and decrease in size towards the top of the stem. In the winter months, the aerial parts of the plants die back, and a large, tuberous rootstock allows the plants to persist underground (Retief *et al.,* 2016).

Identifying potential pollinators

Insect observations and collections were done at Modderfontein Nature Reserve to identify what insects serve as pollinators for this species. Dedicated observations were done on fifteen sunny days between 8h00 and 17h00 as low temperatures decrease insect activity (Mellanby, 1939). Any spatial or temporal variation in insect visitors was noted and no more than 10 specimens of each insect visitor were collected (differentiation was based on morphology). The insects were collected individually in glass vials with tissue paper dipped in ethyl acetate to rapidly kill the insects. They were thereafter stored at –20ºC to prevent decay. A Zeiss Stereo Discovery V12 dissecting microscope was used to examine the insects for pollen grains at a magnification of 100x. The location of pollen on each visitor was noted and the pollen grains were matched to a pollen sample from *C. macrocephalum*.

The pollen load on each insect was determined by dropping small amounts of ethanol into the glass vials to wash the pollen off the insects. A dropper was then used to deposit the ethanol onto a microscope slide and a coverslip was placed over it. The number of pollen grains were then counted using a 25 mm x 25 mm grid transect placed over each slide on a compound microscope at 400x. This process was repeated until all the ethanol in the vials was depleted. The total numbers of pollen grains from all slides were added together to obtain the total pollen load per insect. The data was tested for normality using a Shapiro Wilk test on R Studio (R

version 4.0.3). The data were found to be normal, therefore an ANOVA followed by a Tukey post hoc test was conducted to compare pollen load amongst the collected insects.

Pictures of the insects were taken using a Zeiss Stereo Discovery V12 dissecting microscope and AxioCam MRc camera. The insects were identified based on morphological characteristics to the lowest possible taxonomic level using insect guides and by consulting experts in the field. Insect specimens were then pinned, labelled, and stored at the Life Sciences Museum at the University of the Witwatersrand.

Determining breeding system

Thirty plants each from each population were haphazardly selected provided that they were at least 1 metre apart from each other. On each plant, a group of capitula (synflorescence, i.e., a small cluster of capitula on a common peduncle) was bagged to exclude insect visitation and hence test for self-pollination/apomixis. A second group on the same plant was left unmanipulated to allow for cross-pollination to occur naturally (Figure 3.1.A). Sheer organza bags were used to bag the capitula (Figure 3.1.B). These allowed light, carbon dioxide and oxygen to pass through but with pore sizes small enough to exclude pollen, thus allowing the capitula to develop normally without insect visitation (Black *et al.*, 2019). The capitula bagged to test for self-pollination and apomixis remained bagged throughout the maturation period. The capitula left open for cross-pollination were bagged once they had set seed to prevent loss of cypselas as they are easily wind dispersed once mature. After the cypselas in both treatments (bagged/unbagged) had matured, they were collected and stored in brown paper bags at room temperature for further analysis.

Figure 3.1. A) Open capitula of *Campuloclinium macrocephalum* visited by an African monarch butterfly (*Danaus chrysippus*) and spotted maize beetles (*Astylus atromaculatus*), B) bagged capitula used to exclude insect visitors during the breeding system experiments.

Cypsela counts

To determine if the species can effectively reproduce via self-pollination and/or apomixis, the number of cypselas formed in the bagged and unbagged treatments were compared. This was accomplished by counting all the cypselas present in the bag and then determining average seed set per capitulum for each synflorescence.

Average seed set per capitulum was calculated as follows:

$$\textbf{Seed set per capitulum per inflorescence} = \frac{\text{Number of cypselas in each bag}}{\text{Number of capitula}}$$

The data were tested for normality using a Shapiro Wilk test and were found to be non-normal. A generalised linear model was used to compare the effect of the open and bagged treatments on seed set per capitulum with individual plants included as a random effect. Values that

deviated substantially from the other values were regarded as outliers. These data points were located outside the whiskers of the boxplot.

Germination trials

To test for germinability, 30 cypselas per treatment from each individual were germinated in Petri dishes. The Petri dishes were lined with filter paper that had been dipped in 2% bleach and thereafter rinsed twice in distilled water to remove as much bleach as possible. This treatment prevented fungal growth on the filter paper and cypselas during the incubation period. The Petri dishes were then placed on a windowsill that received direct sunlight. The filter paper was kept moist throughout the incubation period using distilled water. Once the cypselas had germinated successfully, as indicated by a 2 mm radicle protrusion, the germination percentage per petri dish was calculated. The seedlings were then transferred to a soil medium, comprising Culterra^{TM} potting soil and vermiculite, in seed trays. The seedlings took four months to grow and had to be moved into a growth chamber (60% humidity with 16 hours of light at 25ºC, Figure 3.2). The seedlings remained in the soil medium until they developed leaves that were large enough to be harvested for AFLP analyses. The germination percentage in each petri dish was calculated as follows:

$$\textbf{Germination } \% = \frac{\text{Number of germinated cypselas}}{\text{Total number of cypselas}} \text{ x } 100$$

The data was tested for normality using a Shapiro Wilk test. The germination percentage was then compared between treatments and amongst the four populations using a Kruskal-Wallis test followed by a Kruskal–Wallis multiple comparison post hoc test in R Studio (R version 4.0.3) using the pgirmess package (Giraudoux, 2018).

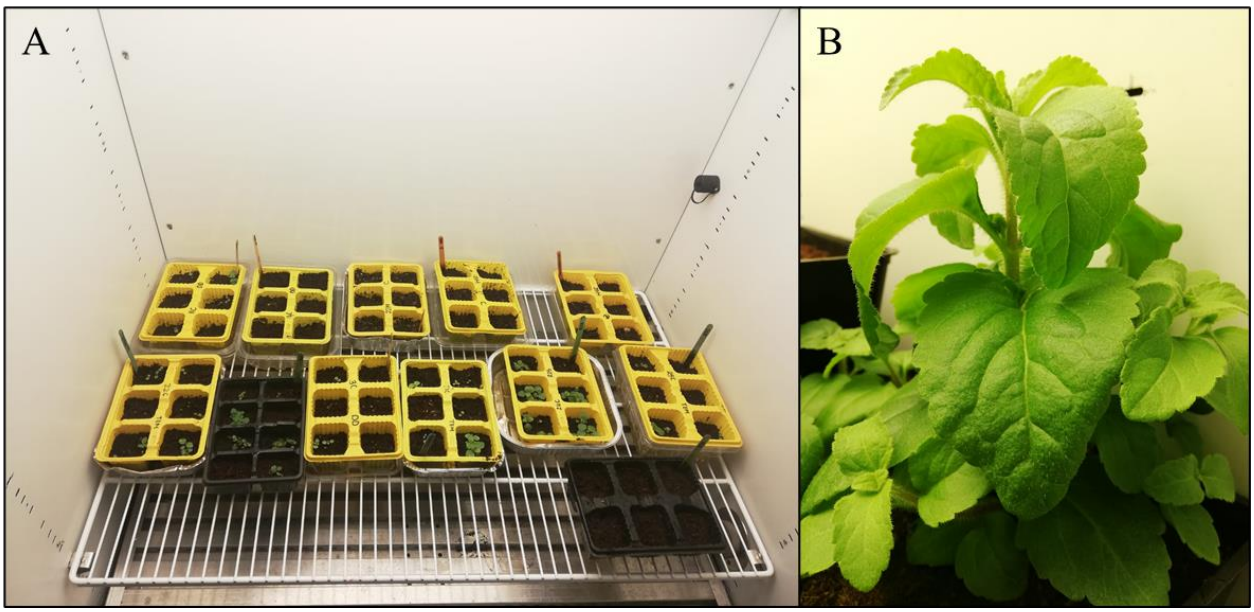


Figure 3.2. A) Seedlings of *C. macrocephalum* in seed trays in the growth chamber, and B) seedling growth after one month in the growth chamber.

AFLP analyses

Leaf tissue from three maternal plants and 15 of their offspring from each population were sampled for AFLP analyses. Young leaves from each maternal plant in the apomixis/self- and cross-pollination treatments were harvested during the fieldwork period (March 2021) and then placed in silica gel to dry rapidly and were stored at −20 °C until DNA extraction. Leaves from the offspring were harvested post-germination and were placed in silica gel until DNA extraction.

DNA extraction

Genomic DNA for the AFLP analyses was extracted from parent and offspring leaves following the modified CTAB DNA extraction protocol (Cullings, 1992; Doyle and Doyle, 1987). DNA samples were quantified using a Thermo Scientific™ NanoDrop OneC Microvolume UV-Vis Spectrophotometer and genomic DNA of good quality was diluted to 200 ng/µL, if necessary, and stored at –80 ºC.

AFLP restriction and ligation

AFLPs were generated following Blignaut *et al.,* (2013). In summary, for each of the individuals, 200 ng of genomic DNA was digested with 5 units of EcoR1 (New England BioLabs, Inqaba Biotec, Pretoria, South Africa) in a 20 µL reaction volume (0.25 µL EcoRI, 2 µL Eco buffer, 16.75 µL distilled water and 1 µL DNA) at 37 ºC for 15 minutes. After EcoRI digestion, 5 units of MseI (New England BioLabs, Inqaba Biotec, Pretoria, South Africa) was added and the buffer mixture was adjusted to 30 µL (0.5 µL MseI, 3 uL Cutsmart Buffer, 6.5 µL distilled water). The mixture was then incubated for 15 minutes at 37 ºC followed by 20 minutes at 65 ºC to denature the enzymes. A 10 µL ligation mix that consisted of 1 unit of T4 DNA ligase (New England BioLabs, Inqaba, Pretoria, South Africa), 1X T4 DNA ligase buffer, 50 mM MseI adapter and 5 mM EcoRI adapter (adaptors from IDT Technologies, Whitehead Scientific, South Africa) was added to the digestion reaction. The digestion–ligation reaction was incubated at 4 ºC overnight.

AFLP amplification

Pre-selective PCR amplification was done in a reaction mix containing 5 µl of the diluted digestion–ligation reaction mix, 1 mM MseI+0 primer, 1 mM EcoRI+0 (IDT Technologies, Whitehead Scientific, South Africa) and 2X µl Quickload *Taq* Master mix (Inqaba Biotec, Pretoria, South Africa) and 10 µg/ml Bovine serum albumin (BSA, Inqaba Biotec, Pretoria South Africa). The pre-selective PCR amplification was conducted under specific conditions: initial denaturation (5s at 94 ºC), denaturation (5 minutes at 94 ºC), annealing (30s at 56 ºC), elongation (30s at 72 ºC) and final elongation (30 min at 60ºC). Successful amplification was determined by running a 4 µL sample of PCR product on 1% agarose gel and observing smears of 100–500 bp. Five µL of the diluted mix were then used for selective PCR amplification. For each selective PCR reaction, a 20 µL reaction volume comprised 2X Quickload Taq master

mix (Inqaba, Pretoria, South Africa), 0.25 µM fluorescently labelled EcoRI+NNN primers (EcoRI–CAT, EcoRI–AAT and EcoRI–ATG) and 1 µM unlabelled MseI+CTT. The PCR conditions followed that of the pre-selective PCR amplification with 30 repeat cycles. After amplification, 5 µL of the fluorescently labelled product were then mixed for each DNA sample in a single tube. The product then underwent electrophoresis to produce AFLP fingerprints at the Central Analytical Facility, University of Stellenbosch.

AFLP analysis

Fragments between 50 to 500 base pairs (bp) were scored using Geneious Prime Version 2022.0.1 (Biomatters Development Team, 2005) and were evaluated manually to ensure fragments were called appropriately. The presence (1) or absence (0) of peaks in the chromatographs was scored for all individuals. A binary (presence/absence) matrix was generated from these scores for the different fragments using Geneious Prime and Microsoft Excel (2019). The matrix was then used to i) to obtain clone estimates, ii) assess clonal genetic diversity (adjusted for presence of clones, using a threshold of two per population), and iii) assess genetic distances (Nei's genetic distance [Nei 1979]) between grouped maternal and offspring per population, and iv) evaluate population differentiation (F_{ST}) among populations using GenoDive v 3.04 (Meirmans, 2020).

The location of stamens and pistils

Evidence of protandry and/or herkogamy was obtained by noting the binary (i.e., above/below) location of the stamen relative to the pistil in all the florets used for pollen tube analyses. In addition, whether the anthers had dehisced or not was also noted. Pictures of their relative locations were taken using Zeiss Stereo Discovery V12 dissecting microscope and AxioCam MRc camera at 100x.

Pollen tube analyses

Pollen tube growth was assessed to determine if *C. macrocephalum* reproduces sexually or asexually. Pollen tube analyses followed the protocol outlined in Kalinganire *et al.,* (2000). Twenty florets from ten individuals per population were removed from the capitula harvested from each treatment. The florets were then fixed in 'Carnoy's' fixative, comprising ethanol: chloroform: acetic acid (6:3:1), for 2 hours. Thereafter, the florets were transferred to 70% ethanol for storage. The pistils were dissected out of the florets under a Zeiss Stereo Discovery V12 dissecting microscope. The pistils were not softened or cleared using NaOH as the delicate tissue disintegrated during this step. The pistils were rinsed with distilled water and stained with aniline blue in a potassium buffer for two hours, longer durations made the tissue difficult to work with. The florets were then placed on a microscope slide with 80% glycerol and dissected using a needle to view the reproductive organs. The method was later modified, and the pistils were squashed using a coverslip rather than dissected. This was done because the pollen grains were attached to the outside of the styles, and they could not be visualised when the styles were dissected. The slides were examined under the BX63 OFM fluorescent microscope to check for pollen tube growth.

To confirm the results obtained from fluorescence microscopy, five pistils from three individuals per population were dissected out and placed on carbon tape adhered to aluminium stubs. The pistils were left to dry overnight and then coated with one coat of carbon and one coat of gold/palladium. The stubs were viewed under a Tescan™ Vega Scanning Electron Microscope (Brno, Czechia).

RESULTS

Potential insect pollinators

The most frequently encountered insect species on capitula of *Campuloclinium macrocephalum* were *Danaus chrysippus* Linnaeus (African monarch butterfly), *Apis mellifera* Linnaeus (honeybee), *Zonocerus elegans elegans* Thunberg (elegant grasshopper), *Astylus atromaculatus* Blanchard (spotted maize/pollen beetle), *Lycus melanurus* Fabricius (hook–winged beetle), *Cheilomenes lunata* Fabricius (ladybird beetle), *Harmonia vigintiduomaculata* Fabricius (chequered ladybeetle), Phlaeothripidae (thrips), Hymenoptera (ants) and *Exochomus flavipes* Thunberg (Coccinellid beetle) (Figure 1 A–J; also see Appendix 3.1).

Pollen loads on the various visitors of *C. macrocephalum* were compared to determine if they were likely pollinators or just floral visitors. There were differences in pollen load amongst the various insect species ($F = 14.07$, df = 9; $P < 0.001$; Figure 2; Table 2.1). A Tukey post-hoc test showed that the pollen loads on *D. chrysippus* and *A. mellifera* were not significantly different than each other ($P = 0.493$) but were significantly higher than all the other insects. The pollen loads on the rest of the insects, however, were not significantly different from each other ($P > 0.05$) (Appendix 3.2).

In addition to pollen load, insect behaviour and the location of the pollen on the insects are important factors to consider when trying to determine pollinator effectiveness (Table 3.1). *D. chyrsippus* and *A. mellifera* frequently moved amongst capitula on a single plant and amongst plants. In contrast, *Z. elegans* rarely moved amongst capitula despite having a high pollen load. The thrips (Phlaeothripidae) and ants (Hymenoptera) were present on all capitula, however they carried small pollen loads. Additionally, the thrips remained within the same capitula throughout the observation period, suggesting that they do not effectively transfer pollen between capitula or plants. Visitation rates of *D. chyrsippus* and *A. mellifera* decreased as the flowering season progressed whereas *L. melanurus* and *C. lunata* only began visiting capitula towards the end of the flowering season (i.e., March). The visitor complex (visitation patterns) of *C. macrocephalum* displayed temporal variation. Some insect species, such as the beetles

H. vigintiduomaculata and *E. flavipes*, were only seen on certain capitula. They would congregate on the capitula of only some individuals.

Most pollen was located on the ventral surface of the insects, which suggests that they may have been transferring pollen when crawling within and amongst capitula. The pollen found in the corbicula (pollen sacs) on the bees were scraped off and not included in the pollen load counts. Despite this, the bees still had the second highest pollen load (Figure 3.2). Collectively, the data suggest that bees and butterflies are the most likely insects to contribute to pollination of *C. macrocephalum.*

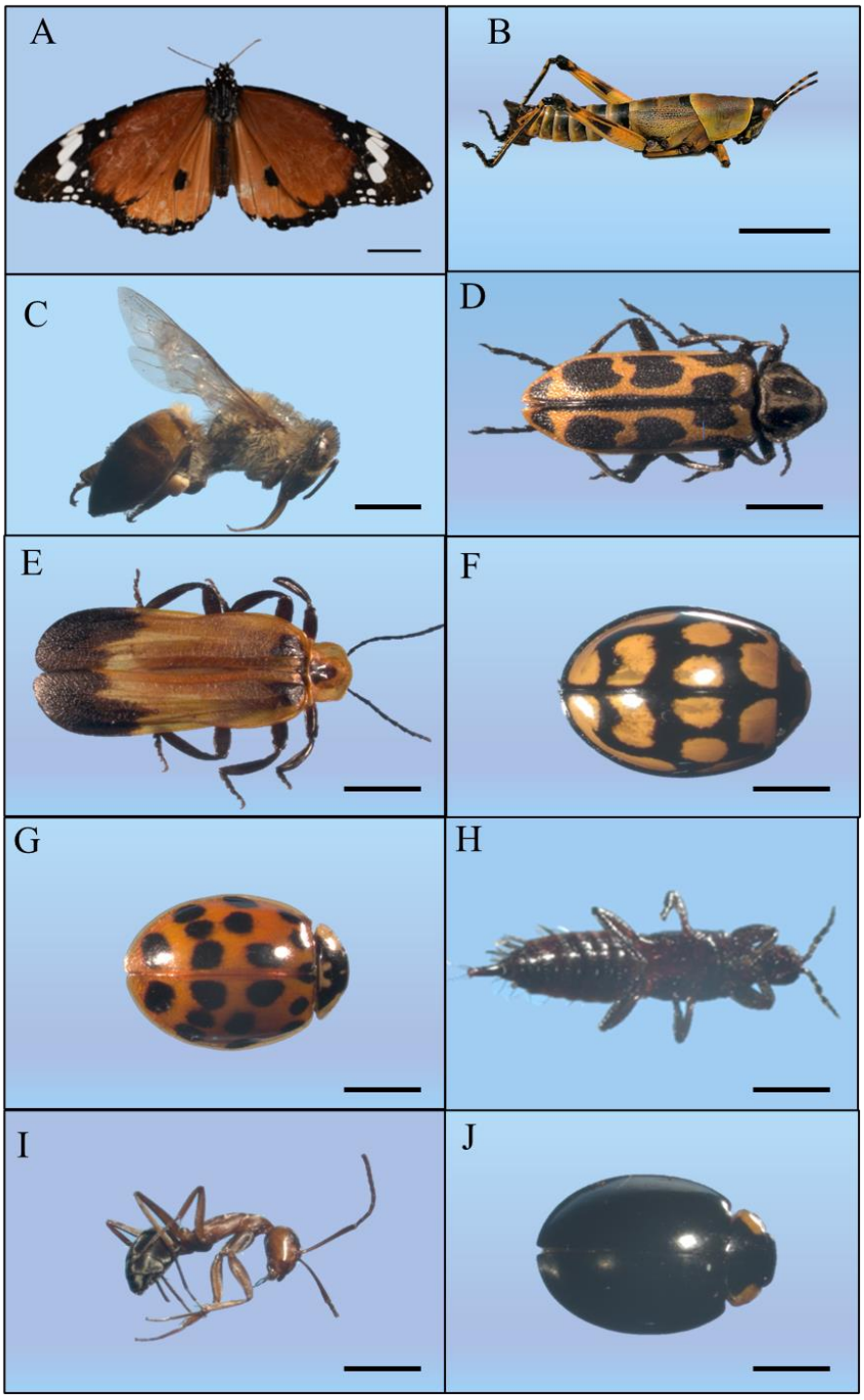


Figure 3.3. A–J: Frequent insect visitors on capitula of *Campuloclinium macrocephalum* during the December 2020–March 2021 flowering period. A) *Danaus chrysippus,* B) *Zonocerus elegans* (Scale bars: A,B = 1cm), C) *Apis mellifera* (Scale bar: C= 0.6 cm) D) *Astylus atromaculatus*, E) *Lycus melanurus* (Scale bar: D, E: 0.4 cm) *Cheilomenes lunata* (Scale bar = 0.25 cm) G) *Harmonia vigintiduomaculata* (Scale bar = 0.16 cm), H) Phlaeothripidae (Scale bar = 0.01 cm), I) Hymenoptera (Scale bar = 0.4 cm) and J) *Exochomus flavipes* (Scale bar = 0.14 cm).

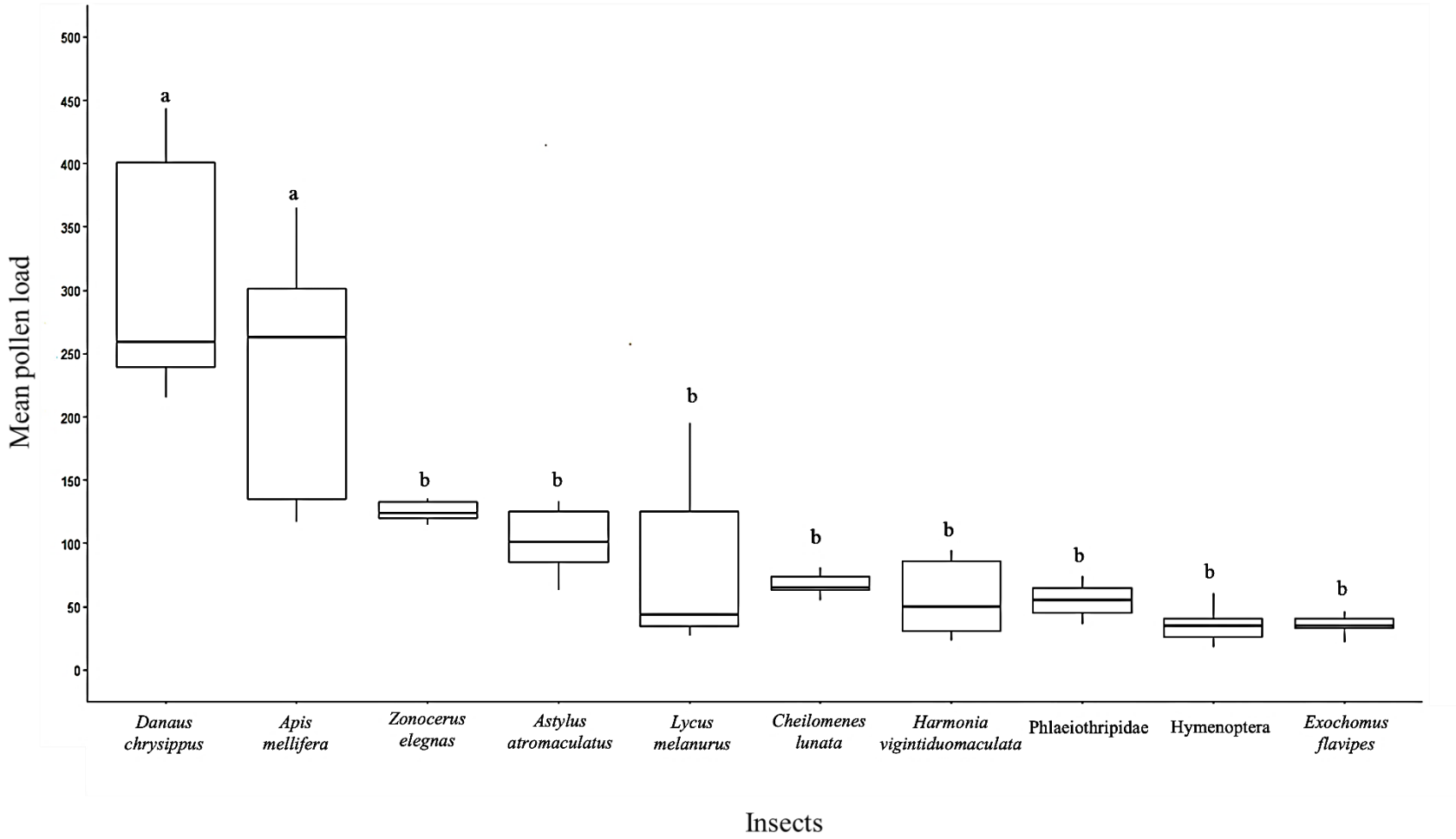


Figure 3.4. Mean pollen load on frequent insect visitors to capitula of *Campuloclinium macrocephalum* from December 2020 – March 2021 at Modderfontein Reserve. The bars represent minimum and maximum values. The horizontal line in each box represents the mean value and the different letters above each box indicate statistically significant differences between taxa. Mean ± standard error is included in Table 3.1.

Table 3.1. Summary of insect activity and pollen load of species observed on capitula of *Campuloclinium macrocephalum* (pers. obs. S. Moodley) at Modderfontein Nature Reserve during the December 2020 – March 2021 flowering season.

Insect	Location of pollen	Mean pollen load (± SE)	Temporal/spatial variation	Movement between capitula or plants	Time spent on capitula
***Danaus chrysippus* (African Monarch butterfly)**	Distributed on the top and sides of the head.	311.4 ± 103.2	Temporal variation. Less visitation was observed towards the end of the flowering season.	Moved between capitula and plants frequently.	~ 20 – 30 s
***Apis mellifera* (Honeybee)**	Corbiculae and on the hairs throughout the bees' bodies**.**	236.2 ± 107.2	Temporal variation. Less visitation was observed towards the end of the flowering season.	Moved amongst capitula and individuals often during observation period.	~ 40 – 50 s
***Zonocerus elegans elegans* (Elegant grasshopper)**	Ventral surface – especially on the legs.	125.4 ± 8.5	Neither. Present throughout the flowering season and on most capitula.	Seldomly moved between capitula or individuals.	Remained on capitula until disturbed.
***Astylus atromaculatus* (Spotted maize/pollen beetle)**	Ventral surface – near mouthparts and on top of the head.	101.4 ± 28.7	Neither. Present throughout the flowering season and on most capitula.	Occasionally, moved amongst capitula and between plants.	~ 4 – 5 min
***Lycus melanurus* (Hook-winged beetle)**	Ventral surface – especially on the legs. Some grains located on top of the head.	85.2 ± 72.9	Temporal variation. Only visited capitula when the plants began setting seed.	Frequent movement amongst plants and capitula.	~ 1 – 2 min
***Cheilomenes lunata* (Ladybird beetle)**	Few grains on the ventral surface.	67.6 ± 10.1	Temporal variation. Visitation increased towards the end of the flowering season.	Frequent movement was observed amongst capitula and between plants.	~ 4 – 5 min
***Harmonia vigintiduomaculata* (Chequered ladybeetle)**	Few grains on the ventral surface.	56.8 ± 32.0	Spatial variation. Only present on certain capitula.	Frequent movement amongst capitula but not plants.	~ 4 – 5 min
Phlaeothripidae (Thrips)	Dorsal and ventral surface.	55.0 ± 15.2	Neither. Present throughout the flowering season and on all capitula.	Remained within individual capitula throughout observation period.	Remained within capitula throughout the observation period.
Hymenoptera (Ants)	Ventral surface.	36.0 ± 16.0	Neither. Present throughout the flowering season and on most capitula.	Frequent movement was observed amongst capitula	~ 20 – 30 s
***Exochomus flavipes* (Coccinellid beetle)**	Few grains found on the ventral surface.	35.4 ± 9.1	Spatial variation. Only present on certain capitula.	Frequent movement amongst capitula but not plants.	~ 4 – 5 m

Cypsela counts and germination trials

Seed set per capitulum was significantly different between the open and bagged treatments (β= 101.60 ± 3.29; *P*= 0.0001), with seed set per capitulum significantly higher in open treatments than bagged treatments (β= 9.86 ± 4.64; *P*= 0.0347) (Figure 3.5). On average, both treatments produced over a hundred cypselas with the open treatment producing ten more cypselas than the bagged treatment.

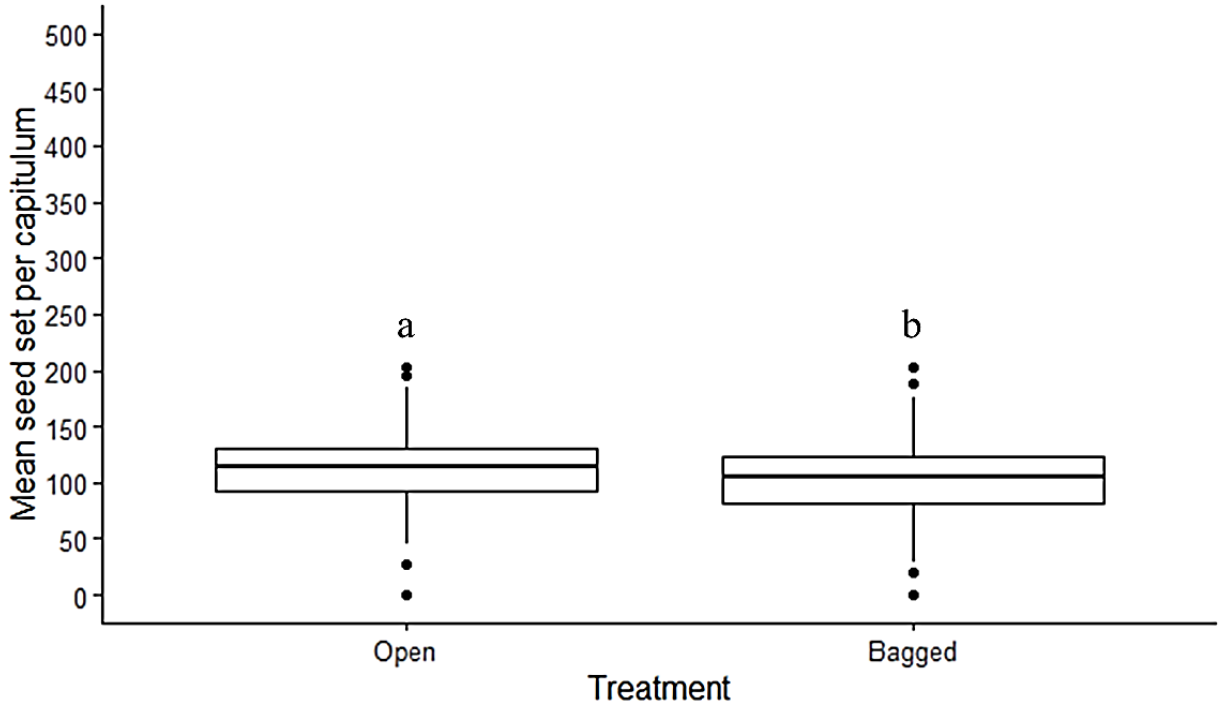


Figure 3.5. Comparison of mean seed set per capitulum in open and bagged treatments of *Campuloclinium macrocephalum* capitula from four populations in Gauteng. The circles represent outliers, the bars represent minimum and maximum values, and the box represents variation in the data and the midline represents the mean.

Mean percentage germination

Mean percentage germination differed significantly between populations ($H_7 = 131.5$; $P < 0.0001$) (Figure 3.6). A Tukey post hoc test showed that the mean percentage germination of the Modderfontein 'open' and 'bagged' treatments were significantly lower than the other three populations ($P < 0.0001$). Additionally, mean percentage germination of the Tembisa bagged treatment was significantly lower than that of the Tembisa open treatment ($P = 0.002$). However, there was no significant difference between the open and bagged treatments in the Modderfontein ($P= 0.889$), Midrand ($P = 0.999$) and Greenstone ($P = 0.943$) populations (Figure 3.6; Appendix 3.3).

The low germination percentages in the Modderfontein population is likely due to that population being more severely affected by resident biocontrol agents than the other populations, viz. the rust fungus (*Puccinia eupatorii*) and thrips (*Liothrips tractabilis*) (Figure 3.7). Cypselas from the other populations germinated in one – two weeks whereas the cypselas from the Modderfontein population took an additional three months to germinate. The Modderfontein seedlings started germinating shortly before the seedlings from the other three populations were moved into the growth chamber. Once the cypselas germinated, few of them survived long enough to grow into seedlings (*pers. obs*. S. Moodley). Despite being grown in a growth chamber, the Modderfontein offspring still showed signs of rust infestation.

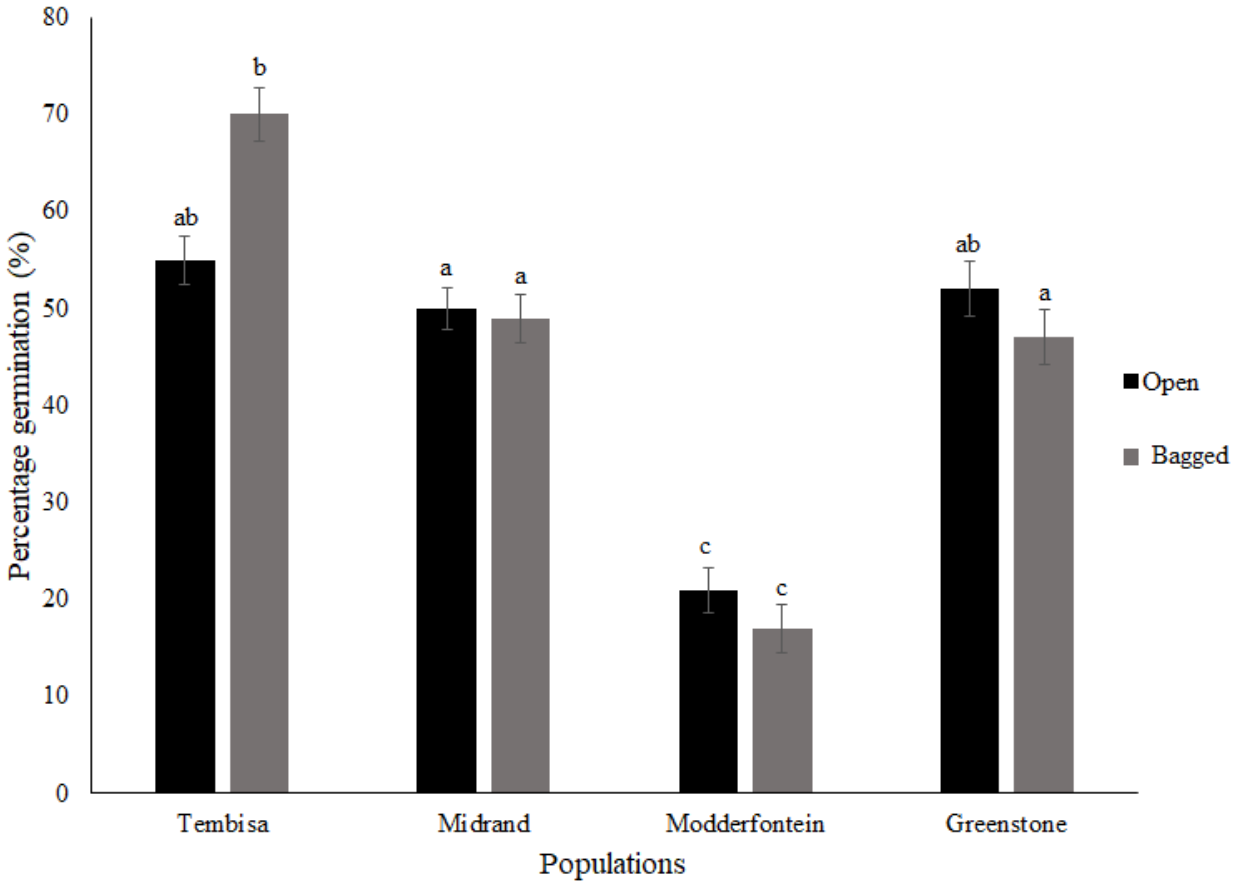


Figure 3.6. Mean percentage germination of open and bagged treatments in four populations of *Campuloclinium macrocephalum* in the Gauteng Province of South Africa. The bars represent standard error, and the letters represent statistically significant differences in mean germination percentages between treatments and populations.

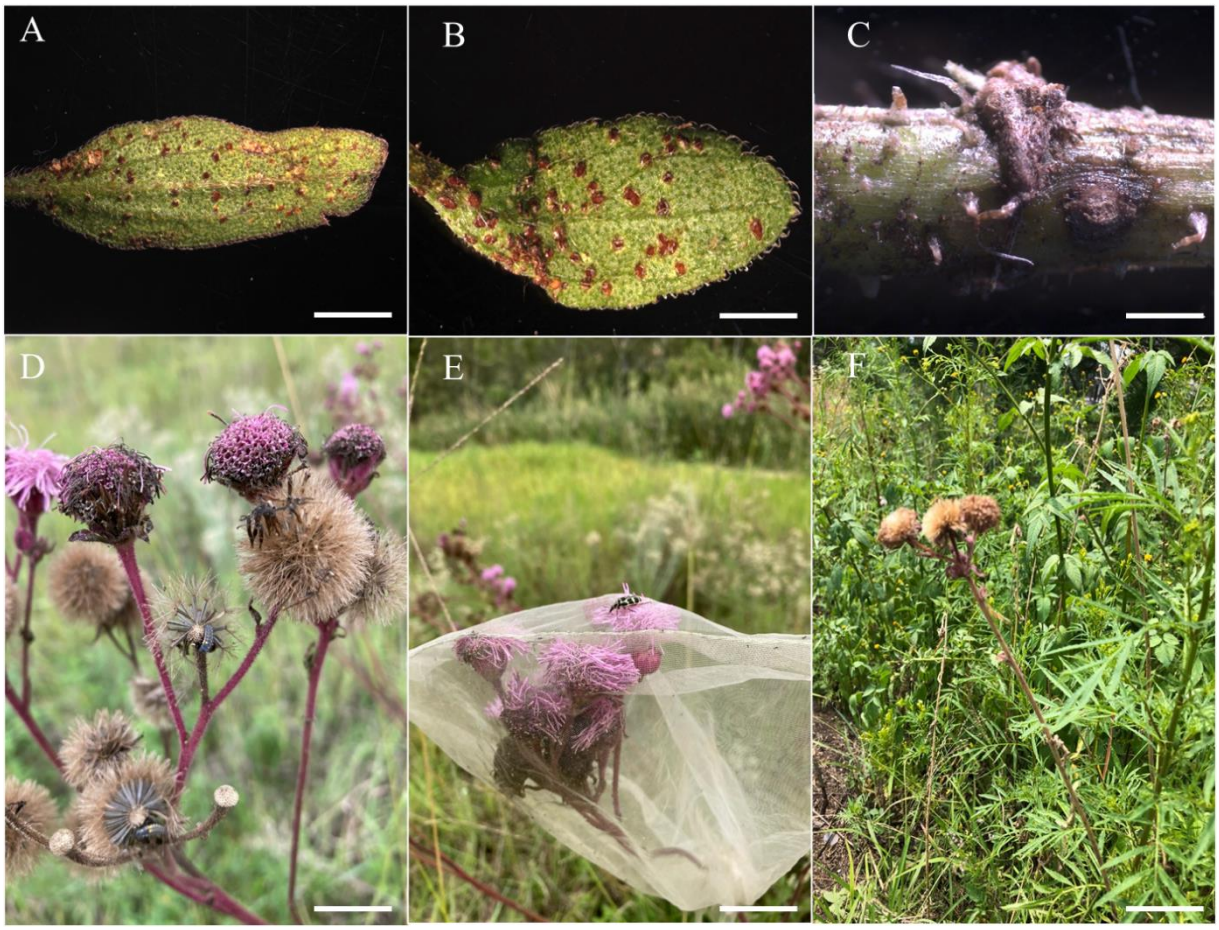


Figure 3.7. The effect of the biocontrol rust fungus (*Puccinia eupatorii)* and thrips (*Liothrips tractabilis)* on leaves and capitula of *Campuloclinium macrocephalum.* A–B) Brown pustules on leaves containing *P. eupatorii* spores (Scale bar= A, B: 1.2 cm), C: Brown pustules containing *P. eupatorii* spores on a stem portion (Scale bar= 1 cm), D–F: The damage caused by thrips to capitula and stem of the plants– characterised by deformed growth, blackened florets, and reduced biomass (Scale bar= D, E: 2 cm; F: 16 cm)

AFLP analyses

All offspring used for the AFLP analyses were found to be triploids using flow cytometry and based on chromosome counts from anther squashes (Chapter 2).

A total of 130 loci were scored from three primer combinations for the 177 individuals sampled. The offspring from one Modderfontein maternal plant (n= 15) showed poor seedling establishment and were excluded. The lower fitness of those individuals could be indicative of a maternal effect. The sample size for Modderfontein was n = 33 (three maternal plants and 30 offspring) instead of n = 48. Among these 177 individuals, there were 173 unique genotypes. Specifically, individual 15M14 matched 25T12 and M25T8 matched 15M14, however they were not from the same maternal plant. Within population genetic diversity, when corrected for the presence of clones, was found to be lower than total genetic diversity for both Nei's and Shannon-Wiener indices (Table 3.2). Additionally, genetic differentiation was also low between the maternal plants and their respective offspring in each population ($H_{s=}$ 0.026 – 0.053). The highest differentiation between the maternal plants and their offspring was observed in Modderfontein (H_s= 0.053) and the lowest differentiation between parent plants and their offspring was observed in Midrand (H_s= 0.026).

The values for the Shannon-Wiener index ranged from 0.28 to 1.65, with the offspring displaying higher genetic diversity than the maternal plants (Table 3.2). Most of the evenness values were equal to or close to one which suggests that frequencies of genotypes were approximately equal within the specified groups (maternal populations and relevant offspring). The corrected Nei's genetic diversity ranged from 0.67 to 1 across populations (Table 3.2). Nei's genetic diversity index was used because we could remove the potential contribution of mutations in order to estimate the recombinant genetic diversity, as we presumed the individuals were potentially clonal due to likely apomictic reproduction in this species.

In general, the genetic differentiation amongst the four populations of *C. macrocephalum* was low (F_{ST} range = 0.006–0.086) with the highest differentiation observed between Midrand and Tembisa (F_{ST} = 0.086) and the lowest between Tembisa and Modderfontein (F_{ST} = 0.006). Nei's genetic distance estimates also showed a low difference among populations (Table 3.3). The overall expected (H_s= 0.253) and total heterozygosity (H_t= 0.257) within populations of *C. macrocephalum* was higher than the observed heterozygosity. Accordingly, the observed frequency of heterozygotes is zero in all populations across all loci. Additionally, inbreeding is likely occurring in all populations (G_{IS} = 1 for all populations).

Table 3.2. Indices of clonal diversity generated from three primer combinations for 177 individuals of *Campuloclinium macrocephalum*. N=population size, Ng= number of genotypes, Ne= effective number of genotypes, He= Nei's genetic diversity, d= Nei's uncorrected genetic diversity, E= evenness, H' = Shannon-Wiener index corrected for sample size and, H= Shannon-Wiener index. The dashes indicate where the correcting bias was not possible.

Population	N	Ng	Ne	He	d	E	H'	H
Midrand Maternal	3.00	3.00	3.00	1.00	0.67	1.00	–	0.48
Modderfontein Maternal	3.00	2.00	1.80	0.67	0.44	0.90	0.46	0.28
Tembisa Maternal	3.00	3.00	3.00	1.00	0.67	1.00	–	0.48
Greenstone Maternal	3.00	3.00	3.00	1.00	1.00	0.67	1.00	0.48
Midrand offspring	45.00	45.00	45.00	1.00	0.98	1.00	–	1.65
Modderfontein offspring	45.00	44.00	43.09	1.00	0.98	0.98	3.00	1.64
Tembisa offspring	45.00	45.00	44.08	1.00	0.98	0.98	3.02	1.65
Greenstone offspring	30.00	29.00	29.00	1.00	0.97	1.00	–	1.46
Average	22.13	21.88	21.50	0.96	0.83	0.94	1.87	1.01

Table 3.3. Pairwise population index using Jost's D index (above diagonal) and F_{ST} (below diagonal) among four populations of *Campuloclinium macrocephalum* in Gauteng, South Africa. Bold values indicate significant difference at $\alpha = 0.05$.

Population	Modderfontein	Tembisa	Greenstone	Midrand
Modderfontein	–	0.002	0.006	0.029
Tembisa	0.006	–	0.004	0.032
Greenstone	**0.017**	0.013	–	0.023
Midrand	**0.071**	**0.086**	**0.057**	–

Protandry and secondary pollen presentation

Capitula of *C. macrocephalum* displayed temporal segregation of maturation of the stamens and pistils, i.e., dichogamy. In young florets (Figure 3.8.A.), the anthers enclose the style (Figure 3.8.C.). However, as the florets develop (Figure 3.8.B), the style elongates through the ring of anthers (Figure 3.8.D). When this process occurs, the anthers have already dehisced and the pollen from the anthers is transferred onto the sides of the style (Figure 3.8.E.). The style apices remained appressed until the style was well above the anthers thereby preventing the transfer of self-pollen onto the stigmatic surface. The dehiscence of the anthers before the style has fully elongated and the stigmatic surface becomes receptive indicates that the florets exhibit dichogamy in the form of protandry.

Figure 3.8. Evidence of protandry in capitula of *Campuloclinium macrocephalum*. A) A young floret before the style emerges (Scale bar: A = 0.20 cm) , B) An older floret in which the style has emerged from the corolla (Scale bar: B = 0.25 cm), C) A young, dissected floret with the style enclosed by a ring of anthers (Scale bar: C= 0.05), D) An older, dissected floret after the style has elongated through the ring of anthers (Scale bar = 0.07 cm) and E) Pollen grains transferred onto the sides of the style branches (Scale bar = 0.18 cm).

Fluorescence microscopy

No pollen grains or pollen tubes were visible from either treatment when the dissected styles were examined under the fluorescence microscope. The only structure visible was vascular tissue – characterised by striations along on its entire length – due to the lignification of the xylem vessels (Figure 3.9A). When the styles were squashed using a coverslip, pollen grains on the outsides of the style were visible, but no pollen tube growth was observed (Figure 3.10). Pollen grains on the stigma were only seen in a few samples, whereas pollen grains were seen on the outsides of the style in every sample (n= 800).

Scanning Electron Microscopy

In a few samples, we observed pollen tube growth on the stigmatic margin and in the middle of the style, suggesting that the pollen grains can effect fertilisation but are being inhibited (Figure 3.11.A). In most samples, however, self-pollen grains had germinated (pollen tubes grew) on the side of the style (Figure 3.11.B–F). Pollen tube growth is usually observed down the inside of the style alongside the vascular tissue as there is less resistance in the surrounding transmitting tissue (Martin, 1959), but the pollen tubes observed in *C. macrocephalum* only grew from pollen grains on the outside of the style and did not penetrate the style.

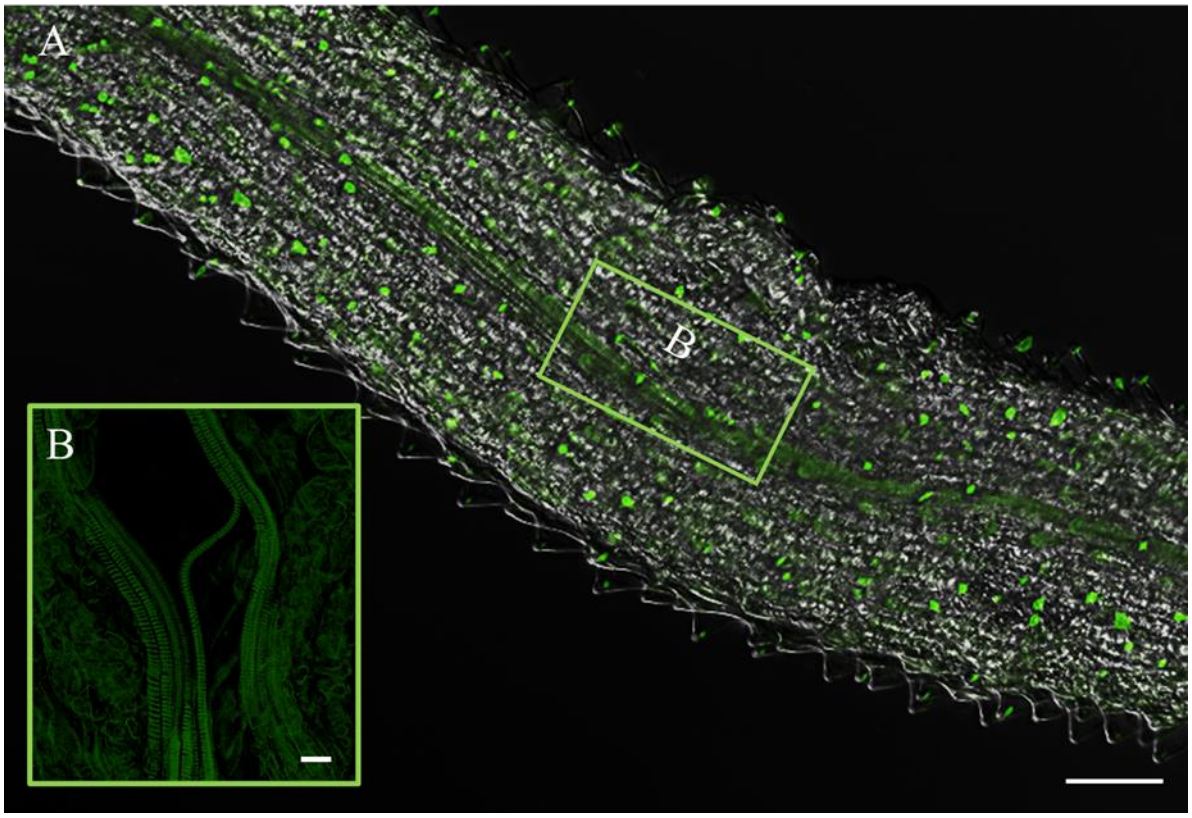


Figure 3.9. A portion of vascular tissue on a *Campuloclinium macrocephalum* style. A) Differential interference contrast used to visualise the vasculature in the style. B) A view of the outlined portion under fluorescence microscopy showing the helical/scalariform secondary cell wall deposition in the xylem vessel elements.

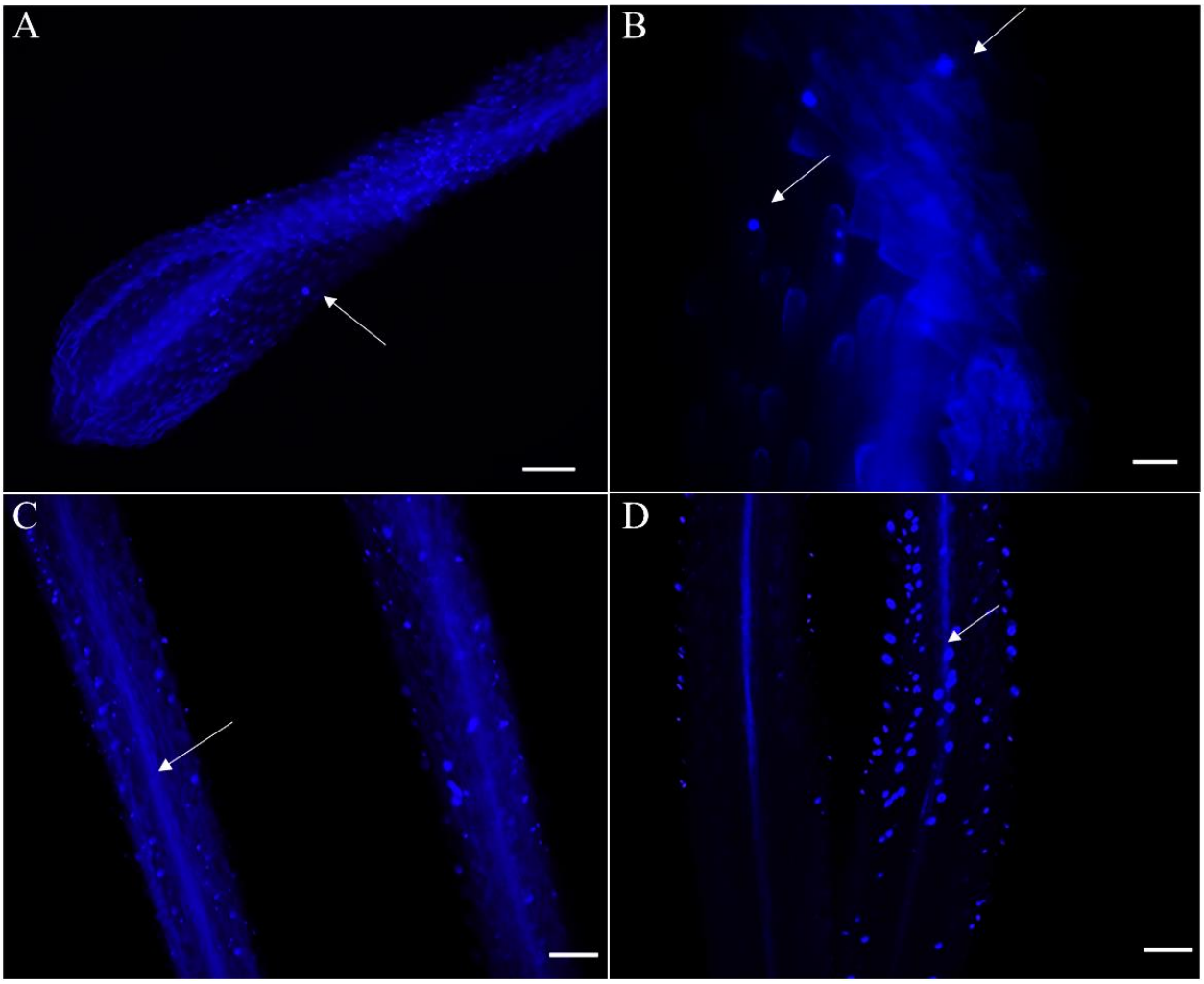


Figure 3.10. The stigma and style of *Campuloclinium macrocephalum* as seen using fluorescence microscopy for the visualisation of pollen grains and pollen tubes. A–B) Fluorescing pollen grains on the style branch apex indicated by the arrows (Scale bar= A: 100 µm; B: 20 µm) C) Fluorescing vascular tissue in the middle of the style branch D) Fluorescing vascular tissue (arrowed) and fluorescing pollen grains on the outer surface of the style branch, near the stigmatic surface, with no visible pollen tube growth (Scale bar = C, D: 100 µm).

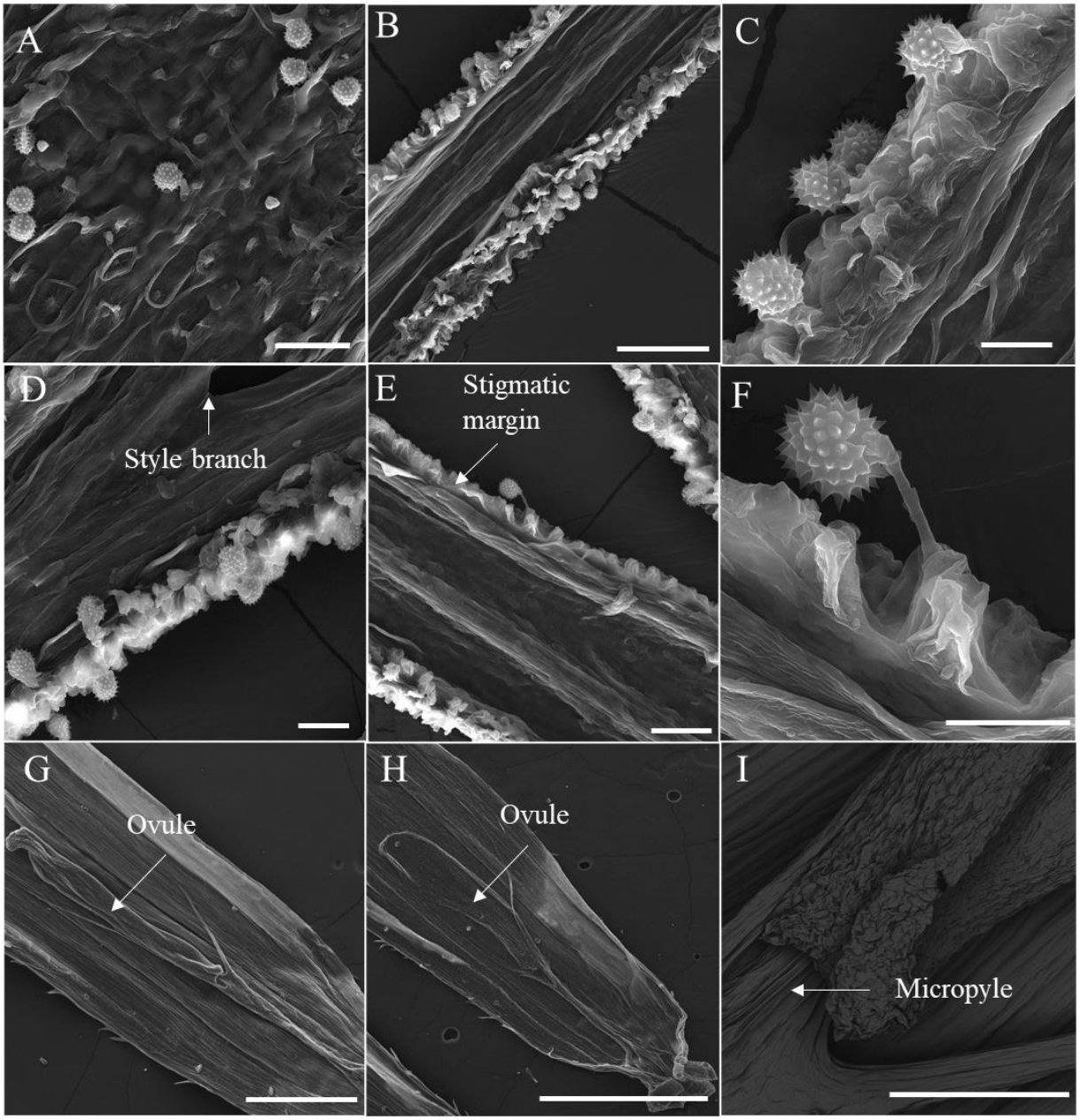


Figure 3.11. Lack of pollen tube growth to the ovules in styles of *Campuloclinium macrocephalum.* A) Pollen grains at the base of the style, near the stigmatic surface, with visible pollen tube growth (Scale bar = 50 µm). B-C) Pollen grains adhered to the sides of the style branches with pollen tube growth evident (Scale bar = B: 100 µm, C: 20 µm). D-F) Pollen tube growth on the on margin of the style branch (Scale bar = D: 50 µm, E: 50 µm, F: 10 µm). G–H) Dissected ovaries revealing ovules with no pollen tubes entering them (Scale bar = G, H: 1 mm). I) Micropylar end of ovule where pollen tubes were expected to enter under a hypothesis of sexual reproduction (Scale bar = 80 µm).

DISCUSSION

The purpose of this study was to determine the mode of reproduction of *Campuloclinium macrocephalum* in its invaded range. This was accomplished by identifying likely pollinators and their effectiveness, assessing pollen tube growth, and estimating the genetic diversity within populations and between maternal plants and their offspring. We also tried to infer links between reproductive mode and polyploidy to determine if a correspondence between reproduction and polyploidy could facilitate invasion success. These results were then used to deduce the implications the species' reproductive mode would have on biocontrol management in South Africa.

Capitula of *C. macrocephalum* were visited by many insect species during the observation period. A generalist pollination syndrome is common in the Asteraceae due to the 'bowl-shaped' inflorescences (Grombone-Guaratini *et al.*, 2004, Torres and Galetto, 2002; Willmer, 2011). The pollen presentation mechanism on the style tips of the florets allows insects to easily access and distribute the pollen as they move around the capitulum – referred to as 'mess pollination'. Mess pollination by various insect groups is effective in daisies because of their exposed sexual organs (Mani and Saravanan, 1999).

Observations are not sufficient to determine if a species is effectively transferring pollen, instead pollen load assessments should be done to assess the relative contribution that insect visitors make to pollination (Primack and Silander, 1975). The pollen load assessments of the insects collected on capitula of *C. macrocephalum* revealed that primarily butterflies and bees appear to play a significantly greater role in pollen transfer. Bees typically moved amongst capitula while butterflies moved amongst individuals. This suggests that the bees were contributing more to geitonogamy, while butterflies contributed more to xenogamy. However,

there was no significant difference between their pollen loads, suggesting that their relative contribution to pollination is the same.

Despite having the third highest pollen load, the elegant grasshopper, *Zonocerus elegans elegans,* did not move between capitula which suggests that the species does not contribute to pollen transfer in *C. macrocephaum.* Similarly, beetles were also found to be inefficient at pollen transfer. Beetles are referred to as 'mess and soil pollinators' as they may use flowers to mate, feed and defecate whilst still transferring moderate amounts of pollen (Willmer, 2011). *Astylus atromaculatus* (maize/pollen beetle), *Lycus melanurus* (hook-winged beetle), *Cheilomenes lunata* (ladybird beetle), *Harmonia vigintiduomaculata* (chequered ladybeetle), and *Exochomus flavipes* (Coccinellid beetle) carried low amounts of pollen and were less mobile than the bees and butterflies. They often moved amongst the capitula of the same individual and therefore would be contributing more to geitonogamy than cross-pollination.

The insect groups observed on the capitula may have been using the capitula as a food source and not contributing to pollen transfer at all (Appendix 3.1) – such as the thrips and ants. The effectiveness of thrips as pollinators have been debated in the past (e.g., Mani and Saravanan, 1999), however various studies have noted their effectiveness as pollinators (e.g., Jürgens *et al.,* 2000; Sakai, 2001; Eliyahu, 2015). In the present study, however, the thrips were not mobile enough to contribute to cross-pollination. Instead, the thrips may have only been eating the pollen and inadvertently contributing to autogamy. Similarly, the ants observed on the capitula may have only been visiting the capitula to obtain pollen as they lack the necessary morphological characteristics, such as hairs for pollen grain adherence, to be effective pollinators (Mani and Saravanan, 1999).

Capitula of *C. macrocephalum* received many insect visitors, however not all served a pollinator function. It is therefore plausible that many pollen grains were lost to non-pompom

weed flowers by these generalist visitors (Mani and Saravanan, 1999; Willmer, 2011). This is mitigated by the plants flowering in great abundance within a specific area so that at least some conspecific pollen is received by individuals of the same species (Willmer, 2011). Most of the insect visitors observed on capitula of *C. macrocephalum* may be contributing to pollination via their movements within and among capitula, however they may not be sufficiently specific in their visits to efficiently affect cross-pollination.

Cypsela counts and germination trials

The higher seed set per capitulum in open treatments in comparison to bagged treatments, shows that reproductive success is lower when pollinators are excluded. This suggests that the insect visitors on the capitula contribute to pollen transfer – either within the capitulum and/or plant, or between individuals. Despite the difference, however, the bagged treatments still produced many seeds. Therefore, capitula still set seed in the absence of pollinators, which is a strong indicator of the species' ability to reproduce via self-fertilisation and/or apomixis. Interestingly, a study conducted by Kao (2007) on the reproductive biology of the autonomous apomictic *Arnica cordifolia* Hook. found a statistically significant difference in seed set between bagged (pollinator exclusion) and open treatments despite the species reproducing via autonomous apomixis. This was attributed to the species still needing pollen from other individuals (cross-pollination) to trigger seed production, even though it was not used for endosperm development. Based on these findings, it is evident that pollinator exclusion has implications for uniparental reproduction too.

Notably, the low germination percentages of seeds in the Modderfontein population may be attributed to the efficacy of biocontrol on these populations. Both the rust fungus (*Puccina eupatorii)* and the thrips (*Liothrips tractabilis*) were present in all four populations, however only the Modderfontein population was severely affected. The main effect of the biocontrol

agent, the rust fungus, on *C. macrocephalum* is premature senescence, however the effect of the rust on cypsela production and viability was previously unknown (Goddall *et al.,* 2012). This study has demonstrated that the rust infestation reduced reproductive success in the Modderfontein population potentially by maintaining a long-term parasitic relationship with the plants (i.e., the rust was present on the offspring even though they were grown in a growth chamber). Additionally, the thrips may have also contributed to the reduced reproductive success in the Modderfontein population. The principal effect of the thrips is plant growth deformities, which reduce height, biomass, and seed production (Besaans, 2014). These observed deformities may account for the reduced reproductive fitness observed in this population. The severity of the infestation at only one of the four populations suggests that either the biocontrol agents are not abundant enough in certain areas, or populations of *C. macrocephalum* display varying levels of resistance to biocontrol agents.

Secondary pollen presentation, protandry and sporophytic incompatibility

In this study, self-pollen was transferred to the style as it elongated through a ring of anthers, which allows the style to present the pollen to biotic vectors. This type of secondary pollination is characteristic of the brush mechanism found in other asteraceous species (Torres and Galetto, 2007). The stigmatic surfaces remain appressed as the style elongates and therefore self-pollination is avoided during this process. Carolin (1960) hypothesised that when the style branches separate and the stigmatic surfaces become receptive, the pollen grains released from the anthers are no longer viable. The style presents pollen during the staminate phase and receives pollen in the pistillate phase in asteraceous species (Howell *et al.,* 1993). The dual purpose of the style is presumably to enhance the exportation and reception of pollen, thereby increasing the fitness of the plant (Howell *et al.,* 1993). Further, dichogamy in the form of protandry was observed in florets of *C. macrocephalum.* The anthers dehisced before the stigmatic surfaces became receptive thereby reducing the probability of self-fertilisation.

Additional evidence for the prevention of self-fertilization is the growth of pollen tubes on the stigmatic surface/margin but their inability to grow down the style to the ovules may be indicative of a sporophytic incompatibility system (Hiscock and McInnis 2003; Takayama and Isogai 2005). Dichogamous protandry and sporophytic self-incompatibility (SSI) have been reported in Asteraceae (Allen *et al.,* 2011). Self-incompatibility is a mechanism that plants use to reduce pollen–pistil interaction thereby reducing self-fertilisation and enhancing outcrossing (Barrett, 2002). Self-incompatibility in Asteraceae usually occurs at the stigmatic surface by preventing the germination of self-pollen grains or arresting pollen tube growth (e.g., Hiscock, 2000; Allen *et al.,* 2011), as appears to be the case in this study.

Pollen tube growth

The presence of pollen tube growth on the stigmatic margins and outer surfaces of the style may be indicative of gametophytic apomixis. Gametophytic apomixis forms part of a developmental continuum with sexual reproduction and sporophytic apomixis (= adventitious embryony) identified as the extremes (Talent, 2009). It is therefore plausible that species may retain sexual traits if apomixis emerged late in the evolutionary history of the genus (Plachno *et al.,* 2015). For example, a comparison of the micropyle structure in sexual and autonomous apomictic *Taraxacum* F.H.Wigg species (dandelions) found that the ovule-transmitting tissue (tissue through which pollen tubes grow to reach the ovules and effect fertilisation) was still active in the apomictic species despite embryo and endosperm formation being independent of fertilisation (Plachno *et al.,* 2015). The presence of pollen tube growth in *C. macrocephalum* may therefore be a remnant of the sexual pathway that could allow for sexual reproduction. The maintenance of structures connected to pollination and fertilisation may be to facilitate gene flow between apomicts and sexually reproducing plants (Plachno *et al.,* 2015). It is unclear, however, whether the benefits of gene flow outweigh the energy benefits associated

with the reduction of unnecessary costs — like maintaining a sexual pathway (Plancho *et al.,* 2015).

The lack of pollen tube growth to the ovules in all samples suggests that endosperm development in the analysed samples was independent of fertilisation. This provides strong evidence for obligate autonomous apomixis occurring in South African populations of *C. macrocephalum.* This is consistent with observations of autonomous apomictic lineages typically being derived from self-incompatible, outcrossing species (Hörandl, 2009). Additionally, autonomous apomixis is considered the rule in Asteraceae (e.g., Noyes, 2006; Janas *et al.,* 2021), with pseudogamy only being expressed in a few genera (Noyes, 2007, Hörandl *et al.,* 2008) such as *Parthenium* L., *Rudbeckia* L., and *Leontopodium* R. Br. Ex Cass (Sokolowska-Kulczycka 1959; Noyes, 2007).

Genetic diversity

Populations of *C. macrocephalum* included in this study were found to be genetically similar. Additionally, genetic differentiation between maternal plants and their respective offspring was also low, however the Shannon-Wiener index revealed higher genetic diversity amongst offspring than between maternal plants. This could be attributed to mutation accumulation in the offspring (Hodač *et al.,* 2019) or the small sample size of the maternal plants. Mutation accumulation typically occurs in the absence of recombination because non-combining genomes cannot eliminate mutations effectively (Muller's ratchet, 1964).

Typically seed-propagated plants exhibit higher genetic diversity than asexually reproducing plants, due to recombination and outcrossing (Olsen and Schaal, 2007). However, the low genetic variability in South African populations of *C. macrocephulm* despite copious seed production, provides further evidence of apomixis within its invaded range. Due to the autonomous endosperm development, seed production in *C. macrocephalum* is independent of

meiosis and fertilization. The lack of recombination could account for the low genetic differentiation between maternal plants and their respective offspring. Despite this, we expected higher levels of heterozygosity than the observed heterozygosity of zero. Apomictic lineages typically have a higher proportion of heterozygotes than outcrossing lineages because the maternal genotype is maintained in the offspring (Peredo, 2013; Grusz and Pryer, 2015). The prevalence of homozygosity within South African of *C. macrocephalum* populations may be linked to the species reproducing via facultative apomixis in its native range (Farco *et al.,* 2012). This suggests that the species often had low recombination frequencies, which could have contributed to the low observed heterozygosity.

While some studies have suggested that genetic variability is important for invasion success (e.g, Barrett, 2000; Lee, 2002), other studies have found low or no genetic variation in successful invasive plant species (e.g., Hollingsworth and Bailey, 2000; Poulin *et al.*, 2005). Low levels of genetic diversity are often attributed to founder effects (i.e., the consequent loss of genetic variation that occurs when a new population is established from a small subset of an existing population) or genetic bottlenecks (i.e., a reduction in population size leading to a loss genetic variation) (Poulin *et al.*, 2005). Despite this, some species with low genetic variation are still successful invaders (e.g., Hagenblad, 2015), a phenomenon referred to as the 'genetic paradox of invasive species' (Allendorf and Lundquist, 2003).

Other than genetic diversity, the success of invasive species has been linked to phenotypic plasticity (Baker, 1965; Davidson *et al*., 2011). Phenotypic plasticity refers to a single genotype's capability to produce different phenotypes in response to environmental conditions (Bradshaw, 1965) thereby enabling an invasive species to establish in a novel habitat (Poulin *et al.*, 2005). Interestingly, polyploidy can increase the plasticity in a species by maintaining intra-individual genetic diversity, even when inter-individual genetic diversity is low (Poulin *et al.*, 2005). Accordingly, despite the low genetic variation in the pompom weed, only four

out of the 177 individuals were identified as clones. This suggests that the interactions of polyploidy and plasticity may be contributing to the pompom weed's invasion success. Therefore, it is evident that there are factors beyond the scope of this study that may account for the success of the pompom weed, despite low genetic variation within and amongst populations.

Invasion success and biocontrol

Autonomous apomictic species are more likely to naturalize in introduced areas and become invasive as they are not restricted by pollinator availability (Rambuda *et al.,* 2004). Asteraceae constitutes a large proportion of invasive taxa worldwide, presumably due to the widespread expression of autonomous apomixis and polyploidy within the family (Dellinger *et al.,* 2016; Zhang *et al.,* 2021). This is in accordance with Baker's law (1955) which states that invasion success is facilitated by a species' ability to reproduce via uniparental reproduction.

Autonomous apomixis is considered to be more advantageous than other uniparental reproductive modes as it is fully independent of pollinator availability and activity (Mraz *et al.,* 2019). As seen in *C. macrocephalum,* despite the inefficient pollen transfer by various insect groups, the species was still able to produce a large seed set. This indicates that reproduction in *C. macrocephalum* would be unaffected even in unpredictable pollinator environments. Autonomous apomixis is therefore contributing substantially to the species' reproductive success and persistence within its invaded region.

Additionally, polyploidy and apomixis often co-occur in asteraceous species (e.g., Bertasso-Borges and Coleman, 2005; Lu *et al.,* 2008; Farco *et al.,* 2012; Zhang *et al.,* 2021). The offspring used for these AFLP analyses were triploids. Triploids are typically characterized by genomic and reproductive instability (Whitton *et al.,* 2008). To mitigate the effect of these instabilities, triploids bypass meiosis and reproduce predominantly via apomixis (e.g., Noyes,

2000; Mraz *et al.*, 2009). Autonomous apomixis contributes to the persistence of triploids by enabling them to maintain reproductive output even in unfavourable conditions (Freeling, 2017). While polyploids can exploit and establish in novel niches, their persistence in these niches may be attributed to the competitive advantage provided by asexual reproduction (e.g, Kirchheimer *et al.*, 2018). The co-occurrence of apomixis and polyploidy in South African populations of *C. macrocephalum* has likely substantially facilitated its invasion success.

Despite the increased colonizing ability provided by both autonomous apomixis and polyploidy, biocontrol using a genotype-specific agents should be successful due to the low genetic variation within and amongst populations. Instead, populations of *C. macrocephalum* appeared to display differential responses to the current biocontrol agents and still had high reproductive outputs. This suggests that another factor, presumably related to environmental conditions or the biocontrol agents' life stages, may be limiting the success of biocontrol agents on South African populations of *C. macrocephalum*.

CONCLUSION

South African populations of *Campuloclinium macrocephalum* are most likely reproducing via gametophytic apomixis with autonomous endosperm development (analogous to autonomous apomixis). Seed set was independent of fertilisation in all the analysed samples thus suggesting that the species is likely using obligate apomixis. Apomixis may be the only form of uniparental reproduction that can be expressed in the pompom weed due to dichogamous protandry, sporophytic incompatibility, and secondary pollen presentation potentially inhibiting self-fertilization. The low genetic differentiation amongst populations and between the maternal plants and their respective offspring is also characteristic of autonomous apomixis. However, this suggests that the populations should not be exhibiting differential responses to biocontrol agents. Therefore, there are unconsidered factors that may be limiting the success of biocontrol

agents on South African populations of the pompom weed. It is evident that polyploidy and apomixis are complex phenomena whose interactions may be greatly facilitating the invasion success of the pompom weed. Based on the findings of this study, however, autonomous apomixis appears to be the most likely explanation for the species persistence and establishment.

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Appendix 3.1: Additional information on common insect visitors to capitula of *Campuloclinium macrocephalum* in Modderfontein Nature Reserve, Gauteng during the December – March 2021 field season. Information on the family, common name, authority, origin, and common food sources have been provided.

Family	Species	Common name	Authority	Indigenous	Common food source	References
Nymphalidae	*Danaus chrysippus*	African Monarch butterfly	Linnaeus (1758)	Yes	Nectar	Smith *et al.* (2005)
Pyrogomorphidae	*Zonocerus elegans elegans*	Elegant grasshopper	Thunberg (1815)	Yes	Herbs and wild plants	Kaufmann (1972)
Apidae	*Apis mellifera*	Honeybee	Linnaeus (1758)	Yes	Nectar and pollen	Gonzales *et al.* (1995)
Melyridae	*Astylus atromaculatus*	Spotted maize beetle or the pollen beetle	Blanchard (1843)	No (Invasive)	Pollen	Van den Berg *et al*. (2008)
Lycidae	*Lycus melanurus*	Hook-winged beetle	Fabricius (1787)	Yes	Nectar, flowers, fungal material	Picker *et al*. (2002)
Coccinellidae	*Cheilomenes lunata*	Ladybird beetle	Fabricius (1775)	Yes	Aphids – especially wheat aphids	Picker *et al*. (2002)
Coccinellidae	*Harmonia vigintiduomaculata*	Chequered lady beetle	Fabricius (1792)	Yes	Soft bodied pest insects	Picker *et al*. (2002)
Phlaeothripidae	–	Thrips	Uzel (1895)	Yes	Pollen	Willmer (2011)
Formicidae	–	Ants	Latreille (1809)	Yes	Pollen, nectar, and floral structures	Picker *et al*. (2002)
Coccinellidae	*Exochomus flavipes*	Coccinellid beetle	Thunberg (1781)	Yes	Insects	Geyer (1947); Picker *et al*. (2002)

Appendix 3.2. Results from a Tukey post-hoc test comparing pollen loads on insect visitors collected from capitula of *Campuloclinium macrocephalum.* Statistically significant values are indicated with an asterisk (*) at $\alpha = 0.05$.

	Exochomus flavipes	**Formicidae**	**Phlaeothripidae**	***Harmonia vigintiduomaculata***	***Cheilomenes lunata***	***Lycus melanurus***	***Astylus atromaculatus***	***Apis mellifera***	***Zonocerus elegans elegans***
Danaus chrysippus	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	0.493	< 0.0001*
Zonocerus elegans elegans	0.253	0.261	0.584	0.618	0.807	0.974	0.999	0.072	
Apis mellifera	< 0.0001*	< 0.0001*	0.0002*	0.0003*	0.0007*	0.003*	0.012*		
Astylus atromaculatus	0.667	0.678	0.938	0.951	0.992	0.999			
Lycus melanurus	0.908	0.914	0.997	0.998	0.999				
Cheilomenes lunata	0.994	0.995	0.999	0.999					
Harmonia vigintiduomaculata	0.999	0.999	1.000						
Phlaeothripidae	0.999	0.999							
Formicidae	1.000								

Appendix 3.3. Results from a Kruskall-Wallis multiple comparisons post hoc test comparing germination percentages in open and bagged treatments amongst four populations of *Campuloclinium macrocephalum.* Statistically significant values are indicated with an asterisk (*) at α = 0.05

		Modderfontein		**Midrand**		**Tembisa**		**Greenstone**
		Apomixis	Open	Apomixis	Open	Apomixis	Open	Apomixis
	Open	<0.001*	<0.001*	0.522	0.841	<0.001*	0.521	0.244
Greenstone	Apomixis	<0.001*	<0.001*	0.507	0.212	<0.001*	0.133	
	Open	<0.001*	<0.001*	0.291	0.498	0.002*		
Tembisa	Apomixis	<0.001*	<0.001*	<0.001*	<0.001*			
	Open	<0.001*	<0.001*	0.522				
Midrand	Apomixis	<0.001*	<0.001*					
Modderfontein	Open	0.231						

Concluding chapter

General overview

Campuloclinium macrocephalum (Less.) DC. (Asteraceae, Eupatorieae), a plant that occurs naturally in tropical South and Central America, is an alien invasive species in South Africa. The species is commonly referred to as the "pompom weed" due to its conspicuous pink flowers during the summer months (December – March). The pompom weed has rapidly invaded roadsides, grasslands, savannas, and wetlands (Goodall *et al.,* 2010) and has significantly reduced native vegetation (Aileen, 2005). Mechanical, chemical and biocontrol management plans have been implemented to reduce the spread of the species (McConnachie *et al*., 2011; Goodall *et al.,* 2012; Ramanand *et al.,* 2016), however the species has still been able to persist and expand its distribution within its invaded range (Trethowan *et al*., 2011).

In accordance with Baker's Law, the pompom weed was hypothesised to reproduce uniparentally in its invaded range. This was corroborated by the species' ability to reproduce in the absence of pollinators (Kgabolese, unpubl. data) and low genetic variation was observed amongst South African populations (Gitonga *et al.,* 2015). Additionally, Gitonga *et al.* (2022) identified triploid and tetraploid cytotypes in South African populations. Polyploids commonly occupy areas outside of their diploid parent populations' ecological range to escape minority cytotype exclusion (Levin, 1975, Fowler & Levin 1984), however parthenogenesis (specifically apomixis) gives them a competitive advantage to persist in their invaded ranges (Rodriguez, 1996). These findings and hypotheses formed the basis of the current study. We aimed to determine the predominant mode of reproduction in South African populations of the pompom weed and explore its link to polyploidy. We investigated the following aspects of its reproduction: male fertility, the efficiency of pollinators, pollen tube growth to the ovules, and

genetic variation. These findings were then used to make inferences on the impact it would have on biocontrol efficacy.

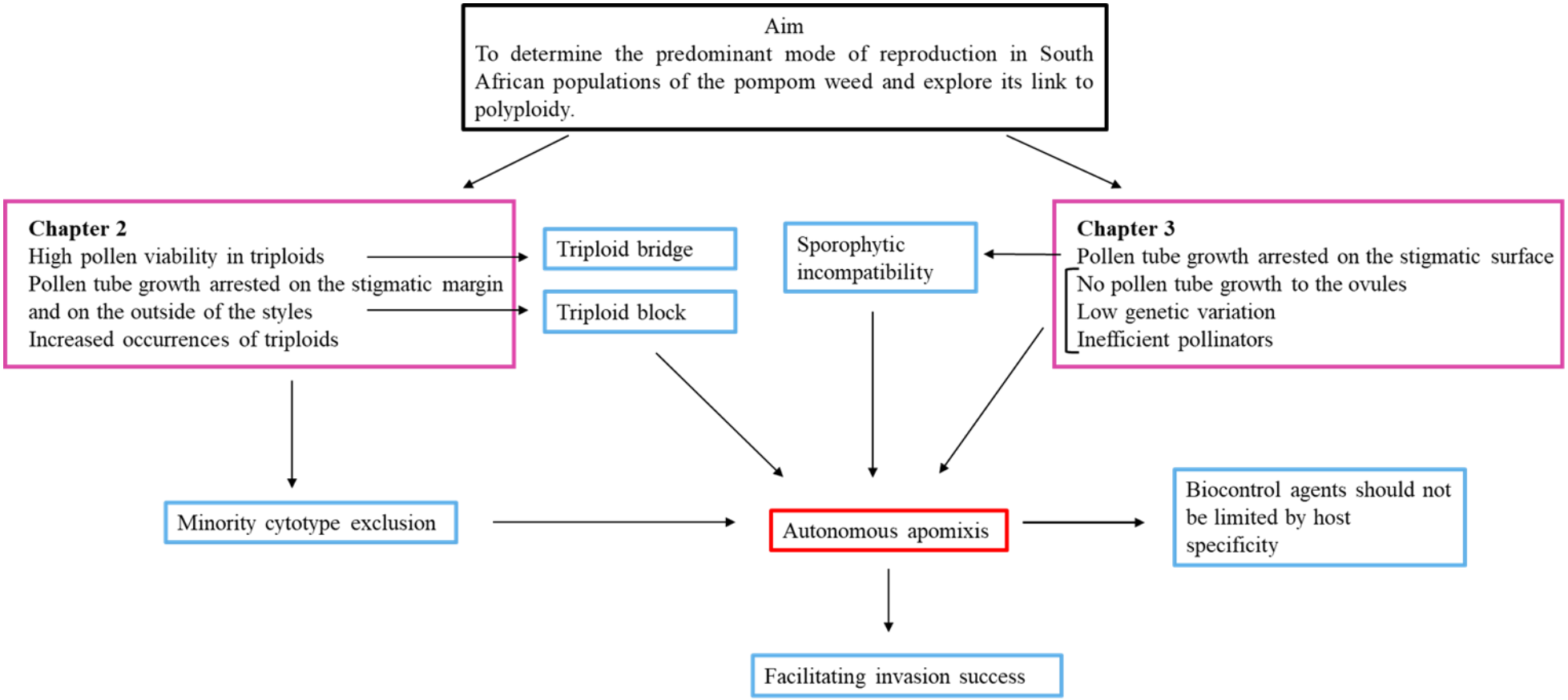

Figure 4.1. A diagram summarising the major findings of this study. Major findings from chapters 2 and 3 are in the pink boxes, potential explanations related to each finding are in the blue boxes, and the likely mode of reproduction of the pompom weed based on inferences from our data is in the red box.

Summary of the study and major findings

In this study, I used a pollinator exclusion experiment to determine if the pompom weed can reproduce in the absence of pollinators. Concurrently, I also assessed the relative contribution insect visitors make to pollination in the open treatments using observations and pollen load assessments. I found that the pompom weed can set seed in the absence of pollinators, however seed set per capitulum was significantly lower than in the open treatments with insect visitation. This result was unexpected as the pollinator observations revealed that most of the insect visitors were not efficient at pollen transfer. Nevertheless, it was concluded that *Apis mellifera (*honeybee) and *Dananus chrysippus (*the African monarch butterfly) contributed the most to pollen transfer in the pompom weed due to their significantly higher pollen loads in comparison to other insect visitors.

To explore what type of uniparental reproduction led to the formation of cypselas in the bagged treatment, I assessed pollen tube growth to the ovules using both fluorescence microscopy and scanning electron microscopy. Since self-fertilization (autogamy and geitonogamy) requires transfer of the sperm to the egg, as does pseudogamy (apomixis with endosperm development dependent on fertilization), we hypothesised that these reproductive strategies would display pollen tube growth to the ovules (e.g, Alves *et al.,* 2001). In contrast, autonomous apomixis (apomixis with endosperm formation independent of fertilization) requires no pollen tube growth to the ovules. I observed no pollen tube growth down the style to the micropyle in multiple (n = 800) analysed samples despite observing pollen tube growth on the style, both near and on the stigmatic surfaces. This suggested that pollen tube growth is being arrested on the stigmatic surface and on the outer surfaces of the style by a sporophytic incompatibility system. Additionally, evidence of dichogamy in the form of protandry supports the hypothesis that self-fertilization is being prevented in the pompom weed, given the position of the

stigmatic surfaces (on the inner surface of the style branches near where the style divides into two, Bremer *et al.*, 1994).

Since no evidence of pollen tube growth to the ovule was found, obligate apomixis appears predominantly to be occurring in South African populations of the pompom weed. Support for this hypothesis was provided by assessing genetic variation within and amongst the four populations used for this study. I found low genetic variation, low heterozygosity, and a high inbreeding co-efficient which are characteristic of a species reproducing by obligate apomixis (e.g., Poulin *et al.*, 2005). Despite the supporting evidence for obligate apomixis, it could not definitively be concluded that no sexual reproduction occurs. Additionally, the genetic marker (AFLPs) used here may not have been the most ideal marker for the assessment of genetic diversity as some variation may not have been recovered. This may occur due to the marker only scanning a small portion of the species' genome (Baydar *et al.*, 2004).

The co-occurrence of triploid and tetraploid cytotypes in South African populations makes it challenging to determine the predominant reproductive mode as both cytotypes could use different reproductive pathways. Various studies have noted that triploid cytotypes reproduce by apomixis while diploids and tetraploids reproduce sexually or by facultative apomixis (e.g., Verduijn *et al.,* 2010; Schinkel *et al.,* 2016; Kutlunina *et al.,* 2017). Despite sampling from populations previously identified as triploid and tetraploid (in 2013-2014), flow cytometry revealed that the offspring germinated for the AFLP analyses were all triploids; therefore, I have limited the inferences to only refer to the reproductive strategies of triploids.

An assessment of male fertility, using staining techniques and pollen germinability as indicators, revealed high pollen viability and the capability of pollen grains to germinate. As noted above however, although pollen tube growth did occur, it was arrested on the stigmatic surface and on the outside of the styles. This might be a consequence of a 'triploid block',

between cytotypes, preventing the formation of an endosperm with an unbalanced gamete ratio (Ramsey and Schemske, 1998). The triploid block is not always 'complete', and triploids can still produce viable gametes that can contribute to tetraploid establishment – commonly referred to as a 'triploid bridge' (Ramsey and Schemske, 1998; Husband, 2004; Yamauchi, 2004). In this study, we obtained results that suggest both processes may be occurring in triploid populations of the pompom weed, however more information is needed to determine what implications these processes have for gene flow. Theoretically, the production of viable male gametes could be contributing to interploidy mating, albeit at low frequencies.

In South African populations of the pompom weed, the increased occurrences of triploids in populations previously identified as tetraploid may be linked to the tetraploids experiencing minority cytotype disadvantage. In mixed ploidy populations, one cytotype is eventually lost due to the production of inviable embryos or infertile offspring (Levin, 1975). It has been hypothesised that minority cytotype disadvantage can be overcome by parthenogenesis (Rodrigues, 1996; Yamauchi *et al.*, 2004). This is likely occurring in South African populations of the pompom weed as the tetraploids may be becoming less frequent due to mate or pollinator limitations, while the triploids are able to reproduce apomictically.

While apomixis and polyploidy may be facilitating the invasion success of the pompom weed, the low genetic variation suggests that biocontrol efficacy should be high. Instead, we found that despite the low genetic differentiation between the sampled populations, only one population was severely affected by any biocontrol agents. The Modderfontein population exhibited lower reproductive success in the form of low seed set per capitulum, low germination percentages and poor seedling establishment. This population was most visibly affected by the rust which resulted in its poor reproduction. In contrast, while the biocontrol agents were present on the other three populations, they were not as severely affected as the Modderfontein population. This suggests that the establishment of biocontrol agents is being

limited by factors other than genetic variation such as environmental factors and time since biocontrol introduction.

Future recommendations

Sporophytic incompatibility system

Determining if there is sporophytic incompatibility system operating on the stigmatic surface (e.g., Hiscock, 2000) in both triploids and/or tetraploids would greatly improve our understanding of why pollen tube growth is being arrested on the stigmatic surface. This would enable us to determine if the pollen tube growth is only inhibited in triploids and may be attributed to a 'triploid block' or if self-fertilization is being prevented in all cytotypes of the species by a sporophytic incompatibility system.

Embryological studies

Embryological studies should be done to confirm the occurrence of autonomous apomixis in South African populations of the pompom weed (e.g., Farco *et al.*, 2012; Xiao *et al.*, 2021). Embryological studies are conducted by preparing longitudinal sections of buds at different stages. These sections are then used to observe the sequence of meiotic division of the megaspore mother cell which enables one to determine which cells give rise to the embryo sacs. Due to the structure and size of the florets, emasculating the anthers to prevent within floret pollen transfer is not possible. It is therefore unlikely male contribution could be completely excluded, which makes embryological studies the most suitable way to confirm apomixis.

Seed cytotyping

Experimental interploidy crosses should be attempted to determine if co-existing triploid and tetraploid individuals in other populations of the pompom weed can produce viable offspring.

Thereafter, the seeds can be cytotyped using flow cytometry to determine the mode of reproduction used to form the seeds (e.g., Matzk *et al.,* 2000). This is accomplished by comparing the DNA content of the embryo to that of the endosperm (see Kao, 2007). This method is particularly useful because one can also estimate the ploidies from which the seeds were derived. This would enable us to infer if interploidy crosses between triploid and tetraploid individuals produce viable offspring.

Genetic variation

As mentioned, the genetic marker used in this study may not have amplified all of the genetic variation present in the analysed samples. Although low genetic variation has been confirmed by two independent studies (Gitonga *et al.,* 2015) and in the present study. Both studies used AFLPs to estimate genetic variation therefore another marker, such as microsatellite analyses, should be used to confirm these results. Microsatellite analyses are species specific, and they provide higher resolution and power than other multilocus techniques (see Selkoe and Toonen, 2006).

Biocontrol efficacy

Based on the findings of this study, it is likely that apomixis and polyploidy facilitate the invasiveness of the pompom weed. However, we expected to find genetic diversity to be the limiting factor for biocontrol efficacy. Instead, we found low genetic variation, which suggests that all populations should be equally susceptible to the biocontrol agents. However, I observed that the rust was dominant in one population. A conceptual framework developed by Myers and Bazely (2003) suggests that abiotic and biotic constraints may affect the efficacy of biocontrol agents, thus resulting in them being successful in one habitat and unsuccessful in another. Biocontrol is the most cost-effective method for the management of invasive species (Barratt, 2018). It is therefore imperative that future studies on the pompom weed determine

what factors may be limiting the success of the biocontrol agents on South African populations of the pompom weed.

Concluding remarks

The complexity of studies involving apomixis and polyploidy makes it difficult to determine the causal mechanism of various reproductive traits, as the occurrence of the two phenomena has differential effects on reproductive strategies. Nevertheless, this study has increased our understanding of the reproductive biology of the pompom weed in South Africa. Based on the evidence from this study, the triploid populations are likely reproducing via obligate autonomous apomixis. This mode of reproduction may be contributing to the establishment, spread, and persistence of triploid cytotypes in South African populations of the pompom weed. Nonetheless, the lack of genetic diversity resulting from apomixis should result in proven biocontrol methods being successful across multiple populations, provided the environmental conditions are also suitable.

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