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Apomixis facilitates invasion success in South African populations of *Campuloclinium macrocephalum* (pompom weed)

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ABSTRACT

Polyploidy and apomixis enable successful plant invasions into new regions. *Campuloclinium macrocephalum* (pompom weed) is a successful alien invasive species in South Africa that has been shown to comprise multiple ploidy levels. Despite the identification of triploid and tetraploid cytotypes in South African populations, the primary reproductive strategy was unknown. We investigated the reproductive strategy used by triploid populations of the pompom weed. We assessed whether insects contributed to pollen transfer using pollinator exclusion experiments, estimated genetic diversity, evaluated pollen tube growth in the styles, and estimated male fertility using pollen grain size and viability. We found evidence that strongly suggests that the pompom weed can reproduce via gametophytic apomixis with autonomous endosperm development. Pollinator exclusion experiments showed that while insects may positively influence reproductive success, the pompom weed can set lower quantities of seed in the absence of pollinators and that overall seed viability did not differ between open and excluded pollinator treatments. Further, while individuals are capable of uniparental reproduction, seed set appears to be independent of fertilization because we did not observe pollen tubes growing down the styles and/or reaching the ovules, despite high pollen viability. The sampled triploid populations also showed low genetic diversity, which may be a result low recombination frequencies. The low genetic variation should result in all of the sampled populations being equally susceptible to the biocontrol agents; however, this is not being observed in the sampled populations. Only one out of the four sampled populations showed a significant reduction in seed set and germination percentages due to the damage from *Puccinia eupatorii* (rust fungus) and *Liothrips tractabilis* (thrips). Nevertheless, the combination of an apomictic reproductive strategy and polyploidy is likely contributing to making pompom weed a formidable invader in South Africa.

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1. Introduction

Invasive plant species pose a significant threat to natural environments and contribute substantially to global biodiversity loss (IPBES, 2023), primarily by interfering with the establishment of native species and altering plant community structure and assembly (Pearson et al., 2018). This alteration occurs through changes in environmental factors such as soil properties, nutrient cycling, and fire regimes, as evidenced by studies (e.g., Brooks et al., 2004; Vilà et al., 2011; Kuebbing *et al.*, 2014). Consequently, invasive species are one of the leading causes of the extinction of indigenous plant species (Pimentel et al., 2000; Pouteau et al., 2022). It is therefore imperative to understand the mechanisms that enable these species to invade and alter areas outside their native range (Levin, 2003).

Successful plant invasions are a multifaceted phenomenon and are often context-dependent (Moravcová et al., 2015), however, the reproductive strategies used by invasive species play a fundamental role in their invasion biology (Barrett and Richardson, 2011). This is because the reproductive strategy of a species is one of the most influential factors that can elicit an evolutionary response due to environmental change (Barrett and Richardson, 2011). The breeding system(s) of species can influence the production, dispersal, and genetic composition of propagules which subsequently influence invasion success (Kinlan and Hastings, 2005).

Plant reproductive strategies are diverse and complex, however, the distinction between uniparental and biparental reproduction is fundamental when considering the effects of reproductive strategies on invasion success (Barrett et al., 2008). Species that use uniparental reproductive strategies (e.g., self-fertilisation and asexual reproduction) are more successful at establishing in new areas than dioecious or self-incompatible species that require biparental reproduction (Baker, 1955; Rambuda and Johnson, 2004). This advantage stems

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from uniparental strategies facilitating colonization by enabling single individuals to found populations and ensure reproduction even in the absence of suitable mates or pollinators (Pannell and Barrett, 1998; Hao *et al*., 2011).

Among the various uniparental reproductive strategies, apomixis is frequently encountered in invasive species, providing distinct advantages in reproductive success and ecological resilience (e.g., Zhang et al., 2021; Hersh et al., 2023). Apomixis (agamospermy) is clonal reproduction through unfertilised seeds by circumventing meiosis and fertilization with the subsequent unreduced egg or somatic cell developing via parthenogenesis (Nogler, 1984; Koltunow and Grossniklaus, 2003). Two apomictic reproductive pathways are recognised: gametophytic and sporophytic apomixis. In gametophytic apomixis, an unreduced embryo sac is either derived from a somatic cell of the nucellus (apospory) or an unreduced megaspore mother cell (diplospory). Thereafter, the unreduced embryo sac 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 (i.e., nucellus) within the ovules (Hörandl, 2010).

Apomixis is a common reproductive strategy used by polyploids (Ramsey and Schemske, 1998) as meiosis is often deregulated in newly formed polyploids, leading to an increase in the production of unreduced gametes (Asker and Jerling, 1992). The unreduced gametes often increase the occurrence of apomixis as it avoids the formation of offspring with lower fitness 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). For example, obligate apomictic plants often have low pollen viability or complete pollen sterility, whereas facultative and pseudogamous apomicts have high pollen viability (Maia et al., 2015).

The distinction between pollen viability and fertility is important for interpreting plant reproductive success (Beaudry et al., 2020). Viability refers to the proportion of pollen grains containing intact cytoplasm and regenerative nuclei, which is often quantified using pollen-staining techniques (e.g., Alexander, 1969; Wizenberg et al., 2021), whereas fertility is assessed by determining the number of pollen grains that can germinate under specific conditions (Heslop-Harrison and Heslop-Harrison, 1987; Soares et al., 2008).

Both polyploidy (e.g., Hamilton and Miller 2016; Moura et al., 2021) and apomixis (e.g., Kumar et al., 2019; Zhang et al., 2021) are frequently encountered in invasive species, however, the association between polyploidy and apomixis is rarely considered in biocontrol management plans. This is presumably due to the difficulty in discerning the causal mechanism of each phenomenon due to their co-occurrence. 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 note that apomixis may promote the establishment of polyploid populations (e.g., Karunarathne *et al*., 2018; Kirchheimer *et al*., 2018). Nevertheless, both polyploidy and apomixis have implications for progeny formation and the subsequent genetic diversity within progeny – factors that may facilitate invasion success (Krahulcová and Krahulec, 2021).

Polyploid establishment is influenced by 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.

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 et al., 2015), a phenomenon referred to as the 'genetic paradox of invasive species' (Allendorf and Lundquist, 2003).

Considering the genetic variability of invasive species is important when developing a biocontrol management plan (Sun et al., 2020). High genetic variability (e.g., Mukwevho et al. 2017) could result in some plant genotypes being resistant or tolerant to a biocontrol agent (van Boheemen *et al*. 2017). It is therefore important to assess the genetic variation within and among populations of plant invaders to determine the long-term efficacy and sustainability of biocontrol management plans. In some instances, a few genotypes or populations of biocontrol agents may need to be introduced (Sun et al., 2020).

The Asteraceae constitute a large proportion of invasive taxa worldwide, possibly due to the widespread expression of autonomous apomixis and polyploidy within the family (Dellinger et al., 2016; Zhang et al., 2021). Apomictic asteraceous lineages are typically derived from self-incompatible, outcrossing species (Hörandl, 2009). Both sporophytic self-incompatibility (SSI) and dichogamous protandry have been found to reduce self-fertilisation and enhance outcrossing (Barrett, 2002). The former prevents the germination of self-pollen grains or arrests pollen tube growth (e.g., Hiscock, 2000; Allen *et al*., 2011), while the latter prevents self-fertilisation by keeping the stigmatic surfaces appressed as the style elongates thereby reducing the likelihood of self-pollination (Torres and Galetto, 2007). *Campuloclinium macrocephalum* (Less.) DC. (Asteraceae, Eupatorieae), commonly known as 'pompom weed', is an alien invasive species in South Africa (Category 1; NEMBA: A&I, 2020). The species is native to Central America, Mexico, and South America, and was introduced into South Africa in the 1960s. The earliest population was found in Pretoria, Gauteng province, however little is known about how the species entered the country (McConnachie et al., 2011). The species reproduces via facultative apomixis in its native range (Farco et al., 2012) and a subsequent study found that meiotic abnormalities reduced the probability of producing viable offspring in four out of the fourteen studied populations in Argentina and Uruguay (Farco and Dematteis, 2014).

The species is a pioneer, occupying disturbed sites, such as road margins and old fields. It thereafter invades grasslands, savannas, and wetlands (McConnachie *et al*., 2011) and transforms these ecosystems to the extent that many indigenous species are eliminated. It does so by increasing soil erosion, interfering with the establishment of grass species, reducing the carrying capacity of invaded areas, and effectively reducing biodiversity by displacing native species (Dixon, 2008). The pompom weed's success may be attributed to its biological characteristics such as resistance to fires, frost, herbivory, and disease (Henderson, 2007; Farco and Dematteis, 2014).

Due to the absence of natural enemies in the non-native region, pompom weed's distribution was predicted to exponentially increase

every year unless a successful biocontrol agent was found (Trethowan *et al*., 2011). However, a study by Farco et al. (2024) contradicted this finding using ecological niche models that demonstrated niche conservatism between the invaded and native range with minimal range expansion. Despite this, high niche unfilling values indicated that there is potential for the expansion of the species into new areas in the southern and south-western regions of South Africa (Preprint, Farco et al., 2024).

While there are two registered herbicides for the control of pompom weed, herbicide use is unlikely to be successful at eradicating the weed due to seedling recruitment (Zachariades *et al*., 2021). Chemical control will only be achievable after following a seven-year programme, thus rendering it economically unfeasible (Zachariades *et al*., 2021). Due to the inefficiency of chemical and mechanical control methods (Goodall and Witkowski, 2023), efforts were shifted to biocontrol approaches. Biocontrol agents seem to have variable effects on pompom populations, with it being successful in some areas and ineffective in others (pers. comm).

The current management programme for the pompom weed in South Africa is an integrated approach that includes herbicides and two biocontrol agents (i.e., the flower-feeding moth *Cochylis campuloclinium* and thrips *Liothrips tractabilis*), yet the species has still been able to persist and spread (McConnachie et al., 2011; Goodall et al., 2012; Ramanand et al., 2016, 2017). The thrips cause significant damage to pompom weed populations where they have been well-established, however, their overall success is limited by environmental factors such as soil type and grass density (Zachariades *et al*., 2021). The flower-feeding moth cultures were lost due to sub-optimal rearing conditions, and efforts are underway to import the species (Zachariades *et al*., 2021). The rust fungus (*Puccinia eupatorii*) 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, Wood *et al*., 2021). The limited success of biocontrol agents on the pompom weed, may be due to its reproductive strategy, polyploid nature, and consequent genetic variation. Although triploid and tetraploid cytotypes were identified in South African populations of the pompom weed, the reproductive mode of the species remained unconfirmed (Gitonga et al., 2022). Here, we focused on identifying the main reproductive strategies of the pompom weed in Gauteng, South Africa as this could inform future deployment of biocontrol agents and explain their effectiveness on local populations.

We aimed to answer the following questions: (i)What is the role of male fertility in the reproductive strategy used by *Campuloclinium macrocephalum*?, (ii) Is cross-pollination necessary for seed set and which insects are effective pollinators of pompom weed?, iii) Is the species capable of self-fertilisation and/or apomixis? and iv) Is there high genetic diversity in populations of the pompom weed, and could it affect biocontrol efficacy?

Flow cytometry, anther squashes, and pollen grain size variability were used to confirm polyploidy. Pollen staining and visualization of pollen grain germination were employed to assess pollen viability and fertility. The role of cross-pollination in pompom weed reproduction was inferred through observations of pollinators and quantification of pollen loads on insect visitors. Pollinator exclusion experiments, visualization of pollen tube growth, and genetic analyses were conducted to investigate the expression of a uniparental reproductive pathway.

2. Method and materials

2.1. Study species

Campuloclinium macrocephalum (pompom weed) is conspicuous during its flowering months from December to March and is characterised by its dense clusters of pink capitula (flowerheads). The leaves form a rosette at the base of the plant, decreasing in number and size up the stem (McConnachie et al., 2011). Adult plants grow to approximately 1.5 m tall. At the end of the flowering season, mature florets each produce a cypsela, resulting in many fruits (each with a single seed) in a single capitulum, dispersed by wind via the pappus (tufts of brown hair) (Henderson et al., 2003). The species has a woody rootstock (xylopodium) with perennial roots that enable it to persist through the winter (Goodall et al., 2010). It displays a secondary pollen presentation brush mechanism characteristic of most Asteraceae species (Torres and Galetto, 2007). Pollen is transferred to the style as it elongates through a ring of laterally connate anthers, which allows the style to present the pollen to biotic vectors.

2.2. Study site

Four populations of *C. macrocephalum* in the Gauteng province of South Africa were selected based on their putative ploidy levels identified in 2014 by Gitonga *et al*. (2022), viz, 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 38 E) and Greenstone (tetraploid, 26.12 S; 28.15 E). When confirming the ploidy level of these four focal populations in 2021 using flow cytometry and anther squashes to count chromosomes, we found that the focal populations of this study currently contain only triploid individuals (Appendix A).

2.3. Male fertility – pollen viability and size, pollen tube germination and growth

Pollen viability and fertility estimates were obtained using pollen staining and visualising pollen grain germination, respectively. Capitula from 10 individuals per population were harvested and fixed in Carnoy's fixative for 2 h, then stored in 70 % ethanol until further examination. Prior to staining, anthers from three capitula per individual were dissected and placed on a microscope slide with a drop of 70 % ethanol. The anthers were then squashed to extract the pollen grains, excess anther tissue was removed, and a drop of aceto-carmine stain was added to the slide, which was then examined under a Zeiss compound microscope at 400×. The aceto-carmine stain was used following the protocol outlined in Farco and Dematteis (2014) as it was found to be a more reliable stain compared to aniline blue. Dark, red-stained nuclei were indicative of viable pollen grains, while unstained or very lightly stained nuclei indicated that they were inviable.

Pollen grain size variability was assessed to determine if there are differences within and amongst ploidy levels. Fifty viable pollen grains and all the non-viable grains (~2 %) from 10 individuals per population were visualised using the Axio Imager connected to a Carl Zeiss compound microscope at 400× and measured using the Zeiss Zen software (version 2.3). Given that pollen grains are circular, we compared pollen grain diameter amongst populations. The variation in pollen grain diameter within populations was also evaluated (mean ± standard deviation) and any qualitative differences in pollen shape between the two ploidies was noted.

Pollen tube growth was visualised following the method of Kalinganire *et al*. (2000). Twenty florets from 10 individuals per population were removed from capitula in both the bagged (n = 800) and open treatments (n = 800) under a Zeiss Stereo Discovery V12 dissecting microscope. The capitula 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 removed from the florets, rinsed with distilled water, and stained with aniline blue in potassium buffer for two hours. They were then placed on a microscope slide with a drop of 80 % glycerol, squashed using a coverslip, and examined under the BX63 OFM fluorescent microscope (Olympus, United Kingdom) to observe pollen

tube growth. To confirm these results, five pistils from three individuals per population were placed on carbon tape adhered to aluminium stubs, left to dry overnight and then coated with one coat each of carbon and gold/palladium. The stubs were viewed under a Tescan Vega Scanning Electron Microscope (Tescan, Brno, Czechia). The presence of pollen tube growth would confirm the fertility of the pollen grains.

2.4. Pollinator observations

Insect observations and collections were undertaken at Modderfontein Nature Reserve (near Johannesburg) to identify potential pollinators for *C. macrocephalum*. Dedicated observations were done in December 2020 on 15 sunny days between 8h00 and 17h00. Any spatial or temporal variation in insect visitors was noted and no >10 specimens of each insect visitor were collected. Only insects that visited the capitula of the pompom weed were captured. Pollen load was determined by counting the number of conspecific pollen grains on each of the collected insect visitors by rinsing the pollen off the insects using 70 % ethanol onto a microscope slide and viewed under a Zeiss compound microscope.

2.5. Breeding system experiments

Thirty plants from each population were randomly selected with a minimum distance of at least 1 m apart. On each plant, a synflorescence (cluster of capitula on a common peduncle) was bagged to exclude insect visitation and test for self-pollination and/or apomixis. The distinction between self-pollination and apomixis was inferred using AFLP analyses as we could not emasculate the florets without damaging them. A second synflorescence on the same plant was tagged and left unmanipulated to allow pollination to occur naturally. The former remained bagged until the end of the flowering season, while the latter were bagged only at the end of the flowering season to prevent the loss of cypselas. After maturation, synflorescences were collected and stored in brown paper bags. Thereafter, seed set per capitulum was calculated and averaged for individuals.

To estimate reproductive success, 30 cypselas per individual from each treatment were placed with moistened filter paper in Petri dishes in direct sunlight on a windowsill until they had germinated (indicated by a 2 mm radicle protrusion). Thereafter, the seedlings were transferred to a soil medium and placed in a growth chamber (60 % humidity with 16 h of light at 25 °C) until their leaves were large enough to be harvested for AFLP analyses. The germination percentage for each treatment per population was calculated.

2.6. Statistical analyses

All statistical analyses were conducted in R v 4.0.3 (RStudio, 2021) and all datasets were assessed for normality using a Shapiro Wilk test to decide on a suitable statistical test. The percentage viability of pollen grains, pollen grain diameter, and germination percentage were compared amongst populations with a Kruskal-Wallis non-parametric test in RStudio using the stats package (Version 4.4.1; Wuertz *et al*., 2017). This was then followed by a Kruskal-Wallis multiple comparison post hoc test using the pgirmess package (Version 1.6.12; Giraudoux *et al*., 2018). The interquartile range (IQR) for all datasets analysed with a Kruskal-Wallis test was represented using boxplots generated using the ggplot2 package (Version 3.5.1; Wickham, 2016)). An ANOVA followed by a Tukey post hoc test was conducted to compare pollen load amongst the collected insects. The seed set data were compared amongst treatments using a generalised linear mixed model (GLMM) with a Poisson distribution and a log link function. Individuals were added as a random effect to account for a potential correlation among seed set counts within the same individual.

2.7. Estimating clonal reproduction using amplified fragment length polymorphism

2.7.1. DNA extraction

Young leaves from three maternal plants per population (total n = 12) in the apomixis/self- and natural-pollination treatments were harvested during March 2021, placed in silica gel to dry rapidly and stored at −20 °C until DNA extraction. Leaves from 15 offspring per maternal plant from each of the four populations were harvested from the germination trials and placed in silica gel until DNA extraction. The cypselas from one maternal plant from the heavily rust-infected Modderfontein population did not germinate; thus only 30 offspring were included in genetic analyses from this population (total offspring for the four populations n = 165). 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 ScientificTM NanoDrop OneC Microvolume UV−Vis Spectrophotometer and genomic DNA of good quality was diluted to 200 ng/μL, if necessary, and stored at −20 °C until analysis.

2.7.2. AFLP restriction and ligation

AFLPs were generated following Blignaut et al. (2013). The following modifications were made to the protocol: DNA was digested with 5 units of EcoR1 (New England BioLabs, Ipswich, MA, USA) 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 °C for 15 min. After EcoRI digestion, 5 units of MseI (New England BioLabs) 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 min at 37 °C followed by 20 min at 65 °C to denature the enzymes.

2.7.3. 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 2× 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 (5 s at 94 °C), denaturation (5 min at 94 °C), annealing (30 s at 56 °C), elongation (30 s at 72 °C) and final elongation (30 min at 60 °C). Successful amplification was determined by running a 4 μL sample of PCR product on 1 % agarose gel to check for smears between 100 and 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 2× Quickload Taq Mastermix (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 each fluorescently labelled product were then mixed for each DNA sample in a single tube, with each tube containing the three primer pairs (EcoRI-CAT, EcoRI-AAT, and EcoRI-ATG). AFLP fingerprints were then generated at the Central Analytical Facility, University of Stellenbosch using an ABI 3100 Sequencer.

2.7.4. Analyses of genetic data

The peaks from the chromatographs were scored manually using Geneious Prime (Version 2022.0.1; Biomatters Development Team, 2005) and translated to a binary matrix that combined all three loci. The matrix was then used to estimate presence of clones, assess clonal genetic diversity (dataset adjusted for the presence of clones, using a threshold of two per population), and assess genetic distances [Nei's genetic distance (Nei 1972)] between grouped maternal plants and their offspring per population and evaluate population

differentiation (F_{ST}) among populations using GenoDive v 3.04 (Meirmans, 2020).

3. Results

3.1. Pollen viability & fertility

The aceto-carmine glycerol stain revealed an average pollen grain viability of 90 % for all four populations with no significant differences amongst them (H_3 = 0.64, df = 1; P = 0.59; Table 1). The pollen grain diameter of non-viable pollen was significantly smaller than that of viable pollen (H_7 = 187.6; df =1, $P < 0.0001$; Table 1; Fig. 1A). Furthermore, viable pollen grain diameter also differed significantly amongst the four populations (H_3 = 204.9, df = 3, $P < 0.0001$, Table 1; Fig. 2). Pollen grain triads and tetrads were found in all the sampled individuals from each population; however, pollen grains forming the triads were found to be non-viable using the aceto-carmine stain (Fig. 1B). The presence of triads is indicative of irregular meiosis occurring in these populations of *C. macrocephalum*.

No pollen tube growth was observed down the style using fluorescence microscopy; therefore, Scanning Electron Microscopy (SEM) was used to see if pollen tube growth was inhibited on the stigmatic surface. Using SEM we observed pollen tube growth on the stigmatic surface in a few samples suggesting that the pollen grains could potentially affect fertilisation but are being inhibited from growing down the inside of style (Fig. 3). In most samples, however, pollen grains were observed to have germinated on the outside of the style (Fig. 3A – D). Pollen tube growth is usually observed down the inside of a style alongside the vascular tissue as there is less resistance in the surrounding transmitting tissue (Martin, 1959), but the pollen tubes observed (using SEM) in *C. macrocephalum* only grew from pollen grains on the outside of the style and did not penetrate the stigma. The location where these pollen grains adhered to the style matched where the laterally-fused anthers enclose the style before it elongates, which suggests that it is self-pollen from the introrse dehiscence of the anthers surrounding the style (Fig. 4A – B). We did not quantify the number of germinated pollen grains, but the pollen tube growth does suggest that pollen fertility could also be high in the pompom weed.

3.2. Pollinator observations

Pollinator observations revealed that the most frequently encountered insect species on capitula of *C. macrocephalum* were *Hypolimnas misippus* Linnaeus 1764 (Common Diadem butterfly; Nymphalidae), *Apis mellifera* Linnaeus 1758 (honeybee; Apidae), *Zonocerus elegans* Thunberg 1815 (elegant grasshopper; Pyrgomorphidae), *Astylus atromaculatus* Blanchard 1843 (spotted maize/pollen beetle; Melyridae), *Lycus melanurus* Fabricius 1787 (hook–winged beetle; Lycidae), *Cheilomenes lunata* Fabricius 1775 (ladybird beetle; Coccinellidae), *Harmonia vigintiduomaculata* Fabricius 1792 (chequered lady beetle; Coccinellidae), Phlaeothripidae (thrips), Hymenoptera (ants) and *Exochomus flavipes* Thunberg 1781 (Coccinellid beetle; Coccinellidae) (Fig. 5).

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

The visitor complex of the pompom weed displayed temporal variation, with the visits of certain species decreasing (e.g., *H. misippus* and *A. mellifera)* or increasing (e.g., L. *melanurus* and *C. lunata)* as the flowering season progressed. When considering insect behaviour and pollen position on the insects' bodies, it is likely that only bees and butterflies contribute to the pollination of the pompom weed in Gauteng.

Table 1
Putative ploidy levels from 2014 and 2021, mean pollen viability percentage, and pollen grain diameter (± SE) of viable and non-viable pollen grains in four populations of *Campuloclinium macrocephalum* in Gauteng province, South Africa.

Population	Putative ploidy level of parent populations in 2014	Putative ploidy level of parent populations in 2021	Mean pollen viability (%)	Diameter of viable pollen grains (mean± SE)	Diameter of non-viable pollen grains (mean ±SE)
Tembisa	**3x**	**3x**	89 ± 28.13	24.05 ± 0.70	21.19 ± 0.82
Midrand	**3x**	**3x**	89 ± 28.23	27.92 ± 0.71	25.58 ± 0.62
Modderfontein	**4x**	**3x**	92 ± 28.98	26.43 ± 0.34	21.62 ± 0.30
Greenstone	**4x**	**3x**	90 ± 28.36	26.98 ± 0.30	23.52 ± 0.41

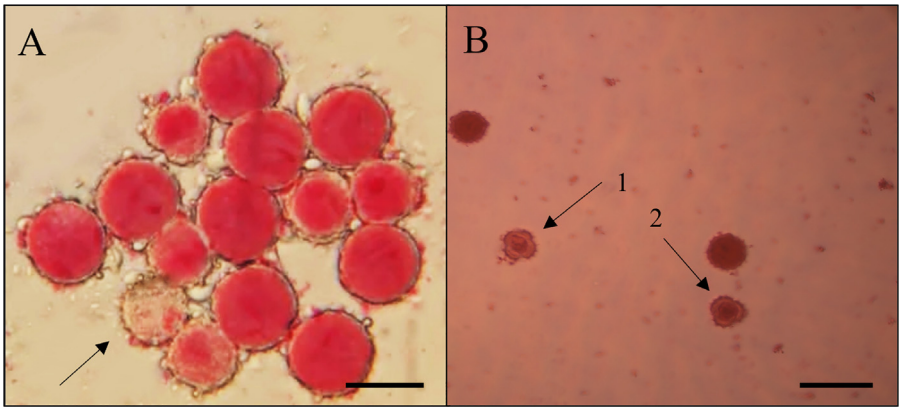


Fig. 1. Pollen grains of *Campuloclinium macrocephalum.* A) Pollen grain stained by aceto-carmine with an arrow indicating a non-viable pollen grain. B) Inviable tetrad and triad pollen grains indicated by arrows 1 and 2 respectively. Scale bars = A: 24 μm and B: 27 μm.

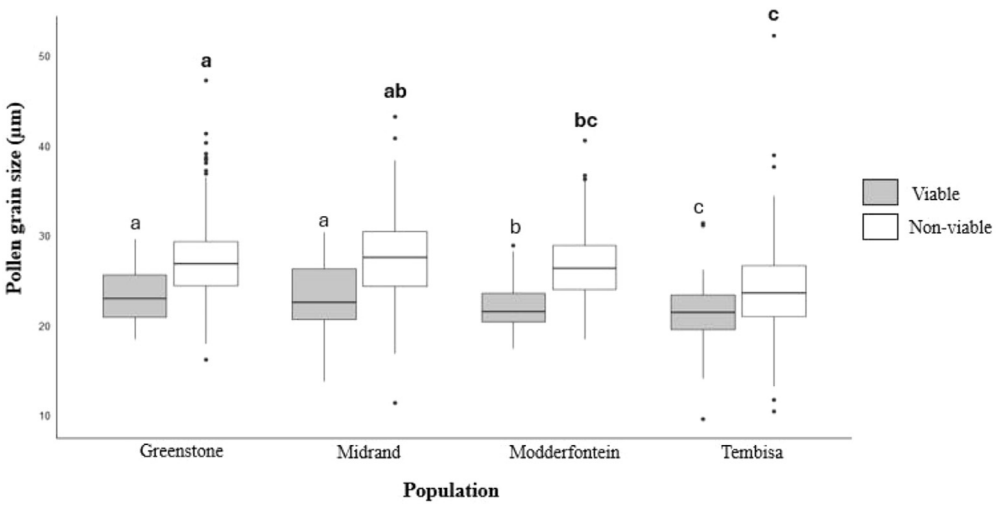


Fig. 2. Mean diameter of viable and non-viable pollen grains in four populations of *Campuloclinium macrocephalum* in Gauteng, South Africa. The circles represent outliers, the horizontal bars represent the lower quartile, median, and upper quartile respectively, the box represents variation in the data, and the whiskers indicate the range of the data. The bold letters denote statistical significance of non-viable grains amongst populations and the non-bold letters denote statistical significance of viable grains.

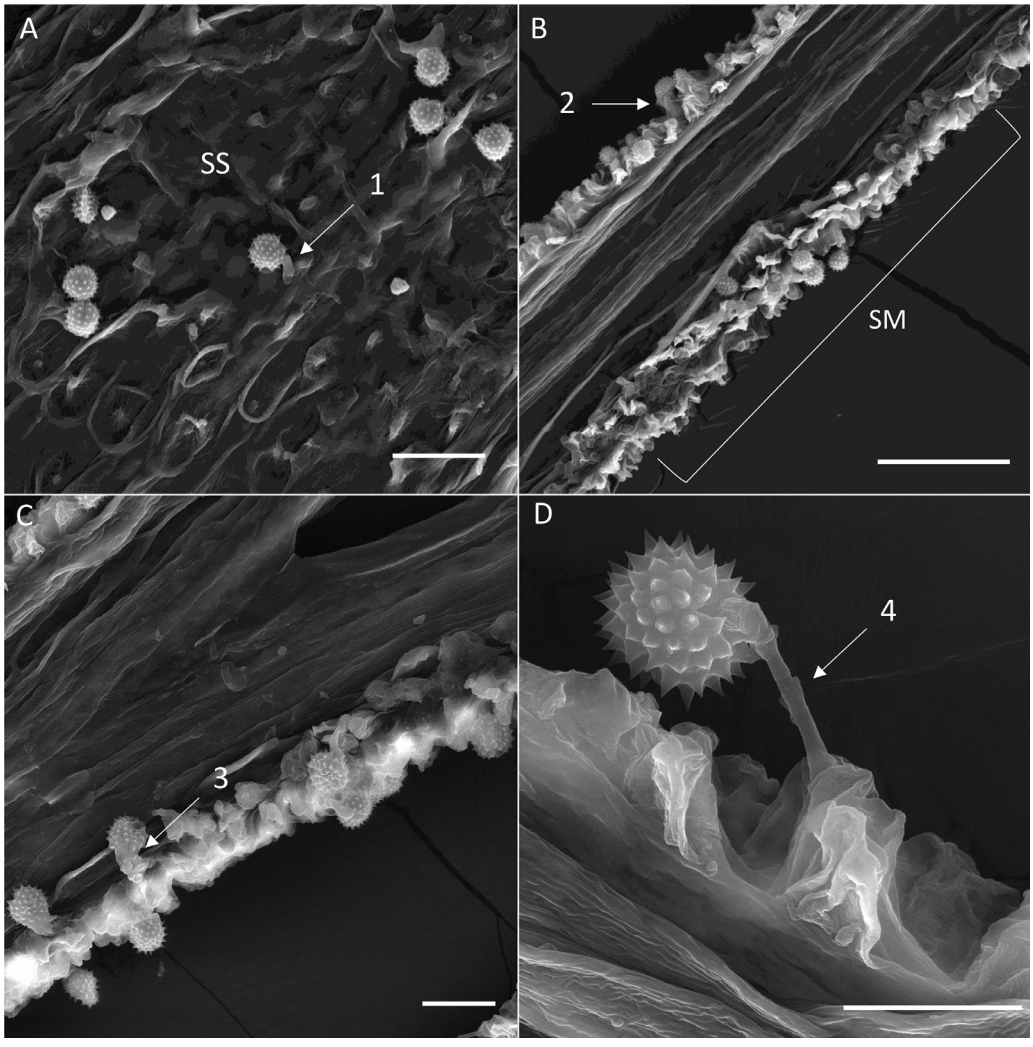


Fig. 3. Lack of pollen tube growth to the ovules in styles of *Campuloclinium macrocephalum.* A) Pollen grains on the stigmatic surface (SS) with visible pollen tube growth, B) and C) Pollen grains adhered to the sides of the styles with pollen tube growth evident, D) Pollen tube growth on the stigmatic margin (SM). Arrows indicate the pollen tubes in germinated pollen grains. Scale bars = *A*: 50 μm, B: 100 μm, C: 20 μm, and D: 50 μm.

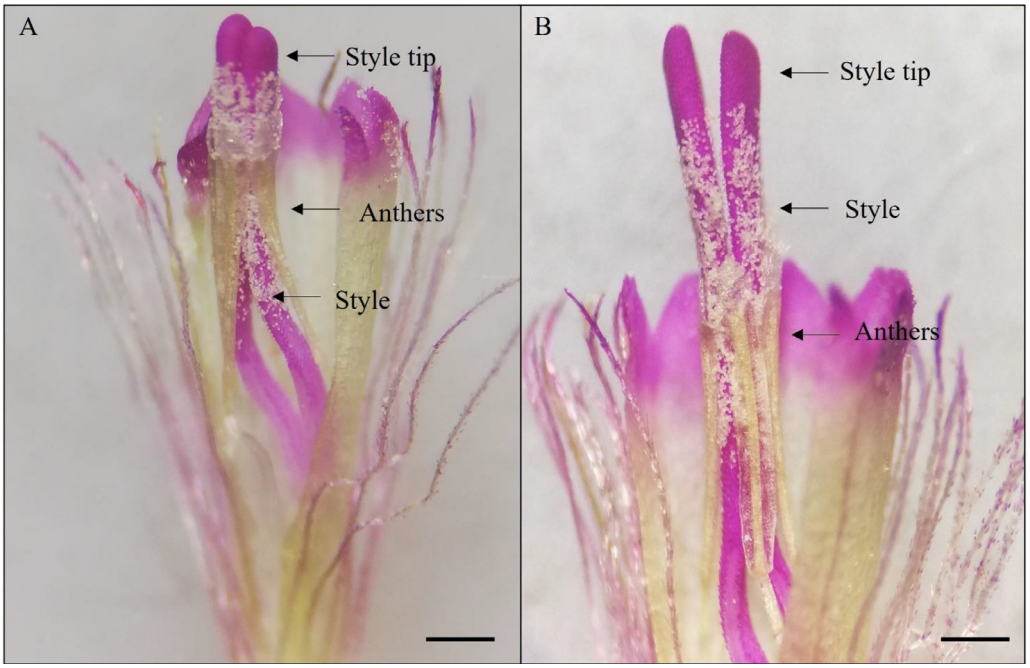


Fig. 4. The position of the anthers relative to the style in developing florets of *Campuloclinium macrocephalum*. A) A ring of laterally fused anthers surrounding the style branches, with appressed style apices before the style elongates and B) Elongated style branches with separated style tips. Scale bars = 1.7 mm.

3.3. Breeding system experiments

Seed set per capitulum was significantly different between the open and bagged treatments, with seed set per capitulum significantly higher in open treatments than bagged treatments (z = 7.11, $P < 0.001$, Table 2). Nonetheless, on average, both treatments produced over 100 cypselas with the open treatment producing 10 more cypselas per capitulum than the bagged treatment (Table 3).

Mean percentage germination differed significantly between populations (H_7 = 131.5; $P < 0.0001$; Fig. 6), however there was no significant difference between the open and bagged treatments in the Modderfontein, Midrand and Greenstone populations. A Tukey post hoc test showed that the mean percentage germination of the Modderfontein 'open' and 'bagged' treatments was significantly lower than the other three populations. Additionally, mean percentage germination of the Tembisa open treatment was significantly lower than that of the Tembisa bagged treatment.

3.4. AFLP analyses

A total of 130 loci were scored from three primer combinations for the 177 individuals sampled (n = 63 EcoRI–CAT loci, n = 35 EcoRI–AAT loci, and n = 32 EcoRI–ATG loci). There was minimal variability among individuals across the three primer combinations. Notably, the offspring from one Modderfontein maternal plant (n = 15) showed poor seedling establishment and was excluded from the analysis. The lower fitness was hypothesised to be due to the presence and effect of the biocontrol rust fungus (*Puccinia eupatorii)* on leaves and capitula. As a result, the sample size for Modderfontein was n = 33 (three maternal plants and 30 offspring) instead of n = 48. Among the 177 individuals, 173 unique genotypes and four clonal individuals were identified. Genetic diversity, after correction for the presence of clones, was found to be lower than total genetic diversity for both Nei's and Shannon-Wiener indices within the groups (viz., the maternal plants and their offspring, Table 4) Additionally, genetic differentiation was also low between the maternal plants and their respective offspring in each population (Hs = 0.026 – 0.053). 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. The overall expected (Hs= 0.253) and total heterozygosity (Ht= 0.257) within populations of *C. macrocephalum* was higher than the observed heterozygosity (H_0 = 0). Accordingly, the observed frequency of heterozygotes is zero in all populations across all loci. The corrected Nei's genetic diversity ranged from 0.67 to 1.00 across populations (Table 4). Nei's genetic diversity index was used because we could remove the potential contribution of mutations to estimate the recombinant genetic diversity. Inbreeding is likely occurring in all populations (G_{IS} = 1 for all populations). These values were expected as the maternal and offspring groups are not representative of the total populations' genetics. These groups were more informative when considering the reproductive biology of the species.

In general, the genetic differentiation amongst the four populations of *C. macrocephalum* was low (F_{ST} range = 0.006 – 0.086; Table 5), 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 were also low among the four populations (Table 5).

4. Discussion

4.1. Autonomous apomixis in the pompom weed

This study aimed to determine the reproductive strategy of the pompom weed in South Africa. Based on the pompom weed's ability to set seed in the absence of pollinators, lack of pollen tube growth to the ovules, and low genetic differentiation and diversity, it is likely that the species produces predominantly through autonomous apomixis.

The expression of apomixis in the pompom weed may be linked to the inefficient pollen transfer by insect visitors. While the Common Diadem (*H. misippus)* and honeybee (*A. mellifera*) had significantly higher pollen loads on their bodies relative to other insect visitors (Appendix B; C), they may not have been sufficiently specific enough

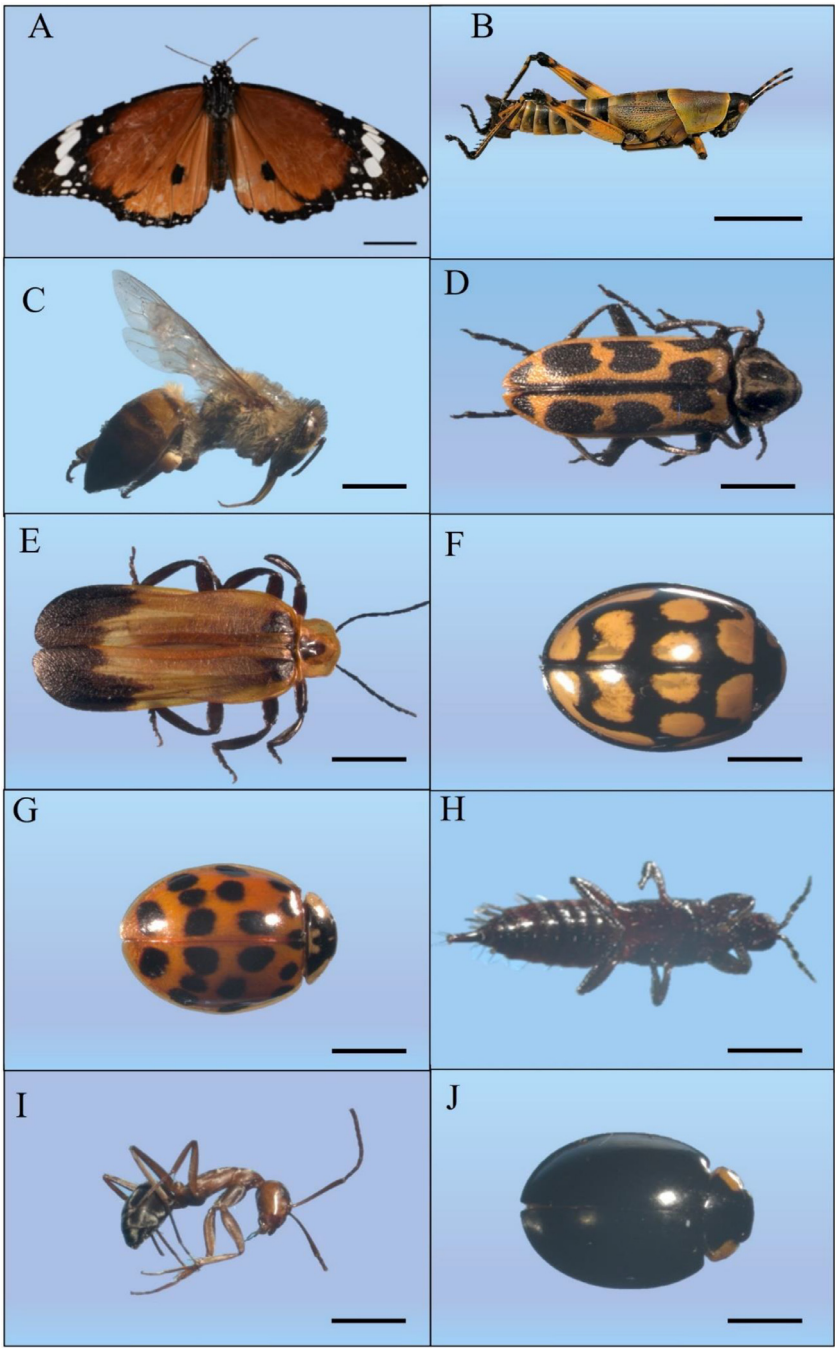


Fig. 5. A–J: Frequent insect visitors to capitula of *Campuloclinium macrocephalum* during the December 2020–March 2021 flowering period at populations in Gauteng, South Africa. A) *Hypolimnas misippus,* B) *Zonocerus elegans,* C) *Apis mellifera,* D) *Astylus atromaculatus*, E) *Lycus melanurus*, F) *Cheilomenes lunata*, G) *Harmonia vigintiduomaculata*, H) Phlaeothripidae, I) Hymenoptera and J) *Exochomus flavipes*. Scale bars = A: 1 cm, B: 1.2 cm, C: 0.5 cm, D, E: 0.4 cm, F: 0.25, G: 0.16 cm, H: 0.01 cm, I: 0.4 cm, and J: 0.14 cm.

Table 2
Statistical summary of a generalised linear mixed model (GLMM) comparing seed set between open and bagged treatments. Significance code (R 4.0.3): 0.001***.

	Estimate	Standard error	z value	Pr (>|z|)
Intercept	4.61207	0.03080	149.75	< 2e-16 ***
Open Treatment	0.08892	0.01251	7.11	1.17e-12 ***
			RS df	**RS dev**
Summary			237	4543.9

Table 3
Mean seed set per capitulum (± SE) in bagged and open treatments in four populations of the pompom weed in Gauteng, South Africa.

Population	Treatment	Seed set per capitulum (mean ± SE)
Tembisa	**Bagged**	99 ± 5,13
	Open	111 ± 6,24
Midrand	**Bagged**	85 ± 9,12
	Open	95 ± 8,45
Greenstone	**Bagged**	113 ± 4,37
	Open	122 ± 4,60
Modderfontein	**Bagged**	110 ± 4,78
	Open	118 ± 6,18

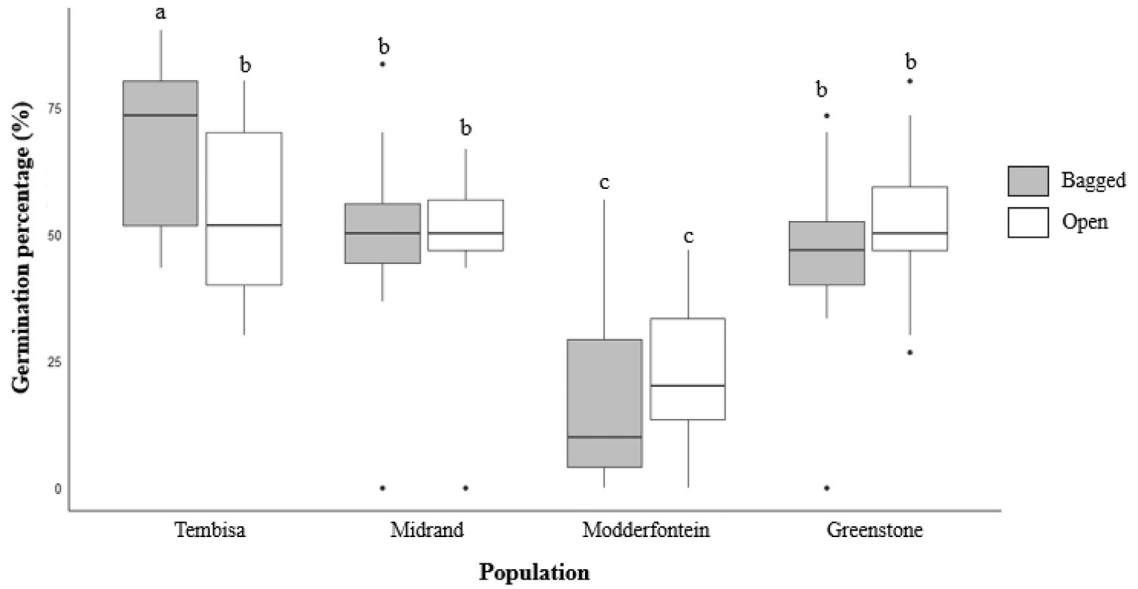


Fig. 6. Mean percentage germination of open and bagged treatments in four populations of *Campuloclinium macrocephalum* in the Gauteng province of South Africa. The circles represent outliers, the horizontal bars represent the lower quartile, median, and upper quartile respectively, the box represents variation in the data, and the whiskers indicate the range of the data.

Table 4
Indices of clonal diversity generated from three AFLP 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 across populations	22.13	21.88	21.50	0.96	0.83	0.94	1.87	1.01

in their visits to effectively cross-pollinate. Consistent with Baker's Law (1955), the pompom weed's ability to autonomously set seed helps the species overcome pollinator limitation.

Despite evidence that male contribution is not needed for seed set in the pompom weed, the species still produces highly viable pollen grains (Table 1). This could be attributed to the expression of both a sexual and an apomictic reproductive pathway (Asker and Jerling, 1992) as there are few examples of truly obligate apomictic species (e.g., Connor and Dawson, 1993; Sorensen et al., 2009; Grusz et al., 2021). Obligate apomixis cannot be confirmed in the present study despite there being no pollen tube growth to the ovules in the open or bagged treatments. While our data suggest obligate autonomous apomixis, it is important to note that only triploid individuals were analysed in this study.

Table 5
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**	–

The maintenance of sexual structures (i.e., viable pollen grains and pollen tube growth) in pompom weed is indicative of gametophytic apomixis. Gametophytic apomixis forms part of a developmental continuum with sexual reproduction and sporophytic apomixis (also known as 'adventitious embryony') at opposite ends of the continuum (Talent, 2009). It is plausible that some species retain sexual traits if apomixis emerged late in the evolutionary history of the genus (Płachno et al., 2015).

Gitonga et al. (2022) found both triploid and tetraploid cytotypes in South African Gauteng populations of pompom weed, however, flow cytometry and anther squashes from the present study found that all sampled individuals were triploid. This was corroborated by significant pollen grain size variability amongst populations of pompom weed (Fig. 2). Pollen grains of different sizes are characteristic of triploids as they produce both haploid and diploid gametes due to the occurrence of irregular meiosis (e.g., Ramsey and Schemske, 1998, Henry *et al.*, 2005; Conceição et al., 2019). Studies have shown that triploid cytotypes reproduce by apomixis, whereas diploids and tetraploids reproduce sexually or by facultative apomixis (e.g., Verduijn et al., 2004; Schinkel et al., 2016; Kutlunina et al., 2017). Therefore, in this study, the inferences on the reproductive biology of the pompom weed are limited to triploid cytotypes.

There was a significant difference in seed set between the open and bagged treatments suggesting that even though pollen may not be needed for endosperm development in autonomous apomixis, it still may trigger seed production. A difference in seed set between

bagged (pollinator exclusion) and open treatments was also observed in *Arnica cordifoliia* Hook. despite the species reproducing via autonomous apomixis (see Kao, 2007). Alternatively, for our study, the bags may have altered the microclimate around the bagged capitula which affected seed set, however, no visible differences were observed between the bagged and open capitula. Nevertheless, there was no significant difference in the germination percentages between the bagged and open treatments in all populations, except Tembisa, suggesting that the species can reproduce in the absence of pollinators albeit at lower frequencies.

Populations of the pompom weed were found to be genetically similar (Table 4), however, only four out of the 177 individuals were identified as clones. 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). While we did find low genetic differentiation between the maternal plants and their respective offspring, characteristic of autonomous apomixis, we expected a higher estimate of heterozygosity in the pompom weed. The prevalence of homozygosity in the pompom weed (Table 4) may be linked to the species' expression of facultative apomixis in its invaded range (Farco et al., 2012). This suggests that the species has always had low recombination frequencies, resulting in low heterozygosity. Moreover, the low levels of genetic diversity may also be attributed to founder effects or genetic bottlenecks (Poulin *et al*., 2005) following an invasion event.

The expression of apomixis in the pompom weed might be linked to a 'triploid block'. This mechanism is an immediate reproductive barrier that prevents backcrossing with progenitors (Köhler et al., 2010). Interploidy crosses typically produce inviable or sterile offspring as an unbalanced ratio of maternal and paternal genomes can lead to endosperm collapse (Ramsey and Schemske, 1998). This mostly prevents incidences of successful fertilisation in sexual triploids. When pollen is not needed for endosperm development, as is the case in most asteraceous species (e.g., Noyes, 2006; Janas *et al*., 2021), an obligate apomictic lineage is selected over sexual reproductive pathways (Hojsgaard and Hörandl, 2019).

Reduced pollen fertility or complete pollen sterility is common in triploids (e.g., Ramsey et al., 2011; Sevilleno et al., 2023) and apomictic species (e.g., Schinkel *et al*., 2017; Conceição *et al*., 2019). In some instances, however, pollen function can be maintained even when it is not being used for endosperm development (Rotreklová and Krahulcová, 2016) or for facultative sexuality (Ortiz *et al*., 2013; Hojsgaard and Hörandl, 2015). An average pollen viability of 90 % (corroborated by pollen grain germination) was an unexpected result for a triploid apomict, thus suggesting that the role of male gametes is not fully understood in apomictic species. Typically, the production of functional gametes in triploids is associated with triploid bridges, but we found no evidence that these gametes are effecting fertilisation. These mechanisms are not strictly delineated, and the production of viable gametes may reduce triploid blocks over time (Burton and Husband, 2001). Gene flow between the triploid and tetraploid populations of *C. macrocephalum* could be possible in contact zones, thereby influencing the genetic variation within the species.

4.2. Sporophytic incompatibility

It is unlikely that pompom weed can reproduce via self-fertilisation based on the presence of dichogamous protandry, secondary pollen presentation, and potentially a sporophytic incompatibility (SI) system. Secondary pollen presentation, assisted by dichogamous protandry, ensures that the stigmatic surface is not receptive until after the self-pollen grains have been released (Fig. 4). While SI was not directly assessed during this study, we found evidence consistent with that of other self-incompatible asteraceous species. For example, *Senecio squalidus* Linnaeus (1753), an invasive species with similar style morphology to the pompom weed, has an SI system that occurs on the stigmatic surface. Incompatible pollen tubes are either arrested before germination or, in rare instances, after penetrating the stigma (Hiscock, 2000).

4.3. Biocontrol efficacy

The low percentage germination of seeds in the Modderfontein population may be attributed to the efficacy of biocontrol agents on this population. Both the rust fungus (*Puccinia 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 rust fungus on *C. macrocephalum* is premature senescence, however, the rust's effect on cypsela production and viability was previously unknown (Goodall *et al*., 2012). This study has demonstrated that the rust infestation reduced reproductive success in the Modderfontein population by potentially 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 seen in plant growth deformities that reduce height, biomass, and seed production (Besaans, 2014), which may account for the reduced reproductive fitness observed in this population.

4.4. Invasiveness of the pompom weed

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 high genetic diversity among populations to be a limiting factor for biocontrol efficacy in the region, as apomictic polyploids typically maintain high levels of heterozygosity (Pellino *et al*., 2013; Karbstein *et al*., 2021). Instead, we found low genetic variation, which suggests that all four populations studied should be equally susceptible to the introduced biocontrol agents (Nissen et al., 1995). This is not what we observed here as the rust infestation only reduced the reproductive success of one out of the four studied populations of pompom weed in Gauteng. Other factors such as phenological (Bean et al., 2007), climatic (Augustinus *et al*., 2020), and/or genetic discrepancies (True-Meadows et al., 2016) between the biocontrol agents and hosts could limit the success of biocontrol programmes. However, these factors were not investigated in this study.

4.5. Future recommendations

Biocontrol is the most cost-effective method for the management of invasive species (Barratt et al., 2018). It is therefore imperative that future studies on the pompom weed determine what factors may be limiting the success of the biocontrol agents in South African populations of pompom weed. Notably, all inferences on the reproductive biology of the pompom weed were limited to triploid cytotypes. This presents a problem when assessing the invasion potential of the species as we cannot predict the reproductive output and genetic variation of tetraploid cytotypes – factors that could influence biocontrol efficacy.

Future studies should focus on determining the reproductive strategy used by tetraploid cytotpes of the pompom weed, understanding the apomictic pathway using embryological studies, and conducting experimental interploidy crosses to confirm the presence of a triploid bridge. The absence/presence of a sexual reproductive pathway should also be confirmed to assess its influence on genetic variation within the species. This information is important for understanding how the reproductive biology of the species is facilitating the invasion success of pompom weed, especially since the species' distribution is expected to expand into new areas in South Africa (Preprint, Farco et al., 2024).

5. Conclusion

We demonstrate that polyploidy and apomixis may be interacting to facilitate the invasion success of the *Campuloclinium macrocephalum,* 'pompom weed', in South Africa. The triploid populations of pompom weed are most likely reproducing via gametophytic apomixis with autonomous endosperm development (analogous to autonomous apomixis). The species is likely using obligate apomixis, as indicated by seed set being independent of fertilization in all analysed samples. Apomixis may be the only form of uniparental reproduction that can be expressed in pompom weed due to protandry, sporophytic incompatibility, and its secondary pollen presentation mode that reduces likelihood of self-fertilization. Our data and interpretation thereof suggest that the sampled populations of *C. macrocephalum* should not exhibit differential responses to biocontrol agents. This suggests that another factor, presumably related to environmental conditions or the biocontrol agents' life stages, may be limiting the success of biocontrol agents in South African populations of *C. macrocephalum*.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Saness Moodley: Writing – original draft. **Kelsey L. Glennon:** Supervision. **Glynis V. Cron:** Supervision.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sajb.2024.08.007.

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