Thecaphora anther-smut fungi: ecology and implications for CFR *Oxalis* species

HELEN R. CURRAN

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DECLARATION

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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H.R. Curran   Date

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SUMMARY

Only a limited number of systems involving anther-smut fungi have been studied, usually due to the economic significance of their crop plant hosts. A smut fungus of the genus *Thecaphora* has been discovered infecting *Oxalis* hosts in South Africa. This pathogenic fungus, *Thecaphora capensis*, produces dark-coloured spores in the anthers of host flowers, rendering it an anther-smut fungus. The host genus is the seventh largest plant genus in the Cape Floristic Region (CFR) and the largest geophytic genus of this region. Nine *Oxalis* species that host *T. capensis* have been identified across a wide distribution in the CFR of South Africa. A preliminary assessment of *T. capensis* infections of *Oxalis* was conducted in 2009, which provided a foundation for further research into the ecological and evolutionary consequences of hosting this fungus.

In this study, a comprehensive host diversity assessment was conducted to determine the extent of infected *Oxalis* individuals within the CFR. Three new *Oxalis* host species for *Thecaphora capensis* were discovered. This brings the total number of known hosts to twelve. The morphological and reproductive effects of the fungus were assessed on two host species (*O. incarnata* and *O. lanata*) by comparing healthy and infected individuals of these species. Infection by *Thecaphora capensis* had a significantly negative effect on both of these factors. Host resources appear to be co-opted for fungal spore production, since floral morphological characters of infected individuals were reduced in size. Furthermore, infection by *T. capensis* ensured near-universal sterility in both hosts.

Differences in floral characters and pollinator preferences for healthy *Oxalis incarnata* and *O. lanata* individuals from disease-free and diseased populations were compared to determine the evolutionary influence of *Thecaphora capensis* infections. It was shown that this pathogen can have a significant evolutionary influence on its hosts, showing its ability to shape flower size and pollinator activity in *O. lanata*, but not in *O. incarnata*. A need has therefore been identified to assess these evolutionary forces independently for each host and its pathogen before making erroneous assumptions for conservation practices.

Plant pollinators play an integral role in plant fitness. Pollinator movements within a population are important when between-flower spore transfer by pollinators increases the likelihood of new infections. Pollinator movements may be influenced by host density and the frequency of diseased individuals, amongst other factors. Pollinators were found to

mediate *Thecaphora capensis* spore transfers within diseased *Oxalis* populations. Host density and disease frequency affected the number of spores transferred under field and standardized conditions. More research is required to investigate confounding factors in these complex systems.

This study highlighted the complexities of a fungal-plant-insect relationship, the evolutionary consequences of such fungal infections and the various factors influencing the likelihood of new infections. This research adds to the limited body of knowledge on multi-organismal interactions in the CFR and provides a base for more detailed future studies on this intriguing system.

OPSOMMING

„n Brandswam, wat deel is van die *Thecaphora* genus, is ontdek in „n *Oxalis* blom waar dit die gasheer plant se blom gebruik om spore in te produseer. Die swam, *Thecaphora capensis*, produseer donker gekleurde spore in die helmknoppe van die blomme van gasheer plante, daarom word dit geklasifiseer as „n brandswam van die helmknop. Die gasheer plante van die swam is deel van die genus *Oxalis*, die sewende grootste plant genus in die Kaapse Floristiese Streek (KFS) en die grootste geofitiese genus in die streek. Nege *Oxalis* species is al klaar identifiseer as gasheer plante van *T. capensis*. Hulle is versprei oor „n groot area van die KFS van Suid Afrika. „n Primêre ondersoek van *T. capensis* infeksies op *Oxalis* is in 2009 onderneem. Hierdie ondersoek het gelei tot meer vrae oor die sisteem en het „n goeie fondasie geskep vir verdere navorsing rakende die ekologiese koste verbonde daaraan om as gasheer plant vir ʼn swam op te tree.

„n Deeglike ondersoek is in die KFS aangepak om die *Oxalis* gasheer plante van die brand swam te identifiseer en om voort te bou op die basiskennis wat in die primêre ondersoek daargestel is. Drie nuwe *Oxalis* gasheer plante van *Thecaphora capensis* is ontdek. Die totale aantal gasheer plante staan nou op twaalf. Gesonde en geinfekteerde individuele gasheer plante is gebruik om die morfologiese en reproduktiewe effekte van die swam te toets in twee *Oxalis* spesies (*O. incarnata* en *O. lanata*). Die negatiewe gevolge om „n gasheer plant van die brand swam te wees was duidelik toe gesonde en geinfekteerde individuele met mekaar vergelyk is. Dit kom voor asof gasheer plante se hulpbronne vir spoor produksie gebruik word, want hulle is morfologies kleiner en meestal steriel.

Die evolusionêre effek van *Thecaphora capensis* op „n populasie is getoets met gesonde individuele in populasies van twee *Oxalis* spesies. Blomkenmerke en insek bestuiwers van gesonde individue in gesonde en geinfekteerde populasies is ondersoek om die effekte van *T. capensis* op populasies te toets. Daar is suksesvol gedemonstreer dat swamme sterk evolusionêre kragte uitoefen, en die vermoë het om plantpopulasies te vorm en te verander, ofskoon nie in alle gevalle ewe sterk nie. Daarom is dit belangrik om die evolusionêre kragte vir elke gasheer plant en sy patogeen onafhanklik te assesseer, sonder om algemene aannames te maak in bewaringspraktyke.

Plantbestuiwers speel „n belangrike rol in die fiksheid van plante. Hulle kan hul fiksheid verbeter deur bestuiwers te lok met blomme en deur aspekte geassosieer met blomme. Die

bewegingspatrone van plantbestuiwers is baie belangrik indien hulle helmknop-geproduseerde spore van brandswamme vervoer instede van stuifmeel, want dit vergroot die kanse vir nuwe infeksies. Die bewegingspatrone van plantbestuiwers word, onder andere, beinvloed deur die digtheid en frekwensie van geinfekteerde individue. Plantbestuiwers speel „n belangrike rol in die vervoer van *Thecaphora capensis* spore in geinfekteerde *Oxalis* populasies. Die digtheid en frekwensie van geinfekteerde blomme het die vervoer van spore geaffekteer onder veld en gestandardiseerde kondisies, alhoewel baie veranderlikes so „n komplekse natuurlike sisteem beinvloed.

Hierdie studie beklemtoon die kompleksiteit van „n fungus-plant-insek verhouding, die gevolge van so „n interaksie en die verskeie faktore wat die waarskynlikheid van nuwe infeksies beinvloed. Tot dusver is daar „n beperkte aantal sisteme soos hierdie bestudeer waarin „n brandswam van die helmknop betrokke is, en die enkele beskikbare studies is onderneem meestal as gevolg van hulle ekonomiese effekte op landboukundig belangrike gasheer plante. Hierdie studie verteenwoordig ʻn belangrike byvoeging tot die inter-organismiese studies in die KFS. „n Holistiese ekologiese oorsig soos hierdie verskaf „n belangrike basis vir toekomstige studies en bewarings- en bestuurspraktyke.

CONTENTS

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Chapter 1

GENERAL INTRODUCTION

The Cape Floristic Region (CFR)

The Cape Floristic Region (Figure 1) (CFR, Goldblatt & Manning 2000) of South Africa, with its winter rainfall pattern and nutrient-poor soils, houses around 9000 vascular plant species in an area of only *ca*. 90 000 km². Astonishingly, nearly 69% of these are endemic to the region (Goldblatt & Manning 2000). The CFR, situated in the southwestern region of South Africa, contains the smallest of the six floral kingdoms and is recognised as one of the ± 30 biological hotspots on earth (Myers *et al*. 2000; Manning 2007). Encompassing less than 0.5 % of the total area of Africa, the CFR at the tip of Africa holds nearly 20% of the plant species on the continent (Goldblatt & Manning 2000).

Figure 1. Map depicting the southwestern part of South Africa. The Cape Floristic Region is indicated by the shaded area (Courtesy Western Cape Nature Conservation Board – http://www.tmg-aquifer.co.za/gw3.shtml).

The flora of the CFR can be divided into distinct vegetation types, included in four different biomes (Fynbos, Succulent Karoo, Albany Thicket and Evergreen Forests) (Mucina & Rutherford 2006). The Fynbos Biome encompasses the largest area and contains the most common and distinctive vegetation within this region, consisting predominantly of the fynbos, renosterveld and strandveld types. Plant families diagnostic of fynbos vegetation

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include the Proteaceae, Ericaceae and Restionaceae (Manning 2007). Renosterveld is characterised by the renosterbos *Dicerothamnus rhinocerotis* (L.f.) Koekemoer (Asteraceae) (Manning 2007) while genera typical of the strandveld include *Aloe*, *Rhus*, *Sideroxylon*, *Crassula*, *Babiana* and *Metalasia*. Proteaceae are absent while Ericaceae are very rare in the strandveld (Mucina & Rutherford 2006).

Fires play an important role in the ecology of the CFR (Goldblatt & Manning 2000) as its cyclic nature provides new niches for many plant species (Linder 2005). These species are allowed the opportunity to exploit a variety of habitats by growing and flowering rapidly before other species mature (Goldblatt & Manning 2000; Linder 2005). The current natural fire regime of the CFR must have been expressed for many years, given the evolutionary development of geophytes and other perennials that flourish for only a few seasons after fire until they are suppressed by larger shrubs with longer above-ground lifespans (Goldblatt & Manning 2000). Fire is therefore considered to be one example of an evolutionary driving force for speciation in the CFR (Linder 2005).

Born *et al*. (2006) proposed an expansion of the CFR to include the entire winter rainfall region into the Greater CFR, leading to increased levels of total endemism. The Greater CFR harbours the largest concentration of geophytic species per surface area in the world, approximately 2096 species, encompassing 20% of the total number of species in the area (Proches *et al*. 2006; Verboom *et al*. 2009).

Oxalis of the CFR

The geophytic genus *Oxalis* L., native to South Africa, is the largest genus of the family Oxalidaceae and the seventh largest genus in the CFR (Goldblatt & Manning 2000). *Oxalis* is by far the largest geophytic genus within both the CFR (Goldblatt & Manning 2000) and the GCFR (Born *et al*. 2006; Verboom *et al*. 2009). It displays two centres of diversity, one in the GCFR (Salter 1944) and the other in South-Central America (Lourteig 1994, 1995, 2000). Unlike most other floral elements in the GCFR, *Oxalis* species are common to both the Fynbos and Succulent Karoo Biomes and occur in both fynbos and renosterveld vegetation of the Fynbos Biome (Oberlander *et al*. 2002).

Southern African *Oxalis* species are perennials with subterranean bulbs (Salter 1944). *Oxalis* bulbs lie dormant during the dry, hot summers in the GCFR, but the first leaves and stems emerge shortly after the onset of winter rains (Dreyer *et al*. 2006). Most GCFR species flower between April and August, with flowering peaks during June and July (Dreyer *et al*. 2006).

All southern African *Oxalis* species have a specialised, tristylous breeding system (Figure 2). Species include three floral morphs based on the height of their respective stigmas (Long, Mid and Short), with the two whorls of anthers arranged reciprocally at the short, medium or high position within each morph (Salter 1944; Dulberger 1992). Successful fertilization is limited to pollen transferral between anthers and stigmas of the same height, thereby enforcing out-crossing.
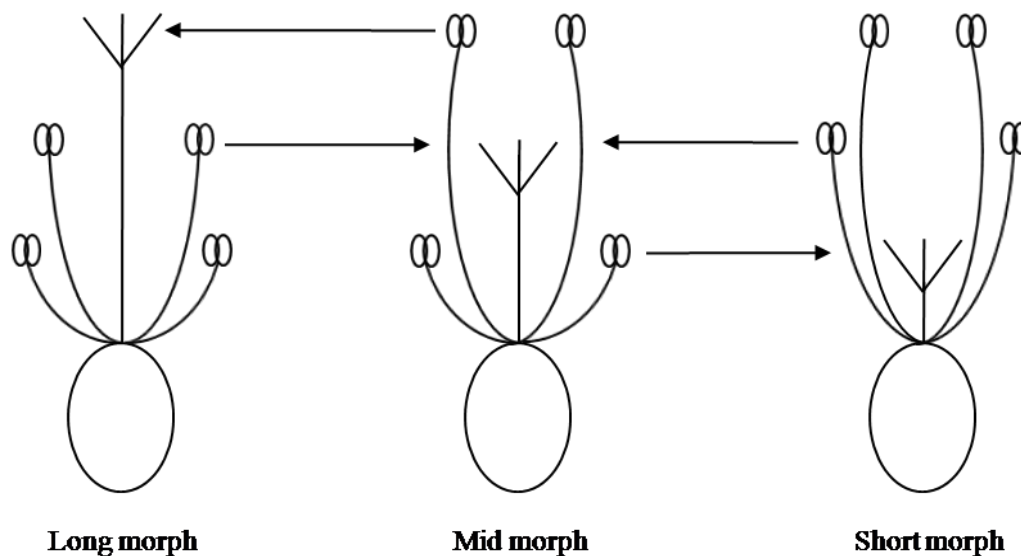
Figure 2. Tristylous breeding system of *Oxalis*. The position of the stigma determines the floral morph, namely Long, Mid or Short. Arrows depict the only methods of legitimate fertilization.

CFR fungal diversity

Plant diversity in the CFR is well-documented, but information on fungal diversity in the region is still very sparse (Crous *et al*. 2004, 2006). This is surprising, since there is an estimated 42 000 unique fungal species in the CFR alone, representing 20% of the estimated total number of fungal species in South Africa (Crous *et al*. 2006). This predicted number of

fungal species may be an underestimate, given that only very few of the possible ecological niches and fungal hosts have been thoroughly investigated (Crous *et al*. 2006). It is predicted that many more fungal species exist in the CFR and will be discovered when all potential hosts have been properly examined and all known isolation techniques utilized (Crous *et al*. 2006). Clearly more studies are needed to focus on documenting and understanding the true fungal diversity in South Africa. Only once these surveys are completed would we be able to adequately assess the importance of plant-fungal interactions within the region.

Smut fungi

The Fungal Kingdom is divided into various phyla including the basidiomycota, ascomycota and zygomycota. The Basidiomycota phylum includes approximately 30 000 species (Prescott *et al*. 2005), many of which are of major economic interest. Well-known groups in this phylum include the smuts, rusts, puffballs, toadstools and mushrooms (Mueller *et al*. 2004).

There are an estimated 1200 known species of smut fungi that have the potential to infect *ca*. 4000 different plant hosts (Bakkeren *et al*. 2008). The group contain many virulent plant pathogens that annually cause major damage to cereal crops (Prescott *et al*. 2005). The grass family (Poaceae), which includes economically important crops such as corn, wheat, oats and barley, contains the plant species most susceptible to fall host to smut fungi (Bakkeren *et al*. 2008). Infected plant parts are characterised by the formation of fruiting structures or tumours containing masses of teliospores (thick-walled inactive spores from sexual reproduction (Kendrick 2000)) that have a dark "smutted" appearance (Bakkeren *et al*. 2008).

Smut fungal species often infect specific non-reproductive plant organs such as roots and stems (Ngugi & Scherm 2006; Vánky 1994). Plant reproductive structures may also host smut fungi. Such is the case in the corn smut fungus (*Ustilago maydis* Roussel), where fungal spores are contained within the grain kernels or inflorescences of host plants, while healthy seed may become infected during harvesting (Bidochka *et al.* 2000). Flowers are highly susceptible to infection due to their thin walled petal (Bowes 1996) and stigmatic surfaces (Heslop- Harrison & Shivanna 1977) and since they represent nutrient-rich habitats (Brysch-Herzberg 2004; Stockwell 2005) that enable flower-infecting fungi to utilize these resources

intended for host reproduction (Ngugi & Scherm 2006). Nutrients from the rest of the plant may similarly be utilized by these fungi for the production of fungal spores, leaving the host plant hungry for nutrients. Results of this deprivation of nutrients include morphological effects such as stunted growth, fewer flowers per plant or smaller flowers (Agrios 1988). In some instances, fungal pathogens may even replace pollen of infected flowers with their spores (e.g. *Microbotryum violaceae* Deml. & Oberw., Jennersten 1988; Alexander 1990). This leads to pollinator-mediated spore transfer from one flower to the next (Marr 1997). Pollinators therefore serve as vectors for these plant pathogens and may cause host infection rates to increase. As a result, infected flowers serve as the reproductive organ for the fungus, while pollinators allow for easy dispersal of spores from one specialised micro-niche to another.

Smut fungi and *Oxalis* hosts

Oxalis species from North America, Europe and Asia are known to be infected by a smut fungus of the genus *Thecaphora* (Glomosporiaceae) (Vánky *et al*. 2007). This species, *Thecaphora oxalidis* Ellis & Tracy, forms reddish-brown teliospores in the seed capsules of its hosts. *Thecaphora capensis* Roets & Dreyer, was recently discovered infecting native southern African *Oxalis* species (Roets *et al*. 2008). This new species of smut fungus forms teliospores within the anthers of two CFR *Oxalis* hosts, *Oxalis lanata* var. *rosea* T. M. Salter and *O. incarnata* Jacq. (Curran *et al*. 2009). A further seven CFR *Oxalis* hosts to *T. capensis* show signs of infection of the anamorphic phase (the asexual reproductive phase, Kendrick 2000) of the same fungus (Curran *et al*. 2009). These include *O. bifida* Thunb, *O. ciliaris* Jacq., *O. depressa* Eckl. & Zeyl., *O. eckloniana* C. Presl., *O. engleriana* Schltr., *O. glabra* Thunb. and *O. tenella* Jacq. (Curran *et al*. 2009). We suspect that these are not the only *Oxalis* species infected by *T. capensis* across the GCFR and therefore, one of the aims of this study is to clarify the extent of infected *Oxalis* hosts.

Oxalis also play host to other members of the Basidiomycota. A smut infection of *Oxalis oregano* Nutt. by *Melanotaenium oxalidis* Dietz & Fisher results in the formation of greyish green to black sori in the petioles and leaf-midribs of infected plants that cause increased or decreased hypertrophy (Dietz & Fisher 1970). A rust fungus, *Puccinia sorghi* Schw., on *Oxalis corniculata* L., *O. bowiei* Lindl., *O. violacea* L. and *O. stricta* L. (Arthur 1904) forms

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spots on the leaves of infected hosts. These effects are also observed in *Zea Mays* infections by *Puccinia sorghi*, resulting in detrimental commercial effects on agricultural industries.

Pollinator exploitation and seed production

Generally, anther-smut fungi exploit flower visitors in order to transmit fungal spores and thereby infect new hosts in a fashion resembling sexually transmitted diseases (Roy 1994). Pollinator behaviour may result in selection for specific floral traits leading to an altered floral morphology (Eckhart 1992), but the opposite selection might be true for a host infected by a sterilizing disease transmitted by pollinators (Elmqvist *et al*. 1993). For example, flower visitors have shown preference for larger, rather than smaller flowers (Cresswell & Galen 1991), since larger flowers usually provide increased nectar volumes (Elmqvist *et al*. 1993). Unfortunately enhanced size may also anticipate the delivery of more pollinator-borne diseases to these individuals (Elmqvist *et al*. 1993). There is therefore a trade-off for insect pollinated plants such as *Oxalis* prone to infection with a pollinator borne disease. Flowers adapted to improve pollination levels by attracting more pollinators endure an equal likelihood of receiving pollinator-vectored plant pathogens (Roy 1994). The presence of a disease may thus drastically impact ecological communities (Dobson & Crawley 1994) and influence evolutionary processes acting within a population (Mauricio & Rausher 1997).

Individuals infected with HIV (Human Immunodeficiency Virus) (another sexually transmitted disease) in a population may lead to increased population level infections and may even lead to the spread across multiple populations in a geographical area (Yerly *et al*. 2001). Similarly, increases in plant density in a diseased population have been shown to increase disease incidence in that population, possibly due to pollinator vector visitation patterns (Anderson & May 1979; May & Anderson 1979). When pollinators show preference for healthy flowers and the frequency of disease in the population is high, infection rates of healthy plants are likely to increase (Real *et al*. 1992), since the probability of the pollinator accidentally visiting a diseased flower is higher. If, in contrast, the frequency of the disease is low, but pollinators show preference for diseased flowers, infection rates of healthy plants are again likely to increase, since the probability of a pollinator accidentally visiting a healthy flower is higher. The ultimate question here is whether pollinator movement patterns in an infected population are random between all flowers or if they show a preference for either healthy or infected individuals.

Gerber *et al.* (2005), studying Population Viability Analyses (PVA) and host-pathogen theory, stated that "the spread of an infectious disease through a population is a function of the density of both the susceptible and infectious hosts". One way in which plants can compensate for this increase in disease spread may be to produce more seeds per successful pollination event (Skogsmyr 1993), thereby producing progeny before sterilisation takes effect. Flowers that receive spores may not necessarily become infected immediately (Alexander 1989), but infection may rather set in within several weeks (Alexander & Antonovics 1988). These flowers receiving spores and pollen may therefore in the interim still be able to produce viable, uninfected seeds. In a different study by Roy (1996) it was suggested that seed set (reproductive potential) may be decreased in the presence of fungal spores due to localised cell death of the stigma as a resistance mechanism to prevent infection.

It is important to note that much of the current knowledge on pollinator exploitation and seed set variations as a result of plant pathogens and anther-smut infections of non-crop plants is focussed on the system of *Microbotryum violaceum* infections of hosts in the Caryophyllaceae family. Prior to the study by Curran *et al.* (2009) there had been no studies focussed on the dynamics of anther-smut fungal infections of *Oxalis* flowers.

Fungal infections of bulbous plants

Systemic fungal infections of bulbous plants are particularly interesting, since they may persist in populations for many generations by surviving in the perennial subterranean structures of such plants. In a fire-prone habitat such as the CFR, this mode of below-ground survival may eliminate the possibility of plant populations regaining a healthy status after fire during the summer months, as the fungus is safely protected from the negative effects of frequent fires when the plant lies dormant. A study by Alexander & Antonovics (1988) showed that *Silene alba* (Miller) plants infected with *Microbotryum violacea* could escape infection if the above-ground plant parts were killed before the infection entered the root system. If the root system became infected though, sterile infected flowers were produced the following year. Clearing of infected vegetation and related disturbances were shown to increase seed production. These disturbances led to the cleansing of the population from the fungus, since the disease could not spread from infected individuals. In this same system,

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there was no transmission of the fungus inside the seeds, since infected flowers were sterile and could not produce seeds (Baker 1947), while healthy flowers were still able to produce seeds. Similarly, *Oxalis* may be able to escape infection by clonally producing uninfected reproductive structures. When above-ground bulbils of infected *Oxalis incarnata* plants were surface sterilised, for example, a few produced disease-free plants when these bulbs sprouted (Curran *et al*. 2009).

Hypotheses

We suspect that *Thecaphora capensis* displays widespread infections of native southern African members of *Oxalis*. A comprehensive host diversity assessment will be conducted in order to determine the extent of *Oxalis* infections throughout the GCFR. Newly discovered infected populations, together with the already known host *Oxalis* populations, will form the basis to investigate the ecological consequences of infection. Each of these consequences will be investigated individually and contribute to answering the overarching question addressed in this thesis, namely: What are the long term survival threats to *Oxalis* species susceptible to infection by *T. capensis,* given the range of normal ecological challenges?

The first hypothesis is that smut fungi have severe adverse effects on *Oxalis* individuals. This will be tested by comparing the morphological and reproductive effects of infection on host species in both healthy and diseased individuals (Chapter 2).

Secondly, it is hypothesised that *Thecaphora capensis* has an evolutionary influence on *Oxalis* species at the population-level. This will be assessed by comparing floral morphologies of healthy plants in healthy populations to that of healthy plants in diseased populations (Chapter 3) using *Oxalis lanata* and *O. incarnata* as test species, as these species include numerous populations known to be infected with *T. capensis* (Roets pers. comm.).

Finally it is hypothesised that flower pollinators act as fungal spore vectors and therefore contribute to disease spread. This hypothesis will be investigated under two main sub-themes (Chapter 4). The first will aim to confirm whether pollinators actually transmit fungal spores between flowers, while the second will assess the effects of host density and disease frequency on disease spread. This will be investigated through pollinator studies under both controlled conditions and through field-based observations.

The results of the present study will contribute to a more thorough understanding of a plant-fungal-animal interaction at work in the CFR. It will also provide knowledge of the likelihood of this fungus spreading and the potential damage it might cause to the genus *Oxalis* as a whole.

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Chapter 2

THECAPHORA CAPENSIS INFECTIONS OF *OXALIS*: DIRECT CONSEQUENCES OF PLAYING HOST TO ANTHER-SMUT FUNGI

ABSTRACT

There is a need for research on the ecology and diversity of fungi in the botanically rich Cape Floristic Region (CFR) of South Africa, since fungi are likely to help shape plant communities. *Thecaphora capensis*, an anther-smut fungus found in the CFR, has a number of host species in the genus *Oxalis* L., the largest geophytic genus within this area. The present study aimed at building on the existing knowledge of this plant-pathogen system through elucidating the known host range of *T. capensis* and assessing the morphological and reproductive consequences of hosting this anther-smut fungus. A further three *Oxalis* host species were identified here, bringing the total number of known host species to twelve. *Thecaphora capensis* has drastic negative effects on the morphology of two species assessed, namely *O. lanata* and *O. incarnata*. Infected individuals of both species were rendered virtually sterile. In addition, the presence of fungal spores on stigmatic surfaces of healthy flowers had no effect on seed set. Determining the full extent of infected hosts is essential for future conservation efforts, as nearly a third of all southern African *Oxalis* species are listed as rare or endangered.

INTRODUCTION

Situated at the southern tip of Africa, the Western Cape Province of South Africa is home to the Cape Floristic Region (CFR) (Goldblatt & Manning 2000) known as one of the world‟s richest regions in terms of botanical diversity (Goldblatt & Manning 2000). With ± 9000 vascular plant species in an area of 90 000 km^2, the CFR has such a unique flora that it represents one of only ± 30 biodiversity hotspots on Earth (Myers *et al.* 2000). The level of endemism among CFR species was calculated to be as high as 69% (Goldblatt & Manning 2000), comparable with the rich island floras of Hawaii or Madagascar (Linder 2003). Often dominated by Proteaceae, Ericaceae and Restionaceae, the vegetation of the CFR also has a remarkably rich geophytic component (Born *et al.* 2006). *Oxalis* L. (Oxalidaceae) represents the largest geophytic genus within the CFR (Goldblatt & Manning 2000). All southern African members of *Oxalis* form true bulbs (Salter 1944). Their leaves and flowers emerge in the wet winter months of the CFR (April to August) (Dreyer *et al.* 2006), while they remain dormant underground for the remaining part of the year. The genus *Oxalis* further displays a specialised breeding system (tristyly), in which individuals in populations produce flowers conforming to one of three different floral morphs (Long, Mid or Short, depending on the position of the stigma) (Salter 1944, Chapter 1). This system enforces outcrossing between individuals of different morphs, such that seed production is limited to crosses in which stigmas receive pollen from anthers of the same height i.e. pollen from a flower of a different morph.

Although information on the botanical diversity of the region abounds, the CFR is understudied in terms of fungal diversity. This is alarming given that the CFR is reportedly better studied in terms of fungal diversity than any other floral region of South Africa (Crous *et al*. 2006). There may be as many as ca. 200 000 fungal species associated with plants in South Africa, while 42 000 of these may be unique to the CFR alone (Crous *et al*. 2006). As fungi form integral parts of almost all ecosystems on earth and are likely to help shape plant communities such as that of the CFR (Marincowitz *et al.* 2008), there is an urgent need for additional research focussed on CFR fungal ecology and diversity.

Salter (1938) reported the first smut fungal infection in the CFR species *Oxalis lanata* var. *rosea* T. M. Salter. This fungus was later re-recorded, described and named *Thecaphora*

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capensis (Roets *et al.* 2008). Smut fungi form fruiting bodies that contain masses of teliospores (thick-walled inactive spores from sexual reproduction (Kendrick 2000)), which gives the infected tissue a dirty, "sooty" appearance (Bakkeren *et al.* 2008). *Thecaphora capensis*, an anther-smut fungus of the Basidiomycota fungal division, forms masses of dark spores that replace pollen within the anthers of infected flowers (Roets *et al.* 2008). Infections of this specialised niche have implicated flower visitors as dispersal agents for *T. capensis* (Curran *et al.* 2009). Interestingly, some *Oxalis* species from Europe, Asia and America are known to host the sister species of *Thecaphora capensis*, *T. oxalidis* Ellis & Tracy (Vánky *et al.* 2008; Roets *et al.* 2008). This species forms teliospores in the seed capsules of its hosts rather than in the anthers. Species of *Thecaphora* may thus infect a great array of different *Oxalis* species. This warrants further investigations into fungus-host relationships of *T. capensis* in the *Oxalis* rich CFR.

Many smut fungi are commercially detrimental, since they infect crop plants such as maize, in the case of *Ustilago maydis* Roussel (corn smut) and barley, in the case of *Ustilago hordei* Pers. (Bakkeren *et al.* 2008). *Ustilago maydis* produces tumours containing fungal spores on all above-ground plant parts, although the bulk of the fungal biomass usually develops in the inflorescences of infected plants (Bakkeren *et al.* 2008). This leads to reduced crop yields and considerable agricultural losses. Although South African *Oxalis* are not crop plants, they are increasing in their popularity as garden plants and are already commercially available in many nurseries in South Africa. This may lead to future problems if infected plants are cultivated, as host jumping and potential future export of this fungus becomes possible. Increased understanding of the ecological and evolutionary consequences of *Oxalis* smut fungus infections is thus important, not only from a scientific perspective, but also in terms of horticulture and commerce.

Flowers are considered an excellent habitat for microbes such as fungi (Brysch-Herzberg 2004), since they contain many nutrient resources intended for host reproduction (Ngugi & Scherm 2006). Pathogens may therefore have the ability to directly affect host fecundity and viability when they tap into these resources (Dobson & Crawley 1994). Flower-infecting fungi must possess phenologies that are synchronised with their hosts flowering cycle (Ngugi & Scherm 2006) in order to infect new hosts and reproduce at the correct time for dispersal. The spread of *Thecaphora capensis* to new *Oxalis* host species may be challenging, since flowering is highly seasonal in southern African *Oxalis* (Dreyer *et al*. 2006). Some species

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flower much earlier than others, while other species show dual flowering peaks during the winter flowering period. Despite this challenge, Curran *et al.* (2009) identified *Thecaphora capensis* from a further eight native CFR *Oxalis* species, including *O. bifida* Thunb., *O. ciliaris* Jacq., *O. depressa* Eckl. & Zeyl., *O. eckloniana* C. Presl., *O. engleriana* Schltr., *O. glabra* Thunb., *O. incarnata* and *O. tenella* Jacq..

Initial results of Curran *et al.* (2009) showed that *Thecaphora capensis* had drastic negative effects on *Oxalis incarnata* individuals when comparing morphology between diseased and healthy individuals. Although sample sizes were limited, results showed that infected plants displayed smaller petal and leaf surface areas and shorter styles and stamens when compared to flowers of healthy plants (Curran *et al.* 2009). Infected *Oxalis lanata* plants displayed an almost complete failure to reproduce sexually. Also, infected *O. incarnata* bulbs that were surface sterilised still contained the fungus in the following flowering season (Curran *et al.* 2009), suggesting that the fungus probably resides in the bulbs while the plants are dormant during summer. Some species in the Caryophyllaceae are also infected by an anther- smut fungus, *Microbotryum violaceum* Deml. & Oberw. (Vánky 2004) (Biere & Honders 1998; Bucheli & Shykoff 1999; Elmqvist *et al.* 1993; López-Villavicencio *et al.* 2007; Shykoff *et al.* 1997). Such plants display morphological differences between healthy and diseased flowers (Baker 1947) congruent with those observed by Curran *et al.* (2009). Similarly, plants in the Caryophyllaceae infected with *M. violaceum* lose their ability to reproduce and their flowers become the site for fungal reproduction (Marr 1997). These two systems may thus prove interesting for the study of comparative biological and evolutionary influences posed by flower infecting fungi and plants. There is therefore a need to quantify the biological effect of the fungi on the host plants and to elucidate the host ranges of these fungi.

The study by Curran *et al.* (2009) initiated investigations into the biology and host range of *Thecaphora capensis* on CFR *Oxalis*. However, due to time restrictions and limited sample sizes they were unable to draw conclusions on the morphological and reproductive effect of the fungus on *Oxalis* in general. The present study aims to address these shortcomings by assessing morphological and reproductive differences between healthy and diseased individuals in two test species. Here we specifically set out to build on a data base of infected *Oxalis* populations within the CFR to elucidate the known host range of *T. capensis*. We also aimed to assess the morphological and reproductive effects of *T. capensis* on the known hosts *O. lanata* and *O. incarnata*.

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MATERIAL AND METHODS

Host diversity assessment

Continuous field based monitoring was conducted across the Western Cape, Namaqualand and the Southern Cape regions of South Africa during the main *Oxalis* flowering season (April to September; Dreyer *et al.* (2006)) in 2009 and 2010. All *Oxalis* hosts of *Thecaphora capensis* were recorded throughout the CFR region. The presence of any alien invasive *Oxalis* species (e.g. *O. corniculata* L.) in close proximity to infected *Oxalis* populations was documented and when present, these alien species were evaluated for the presence of *Thecaphora*, as the possibility of pathogens jumping from alien hosts to our native flora cannot be ruled out at this stage. Environmental disturbances were noted, since the clearing of vegetation can lead to the removal of infected plant material or the transmission of fungal spores across the landscape. In order to monitor the preferred climatic conditions associated with *T. capensis* infection, the soil moisture regime was identified at each site as wet, moist or dry, since fungi mostly prefer damp situations. Populations were further categorized as being shaded or exposed to direct sunlight.

Morphological effects of infection on host

Healthy and diseased individuals of infected *Oxalis lanata* and *O. incarnata* populations were compared morphologically to test the morphological effect of *Thecaphora capensis* on its host. Populations of *O. lanata* and *O. incarnata* were categorised according to infection status, namely a) healthy (no signs of infection) and b) diseased (> 0% of the population infected). A collective total of 16 populations were identified for *O. lanata* and *O. incarnata* and used for further study (Appendix 1) (Figure 1). Nine populations were identified for *O. lanata*: three within the healthy category and six within the diseased category. Seven populations were identified for *O. incarana*: three within the healthy category and four within the diseased category (Appendix 1) (Figure 1). Whole plants (all above-ground plant parts) (20 healthy and 20 diseased (where possible)) were collected from these populations for morphological examination. For each of these plants the total number of flowers per plant and the total dry mass was established. Additional flowers and leaves (20 healthy and 20

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diseased (where possible) from separate individuals) were also collected from each of these 16 populations and the following measurements were taken: style length, stamen heights and petal and leaf surface areas. Style and stamen lengths were measured using electronic callipers. Single petal and leaf circumferences were traced onto transparency film and later filled with an overhead projection marker. These pictures were cut out and their surface areas were measured using a planimeter (Model LI-3000, Lambda Instrument Corporation, USA).

All morphological data were analysed in Statistica 10 (StatSoft Inc, Tulsa, OK, USA) using ANOVA and t-tests for normally distributed data, and Mann-Whitney U-tests and Kruskal-Wallis for non-parametric data.
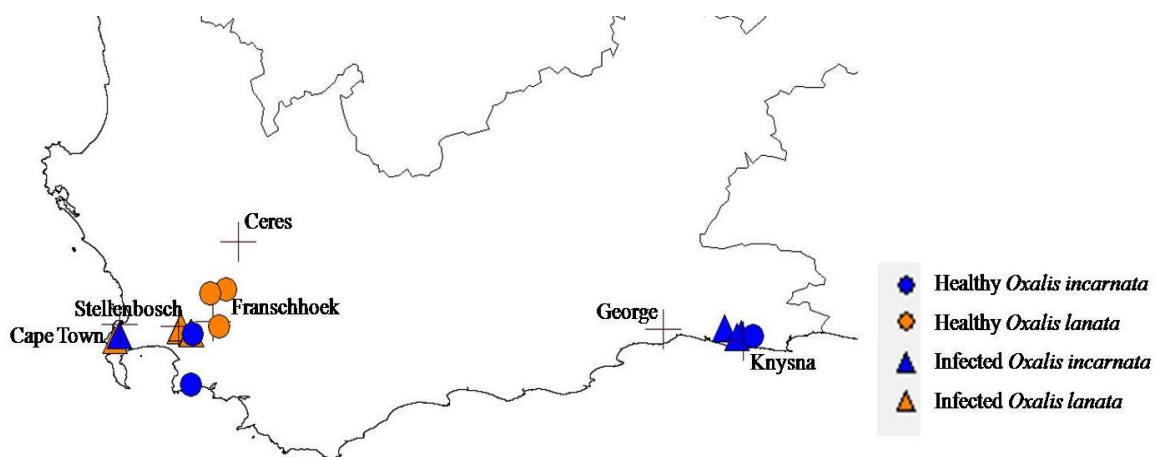
Figure 1. Localities of healthy and infected populations of *Oxalis lanata* and *O. incarnata* that were used to assess the morphological and reproductive effect of *Thecaphora capensis* on its hosts.

Reproductive effects of infection on host

The reproductive potential of healthy and diseased plants was compared in the same 16 populations of *Oxalis lanata* and *O. incarnata* mentioned above (Appendix 1) (Figure 1). For each diseased population, 20 healthy and 20 diseased plants were hand pollinated in the field. Legitimate crosses were made with pollen from healthy plants only. Only young, newly-opened, healthy flowers were pollinated to control for the presence of fungal spores on the stigmas. In cases where newly opened healthy flowers could not be found, healthy flowers were first examined for the presence of fungal spores on their stigmas using a hand-held

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magnifying glass. Hand pollinated flowers were covered with fine gauze to control for external flower visitors. Seeds produced by flowers covered with fine gauze were collected three weeks later. The mean number of seeds produced per capsule by healthy and diseased plants was calculated and statistically compared.

For each of the infected populations of both *Oxalis lanata* and *O. incarnata*, 20 healthy plants were selected to investigate the effect of the presence of fungal spores on healthy stigmatic surfaces on seed set (reproductive potential). Both fungal spores and legitimate pollen from sympatric individuals were applied to the stigmas of one flower per plant. These flowers were again covered with fine gauze to exclude other pollinators. Seeds produced by flowers covered with fine gauze were collected three weeks later and the mean number of seeds produced was calculated. Numbers of seeds produced per capsule by individuals in this experiment were compared with the number of seeds produced per capsule following hand pollination as described above.

All data were statistically compared in Statistica 10 (StatSoft Inc, Tulsa, OK, USA) using ANOVA and t-tests for normally distributed data, and Mann-Whitney U-tests and Kruskal-Wallis for non-normally distributed data.

RESULTS

Host diversity assessment

A further three *Oxalis* host species of *Thecaphora capensis* were identified during this study, namely *O. nidulans* Eckl & Zeyl.*, O. polyphylla* Jacq. and *O. truncatula* Jacq. This brings the total number of known host species to twelve, with *O. bifida, O. ciliaris, O. depressa, O. eckloniana, O. engleriana, O. glabra, O. incarnata, O. lanata* and *O. tenella* previously identified as host species (Curran *et al.* 2009) (Figure 2).
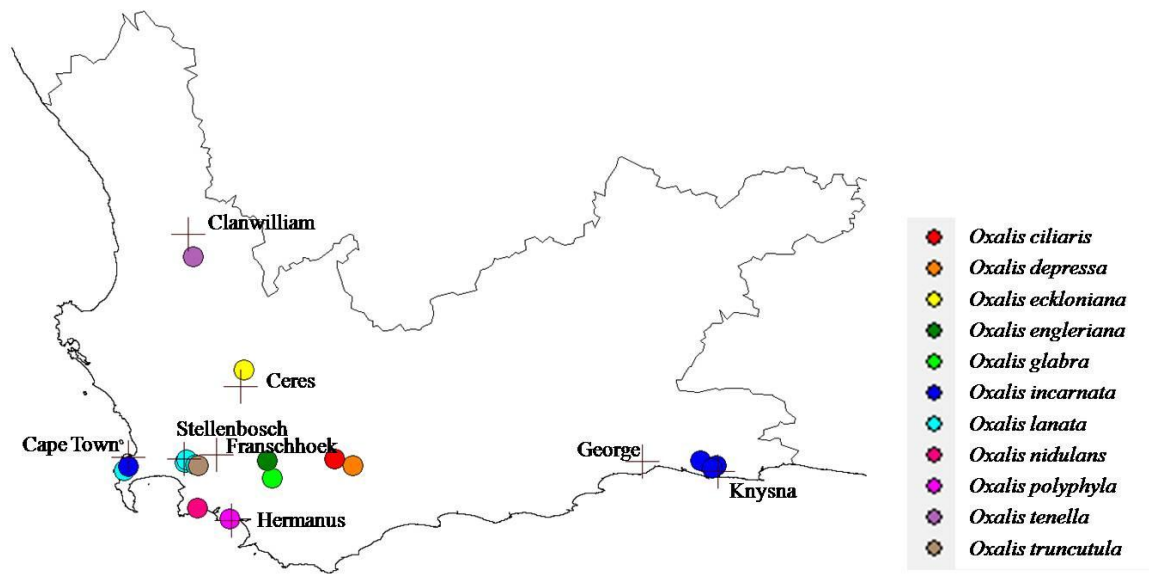
Figure 2. Localities of all known populations of *Oxalis* species infected with *Thecaphora capensis* in the Cape Floristic Region of South Africa.

No invasive *Oxalis* species were present at any of the sites where infected native species were found. Environmental disturbance was evident near almost all infected populations. These included frequent signs of recent fires, the clearing of roadside cuttings or the cutting of grass (Appendix 2). Soils were very moist at all of the collection sites, but this was not unexpected, as collections were exclusively made in the flowering season during the wet winter months of the CFR (Dreyer *et al.* 2006). Interestingly, all infected populations displayed a noticeable degree of shade cover (Appendix 2); infected individuals within populations were also more often found clustered under larger trees or bushes than healthy individuals in the same population.

Morphological effects of infection on host

Infected *Oxalis lanata* populations showed many obscure floral mutations (Figure 3). Although not explicitly tested, it appears as though flowers may display varying degrees of disease expression, ranging from healthy to partially infected to completely infected, with some flowers displaying extreme mutations probably as a result of the infection (Figure 3e). Partially infected flowers contained anthers with healthy pollen in combination with others

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containing fungal spores (Figure 3c). Some of the observed mutations included styles within visibly healthy flowers that radiated outwards (Figure 3b) and sepals growing as additional whorls within the petals of infected flowers (Figure 3e).
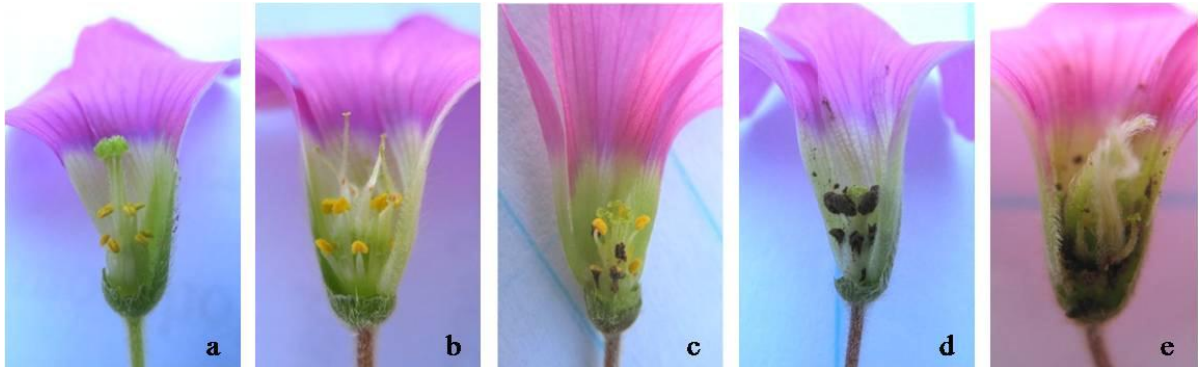
Figure 3. An array of *Oxalis lanata* flowers displaying different degrees of disease expression and mutations: a) A healthy Long morph flower with anthers containing pollen only; b) seemingly healthy flowers with styles no longer grouped in the central column of the flower, but radiating outward in different directions; c) healthy and diseased anthers within a single flower, containing pollen and spores, respectively; d) a completely infected flower containing only diseased anthers with fungal spores and e) a mutation, possibly as a result of infection, with a second whorl of sepals growing within the whorl of petals.

Thecaphora capensis had drastic negative effects on both *Oxalis lanata* and *O. incarnata* plants. Infected *O. incarnata* plants produced significantly fewer flowers compared to healthy plants (Table 1). Healthy *O. lanata* plants had a significantly higher total dry mass than infected plants. Healthy and diseased plants of both *O. lanata* and *O. incarnata* displayed significant differences in style and stamen lengths and petal and leaf surface areas (Table 1). Healthy plants of both species produced longer styles and stamens and larger petals and leaves than their infected counterparts.

Table 1. Morphological effect of *Thecaphora capensis* infection on *Oxalis lanata* and *Oxalis incarnata.* Results are reported as either mean ± standard deviation (parametric data) or median ± standard deviation (nonparametric data).

Morphological Character	*Oxalis lanata*				*Oxalis incarnata*			
	Healthy	Diseased	Test value (d.f.)	p	Healthy	Diseased	Test value (d.f.)	p
Number of flowers per plant	5.0 ± 5.58	5.0 ± 3.51	U (204) = 4715.50; Z = 1.38	>0.05	4.5 ± 4.17	3.0 ± 2.77	U (159) = 2419.50; Z = 2.77	<0.05
Total dry mass (g)	0.25 ± 0.32	0.16 ± 0.16	U (198) = 3198.50; Z = 4.40	<0.05	0.15 ± 0.40	0.11 ± 0.17	U (162) = 2848.50; Z = 1.68	>0.05
Style length (mm)	7.28 ± 0.56	4.98 ± 1.07	U (198) = 612.00; Z = 10.72	<0.01	7.55 ± 0.76	5.35 ± 1.52	U (159) = 499.00; Z = 9.27	<0.01
Mid level stamen (mm)	5.23 ± 0.38	4.04 ± 0.77	t (198) = 13.79	<0.01	4.92 ± 0.36	3.22 ± 0.35	t (159) = 30.07	<0.01
Short level stamen (mm)	3.75 ± 0.38	2.94 ± 0.42	t (198) = 14.25	<0.01	3.44 ± 0.30	2.63 ± 0.26	t (159) = 18.28	<0.01
Petal surface area (cm²)	1.01 ± 0.28	0.74 ± 0.27	t (198) = 7.03	<0.01	0.51 ± 0.15	0.43 ± 0.13	U (181) = 2926.00; Z = 3.51	<0.01
Leaf surface area (cm²)	1.70 ± 0.68	1.13 ± 0.43	U (198) = 2551.00; Z = 5.98	<0.01	1.69 ± 1.02	1.36 ± 0.63	U (167) = 2488.50; Z = 3.40	<0.01

Reproductive effects of infection on host

Healthy and diseased *Oxalis lanata* and *O. incarnata* plants differed significantly in seed set. Legitimate crosses using pollen from healthy plants resulted in significantly more seeds being produced by healthy flowers of *O. lanata* (Mann-Whitney U Test U = 1334.50; d.f. = 190; Z = 8.50; p<0.01) (Figure 4i) and *O. incarnata* (Mann-Whitney U Test U = 1407.00; d.f. = 130; Z = 3.50; p<0.01) (Figure 4ii) compared to diseased flowers.

The number of seeds produced by healthy flowers pollinated with a mixture of both *Thecaphora capensis* spores and legitimate pollen did not differ significantly to flowers that received legitimate pollen only. This was true for both *Oxalis lanata* (Mann-Whitney U Test U = 4097.50; d.f. = 191; Z = 1.44; p>0.05) (Figure 5i) and *O. incarnata* (Mann-Whitney U Test U = 1954.00; d.f. = 126; Z = -0.44; p>0.05) (Figure 5ii).

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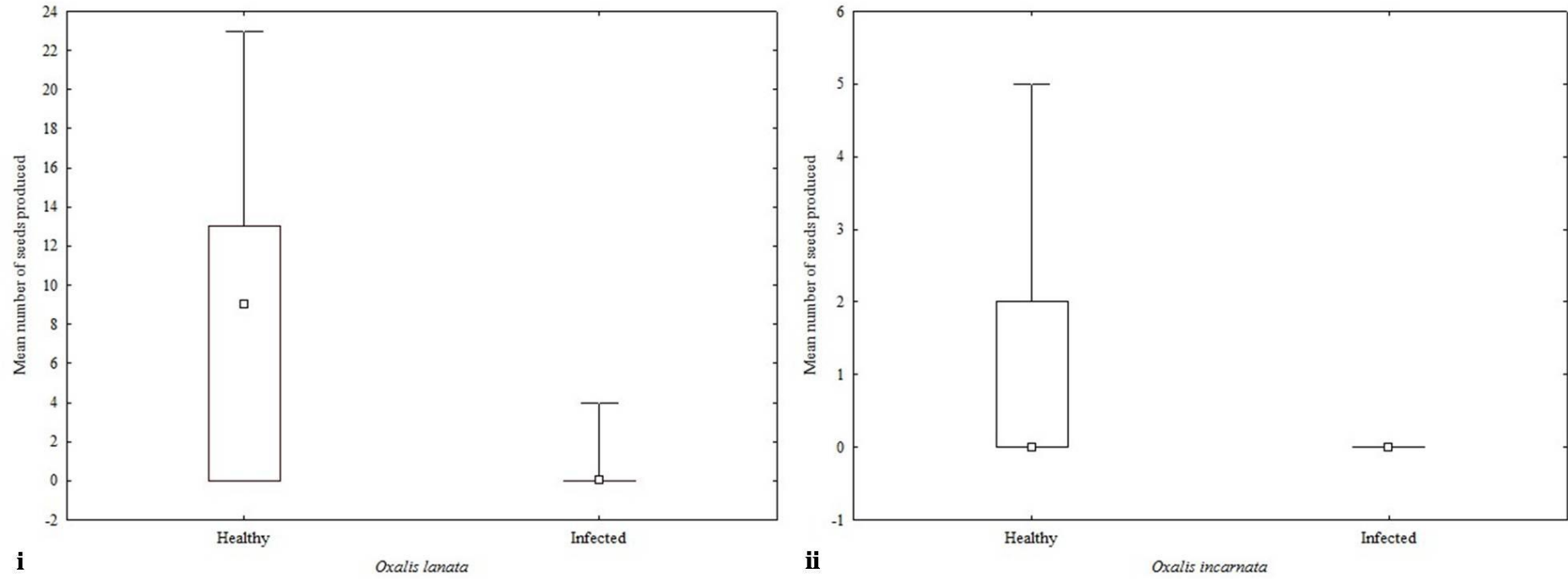
Figure 4. A comparison between the number of seeds produced by healthy and infected flowers of (i) *Oxalis lanata* and (ii) *O. incarnata*. The median and 25% - 75% percentile are indicated by the small and large squares, respectively, while the minimum and maximum are indicated by the error bars.
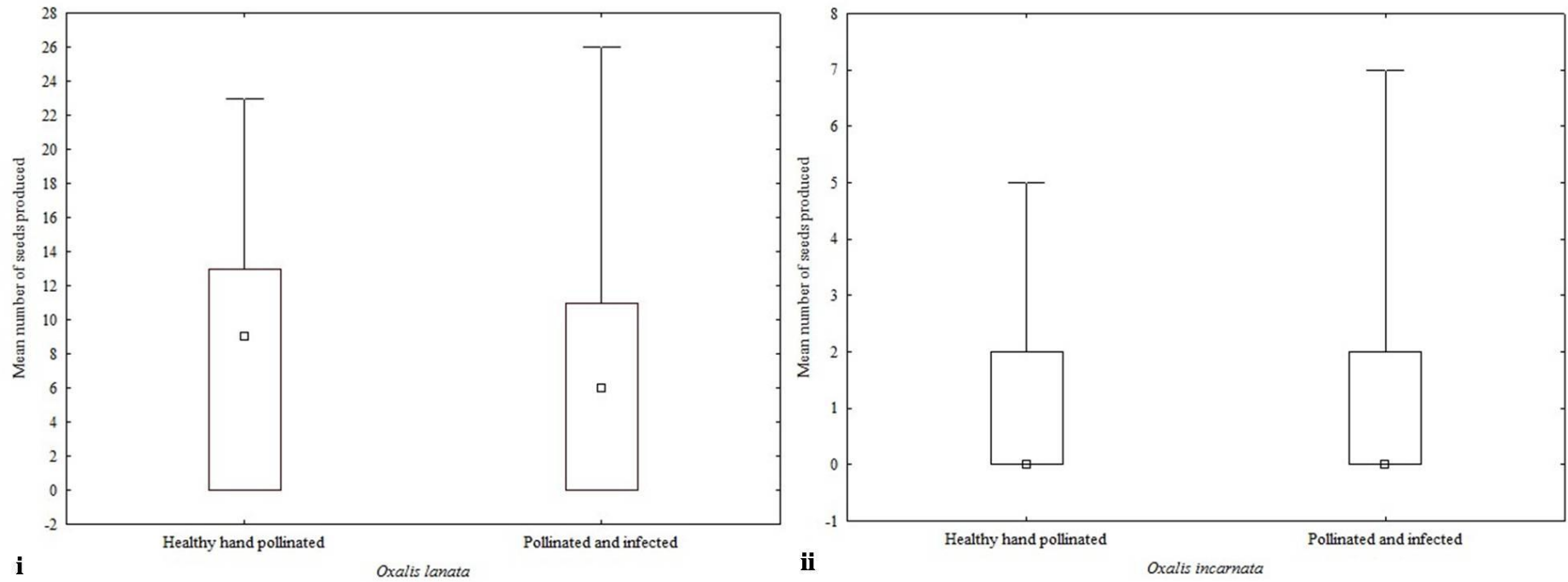
Figure 5. A comparison between the number of seeds produced by healthy flowers that were hand pollinated and others that were exposed to a combination of pollen and spores. This comparison was made using (i) *Oxalis lanata* and (ii) *O. incarnata*. The median and 25% - 75% percentile are indicated by the small and large squares, respectively, while the minimum and maximum are indicated by the error bars.

DISCUSSION

The known range of *Thecaphora capensis* has been expanded by three new hosts, now to include 12 *Oxalis* host species. This expansion suggests that more hosts may await discovery, and thus emphasizes the need for continuous monitoring of *Oxalis* populations in the CFR. When the known hosts are plotted onto the phylogeny of southern African *Oxalis* (Oberlander 2009), it is clear that host species are distributed across this phylogeny and are not confined to any specific clade or closely related groups of species. It can therefore be assumed that all *Oxalis* species are equally susceptible to infection by *T. capensis*, and, since infected species were found across a broad geographic range, many species may be at risk of contracting this disease.

Oxalis corniculata and *O. stricta* are both included in the American *Oxalis* section *Corniculatae* (Eiten 1963). The latter species is the known host of the smut fungus *Thecaphora oxalidis* Ellis & Tracy (Vánky *et al.* 2008) which is the closest known relative to *T. capensis* (Curran *et al.* 2009). *Oxalis corniculata* is a global weed (Eiten 1963) and is one of only two invasive *Oxalis* species in South Africa (Dreyer pers. comm.). Therefore, although no invasive *Oxalis* species were found in close proximity to infected native populations and no non-native *Oxalis* species have yet been found to host *Thecaphora capensis,* the possibility of a host shift from an invasive species cannot yet be ruled out, since sympatric infected native and invasive populations may still be discovered.

Some form of environmental disturbance was noted at almost all infected populations. This disturbance could be detrimental to the population if fungal spores were air borne and could enter new hosts through vegetative tissue (e.g. *Microbotryum violaceum*) or soilborne and infected new seedlings (e.g. *Tilletia controversa* Kühn, causing dwarf bunt of wheat) (Ngugi & Scherm 2006). Such threats are real, given that many *Oxalis* species are exendospermous (Salter 1944), which causes seeds to germinate within the same flowering season as their production. In addition, it may be likely that *Thecaphora capensis* is capable of host jumping from one *Oxalis* species to another, as seen in *Microbotryum violaceum* anther-smut infections of various members of the Caryopyllaceae (Vánky 2004). The soil moisture content in all infected populations was moist to wet as a result of the wet winter months in which *Oxalis* species flower. Heightened soil moisture and increased shade cover both

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contribute to less chance of spore desiccation with an associated increased chance of spore viability once it reaches a new potential host. However, as the rainy season comes to an end, *Oxalis* plants are drought-stressed and they begin to die back, which may make them vulnerable to fungal penetration. A study on flower smut fungi of grasses suggested that populations located in disturbed roadside habitats were more likely to be infected than those in preserved areas (García-Guzmán *et al.* 1996). The same may hold true here, as many populations included in the diversity assessment occurred along roadsides.

Another mode of transmission of the disease is probably through insect vectors. Fungi vectored by insects can usually prevail even under drier conditions (García-Guzmán & Morales 2007). Although insects have been identified as carriers of *Thecaphora capensis* spores (Curran *et al.* 2009), the specific site of infection has not yet been identified. However, since the fungus has evolved to produce spores in such a specialised niche, it can be assumed that flower-visiting insects play a major part in fungal transmission. Naturally occurring, undisturbed populations of *Oxalis* in the southwestern Cape may house between three and eight species sympatrically (De Jager *et al.* 2010; Dreyer pers. comm.). Sympatric *Oxalis* species that showed no signs of infection were found in most populations in which individuals of a species were found to be infected with *Thecaphora capensis.* No populations comprised of more than one infected *Oxalis* host. This is surprising if we assume each *Oxalis* species to be equally susceptible to infection by *T. capensis*. Pollinator movement between different *Oxalis* species within the same population may explain this. De Jager *et al.* (2010) showed that pollinators of *Oxalis*, specifically honeybees, were more likely to alternate between sympatric *Oxalis* species that displayed similar flower colours. However, other floral traits, including flower size, may also influence pollinator preferences when flower colours of co-occurring *Oxalis* species are identical (see Chapter 3). These pollinator preferences could ultimately lead to the movement of *T. capensis* spores and the infection of new hosts.

Morphologically, *Thecaphora capensis* induced negative effects on infected *Oxalis lanata* and *O. incarnata* plants. Populations of *O. lanata*, known to have shown signs of infection since 1938 (Salter 1938), now display high disease incidences and plants with strange malformations. A similar anther-smut fungus, *Microbotryum violaceum*, has also been shown to affect hosts by inducing stunted growth and asymmetrical flowers with elongated petal claws (Baker 1947), while initiating early and extended periods of flowering (Jennersten 1988). Alexander & Antonovics (1988) suggested that this fungus (*M. violaceum*) may take

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more than one season to fully infect its host, *Silene alba* Miller. A similar system may hold true for *Oxalis* infections by *T. capensis,* which might explain the observed mutations in *O. lanata.* This hypothesis will require further detailed testing.

Diseased plants were easily identifiable within a population as they visibly appeared sickly, bearing smaller petals and leaves. This reduction in size may be attributed to the utilization of host resources (Ngugi & Scherm 2006) by *Thecaphora capensis* for production of its own spores. Shorter styles and stamens in infected flowers may also result from resource allocation towards the fungus. Since infected flowers are virtually sterile, there is no need for female reproductive organs and there is a resultant reduction in style length. Longer stamens with anthers containing fungal spores may result in spore losses through wind dispersal, which may explain the shorter stamens of infected flowers. Shorter stamens with anthers may also be advantageous to *T. capensis* in terms of successful insect vectoring, since pollinators would have to enter the flower to reach the potential reward and collect more spores in doing so.

Infected *Oxalis lanata* and *O. incarnata* flowers were rendered virtually sterile. These flowers now only appear to facilitate fungal reproduction, and are no longer capable of seed production. Infected hosts are therefore now totally reliant on clonal reproduction through bulbil formation. Importantly, all infected populations still contained some healthy plants that still produced healthy flowers and seeds. Such plants are thus able to both persist and produce healthy progeny in an infected population, until they too, may become infected. These healthy flowers create the only viable seeds to increase the population size of healthy individuals and therefore form the basis of the long-term survival of such populations.

Healthy flowers that received both fungal spores and legitimate pollen displayed no variation in the number of seeds produced compared to healthy flowers that received only legitimate pollen. This seemingly unaffected production of seeds by flowers that received both legitimate pollen and fungal spores may be ascribed to the required time for the fungus to infect the plant, since it has been suggested that fungal infections are a two-year process (Alexander & Antonovics 1988) in which the first season sees normal seed production without being affected by fungal spores present on the stigma. Opposite results were shown by Marr (1997), where healthy flowers of *Silene acaulis* L. that received both *Microbotryum violaceum* spores and healthy pollen produced less seeds than flowers receiving only pollen.

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In this study we have contributed to a growing base of knowledge on the ecology of *Thecaphora capensis* on *Oxalis* hosts (Roets *et al.* 2008, Curran *et al.* 2009) and provide further insight into this anther-smut fungus-plant interaction in general. The number of known *Oxalis* host species to *Thecaphora capensis* has increased and we suggest that further monitoring for this fungus in the CFR is needed. As this fungus has drastic negative effects on host morphology and reproduction, it is essential to identify further infections and to monitor the extent of these infections into the future. This is especially true when planning for the future conservation of the numerous red listed species in the genus, since more than a third of all southern African *Oxalis* species are listed as either rare or endangered (Raimondo 2011).

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Appendix 1. Locality details for healthy and diseased *Oxalis lanata* and *O. incarnata* populations used in this study

Population type	Species					
	Oxalis lanata			*Oxalis incarnata*		
	Population	Grid reference		Population	Grid reference	
Healthy	Franschoek	S: 33˚ 56, 252'	E: 19˚ 09, 655'	Jonkershoek	S: 33˚ 59, 549'	E: 18˚ 57, 871'
	DuToitskloof Orchid site	S: 33˚ 41, 304'	E: 19˚ 12, 845'	Knysna R339	S: 34˚ 00, 204'	E: 23˚ 07, 101'
	DuToitskloof Trout farm	S: 33˚ 43, 124'	E: 19˚ 05, 570'	Kogelberg Reserve	S: 34˚ 19, 961'	E: 18˚ 57, 064'
Diseased	Stellenbosch Mountain	S: 33˚ 56, 657'	E: 18˚ 52, 815'	Knysna Phantom Pass	S: 34˚ 00, 623'	E: 23˚ 00, 199'
	Jonkershoek 1	S: 33˚ 59, 400'	E: 18˚ 57, 873'	Knysna Homtini Pass	S: 33˚ 57, 144'	E: 22˚ 54, 840'
	Jonkershoek 2	S: 33˚ 59, 027'	E: 18˚ 57, 395'	Knysna Gouna	S: 33˚ 59, 590'	E: 23˚ 02, 270'
	Paradyskloof	S: 33˚ 58, 039'	E: 18˚ 51, 954'	Cecelia Forest	S: 33˚ 59, 812'	E: 18˚ 25, 339'
	Brandwacht	S: 33˚ 57, 918'	E: 18˚ 52, 624'			
	Constantia	S: 34˚ 00, 235'	E: 18˚ 24, 532'			

Appendix 2. Site specific data for all known *Thecaphora capensis* - infected *Oxalis* species and populations.

Species	Location	Environmental disturbance Yes / No	Soil moisture Wet / moist / dry
Oxalis bifida	Cultivated specimen	Yes	Wet
Oxalis ciliaris	Bonnievale	Yes	Moist
Oxalis depressa	Bonnievale	Yes	Moist
Oxalis eckloniana	Ceres, Gydo Pass	Yes	Moist
Oxalis engleriana	Jonaskop	No	Moist
Oxalis glabra	Caledon	Yes	Wet
Oxalis incarnata	Knysna Phantom Pass	No	Wet
"	Knysna Homtini Pass	Yes	Wet
"	Knysna Gouna	No	Wet
"	Cecelia Forest	Yes	Wet
Oxalis lanata	Stellenbosch Mountain	No	Moist
"	Jonkershoek 1	Yes	Wet
"	Jonkershoek 2	Yes	Wet
"	Paradyskloof	Yes	Wet
"	Brandwacht	Yes	Wet
"	Constantia	Yes	Moist
Oxalis nidulans	Betty's Bay	Yes	Wet
Oxalis polyphylla	Hermanus	No	Moist
Oxalis tenella	Clanwilliam	Yes	Wet
Oxalis truncatula	Jonkershoek	No	Wet

*Note: No alien invasive *Oxalis* species were found at any locations listed about. All field sites were recorded as having shade cover.

Chapter 3

CONTRASTING PATTERNS IN THE EVOLUTIONARY INFLUENCE OF A POLLINATOR-TRANSMITTED PATHOGEN ON TWO *OXALIS* HOST SPECIES

ABSTRACT

Pathogens are powerful evolutionary forces that have the ability to shape the structure and dynamics of species, populations and communities. The Cape Floristic Region (CFR) of South Africa houses a system in which the evolutionary influence of a pollinator-transmitted anther-smut fungus, *Thecaphora capensis*, on host *Oxalis* species could be determined. Two *Oxalis* species (*O. lanata* and *O. incarnata*) were used to assess the evolutionary consequences of *Thecaphora capensis* infections on reproduction and attractiveness of flowers to pollinators in healthy individuals from disease-free and diseased populations. To this end, natural seed production was measured in healthy flowers that had been allowed pollinator visitors and covered with fine gauze while the number of flowers per plant were counted, flower morphological characters were measured and pollinator preferences for healthy flowers of these two population disease categories were assessed through pollen and spore transfers in a randomized block design of flowers in the field. Results implicated the evolution of pathogen avoidance strategies by one host species, *O. lanata*, while the second host species, *O. incarnata*, displayed no evidence of this. These results highlight the need to independently evaluate the effects of pathogens on hosts without making generalised conclusions and recommendations for conservation practices.

INTRODUCTION

Burdon *et al.* (2006) described pathogens as powerful evolutionary forces that can shape both the structure and dynamics of individual species and their communities at genetic, ecological, spatial and temporal scales. Although research on pathogens has expanded over the past few decades (Gilbert 2002), very few studies have focussed on the population-level evolutionary effects of pathogens on their hosts.

Pathogens have been identified as having direct effects on individuals within a population, affecting flowers size (e.g. *Microbotryum violaceum* Deml. & Oberw. infections of *Silene latifolia* Poir. (Alexander & Maltby 1990)), flower numbers (Shykoff *et al.* 1997) and reproduction (Marr 1997). This may have cascading effects on the successful pollination of such individuals, as petals are important in pollinator attraction (Ngugi & Scherm 2006). Larger flowers have been shown to draw more attention from pollinators (Shykoff *et al.* 1997). If pollinators were implicated as transmitting a flower-infecting pathogenic fungus within a population, larger flowers would attract more attention and therefore have an increased risk of receiving fungal spores. Plants would face a trade-off between pollinator attractiveness that may lead to larger pollen deposits and increased fitness, and the negative consequence of receiving more detrimental pathogens (Shykoff *et al.* 1997). Due to high pathogen pressure, the less attractive floral phenotypes (e.g. smaller flowers) within a diseased population would experience positive directional selection to avoid infection (Biere & Antonovics 1996). If the pathogen persists in a population over many generations, this pollinator-pathogen-host interaction can direct selection on floral (Elmqvist *et al.* 1993) and reproductive characters (Marr 1997), with distinct evolutionary consequences for the population as a whole. Pathogens can thus shape the evolutionary trajectories of species, populations and communities (Burdon *et al.* 2006).

The Cape Floristic Region (CFR) of South Africa, known for its rich botanical diversity and high levels of endemism (Goldblatt & Manning 2000), houses at least one system in which a fungal pathogen may have direct evolutionary consequences for populations of its host plant species. The recently described anther-smut fungus *Thecaphora capensis* Roets & Dreyer (Roets *et al.* 2008) infects a number of different *Oxalis* species across the CFR (Curran *et al.* 2009). Host plants are unable to reproduce normally, as flowers of infected plants are induced

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to produce fungal spores within their anthers (Roets *et al.* 2008) and produce significantly less seed than healthy individuals (Chapter 2). Flowers of infected plants have smaller petal surface areas and shorter style and stamen lengths than healthy flowers within the same population (Curran *et al.* 2009, Chapter 2). In addition, pollinators have been identified as carriers of these pathogenic spores (Curran *et al.* 2009).

All southern African species of *Oxalis* have bulbs (Salter 1944; Oberlander *et al.* 2009), enabling them to remain dormant during the dry summers in the CFR, only to emerge at the onset of winter (April to August) (Dreyer *et al.* 2006). The bulbs of plants in which the above-ground parts die back during the dry season would have to contain the fungus in order for the plant to display signs of infection in the following flowering season (Alexander & Antonovics 1988). Infected *Oxalis* plants display symptoms repeatedly over many flowering seasons. It has also been shown that surface sterilised bulbs from infected *Oxalis* plants produce plants with disease symptoms indicative of the fungus receding into the bulb (Curran *et al.* 2009). This permanent infection of plants may lead to increased levels of the fungus in populations over many generations if the rate of infection is greater than the death rate of diseased individuals. If selection had been operational on floral and reproductive characters over many generations, we hypothesize that this fungus brought about population-level evolutionary changes within diseased populations.

Flowers of non-crop plants in unmanaged systems are frequently infected by numerous smut fungi which influence species distribution patterns, abundance and evolution (Burdon *et al.* 2006; Ngugi and Scherm 2006). The present study aims to explore evolutionary changes brought about by the presence of this fungus in populations of two *Thecaphora capensis* hosts, *Oxalis lanata* and *O. incarnata*. To this end we compared 1) reproductive potential of seemingly healthy individuals from diseased and healthy individuals from disease-free populations; 2) floral morphological differences between healthy individuals from diseased and disease-free populations and 3) pollinator preferences for healthy flowers from diseased or disease-free populations. These results will clarify the magnitude of selection pressure by pollinators due to the presence of this disease in *Oxalis* populations.

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MATERIAL AND METHODS

Evolutionary influence of *Thecaphora capensis* on *Oxalis* reproductive potential

Reproductive differences between flowers of healthy plants from disease-free and diseased populations of *Oxalis lanata* and *O. incarnata* were compared during 2009 and 2010. It was hypothesized that healthy flowers from diseased populations would receive less pollen than healthy flowers in disease-free populations and that this would lead to an overall reduction in natural seed set (reproductive potential) for plants in diseased populations. This was tested by assessing the differences in natural seed production of healthy plants in diseased populations and disease-free populations. A total of 12 populations were included in these studies, including 7 populations of *O. lanata* and 5 populations of *O. incarnata* (Table 1). Natural seed set in each of these populations was determined for 20 randomly selected flowers (one flower per plant) that were beyond the pollination receptive stage, from healthy individuals. In addition, we determined whether observed seed set in the field was dictated by pollen limitation by hand-pollinating 20 randomly selected healthy flowers from each of the above-mentioned populations with legitimate pollen. We thus assumed that we would overcome limitations in pollen transfer posed by pollinators if we pollinated flowers by hand. Under these circumstances an increase in seed set for hand pollinated flowers vs. naturally pollinated flowers would indicate that seed set in the field is limited by pollinator movement.

To measure seed set, each experimental flower was covered with fine gauze and allowed to set seed (Figure 1). Fruits were collected three weeks later and the number of seeds per fruit was counted. The mean number of seeds per fruit was calculated and statistically compared using ANOVA with an LSD *post hoc* test for the normally distributed data and Mann-Whitney U-tests and Kruskal-Wallis ANOVA with a Multiple Comparisons z" value *post hoc* test for non-normally distributed data in Statistica 10 (StatSoft Inc, Tulsa, OK, USA).

It is possible that diseased populations may have evolved mechanisms to compensate for reduced numbers of sexually reproducing individuals. Accordingly, we tested for this by comparing the number of flowers produced and differences in seed set of flowers from healthy individuals in diseased and non-diseased populations respectively. A significant difference in seed set between hand-pollinated flowers of healthy individuals from diseased

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and disease-free populations could indicate differences in reproductive potential caused by evolutionary processes. Similarly, significant differences in the numbers of flowers produced by healthy individuals in diseased vs. disease-free populations would indicate differences in reproductive potential. These factors were again statistically compared using the above-mentioned procedures.

Evolutionary influence of *Thecaphora capensis* on *Oxalis* flower size

To test whether the presence of *Thecaphora capensis* has an evolutionary influence on flower size (i.e. pollinator attractiveness) we compared the sizes of flowers from healthy individuals of *Oxalis lanata* and *O. incarnata* collected from all populations tabulated in Table 1. From each of these populations we collected 20 fresh flowers from healthy plants (one flower per individual plant). Significantly larger flowers produced by healthy individuals in disease-free populations vs. diseased populations would indicate natural selection towards smaller flower size in the presence of the disease (i.e. less attractive flowers). Petal surface areas were measured using a planimeter (Model LI-3000, Lambda Instrument Corporation, USA), while style and stamen heights were measured using digital callipers. Results were analysed in Statistica 10 (StatSoft Inc, Tulsa, OK, USA) using ANOVA with an LSD *post hoc* test for the normally distributed data.

Table 1. Locality information for all *Oxalis* populations assessed in this study. Populations indicated in bold type face were assessed for seed production.

| *Oxalis lanata*			*Oxalis incarnata*		
Population	Grid reference		Population	Grid reference	
Disease-free populations					
Franschhoek	**S: 33˚ 56, 252'**	**E: 19˚ 09, 655'**	Jonkershoek	S: 33˚ 59, 549'	E: 18˚ 57, 871'
Du Toitskloof 1	S: 33˚ 41, 304'	E: 19˚ 12, 845'	**Knysna, R339**	**S: 34˚ 00, 204'**	**E: 23˚ 07, 101'**
Du Toitskloof 2	S: 33˚ 43, 124'	E: 19˚ 05, 570'	Kogelberg Reserve	S: 34˚ 19, 961'	E: 18˚ 57, 064'
Diseased populations					
Stellenbosch Mountain	**S: 33˚ 56, 657'**	**E: 18˚ 52, 815'**	**Knysna, Phantom Pass**	**S: 34˚ 00, 623'**	**E: 23˚ 00, 199'**
Jonkershoek 1	**S: 33˚ 59, 400'**	**E: 18˚ 57, 873'**	**Knysna, Homtini Pass**	**S: 33˚ 57, 144'**	**E: 22˚ 54, 840'**
Jonkershoek 2	**S: 33˚ 59, 027'**	**E: 18˚ 57, 395'**	**Knysna, Gouna**	**S: 33˚ 59, 590'**	**E: 23˚ 02, 270'**
Paradyskloof	**S: 33˚ 58, 039'**	**E: 18˚ 51, 954'**	**Cecelia Forest**	**S: 33˚ 59, 812'**	**E: 18˚ 25, 339'**
Brandwacht	**S: 33˚ 57, 918'**	**E: 18˚ 52, 624'**			
Constantia	**S: 34˚ 00, 235'**	**E: 18˚ 24, 532'**			

Figure 1. *Oxalis lanata* flower that has been covered with fine gauze in order to determine natural seed set.

Evolutionary influence of *Thecaphora capensis* on attractiveness of flowers to pollinators

We determined whether healthy flowers from disease-free populations received more pollen and/or spores than healthy flowers from diseased populations due to altered pollinator behaviour under controlled conditions. Virgin healthy flowers of *Oxalis lanata* from a disease-free population in Franschoek and a diseased population in Brandwacht were picked on the first day of anthesis and placed in water in micro-centrifuge tubes. Only flowers with no pollen or *Thecaphora capensis* spores on their stigmas, determined with the aid of a hand lens, were collected. A further sample of similar aged flowers showing disease symptoms (pollen in anthers replaced by spores) was picked and also placed in water in micro-centrifuge tubes. For consistency and to ease pollen and spore counts, we used only flowers from Long-morphed individuals. These micro-centrifuge tubes with healthy and diseased flowers were mixed in a randomized block design (n= 12 flowers; four from each of the three different treatments) and spaced at equal distances (8 cm) on the back of a shallow cardboard box (Figure 2). During September of 2010 these boxes were placed in a field in Brandwacht where no natural populations of *O. lanata* or diseased *Oxalis* populations occurred. After 3 days, and presumably after visitation by pollinators that had no prior contact with *O. lanata* or *T. capensis*, the flowers were collected and pollen and spore loads present on their stigmas determined. This experiment was replicated nine times on consecutive occasions.

For pollen and spore counts, stigmas were removed from flowers and placed in micro-centrifuge tubes containing 0.1 ml of 70% ethanol. Tubes were vortexed for 1 minute in order to loosen pollen grains and spores from stigmatic surfaces. The number of spores and pollen grains in the solution was determined using a haemocytometer. For this, the mean of six counts (six chambers) were used to estimate the number of propagules in the original volume. Results were analyzed with the software package SAS 9.1 (SAS Institute Inc., Cary, U.S.A.), using a Generalized Linear Mixed Model with Poisson distribution and Identity-link Function.
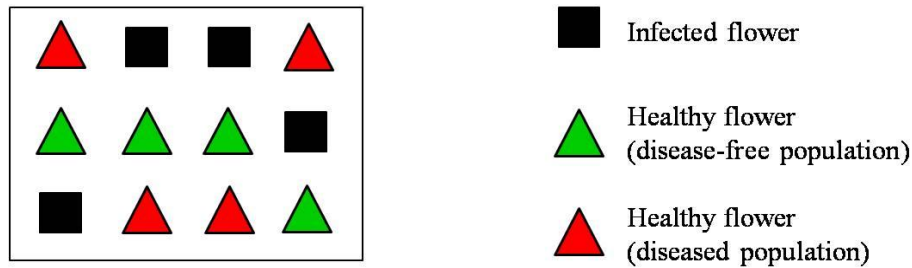
Figure 2. A schematic representation of the randomized block design used to test the evolutionary influence of *Thecaphora capensis* on the attractiveness of flowers to pollinators.

RESULTS

Evolutionary influence of *Thecaphora capensis* on *Oxalis* reproductive potential

Two of only three known disease-free *Oxalis lanata* populations were very inaccessible (Du Toitskloof site 1 and 2) and therefore could not be included in seed set investigations. The disease-free Franschhoek population of *O. lanata* produced no natural seed set. Although small, a significant difference was found when comparing the natural number of seeds produced by healthy *O. lanata* plants from diseased and disease-free populations (U (138) = 4.66; Z = -1.797; P < 0.05, Table 2). The number of naturally produced seeds by *Oxalis incarnata* plants of the disease-free and diseased populations did not differ significantly (U (88) = 533.5; Z = 1.07; P > 0.05, Table 2).

When healthy *Oxalis lanata* flowers were hand-pollinated and their seed set compared with those of natural seed set, significant differences were observed. Flowers that were hand-pollinated produced significantly more seeds than those that were allowed to naturally set seed (Table 2). Hand-pollinated *O. incarnata* flowers produced similar numbers of seeds as flowers that were allowed to set seed naturally. These results were similar for plants from both diseased and disease-free populations (Table 2).

The number of flowers produced by healthy individuals from diseased and disease-free populations differed significantly for both *Oxalis lanata* and *O. incarnata* (Figure 3). Individuals from diseased populations produced significantly more flowers than individuals from disease-free populations. There was no significant difference between the number of

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seeds produced by hand-pollinated flowers from the diseased and disease-free populations of *O. lanata* (U (133) = 968.5; Z = 1.07; P > 0.05, Table 2) and *O. incarnata* (U (83) = 488.0; Z = 1.38; P > 0.05, Table 2).

Using above-mentioned data, we were able to calculate a rough estimate of the total reproductive potential of individuals from diseased and disease-free populations respectively by calculating the value: *mean number of flowers produced per individuals* x *mean number of seeds produced per flower = mean reproductive potential per individual at a given time*. Substituting the values we obtained in our assay into this equation revealed that healthy individuals of *Oxalis lanata* in diseased populations have the potential to produce an average of *ca*. 16.84 offspring, whilst those from disease-free populations have the potential to produce slightly above 0 offspring at a given time. Individuals of *O. incarnata* in diseased populations have the potential to produce an average of *ca*. 8.2 offspring, whilst those from disease-free populations have the potential to produce a mean of 6.12 offspring at a given time.

Evolutionary influence of *Thecaphora capensis* on *Oxalis* flower size

Oxalis lanata displayed visible macro-morphological differences between disease-free and diseased populations. When observed in the field, healthy flowers from disease-free populations appeared larger than healthy flowers from diseased populations. Statistically, the flower sizes, as measured by the petal surface areas and the lengths of reproductive structures, differed significantly for *O. lanata* from diseased vs. disease-free populations (Table 3). In contrast, all measured flower morphological characters were similar between diseased and disease free populations of *O. incarnata* (Table 3).

Table 2. Reproductive data from the field assessments of *Oxalis lanata* and *O. incarnata*. Natural seed set is the mean number of naturally produced seeds by healthy flowers. Hand-pollinated seed set is the mean number of seeds produced by healthy flowers that were artificially pollinated in each of the population infection categories. Results are reported as median ± standard deviation. A statistically significant difference is indicated in bold type face at $P < 0.05$.

| Disease status	Natural seed set	Hand pollinated seed set	Test value (d.f.); P value
Oxalis lanata			
Disease-free	0.0 ± 0.00	5.5 ± 8.74	U (39) = 60.00; Z = 3.77; **P < 0.01**
Diseased	0.0 ± 4.94	7.0 ± 6.51	U (232) = 3921.00; Z = 5.56; **P < 0.01**
Test value (d.f.); P value	U (138) = 4.66; Z = -1.797; **P < 0.05**	U(133) = 968.5; Z = 1.07; P > 0.05	
Oxalis incarnata			
Disease-free	1.5 ± 1.95	1.0 ± 1.50	U (36) = 149.5; Z = -0.64; P > 0.05
Diseased	1.0 ± 1.74	0.0 ± 1.53	U (155) = 1930.0; Z = -1.64; P > 0.05
Test value (d.f.); P value	U (88) = 533.5; Z = 1.07; P > 0.05	U (83) = 488.0; Z = 1.38; P > 0.05	

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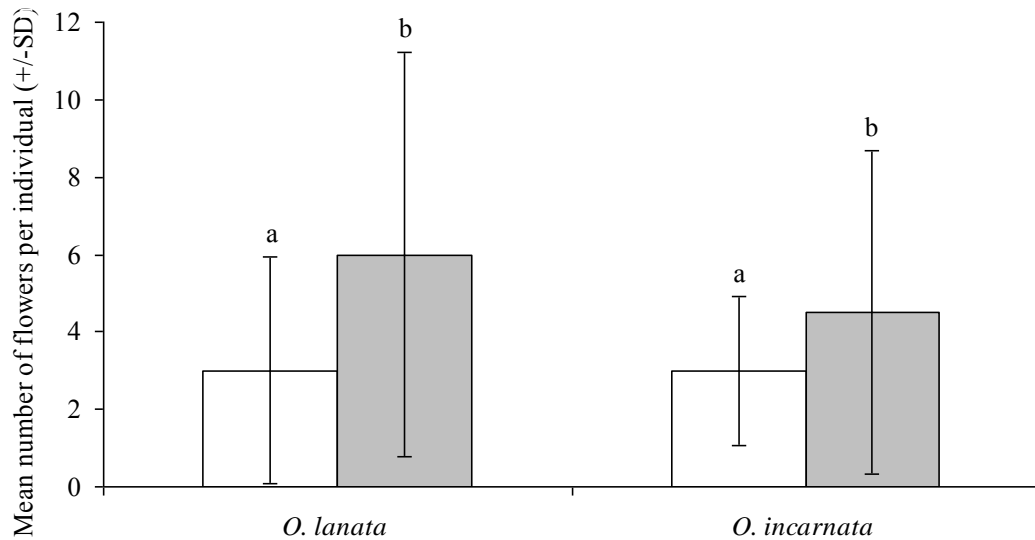
Figure 3. Mean number of flowers produced by healthy individuals in disease-free (clear bars) and diseased (grey bars) populations of *Oxalis lanata* and *O. incarnata*. Different letters indicate a statistically significant difference.

Table 3. Comparison between size of flower morphological characters for healthy *Oxalis lanata* and *O. incarnata* individuals from diseased and disease-free populations. Results are reported as mean ± standard deviation. Statistically significant differences are reported at P < 0.05.

Morphological Character				
	Oxalis lanata			
	Disease-free	Diseased	Test value (d.f.)	P
Petal surface area (cm²)	1.65 ± 0.33	0.97 ± 0.28	F (180) = 215.37	<0.01
Style length (mm)	8.38 ± 0.54	7.27 ± 0.52	F (180) = 156.86	<0.01
Mid level anther length (mm)	6.09 ± 0.40	5.32 ± 0.41	F (180) = 143.56	<0.01
Short level anther length (mm)	4.54 ± 0.45	3.80 ± 0.37	F (180) = 116.88	<0.01
	Oxalis incarnata			
Petal surface area (cm²)	0.52 ± 0.14	0.51 ± 0.15	F (134) = 0.614	>0.05
Style length (mm)	7.67 ± 0.49	7.48 ± 0.57	F (119) = 0.46	>0.05
Mid level anther length (mm)	4.99 ± 0.27	4.92 ± 0.36	F (119) = 0.13	>0.05
Short level anther length (mm)	3.51 ± 0.22	3.44 ± 0.30	F (119) = 0.48	>0.05

Evolutionary influence of *Thecaphora capensis* on attractiveness of flowers to pollinators

High numbers of *Oxalis lanata* pollen grains and *Thecaphora capensis* spores were observed on the stigmatic surfaces of numerous flowers placed on the boxes and left for three days. This provides evidence that pollinators visited these experimental flowers regularly. There were significantly more pollen grains deposited on healthy flowers from the disease-free population (i.e. larger flowers) compared to healthy flowers from the diseased population (i.e. smaller flowers) (d.f. = 1; Wald Statistic = 47.01; p < 0.001) (Figure 4). The numbers of spores deposited on stigmatic surfaces of flowers from both population categories was statistically similar (d.f. = 1; Wald Statistic = 0.57; p = 0.451) (Figure 4).
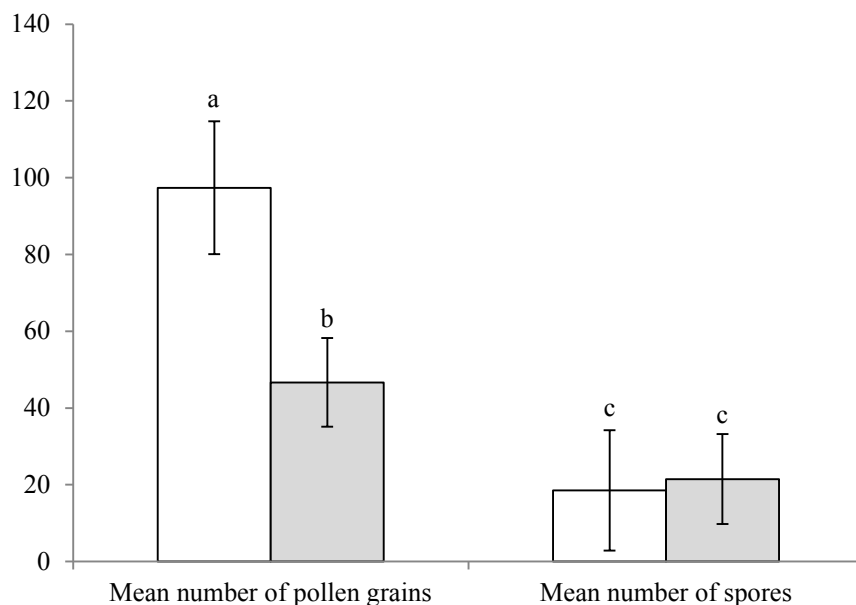
Figure 4. Mean number of pollen grains and spores deposited by pollinators on the stigmatic surfaces of healthy flowers from disease-free (clear bars) and diseased (grey bars) populations of *Oxalis lanata* that had been placed in micro-centrifuge tubes in boxes in the field. Results were obtained using a Generalized Linear Mixed Model with a Poisson distribution (Identity Link Function) and are represented as mean ± S.E. Different letters represent a statistically significant difference at P < 0.001.

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DISCUSSION

Results from this study show that the presence of *Thecaphora capensis* in *Oxalis* populations can have a profound influence on evolutionary processes. For example, seemingly healthy flowers in diseased populations of *O. lanata* produced smaller flowers than healthy flowers in disease-free populations. Also, healthy flowers from diseased populations (smaller flowers) on average receive less pollen and presumably less pollinator visitations when compared to healthy flowers from disease-free populations (larger flowers). Disease-free populations have not been exposed to the disease and therefore face no risk of becoming infected. Plants within these disease-free populations thus would experience directed selection towards better pollinator attraction (larger flowers) to increase seed set (Carter & Thornburg 2004). Conversely, there seems to be strong selection for the production of smaller flowers in diseased populations of *O. lanata*. Thus, it appears that individuals from diseased populations of *O. lanata* have evolved mechanisms to avoid strong pollinator visitation (e.g. smaller flower size) and in turn, avoid receiving large spore deposits on their stigmas (see Chapter 4).

In diseased populations, as the number of healthy plants producing healthy flowers with pollen become increasingly scarce, plants are at risk of low recruitment rates due to decreased pollination within the population. This may lead to selection on plants with more attractive floral traits in order to produce at least some seed set, despite the risk of becoming infected (Roy 1994). A decrease in pollinator attractiveness in diseased populations of *O. lanata* was coupled with an increase in overall fecundity of the remaining individuals. Healthy *O. lanata* individuals from diseased populations produced significantly more flowers linked to potential higher seed set, than those from disease-free populations. Thus plants from diseased populations have evolved mechanisms to compensate for lowered attractiveness of smaller flowers. The selective pressures resulting from the fluctuation of pathogen abundance and severity of impact across space and time has been shown to lead to the evolution of resistance diversity in populations of other plant species (Burdon *et al.* 2006). The remaining healthy plants in the diseased populations of *O. lanata* may thus have evolved additional mechanisms to cope with the presence of the disease and should be an interesting field for future study.

In contrast to *Oxalis lanata*, *O. incarnata* populations did not seem to experience the same selective forces towards smaller flower sizes in diseased populations. Flowers produced by

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healthy individuals in diseased populations were the same size as those from disease-free populations. Even though healthy *O. incarnata* individuals in diseased populations produced more flowers than individuals from disease-free populations, selection towards an increase in fecundity of *O. incarnata* individuals from diseased populations was only slight. A possible explanation for this reduced selective force on *O. incarnata* as opposed to *O. lanata* may be that seeds are only rarely produced and therefore selection will be reduced. This was evident from our data as artificially pollinated flowers of *O. incarnata* produced far less seed than artificially pollinated *O. lanata* flowers. Even if the selective forces acting on *O. incarnata* was similar to that on *O. lanata*, one would expect the selection process would take much longer to result in phenotypic changes under these conditions. Possible differences in pollinator movements between these two species may also have an effect on selection pressures.

Natural seed production was low for both *Oxalis lanata* and *O. incarnata*. This may be due to pollinator limitation, which is known to lead to decreased pollination efficiency and thus decreased seed set (Carter & Thornburg 2004). Natural seed set did not differ between healthy individuals from diseased and disease-free populations of *O. incarnata*. For *O. lanata* there was a slight increase in natural seed set in diseased populations. When flowers were artificially pollinated, there was no significant increase in the numbers of seeds produced by *O. incarnata*. Thus, pollinator limitation does not play a role in the fecundity of this species and other mechanisms probably dictate the low natural seed set. In contrast, artificially pollinated flowers of *O. lanata* produced significantly more seed than those that were naturally pollinated. Natural seed set is therefore limited by pollinator movement in this species.

Honeybees have been identified as the predominant flower visitor to many *Oxalis* species (De Jager *et al.* 2010). Pollen and spore transfers to healthy flowers in the randomized block design experiment confirmed pollinator visits to the block. The increased level of pollen transfer to healthy flowers from disease-free populations within the experimental block confirmed that pollinators are attracted to larger flowers (Shykoff *et al.* 1997). Although these larger flowers did not receive increased numbers of spores, spores were transferred to the healthy flowers from both the disease-free and diseased populations within the block. This suggests that pollinators may display a learned behavior and may be able to discriminate between healthy and infected plants, possibly due to differences in nectar quality and quantity

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(Shykoff & Bucheli 1995). Thus, similarity between spore numbers here could have resulted from accidental movement to healthy flowers by a pollinator that preferred diseased flowers.

In this study we have successfully shown that there are differences in the healthy individuals from natural populations of *Oxalis lanata* collected from populations with varying degrees of infection. Selection pressures on healthy plants within these populations may have led to their evolutionary adaptation to avoid infection by *Thecaphora capensis* and also to mechanisms that compensate for lowered numbers of reproductive individuals within diseased populations. Flower size data and our pollination experiment suggested that there has been positive selection by pollinators for smaller flower sizes in diseased populations, which may be the underlying driver of these adaptations. These same selective forces have not resulted in changes of above-mentioned characters of *O. incarnata*. Our results thus highlight the need to independently evaluate the effect of sexually transmitted diseases on a species-by-species basis as to avoid making erroneous general conclusions regarding consequences, patterns and processes of disease spread.

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Chapter 4

FUNGAL SPORE VECTORS: HOST DENSITY AND FREQUENCY EFFECTS ON DISEASE SPREAD

ABSTRACT

Many plant species rely on insect pollinators for reproductive success. Plants have adapted a variety of mechanisms to attract these insect pollinators for the receipt and deposition of pollen. However, when pollinators also spread pathogenic fungal spores they are likely to increase infection rates in a population. Some South African *Oxalis* species host the anther-smut fungus, *Thecaphora capensis*. Pollinators have been implicated as fungal spore vectors of this fungus. In order to investigate the response of pollinators to host density and disease frequency within infected populations, we determined the numbers of spores and pollen grains that were deposited on stigmatic surfaces of flowers under natural and standardized conditions. The visitation patterns of pollinators between flowers of different disease statuses were also evaluated. Host density and disease frequency effects on pollen and spore transfers were variable under natural conditions, as these factors probably interact significantly. However, an increase in host density and disease frequency significantly increased pollen and spore deposits under standardized conditions. Pollinators were found to be important in fungal spore transfers within populations, since they showed no preference for flowers of a specific disease status, but did not favour oscillating between healthy and infected flowers. This complex host-pathogen system is influenced by many variables, each contributing to the infection of new hosts through the transmission of pathogenic spores.

INTRODUCTION

Many plant species rely on insects for pollination to increase their reproductive success (Carter & Thornburg 2004). Pollinators are attracted to these plants by their floral displays and odours, and are rewarded with nutritious pollen and sometimes sugary nectar (Pacini *et al.* 2003). Floral diversification can result in variations in pollinator attractions and have directly resulted in the explosion of flowering plant numbers in the recent evolutionary history of the planet (Barrett 2002; Johnson 1996; Sargent 2004; Van der Niet & Johnson 2009). Similarly, nectar can be highly variable in quality and quantity (Shykoff & Bucheli 1995), which results in variations in pollinator visitations (Zimmerman 1982), time spent on flowers (Elmqvist *et al.* 1993) and size of pollen deposits (Galen & Plowright 1985; Thomson 1986).

Although pollinators are defined as pollen dispersers, they provide a similar service to flower-infecting pathogens by dispersing their reproductive propagules (Roy 1994). Dispersal of fungal propagules by flower visitors, in combination with increased or longer visitations, may result in greater infection rates. Some pathogens ensure the dispersal of their reproductive spores by contributing towards the floral displays (e.g. *Microbotryum violaceum* Deml. & Oberw. infections of *Dianthus silvester* Wulf., Shykoff *et al.* 1997), flowering times (e.g. *Microbotryum violaceum* infections of *Viscaria vulgaris* L., Jennersten 1988), olfactory cues and nectar rewards (Shykoff & Bucheli 1995) of their hosts in order to enhance attractiveness to pollinators. The enhanced attractiveness of flowers to pollinators brought about by flower-infecting pathogens and the ability of pollinators to disperse fungal propagules place flowers in a vulnerable position for infection by fungi. Increased nectar volumes are likely to lead to extended foraging episodes or multiple short visits to flowers by pollinators carrying fungal propagules (Thomson 1986). Spore deposits on flowers may, therefore, increase if nectar volumes are increased. Increased spore deposits have consequently proven to lead to the increased likelihood of plants becoming infected in the next flowering season, while plants receiving small spore loads have a high probability of escaping infection (Elmqvist *et al.* 1993).

There are three possible outcomes for plant populations containing vector-borne diseases. The offspring of the population will be free of infections due to host reproduction rates

exceeding transmission rates, or both host and parasite will be driven to extinction (Alexander & Antonovics 1988), or the host and pathogen may co-exist. May & Anderson (1983) questioned whether pathogens evolve to a less virulent state to allow for the stable co-existence of hosts and their pathogens. For a stable co-existence between hosts and their pathogens, density-dependent population regulations must be taken into account (Thrall & Jarosz 1994). In the case of *Microbotryum violaceum* infections of *Silene alba* (Miller) the physiology of the host plant remains largely unaffected, as they reproduce pathogenic spores for several years (Alexander & Antonovics 1988). Thrall & Jarosz (1994) showed that more resistant populations of *S. alba* had the ability to regain a healthy status, while populations of *S. alba* susceptible to *M. violaceum* can co-exist with these pathogens. Plants differ in their genetic make-up with respect to resistance alleles, which could explain the variability in their susceptibility to fungal infections (Biere & Honders 1998; Burdon *et al.* 2006). Host age, phenology, size and nutritional status also contribute to this susceptibility (Biere & Honders 1998). In addition, plants have also adapted by developing different defence mechanisms against pathogen infections. The biochemistry of nectar in ornamental tobacco (*Nicotrana alata* Link & Otto) plants protect the gynoecium from infection by micro-organisms (Carter & Thornburg 2004), while ovary abortion occurs in female *Silene latifolia* (Miller) plants when healthy flowers receive fungal spores (Baker 1947). Other species experience localised cell death of the stigmatic surface in the presence of fungal spores, which leads to a decrease in seed production (Roy 1996).

Factors that influence fungal spore transmissions by insects have been identified in a limited number of study systems, many including the fungal pathogen *Microbotryum violaceum* and members of the plant family Caryophyllaceae (Biere & Honders 1998; Bucheli & Shykoff 1999; Elmqvist *et al.* 1993; López-Villavicencio *et al.* 2007; Shykoff *et al.* 1997). These include the density (Anderson & May 1979; May & Anderson 1979) and frequency (Biere & Honders 1998; Bucheli & Shykoff 1999; Gerber *et al.* 2005; Shykoff *et al.* 1997) of hosts within a population. Bucheli and Shykoff (1999) and Rudolf & Antonovics (2005) suggested that the frequency of diseased individuals is a more important variable than density in systems where transmission is an active process facilitated by insect vectors. Lower disease frequencies would present larger numbers of healthy individuals and increased infection rates if flower visitors were active spore dispersers and made only a single transition to an infected flower during its foraging run of healthy flowers. This is due to the fact that flowers, visited

late in the foraging run of a pollinator, still have the potential to receive infectious spores (Shykoff & Bucheli 1995). Host density within a population can be measured as the number of host individuals per unit area. When the density or spacing between host individuals is altered, both the time spent searching for individuals and visiting individuals is altered. Therefore, as the distance between host plants is increased, the mode of transmission of infectious spores may switch from frequency to density dependent (Biere & Honders 1998; Bucheli & Shykoff 1999).

In a recently described system a smut fungus, *Thecaphora capensis* Roets & Dreyer, was found to be transmitted between *Oxalis* L. flowers by flower visitors in the southwestern Cape of South Africa (Roets *et al.* 2008; Curran *et al.* 2009). It represents the first known *Thecaphora* infection in Africa (Roets *et al.* 2008). This sexually-transmitted disease affects the anthers of its host by inducing the production of infectious spores instead of pollen in the anthers of its hosts (it is thus known as an anther-smut fungus). Flowers of 12 *Oxalis* species have been found infected by *T. capensis* (Chapter 2). Infected flowers of two of these species were morphologically examined and showed drastic differences to healthy flowers of the same species, potentially influencing the behaviour of pollinators that transport fungal spores between flowers (Chapter 3).

Oxalis is the largest geophytic genus in the Cape Floristic Region (CFR) (Goldblatt & Manning 2000; Proches *et al.* 2006) of South Africa. Flowers of the genus display a tristylous breeding system, comprised of three different floral morphs. Morphs differ with respect to the position of stigmas (carried in the Long, Mid or Short position). Each morph also has two whorls of anthers borne in reciprocal positions relative to stigmas. Tristyly only allows successful fertilization to occur between pollen and stigmas of the same height (Salter 1944; Dulberger 1992). Although southern African *Oxalis* has been the focus of extensive recent systematic study, not much is known about the pollinators of the *ca*. 210 species (Oberlander *et al*. 2009; Dreyer pers. comm.). Similarly, very little is known about the pollinator-mediated fungal spore transfers within *Oxalis* populations infected with *Thecaphora capensis*. De Jager *et al.* (2010) ascribed honeybees (*Apis melifera capensis* Esch.) to be the predominant pollinators of the *Oxalis* species he studied, while Curran *et al.* (2009) also commonly observed Tenebrionid beetles and Halictid bees visiting infected flowers.

Microbotryum violaceum, an anther-smut fungus with hosts in the Caryophyllaceae, has been well-studied (Biere & Honders 1998; Bucheli & Shykoff 1999; Elmqvist *et al.* 1993; López-Villavicencio *et al.* 2007; Shykoff *et al.* 1997). *Microbotryum violaceum* infections can therefore be used as a model system for studies on *Thecaphora capensis,* given the similarities in their growth forms and impacts on their hosts. Studies on pollinators and the influence of disease frequency and host density on disease spread within populations infected by *M. violaceum* (Alexander & Antonovics 1988; Baker 1947; Biere & Honders 1998; Bucheli & Shykoff 1999; Jennersten 1988; Marr 1997; Shykoff & Bucheli 1995; Sloan *et al.* 2008), in particular, pose interesting questions and offer exciting comparisons with *T. capensis* infections of *Oxalis* species in the CFR.

The main objective of this study was to evaluate the effects of disease frequency and host density on disease spread within *Oxalis* populations. Two distantly related *Oxalis* taxa, *Oxalis lanata* T. M. Salter and *O. incarnata* Jacq., were used as case studies. They were selected because of the differences in their habitat preferences, associated pollinators and floral morph frequencies. *Oxalis lanata* inhabits sun to semi-shaded areas, while. *O. incarnata* prefers fully shaded environments associated with natural forest patches. Pollinators of the two species are thought to differ due to their different habitat preferences, which were confirmed through field observations (H. Curran; F. Roets). In addition, in this study we aimed to determine pollinator visitation patterns within diseased populations.

MATERIAL AND METHODS

The influence of host density and disease frequency on disease spread under field conditions
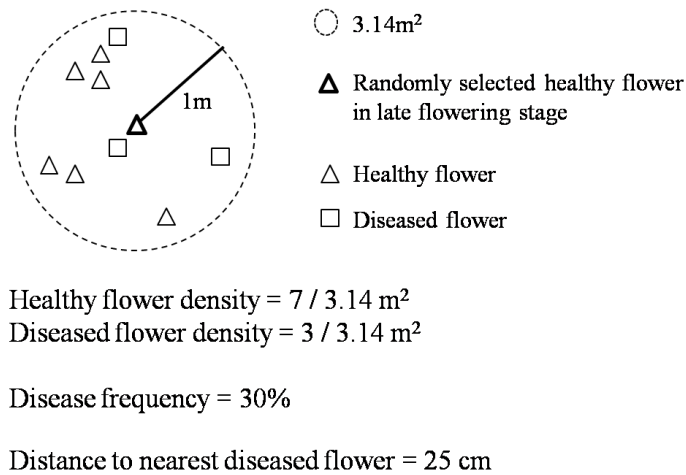
The effects of host density and disease frequency on disease spread were assessed in the field in four populations of *Oxalis lanata* and one population of *O. incarnata* (Table 1). Healthy Long morph flowers (n = 120 for *O. lanata* and n = 100 for *O. incarnata*) in the late flowering stage (petals starting to show signs of aging) were randomly selected in diseased populations. The number of healthy and diseased flowers within a one meter radius (3.14 square meters) of each of these randomly selected flowers was counted. The distance from

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this randomly selected healthy flower to the nearest infected flower was also recorded. The total number of flowers within the area around the selected flower provided a host density estimate, while the percentage of healthy and infected flowers provided an estimate of healthy and diseased frequencies (Figure 1).

The randomly selected flowers were picked and the stigmas were removed using a pair of sterilised scissors and tweezers. The stigmas were placed in micro-centrifuge tubes containing 0.1 ml of 70% alcohol and vortexed at high speed for 30 seconds to loosen pollen grains and spores from the stigmas. The number of pollen grains and spores present within each tube were determined using a haemocytometer. A total of six haemocytometer chambers were counted per micro-centrifuge tube. An estimate of the original number of pollen grains and spores per micro-centrifuge tube was then calculated by adding the counts per tube and multiplying these by a factor to revert the volume of liquid in the six haemocytometer chambers back to the original volume in the micro-centrifuge tube. Results were analysed using a Generalized Linear Mixed Model with a Poisson distribution (log link function) in SAS 9.1 (SAS Institute Inc., Cary, U.S.A.).

Table 1. Collection data of healthy flowers from various populations that were assessed for pollen and spore deposition on their stigmas.

Species	Population	Grid reference		Number of randomly selected flowers
Oxalis lanata	Brandwacht	S: 33˚ 57, 918'	E: 18˚ 52, 624'	90
"	Constantia	S: 34˚ 00, 235'	E: 18˚ 24, 532'	10
"	Jonkershoek	S: 33˚ 59, 400'	E: 18˚ 57, 873'	10
"	Paradyskloof	S: 33˚ 58, 039'	E: 18˚ 51, 954'	10
Oxalis incarnata	Cecelia Forest	S: 33˚ 59, 812'	E: 18˚ 25, 339'	100

Healthy flower density = 7 / 3.14 m²
Diseased flower density = 3 / 3.14 m²

Disease frequency = 30%

Distance to nearest diseased flower = 25 cm

Figure 1. Diagram of the conceptual design of the experimental procedure used in the field to determine the effects of host density and disease frequency on the disease spread. The total number of healthy (triangles) and infected (squares) flowers within the area around the randomly selected flower (bold triangle) provided estimates of the total density of flowers, while the percentage of healthy and diseased flowers of the total number of flowers in the circle provided an estimate of healthy and diseased flower frequencies, respectively.

The influence of host density and disease frequency on disease spread under standardized conditions

1. The influence of host density on disease spread

The effect of host density on disease spread was tested using an organised block design experiment in the Assegaaibosch Nature Reserve, Stellenbosch (S 33˚ 57" 59.64"; E 18˚ 55" 24.46"). Healthy and infected *Oxalis lanata* flowers were collected from the severely diseased population at Brandwacht, Stellenbosch (Chapter 3). Only healthy flowers with no pollen or *Thecaphora capensis* spores on their stigmas, determined with the aid of a hand lens, were collected. Flowers were transported to the Assegaaibosch Nature Reserve in micro-centrifuge tubes filled with distilled water. Four infected and five healthy *O. lanata* flowers were used in each experiment. In this way the frequency of diseased flowers in relation to healthy flowers in each experiment was kept constant at 44.44 %. Single flowers in micro-centrifuge tubes filled with water were mounted on 20 cm long black plastic poles using brown tape (Figure 2). These were arranged in a block (Figure 3) with varying interspacing distances of: a) 5 cm, b) 50 cm, c) 100 cm and d) 200 cm. For each experiment the nine flowers were arranged within the square such that the five healthy flowers occupied

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the four corner positions and the central position of the square, while the four infected flowers occupied the middle positions of the four sides of the square (Figure 4). Each interspacing experiment was replicated three times and experiments were separated in space (>100 metres apart) and time to avoid pollinator interference. Squares were erected in fields where no natural populations of *Oxalis lanata* were present. It is estimated that *Oxalis* flowers have a vase life of approximately five days. After three days stigmas from all healthy flowers were removed with a sterilized pair of scissors and collected in micro-centrifuge tubes containing 0.1 ml of 70% alcohol. Pollen and spore counts were calculated as outlined above. A Generalized Linear Mixed Model with a Poisson distribution (log link function) was used to analyse the results in SAS 9.1 (SAS Institute Inc., Cary, U.S.A.).


Figure 2. Single flowers were placed in micro-centrifuge tubes filled with water and mounted on 20 cm long black plastic poles with brown tape. These poles were used in the block design with varying interspacing distances.

Figure 3. Picture of flowers in micro-centrifuge tubes mounted on 20 cm long black plastic poles that were arranged in a block design at various set interspacing distances.
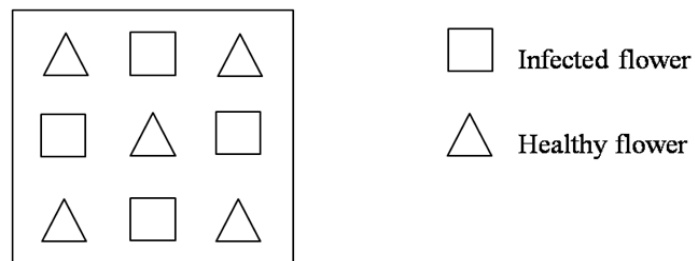
Figure 4. Conceptual design of the organised block in which the effect of host density on disease spread was tested. The four corners and central position of the block were occupied by healthy flowers, while the centre position of each side of the block was occupied by diseased flowers.

2. The influence of disease frequency on disease spread

The effect of disease frequency on the spread of spores was tested using a randomised block design containing 20 *Oxalis lanata* flowers. Flowers were placed in micro-centrifuge tubes filled with distilled water and inserted into a box with equal distances between each flower (5 cm) (Figure 5). Boxes were placed in the field at the Jan Marais Park, Stellenbosch (S 33˚ 55" 59.51"; E 18˚ 52" 27.67") in areas where no natural populations of *O. lanata* were present to avoid interference with the experiment. Healthy and diseased flowers were mixed randomly in three boxes to create disease frequencies of 20% (four diseased flowers), 50% (ten diseased flowers) and 80% (16 diseased flowers), respectively. In the case of healthy flowers, only those with no pollen or *Thecaphora capensis* spores on their stigmas, determined with the aid of a hand lens, were collected. Boxes, each with flowers at one of the three disease frequencies, were placed in the field simultaneously and separated by distances of at least 100 metres to control for pollinators visiting multiple boxes. Each experiment was replicated three times. Stigmas from the healthy flowers within the boxes were again collected in micro-centrifuge tubes containing 0.1 ml of 70% alcohol after three days. The number of pollen grains and spores on these stigmas were again determined as described above. Results were analysed in SAS 9.1 (SAS Institute Inc., Cary, U.S.A.) using a Generalized Linear Mixed Model with a Poisson distribution (log link function).

Figure 5. In order to test the influence of disease frequency on disease spread under standardized conditions, flowers in micro-centrifuge tubes filled with distilled water were placed in a box in the field for three days to allow for visitation by pollinators.

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Pollinator movement between flowers of different disease statuses

The patterns of pollinator visitations within infected populations of *Oxalis lanata* and *O. incarnata* were monitored. Flower visitors were identified and their movements were recorded as the sequence of transitions between healthy and infected flowers. To count as a visitation, a potential pollinator had to physically land on a flower. Flowers on which the flower visitor landed on but did not enter were also recorded in this sequence. A minimum of 10 transitions (including flowers not visited) were recorded for each visitor or until the visitor moved out of visual range. Data were analysed using G-statistics for goodness of fit to randomly expected visitation patterns. To determine whether flower visitors had a preference towards healthy or diseased flowers, the total number of visits by pollinators to healthy and infected flowers was used as observed values, while flowers that were not visited but occurred directly in the flight path of these visitors were included in the expected count. To determine whether individual flower visitors tended to visit flowers of a specific disease status, the total number of same status movements and transitions between flowers of different disease statuses (in the sequence of visitation) was used as observed counts. The flowers that the flower visitors landed on but did not enter were included in the movement sequence for expected counts.

<div align="center">RESULTS</div>

The influence of host density and disease frequency on disease spread under field conditions

The distance between the selected healthy *Oxalis lanata* flowers and their nearest infected individual had no significant effect on the number of spores deposited on their stigmas (df = 3; Wald Statistic = 0.32; p = 0.9553). In contrast, the distance between the selected healthy *O. incarnata* flower and the nearest infected flower had an inversely proportional effect on the number of spores deposited on the stigmas of the healthy flower (df = 7; Wald Statistic = 355.47; p < 0.0001) (Figure 6). The number of spores present on the stigmas of the healthy flowers was highest at the shortest distance between this healthy flower and the nearest infected flower, with decreasing spore transfers at increasing distances to infected flowers.

When both pollen grains and spores present on stigmatic surfaces were combined, a significant relationship was observed for both *Oxalis incarnata* (df = 5; Wald Statistic = 2998.03; p < 0.0001) and *O. lanata* (df = 5; Wald Statistic = 1037.23; p < 0.0001) (Figure 7). For *O. incarnata* there was an increase in the number of pollen grains and fungal spores transferred with an increase in the total numbers of flowers up to a maximum of between 31 and 40 flowers per 3.14 m^2. Total pollen and spore transfer decreased with further increase in flower density. For *O. lanata* there was no clear peak, however, there was an overall decrease in the total number of pollen grains and fungal spores transferred with an increase in flower density (Figure 7).

When data for pollen grain and fungal spore depositions were analysed separately, host density showed significant effects in terms of the number of spores (df = 5; Wald Statistic = 367.33; p < 0.0001) and pollen grains (df = 5; Wald Statistic = 1242.44; p < 0.0001) deposited on the stigmas of the selected healthy *Oxalis lanata* flowers (Figure 8). Similarly, host density had a significant influence on the number of spores (df = 5; Wald Statistic = 3264.21; p < 0.0001) and pollen grains (df = 5; Wald Statistic = 739.45; p < 0.0001) deposited on the randomly selected healthy *O. incarnata* flowers (Figure 8). A density of between 31 and 40 infected flowers per 3.14 m^2 increased the numbers of spores deposited on the stigmas of healthy *O. incarnata* flowers significantly. A density of between 21 and 30 infected flowers per 3.14 m^2 significantly increased the numbers of spores deposited on the stigmas of healthy *O. lanata* flowers. At higher densities of flowers per 3.14 m^2 the numbers of spores deposited on stigmas again decreased significantly in both species. The effect of flower density on the deposition of pollen grains was far less clear (Figure 8).

The frequency of diseased *Oxalis lanata* flowers within the area around the selected flowers had a significant impact on the number of spores deposited on their stigmas (df = 3; Wald Statistic = 93.9; p < 0.0005) (figure 9i). With an increasing disease frequency in the area around the selected healthy flowers, the number of spores deposited on their stigmas increased until the frequency reached 50%. A further increase in the disease frequency showed a significant decrease in the number of spores deposited on the stigmas of the healthy *O. lanata* flowers within the area. The lowest number of spores transferred was observed at the highest disease frequencies of between 76% and 100%. Diseased flower frequencies of *O. incarnata* also showed significant influences on the number of spores deposited on the randomly selected healthy flowers (df = 3; Wald Statistic = 4929.7; p < 0.0001) (Figure 9ii).

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The highest spore transfers were found in areas where infected flowers reached frequencies of between 51% and 75%, with the lowest spore transfers found in regions where infected flowers were at a frequency of between 0 and 25%.

When assessing the effect of healthy *Oxalis lanata* flower frequencies on the number of pollen grains deposited on the randomly selected healthy flowers, there was no significant difference across any frequency category (df = 3; Wald Statistic = 4.32; p = 0.2294). The frequency of healthy *O. incarnata* flowers, however, influenced the number of pollen grains transferred to the healthy randomly selected flowers (df = 3; Wald Statistic = 23.24; p < 0.0005) (Figure 10). Healthy flower frequencies of between 0 and 25% showed lower pollen transfers than higher healthy flower frequencies of between 26% and 100%.
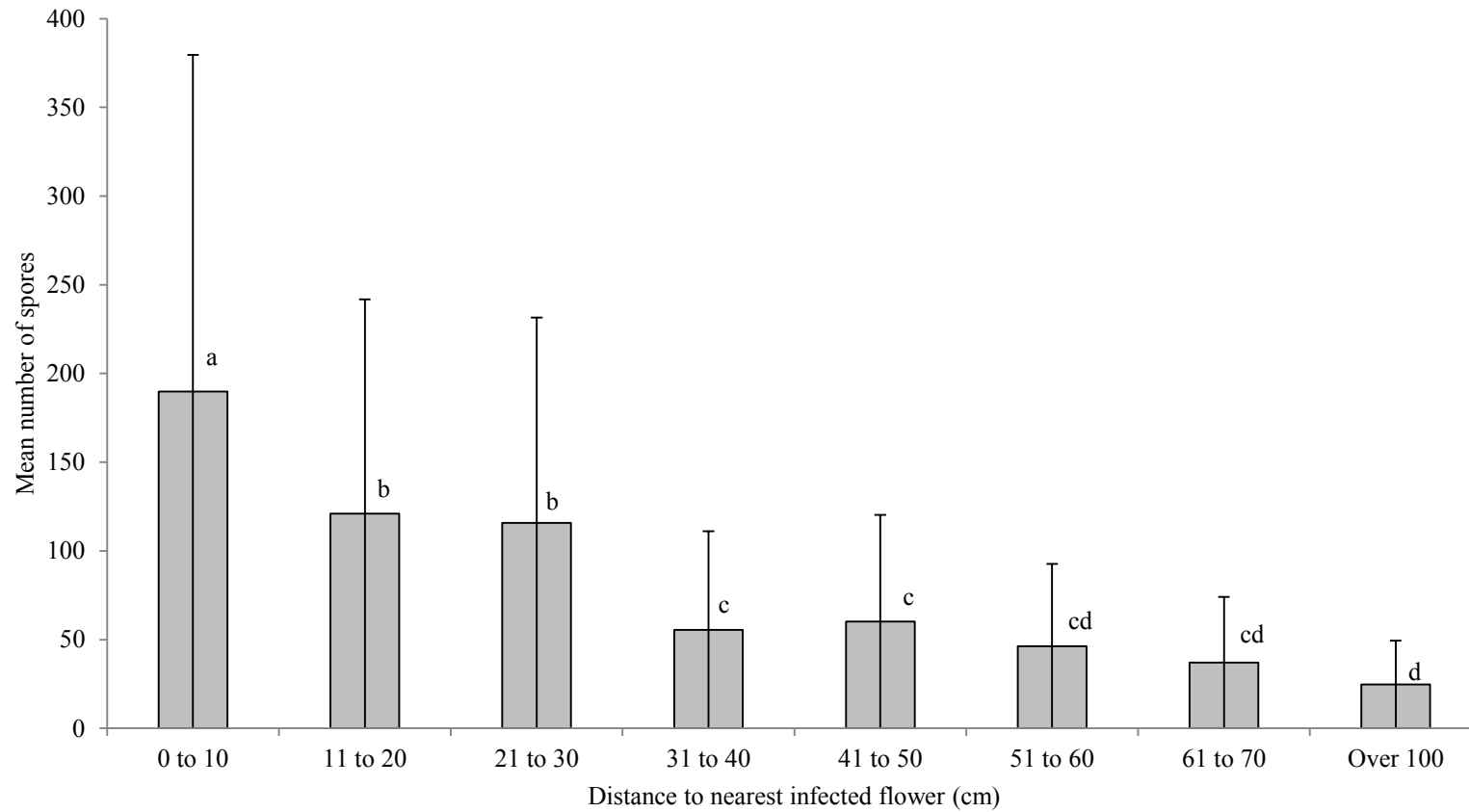
Figure 6. Summary of the effect of the distance to the nearest infected flower on the mean number of spores deposited on the stigmas of the randomly selected healthy *Oxalis incarnata* flowers in the field assessment experiment, using the Generalized Linear Mixed Model with a Poisson distribution (identity link function). Results are indicated as mean ± S.E., while different letters represent a statistically significant difference at $p < 0.0001$.
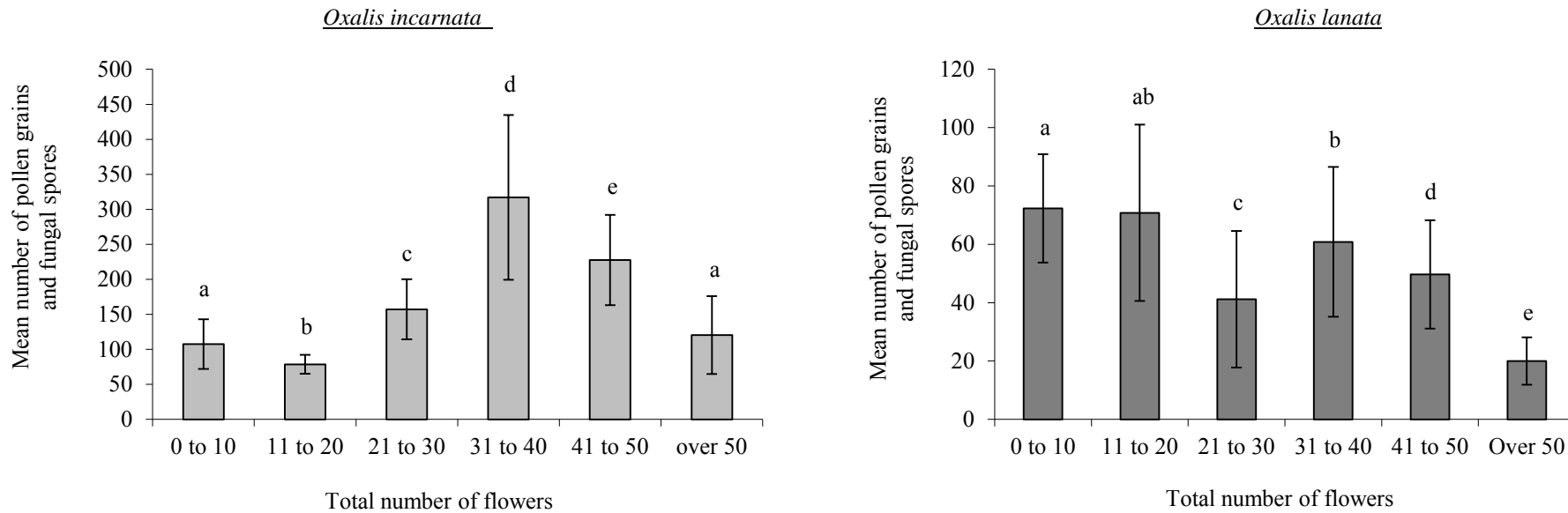
Figure 7. The effect of total density of flowers (within 3.14 m2) in populations of *Oxalis incarnata* (left) and *Oxalis lanata* (right) on the mean number of spores and pollen grains (total of the two) transferred to the stigmas of the randomly selected healthy flowers by pollinators (i.e. pollinator activity). Results were obtained using a Generalized Linear Mixed Model (Poisson distribution, log link function). Results are indicated as mean ± S.E., while different letters represent a statistically significant difference at p < 0.001.

FUNGAL SPORES

POLLEN GRAINS

Oxalis incarnata
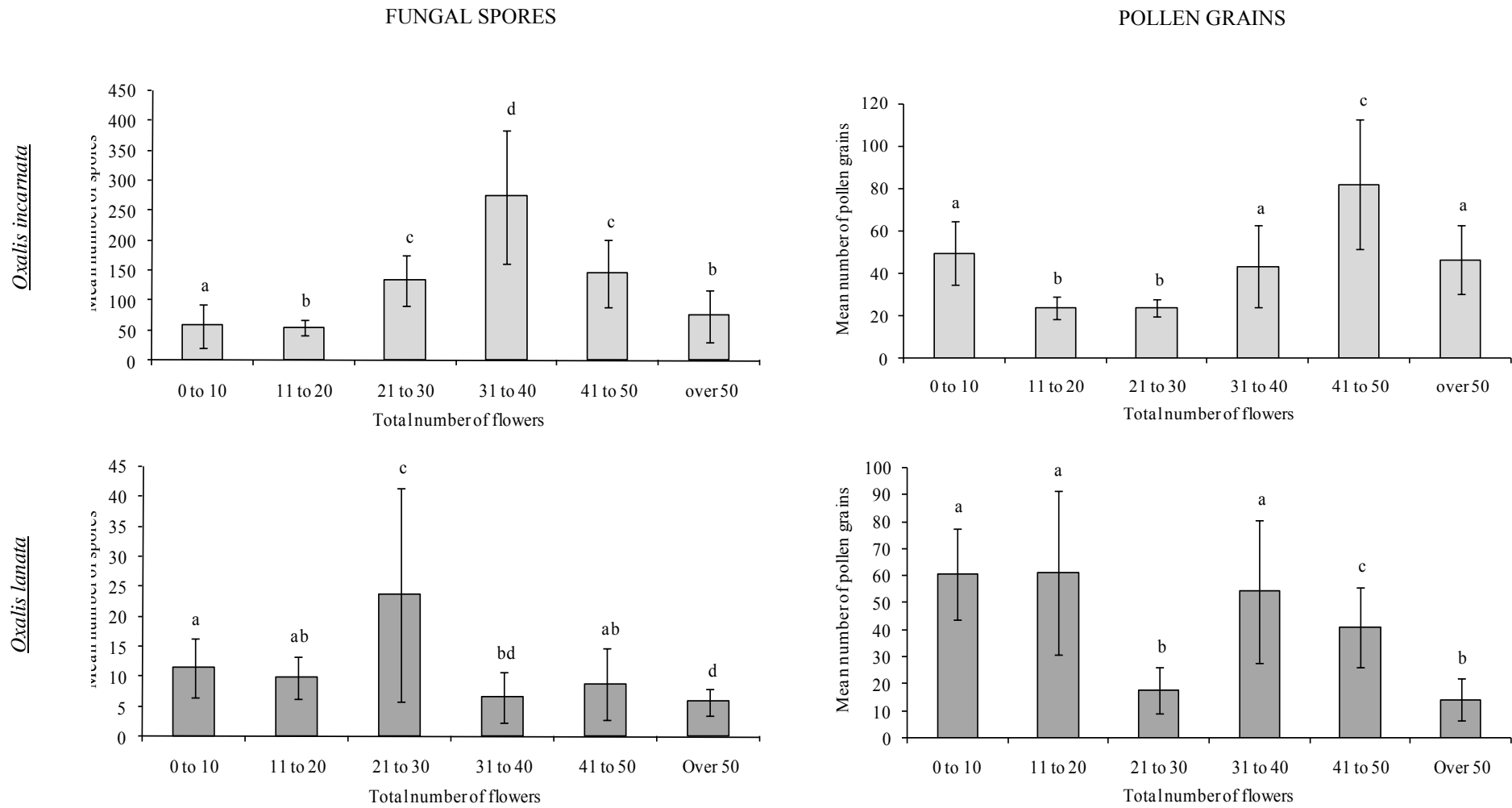
Oxalis lanata

Figure 8. Summary of the effect of flower densities on the mean number of pollen grains (right) and fungal spores (left) deposited on the stigmas of the randomly selected healthy *Oxalis incarnata* (top) and *Oxalis lanata* (bottom) flowers in the field. Results were obtained using a Generalized Linear Mixed Model with a Poisson distribution (log link function). Results are indicated as mean ± S.E., while different letters represent a statistically significant difference at $p < 0.0005$.
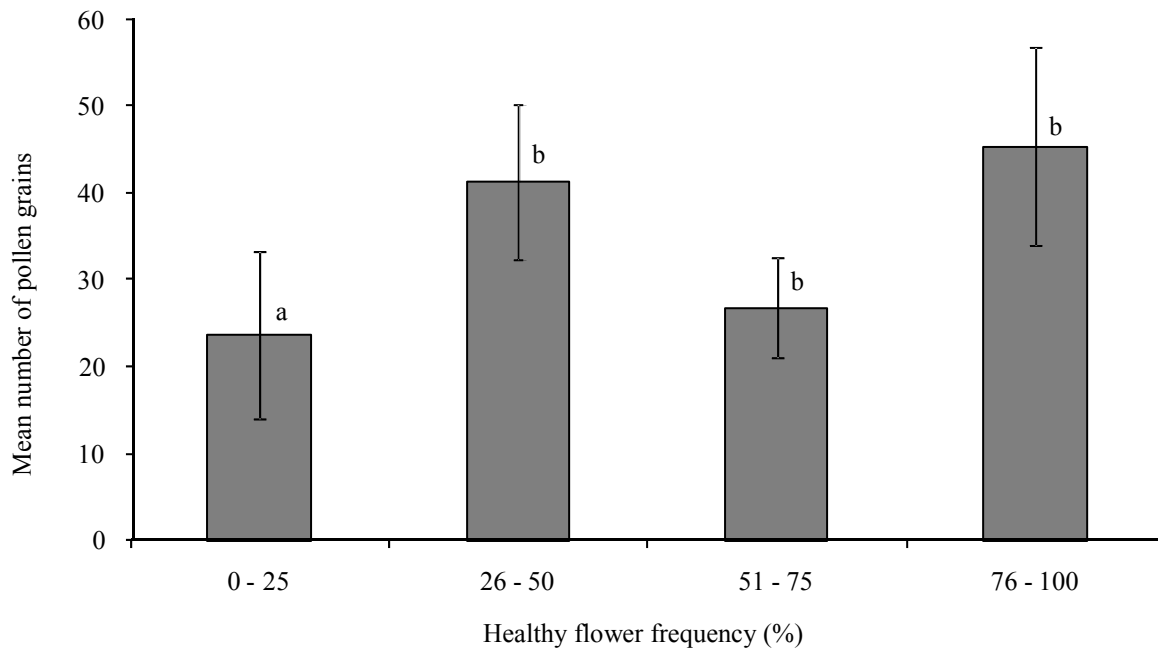
(i)

Oxalis lanata

(ii)
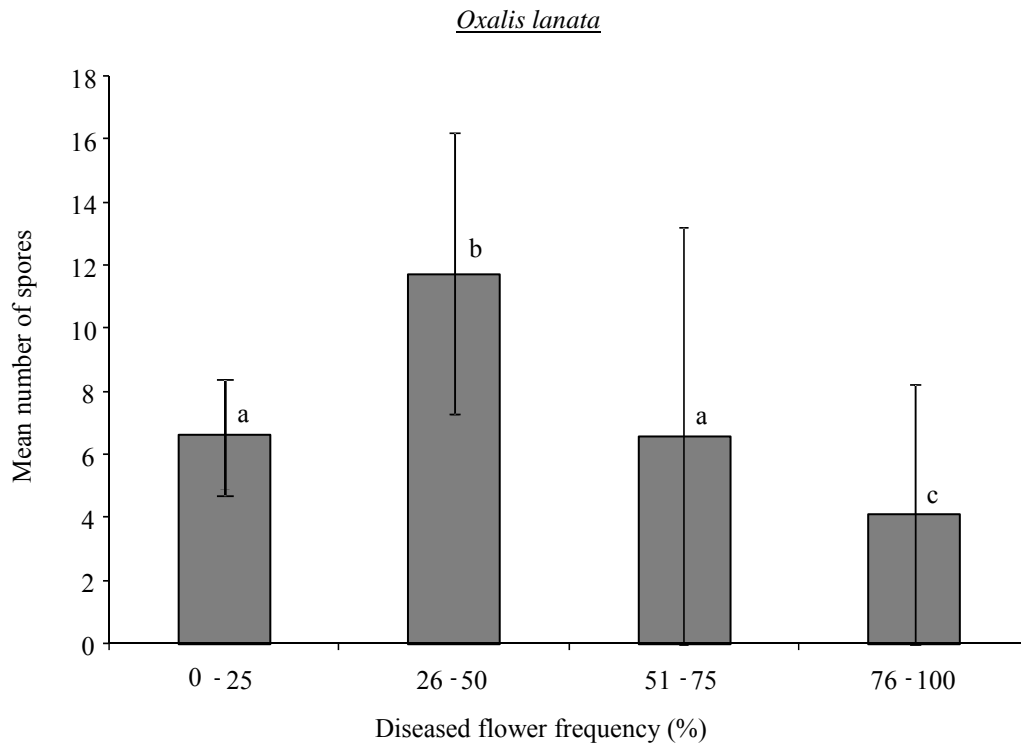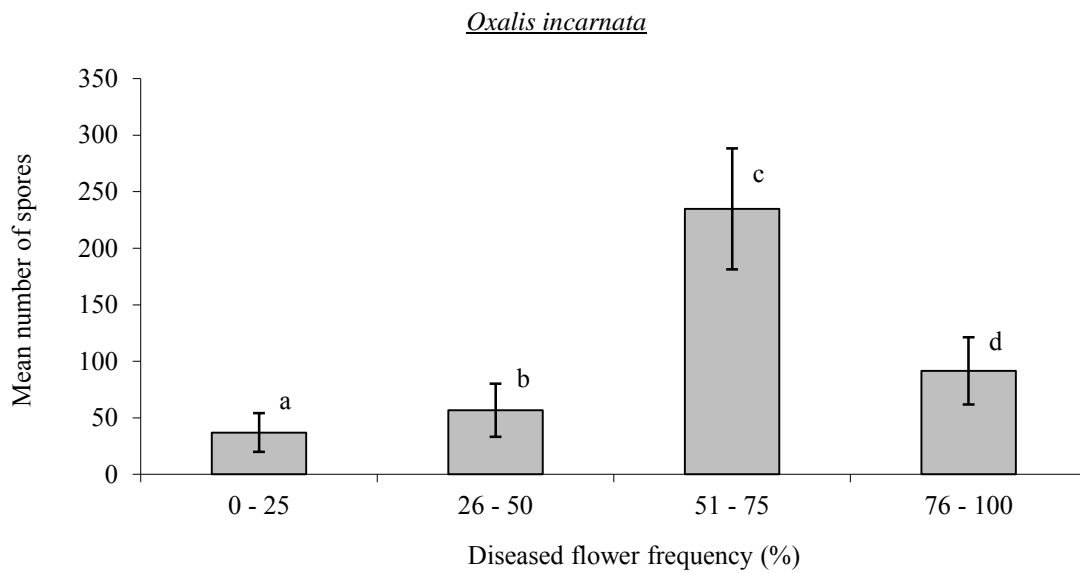
Oxalis incarnata

Figure 9. The effect of diseased flower frequency on the mean number of spores deposited on the stigmas of the randomly selected healthy **(i)** *Oxalis lanata* and **(ii)** *O. incarnata* flowers in the field. Results were obtained using a Generalized Linear Mixed Model with a Poisson distribution (identity link function). Results are indicated as mean ± S.E., while different letters represent a statistically significant difference at **(i)** p < 0.0005 and **(ii)** p < 0.0001.

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Figure 10. The effect of healthy *Oxalis incarnata* flower frequencies within a 1 meter radius on the mean number of pollen grains deposited on the stigmas of the randomly selected healthy flowers. Results were obtained from a Generalized Linear Mixed Model with a Poisson distribution (identity link function). Results are indicated as mean ± S.E., while different letters represent a statistically significant difference at $p < 0.0005$

The influence of host density and disease frequency on disease spread under standardized conditions

1. The influence of host density on disease spread

Experiments, in which the host density was manipulated, while the disease frequency remained constant, provided significant results. The distance categories of 50 and 100 centimetres between flowers resulted in the most pollen being transferred to these flowers (df = 3; Wald Statistic = 45.19; $p < 0.0001$) (Figure 11i). Significantly less pollen was transferred between flowers that were five centimetres apart than between flowers that were 50 and 100 centimetres apart. Flowers that were 200 centimetres apart received the least amount of pollen from pollinators within the square design.
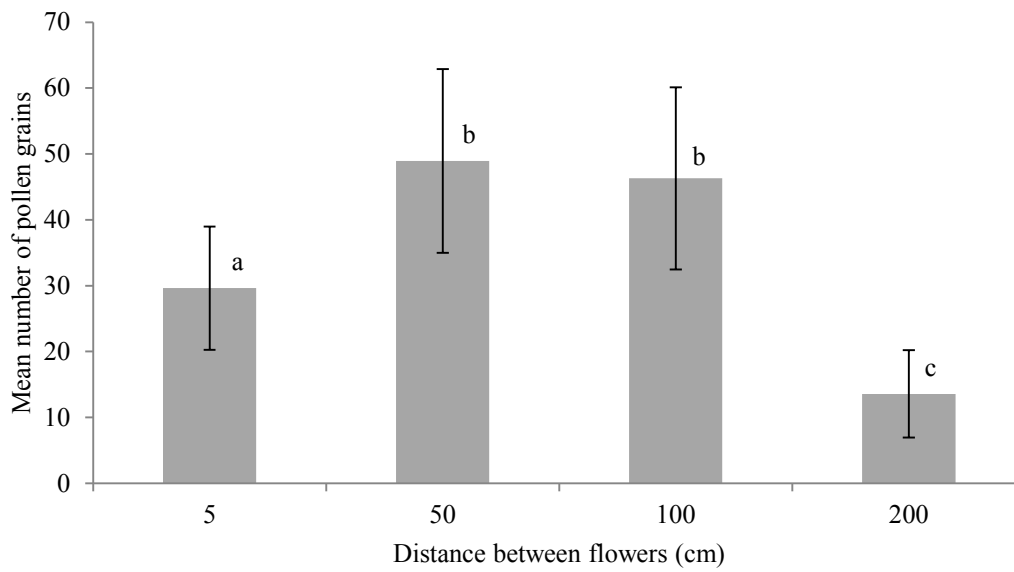
The most spores transferred to healthy flowers within the square design were observed when flowers were 100 centimetres apart (df = 3; Wald Statistic = 129.75; $p < 0.0001$) (figure 11ii). This was significantly more than when flowers were five or 50 centimetres apart. The flowers placed 200 centimetres apart had the least spores transferred to their stigmas.

2. The influence of disease frequency on disease spread

Experiments in which the influence of disease frequency on pollen and spore transfer was tested, while flower density was kept constant, yielded significant results. The mean number of pollen grains transferred to the healthy flowers within the block decreased significantly as the frequency of infected flowers within the block increased (df = 2; Wald Statistic = 146.8; $p < 0.0001$) (Figure 12i). The largest pollen transfers were experienced when 80% of the flowers within the block were healthy, while the lowest pollen transfers were seen at a disease frequency of 80%.

The mean number of spores transferred to the healthy flowers within the randomised blocks revealed an increase with an increase in disease frequency (df = 2; Wald Statistic = 210.52; $p < 0.0001$) (Figure 12ii). The largest number of spores transferred to healthy flowers within the block were seen at the highest disease frequency of 80%, while the lowest number of spores transferred were observed when the frequency of infected flowers was 20%. This is opposite to what was observed for pollen transfer within these blocks (Figure 12i).

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(i)

(ii)
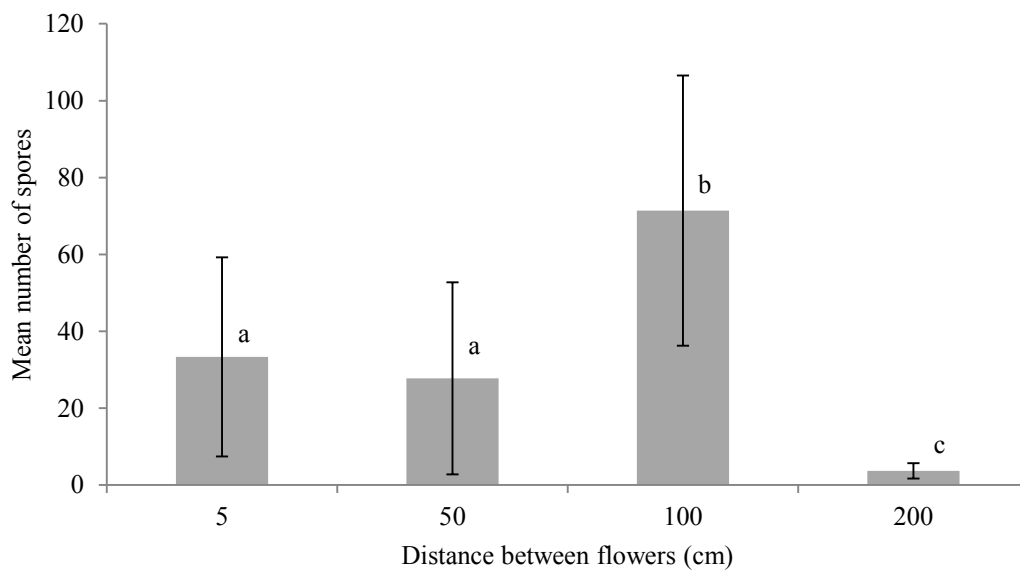
Figure 11. Summary of the mean number of **(i)** pollen grains and **(ii)** spores transferred to healthy *Oxalis lanata* flowers within a square design with varying distances between flowers. Results were obtained using a Generalized Linear Mixed Model (Poisson distribution, identity link function). Results are indicated as mean ± S.E. while different letters represent a statistically significant difference at p < 0.0001.
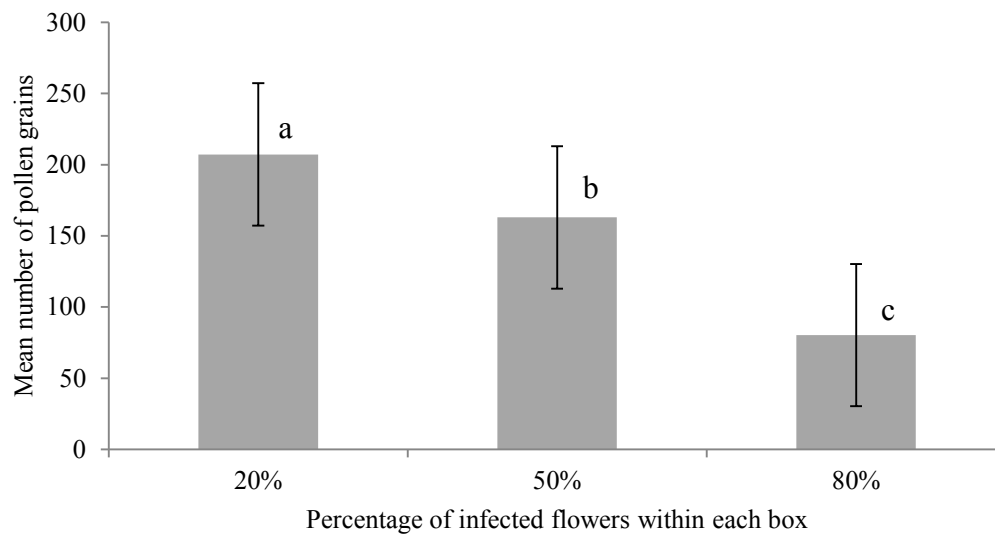
72

(i)

(ii)
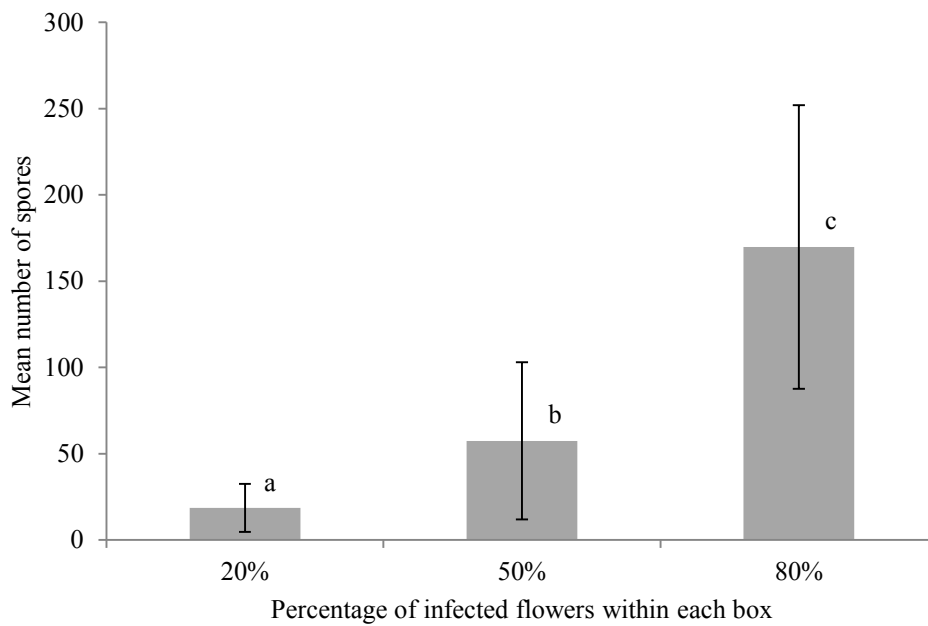
Figure 12. Summary of the mean number of **(i)** pollen grains and **(ii)** spores transferred to healthy *Oxalis lanata* flowers within a randomised block design with varying disease frequencies. Results were obtained using a Generalized Linear Mixed Model (Poisson distribution, identity link function). Results are indicated as mean ± S.E., while different letters represent a statistically significant difference at $p < 0.0001$.

Pollinator movement between flowers of different disease statuses

Although *Oxalis lanata* and *O. incarnata* display differences in their habitat preferences (*O. incarnata* grows in shaded forest areas, while *O. lanata* prefers full sun to semi-shaded locations) honeybees (*Apis melifera capensis*) were found to be the main flower visitors to both species. Honeybees were seen visiting infected flowers of *O. lanata* (Figure 13) and *O. incarnata* and transporting fungal spores between flowers during a single foraging run. In addition to the honeybees, solitary bees were also observed visiting *O. incarnata* individuals. It is important to note that pollinator visitations were low at all sites.

Overall, visitors showed no significant preference for visiting either healthy or infected flowers (Table 2). However, honeybees showed a significant tendency to visit flowers of the same disease status (Table 3). Six of the nine honeybees observed showed a repeated preference to visit flowers of the same disease status, whether healthy or diseased, instead of oscillating between healthy and diseased flowers (Table 3). Solitary bees displayed no significant preference to keep to flowers of the same disease status or to oscillate between flowers of different disease status.
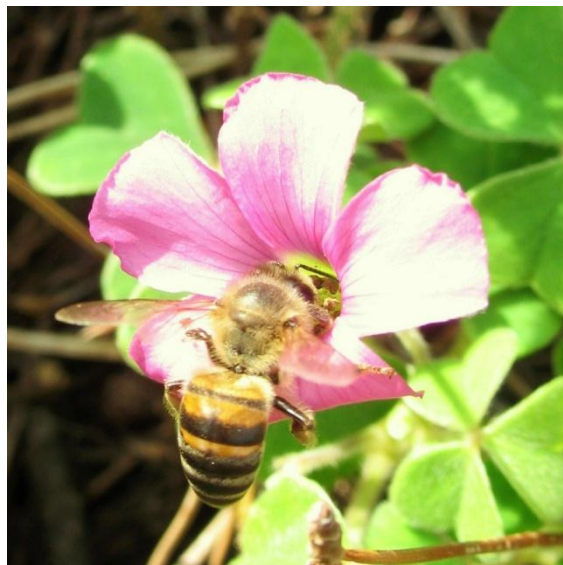
Figure 13. A honeybee (*Apis melifera capensis*) visiting a diseased *Oxalis lanata* flower during a foraging run through the Brandwacht population, Stellenbosch.

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Table 2. Summary of the G- statistics analyses of the visitation patterns of bees on healthy and infected flowers of *Oxalis lanata* and *O. incarnata*. Significance was determined from $\chi2$-square distribution with 1 degree of freedom. NS = not significant.

Pollinator	Total observed healthy visits	Total expected healthy visits	Total observed infected visits	Total expected infected visits	G - value	Significance level
Honeybee # 1	13.00	13.00	4.00	4.00	0.00	NS
Honeybee # 2	19.00	19.46	4.00	3.57	0.00	NS
Honeybee # 3	5.00	5.83	29.00	28.17	0.15	NS
Honeybee # 4	2.00	6.21	16.00	11.79	5.24	P < 0.05
Honeybee # 5	1.00	2.60	12.00	10.40	1.52	NS
Honeybee # 6	16.00	20.70	35.00	30.30	1.85	NS
Honeybee # 7	31.00	24.80	1.00	7.20	9.89	P < 0.05
Honeybee # 8	14.00	9.55	1.00	5.45	3.66	P < 0.05
Honeybee # 9	31.00	30.35	3.00	3.65	0.14	NS
Total for all honeybees	**132**	**133**	**105**	**104**	**0.02**	**NS**
Solitary bee # 1	4.00	4.40	18.00	17.60	0.05	NS
Solitary bee # 2	13.00	13.00	7.00	7.00	0.00	NS
Total for all solitary bees	**17**	**17.4**	**25**	**24.6**	**0.02**	**NS**

Table 3. Summary of the G- statistics analyses of the movement of bees between *Oxalis lanata* and *O. incarnata* flowers of the same and different disease status. Significance was determined from χ2-square distribution with 1 degree of freedom. NS = not significant.

Pollinator	Observed same status movement	Expected same status movement	Observed different status movement	Expected different status movement	G - value	Significance level
Honeybee # 1	10.00	10.00	6.00	6.00	0.00	NS
Honeybee # 2	15.00	15.83	7.00	6.16	0.17	NS
Honeybee # 3	25.00	25.24	8.00	7.76	0.01	NS
Honeybee # 4	13.00	9.09	4.00	7.91	3.85	P < 0.05
Honeybee # 5	11.00	6.37	0.00	4.63	12.02	P < 0.05
Honeybee # 6	49.00	45.02	2.00	5.99	3.91	P < 0.05
Honeybee # 7	30.00	20.44	2.00	11.22	16.12	P < 0.05
Honeybee # 8	13.00	7.53	0.00	5.47	14.20	P < 0.05
Honeybee # 9	32.00	27.01	1.00	5.99	7.27	P < 0.05
Total for all honeybees	**198.00**	**166.53**	**30.00**	**61.13**	**25.84**	**P < 0.05**
Solitary bee # 1	17.00	15.75	4.00	5.52	0.02	NS
Solitary bee # 2	15.00	15.00	3.00	3.00	0.00	NS
Total for all solitary bees	**32.00**	**31.75**	**7.00**	**9.52**	**3.80**	**NS**

DISCUSSION

The effects of disease-transmission processes on the probability of infection of a host plant, in this case density-dependent and frequency-dependent disease transmission processes, should be viewed as two extremes of a general functional response curve (Antonovics *et al.* 1995). The process of infection would be unsuccessful without spore transmission to a potential host (Bucheli & Shykoff 1999). The most important aspect influencing spore transmission, within and among populations, is vector behaviour (Bucheli & Shykoff 1999).

In this study we measured spore and pollen deposition in response to host density and disease frequency variations under natural and standardized conditions. In addition we studied pollinator movement patterns within diseased populations of two *Oxalis* species. Host density and disease frequency effects on pollen and spore deposits were variable under natural conditions, since they are influenced by many factors. The distance between a healthy *Oxalis incarnata* flower and the nearest infected flower in the field influenced the number of spores deposited on its stigmas. We have successfully shown that when the distance between a healthy and infected *O. incarnata* flower is reduced, there is a greater likelihood of an increased quantity of spores deposited on the stigmas of the healthy flower. This result seems realistic if pollinators are the mediators of spore transfers between flowers and their trajectories were of such a nature that they visited the next nearest flower irrespective of its disease status. Interestingly, the same was not true in field populations of *O. lanata*. These differences may indicate that these two species are pollinated by different pollinators, or that pollinators react differently towards the two *Oxalis* species. Some evidence for the former was found as, in addition to honeybees, *O. incarnata* flowers were also visited by solitary bees that were never observed on *O. lanata* flowers. Additional factors may influence pollinator movements and their associated potential increase in spore transfers between flowers. Floral displays by flower sizes (*Microbotryum violaceum* infections of *Silene latifolia* (Alexander & Maltby 1990)) (Chapter 2), numbers of flowers within a given area (density) (Shykoff *et al.* 1997) and the percentage of healthy and diseased flowers within a given area (frequency) may impact on distances to be traversed by a pollinator between flowers. Nectar quality and quantity (Shykoff & Bucheli 1995) may further affect the time spent by a pollinator on a single flower.

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Large variations were observed when the influence of host densities in the field on pollen and spore deposits on healthy flowers was compared. Overall, field populations with a higher disease density appeared to be less attractive to pollinators. The significant decline in the number of spores deposited on the stigmas of the healthy flowers in this case may be due to the unattractive nature of the infected flowers to pollinators because of their reduced size (Chapter 2 & 3). Healthy individuals in these populations are in competition for the limited number of pollinators, which is reflected in their low natural seed production (Chapter 2), while in a position of increased risk to receiving fungal spores.

Disease frequency played a significant role in pollen and spore loads deposited on the stigmas of healthy *Oxalis incarnata* flowers in the field, while the frequency of infected flowers influenced the number of spores deposited on the healthy *O. lanata* flowers. A disease frequency of between 26% and 50% resulted in the largest number of spores being transferred to healthy *O. lanata* flowers within the given area, while a disease frequency of between 51% and 75% resulted in the largest number of spores being transferred to healthy *O. incarnata* flowers within the given area. A further increase in disease frequency led to a decline in the number of spores transmitted to healthy flowers in the area, possibly due to the unattractive nature of diseased plants to plant visitors (Chapter 3) or due to pollinator movements within a population (as discussed below). It would be interesting to test the impact of disease frequency on spore deposition at different interspacings in the field, as this would indicate whether the disease transmission mode for *Thecaphora capensis* is density or frequency dependent (Biere & Honders 1998).

Since these assessments were conducted in the field under natural conditions it is very difficult to tease apart the specific aspects influencing the observed patterns. It would be more realistic to accept a combination of aspects participating in the process of driving pollinators to visit certain flowers more than others. Pollinator flight patterns and direction, flower attractiveness (e.g. *Microbotryum violaceum* infections of *Dianthus silvester*, Shykoff *et al.* 1997), nectar volumes and quality (Shykoff & Bucheli 1995) and flower scent (Pacini *et al.* 2003) are all important variables that cannot be controlled under natural conditions. Future research might aim to assess the effects of these variables on spore transfers independently within populations in order to ascertain the main drivers of new individuals, populations and species becoming infected by *Thecaphora capensis*.

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Standardized experimental conditions showed that host density significantly affects pollen and spore dispersal within populations of *Oxalis lanata*. The highest pollen transfers were observed when flowers were 50 cm and 100 cm apart, while the highest spore transfers were observed when flowers were 100 cm apart. An interspacing distance of 200 cm led to the lowest pollen and spore depositions. These results suggest inter-floral distances that pollinators are prepared to traverse between *Oxalis* flowers in order to receive floral rewards. The successful, yet low, dispersal of *Thecaphora capensis* spores over the 200 cm distance questions the theory that *T. capensis* is solely wind dispersed, since pollen grains were found on the same flowers. In addition, most of the reproductive structures of *O. lanata* flowers are enclosed within the floral tube. The standardized conditions ensured pollinators had not been previously exposed to *T. capensis* infected *Oxalis lanata* flowers and the learned behaviour of pollinators could therefore be ruled out. Our experimentally obtained data do not, however, rule out the possibility of multiple visitations per flower or extended visitation periods, which could influence the number of pollen grains and spores deposited during each visit (Elmqvist *et al.* 1993). This variation in visitation time might be attributed to the differences in nectar volumes, a variable that may need to be standardised in future experiments.

Disease frequency effects on pollen and spore depositions under standardized conditions yielded significant results. An increase in disease frequency led to an increase in spore depositions and a decrease in pollen depositions to the healthy *Oxalis lanata* flowers within the box. The number of pollen grains and spores transferred was high, suggesting either high pollinator availability or multiple visits per flower by single pollinators. What is important to note here is that flower interspacing was small, therefore even at a high disease density and frequency flower visitation rates and spore transfers were high. When pollinators show preference for healthy flowers and the frequency of disease in the population is high, infection rates of healthy plants are likely to increase (Real *et al.* 1992), since the probability of the pollinator accidentally visiting a diseased flower is higher. If, in contrast, the frequency of the disease is low, but pollinators show preference for diseased flowers, infection rates of healthy plants are again likely to increase, since the probability of a pollinator accidentally visiting a healthy flower is higher. The ultimate question here is whether pollinator movement patterns in an infected population are random between all flowers or if they show a preference for either healthy or infected individuals. Biere and Honders (1998) showed sharp increases in infection rates with increasing disease frequencies in areas with short inter-

plant distances for *Silene dioica* and *S. latifolia* infected by *Microbotryum violaceum.* This places infected *Oxalis lanata* populations in a vulnerable position of being driven to extinction if host and pathogen cannot co-exist for an extended period of time. Future studies may aim to determine the duration of co-existence between host and pathogen before the plant eventually dies, in order to estimate the life-span of infected populations at the current infection rate. This will direct management and mitigation measures to be put in place for the conservation of this species and other potential hosts.

Pollinators were found to play an important role in the transfer of fungal spores within populations of both *Oxalis incarnata* and *O. lanata*. Honeybees were identified as the main flower visitor of *Oxalis lanata* and *O. incarnata*, confirming results by De Jager *et al.* (2010). In this study, honeybees were also observed transferring spores of *Thecaphora capensis*, a finding that adds to the growing evidence that pollinators act as fungal spore vectors in this system (Curran *et al.* 2009). Solitary bees were only observed visiting flowers of *O. incarnata.* It is important to note that pollinator visitation rates were low in populations of *O. lanata* and *O. incarnata*, a possible reason for the low natural seed set (reproductive potential) in both species (Chapter 2). Pollinators visited only for short periods at different times on specific days, with less activity on colder days. This was an important observation, given that *Oxalis* species flower during the wet winter months of the CFR (Dreyer *at al.* 2006). Hampered pollination activity during cold weather could further reduce natural seed production.

Pollinators of *Oxalis lanata* showed no overall preference for healthy or infected flowers, but individual honeybees showed a preference to visit flowers of the same disease status and did not favour oscillating between healthy and infected flowers. Preliminary observations of solitary bees visiting *Oxalis incarnata*, in contrast, did not preferentially visit flowers of the same disease status, but randomly moved between flowers of different disease statuses. Insect pollinators, specifically honey bees, have previously been shown to visit flowers of a certain type even though other flower types may offer more rewards (Gegear & Laverty 2004). This flower constancy is not, however, a generalized response among bees (Gegear & Laverty 2004) and solitary bees might not display such strict flower constancy. This could explain the variations in spore and pollen deposits on healthy *O. incarnata* flowers in the field, since they are frequently visited by solitary bees. Flowers of two colours separated enough in bee colour space have the ability to trigger an individual constancy response (Gegear & Laverty 2004).

It would be interesting to test whether infected *Oxalis* flowers with their dark, spore-filled anthers appear different or similar to healthy flowers containing yellow pollen in bee colour space.

Although visitors did not always enter infected flowers, they could still possibly obtain fungal spores simply by landing on the petals scatted with spores by a previous visitor or disturbance. It is also possible for pollinators to make mistakes by accidentally crossing between healthy and diseased flowers during their foraging run (Roy 1994). This would have dire consequences for the healthy plants in the population, since a single visit to an infected flower could lead to large numbers of spores deposited on healthy flowers visited late in the foraging run (Shykoff & Bucheli 1995).

Here we have shown that honeybees do transfer *Oxalis* pollen and *Thecaphora capensis* spores in infected populations of *Oxalis lanata* and *O. incarnata*. The distance between infected and healthy flowers sometimes affects spore deposition, but it should be assessed in conjunction with host density and disease frequency in natural populations. Spore transfer is frequency-dependent at short plant interspacing distances and seems to be density-dependent at medium to high plant interspacing distances under standardized conditions. Results indicate that this is a complex system with many variables at work, each of which plays an important role in the transmission of pathogenic spores in the infection of new hosts, especially since different *Oxalis* species are visited by various pollinators, each with a unique movement pattern within a population.

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Chapter 5

GENERAL CONCLUSIONS

This study elucidated the long-term effects on *Oxalis* species subjected to *Thecaphora capensis* infection. Results demonstrated that the likelihood of this fungus spreading and the potential evolutionary effect on the genus *Oxalis* is host species dependant. The acquired more detailed understanding of this system can provide feedback for conservation management to minimize new infections and avoid the fungus spreading to new host species, especially since numerous extant species are rare and/or range restricted. Further monitoring of the extent of infections in the Cape Floristic Region (CFR) is essential in determining the level of conservation measures required to protect this key geophytic genus.

Thecaphora capensis has far-reaching detrimental effects on *Oxalis* individuals. Floral morphological characters of host plants are drastically reduced, while malformations probably resulting from infections have been observed. Host plant fitness has been reduced to virtually zero, while the production of fungal spores continues in the anthers of infected individuals. *Thecaphora capensis* influences evolutionary processes in diseased populations of some *Oxalis* species. Healthy individuals in diseased populations are at risk of low recruitment rates due to the reduced availability of pollen, since healthy individual numbers decline with increasing infections. In an attempt to attract greater pollinator visitation through improved floral displays, plants are at risk of contracting the disease, which may prove fatal in the longer run. Individuals therefore face a trade-off between pollinator attractiveness and disease avoidance, resulting in selection pressures on less attractive individuals displaying smaller flowers. A reduction in flower size has cascading effects on pollinator attraction and host fecundity, while it has been shown that all flowers are at risk of receiving spores on their stigmas, despite their size. Pollinators are therefore seen as the drivers of this selection pressure in some *Oxalis* species, although this is not necessarily so in all *Oxalis* host species. Species-level evaluations of sexually-transmitted diseases such as *T. capensis* are essential to avoid generalized conclusions about their spread within a population and beyond. This is important when preparing a management plan for infected populations of rare species.

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Flower visitors of *Oxalis lanata* and *O. incarnata,* however different, were identified as *Thecaphora capensis* fungal spore vectors. Interestingly, individuals of the main flower visitors (*Apis mellifera capensis*) preferred not to oscillate between flowers of different disease statuses and displayed no preference for flowers of a single disease status. This could prove to be the crux of new fungal infections if individuals accidentally did oscillate between healthy and infected flowers, thereby potentially infecting numerous individuals in one foraging run. The influence of nectar quality and quantity on pollinator visitation and its variation between healthy and diseased individuals remains an interesting topic for future research and might elucidate pollinator movement patterns within diseased populations. The frequency and density of infected individuals impacted on the transfer of fungal spores to healthy individuals within close proximity. This highlights a chain reaction effect with infections radiating from a single point, provided that fungal spore transfers by pollinators lead to the infection of new hosts. Future research should aim to identify the site of infection of new host individuals as a matter of urgency.

Populations of *Oxalis* species known to host *Thecaphora capensis,* but not yet displaying disease symptoms, should be monitored for fungal spread to these populations. Populations of *Oxalis* species not identified as hosts of *T. capensis*, especially those with a heightened conservation status, should be monitored closely for indications of the disease, and once disease symptoms appear they should be placed under strict management. Future research should also aim to explain why certain *Oxalis* populations of known hosts are prone to infection, while others within areas with different infected species appear immune. Also, a future research objective should be to evaluate the possibility of using this fungus in the biological control of *Oxalis* species that are invasive in other parts of the world.