The role of competition and mutualism in shaping microbial communities in *Protea* flowers

by

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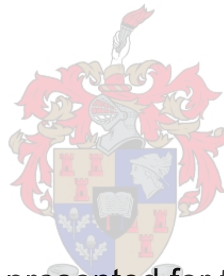

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Summary

Protea is a keystone member of the Cape floristic region and a crucial part of the ecological functioning of the fynbos ecosystem. These plants structurally dominate fynbos vegetation and they maintain large numbers of phytophagous organisms and pollinators. Their iconic inflorescences form the basis of a thriving cut flower industry, but this is under threat from pests and pathogens. *Protea* inflorescences and infructescences are also colonised by saprobic fungi that are of phytosanitary concern. These are dominated by ophiostomatoid fungi in the genera *Knoxdaviesia* and *Sporothrix* that form complex, often mutualistic, interactions with mites, pollinating insects and pollinating birds. How these fungi affect their host plants are not currently known. Also, it is unknown how they are able to dominate fungal communities within an environment optimal also for the dominance of common contaminant saprobic fungi. The fourteen described species of ophiostomatoid fungi from *Protea* inflorescences have well-defined host ranges and may even be associated with specific tissue types. Here I test various hypotheses related to fungal competitive abilities to explain patterns of association between ophiostomatoid fungal species, 'environmentally acquired' fungal taxa, and their hosts. I showed that host chemistry partially explain host exclusivity of ophiostomatoid fungi, but that differences in the actions of spore vectors may be more important. I found that without ophiostomatoid fungi, infructuscences are dominated by 'environmentally acquired' fungi such as *Penicillium*, *Cladosporium* and *Fusarium*. Even though the ophiostomatoid fungi are comparatively weak competitors, they are able to defend captured space against these when they colonise structures early and when they grow on their usual hosts. Although ophiostomatoid fungi do not increase numbers of viable seeds, they prevent seed release when recruitment will be suboptimal. This is because infructuscences containing ophiostomatoid fungi persist longer on plants. There is therefore mutual benefit for the association between *Protea* and ophiostomatoid fungi. I also uncovered complex interactions between different ophiostomatoid fungi within individual infructescences. Some species are neutral competitors and they can occupy the same tissue types within individual infructescences, while others are strong competitors on specific tissue types and can exclude competing species. Again the actions of spore vectors likely explain the persistence of weaker competitors in this scenario, but the actions of possible bacterial mutualists or other microbes should not be ignored in future studies. In this work I demonstrated the use of fungal competition studies for investigations into host relations and dispersal ecology of microbes in

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an atypical ecosystem, but these same techniques can be adapted to investigate associations between microbes in multiple other systems.

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Opsomming

Protea is ’n sleutel lid van die Kaapse Floristiese streek en ’n uiters belangrike deel van die ekologiese funksionering van die fynbos ekosisteem. Hierdie plante domineer fynbos plantegroei struktureel, en hulle onderhou groot getalle fitofae organismes en bestuiwers. Hulle ikoniese bloeiwyses vorm die basis van ’n suksesvolle snyblom industrie, maar dit word bedreig deur peste en patogene. Protea bloeiwyses en saadkeëls word ook gekoloniseer deur saprofitiese fungi wat ’n fitosanitêre probleem skep. Hierdie word gedomineer deur ophiostomatiede fungi in die genera *Knoxdaviesia* en *Sporothrix* wat komplekse, dikwels mutualistiese interaksies met myte, bestuiwer insekte en bestuiwer voëls vorm. Dis steeds onbekend hoe hierdie fungi hul gasheerplante beïnvloed. Dis verder onbekend hoe hulle in staat is om fungi gemeenskappe te domineer binne ’n omgewing wat ook optimaal is vir dominansie deur algemene, kontaminerende saprofitiese fungi. Die veertien beskryfde spesies van ophiostomatiede fungi vanaf Protea bloeiwyses het goed gedefinieerde gasheer reekse en mag selfs met spesifieke weefseltipes geassosieer wees. Hier toets ek verskeie hipoteses wat verban hou met fungi se kompeterende vermoëns om die patrone van assosiasie tussen ophiostomatiese fungs spesies, kontaminerende fungus taxa en hulle gashere te verduidelik. Ek wys dat gasheer chemie gasheer eksklusiwiteit van ophiostomatiede fungi ten dele verduidelik, maar dat verskille in die aksies van spoorvektore meer belangrik mag wees. Ek het gevind dat sonder ophiostomatiede fungi, bloeiwyses gedomineer word deur kontaminerende fungis soos *Penicillium*, *Cladosporium* en *Fusarium.* Al is die ophiostomatiede fungi vergelykend swak kompeteerders, is hulle in staat om geannekseerde ruimte te beskerm teen hierdie genera wanneer hulle strukture vroeg koloniseer en wanneer hulle op hulle gewone gashere groei. Alhoewel ophiostomatiede fungi nie die aantal lewensvatbare sade vermeerder nie, verhoed hulle saadvrystelling wanneer suksesvolle ontkieming suboptimaal is. Daar is dus mutualistiese voordeel vir die assosiasie tussen Protea en ophiostomatiede fungi. Ek het ook die komplekse interaksies tussen verskillende ophiostomatiede fungi binne individuele saadkeëls ontrafel. Sommige spesie is neutrale kompeteerders en hulle kan dieselfde weefseltipes binne individuele saadkeëls bewoon, terwyl ander sterk kompeteerders is op spesifieke weefseltipes en kan kompeterende spesie uitsluit. Weereens verduidelik die aksie van spoorvektore waarskynlik die behoud van swakker kompeteerders in hierdie senario, maar die aksies van moontlike bakteriële mutualiste of ander mikrobe moet nie geïgnoreer word in toekomstige studies nie. In hierdie werk het ek gedemonstreer hoe fungus kompetisie studies in ondersoeke na

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gasheerverwantskappe en verspreidingsekologie van mikrobe in ŉ atipiese sisteem gebruik kan word, maar hierdie selfde tegnieke kan aangepas word om assosiasies tussen mikrobe in verkeie ander sisteme te ondersoek.

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Chapter 2: Early colonization helps weaker competitors dominate saprobic fungal communities in *Protea* flowers, to the possible benefit of their hosts

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Chapter 2: Early colonization helps weaker competitors dominate saprobic fungal communities in *Protea* flowers, to the possible benefit of their hosts.

Table 1: ANOVA statistics for tests of deviation from linearity in relationships between the areas occupied by competing fungal species in a de Wit replacement series on media prepared from *P. repens* and *P. neriifolia* host tissues. The competitive influence of each separate species in an interacting pair, or relative crowding coefficient (RCC) and the product of the RCC values of the interacting pairs (in brackets) are also provided. *df* = Degrees of freedom, SS = Sum of squares, MS = Mean square.

Chapter 3: Co-occupancy of a restricted niche by ecologically similar fungi.

Table 1: ANOVA statistics for tests of deviation from linearity in relationships between the areas occupied by competing fungal species in a de Wit replacement series on media prepared from senescent tissues within infructescences of *P. repens* and *P. neriifolia*. The competitive influence of each separate species in an interacting pair, or relative crowding coefficient (RCC) and the product of the RCC values of the interacting pairs (in brackets) are also provided. *df* = Degrees of freedom, SS = Sum of squares, MS = Mean square.

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Introduction

The Cape floristic region (CFR), as defined by Goldblatt & Manning (2002), is situated in the southwestern part of the African continent in an area of 90 000 km^2. It displays a species-level endemism of 68.8%, while genus- and family-level endemism is so high that it has been described as one of 6 global Floral Kingdoms (Good 1947; Takhtajan 1986), and it has been listed as one of 25 global biodiversity hotspots (Myers *et al*. 2000). It has a Mediterranean climate characterised by wet, cold winters and hot, dry summers. The base substrate is sandstone-derived soils, which are nutrient poor, well leached and acidic (Linder 2003). The Fynbos Biome is one of four biomes recognized in the Greater Cape Floristic Region (Bergh *et al.* 2014; Born *et al.* 2007), and includes Fynbos, Strandveld and Renosterveld vegetation types (Bergh *et al.* 2014). Fynbos vegetation is defined by the co-occurrence of proteoid, ericoid and restionoid elements (Linder 2003). Defining families include the Proteaceae, Ericaceae and Restionaceae, while the vegetation is also characterized by unusually high numbers of geophytes, mostly belonging to the Iridaceae (Manning & Goldblatt 2013).

The Proteaceae is one of the most important plant families in the Southern Hemisphere, including >1700 species distributed in South Africa, Australia and South America (Rebelo 1995; Valente *et al*. 2010). Although members of Proteaceae are also found in tropical rainforests, sclerophyllous forests and open shrublands, the family is most prominent in the Mediterranean ecoregions of South Africa and southwestern Australia (Reyes *et al*. 2015). This plant family is considered a flagship of conservation and has drawn global attention for biodiversity research and conservation (Schurr *et al*. 2012). However, members of the Proteaceae are also economically important in, for example, the South African cut flower industry (Turpie *et al*. 2003). In fact, South Africa is one of the biggest producers of commercially cultivated *Protea* L., *Leucospermum* R.Br. and *Leucadendron* R.Br. flowers (Littlejohn 2000, 2001). Other African Proteaceae genera commonly used in the cut flower

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industry includes *Serruria* Burm. ex Salisb., *Mimetes* Salisb., *Aulax* Berg. and *Paranomus* Munroe & Mutuura (Coetzee & Littlejohn 2007; Conradie & Knoesen 2010; Littlejohn 2001). The cut flower industry is one of the most important contributing sectors to the economy of the Western Cape Province and creates thousands of jobs (Van Rooyen & Van Rooyen 1998; Conradie & Knonsen 2010). Internationally, South Africa ranks 17th in terms of cut flower exports and is valued at R400 million per year (Van Rooyen & Van Rooyen 1998).

Protea (Fig. 1) is the largest African Proteaceae genus (Rebelo 1995; Valente *et al.* 2010), including about 400 species (Rebelo 1995). Of these, about 360 occur in southern Africa, and more than 330 species are present in the Fynbos Biome (Rebelo 1995). *Protea* species are trees, shrubs or creepers, all with leathery, sclerophyllous leaves (Rebelo 1995). Flowers are small and inconspicuous, but are borne in large inflorescences surrounded by very colourful bracts (Fig. 1). After seed formation, the inflorescences of many species mature to form fire resistant woody cone-like structures (infructuscences; Fig. 1). These *Protea* species are serotinous, as the seeds are stored above ground and their release is triggered by fire (Bond 1984; Rebelo 1995).

Protea is crucial to the ecological functioning of the Fynbos Biome, and is regarded as a keystone genus (Cowling & Holmes 1992) that often structurally dominate plant communities (Fig. 1) and a large number of other organisms are directly or indirectly affected by their presence. This also leads to numerous problems in terms of pests and diseases associated with this genus in cultivation, as the plants are grown in areas where native antagonistic organisms abound. Some of the best known biotic interactions between *Protea* and other organisms are summarized below, but this remains an understudied research topic.

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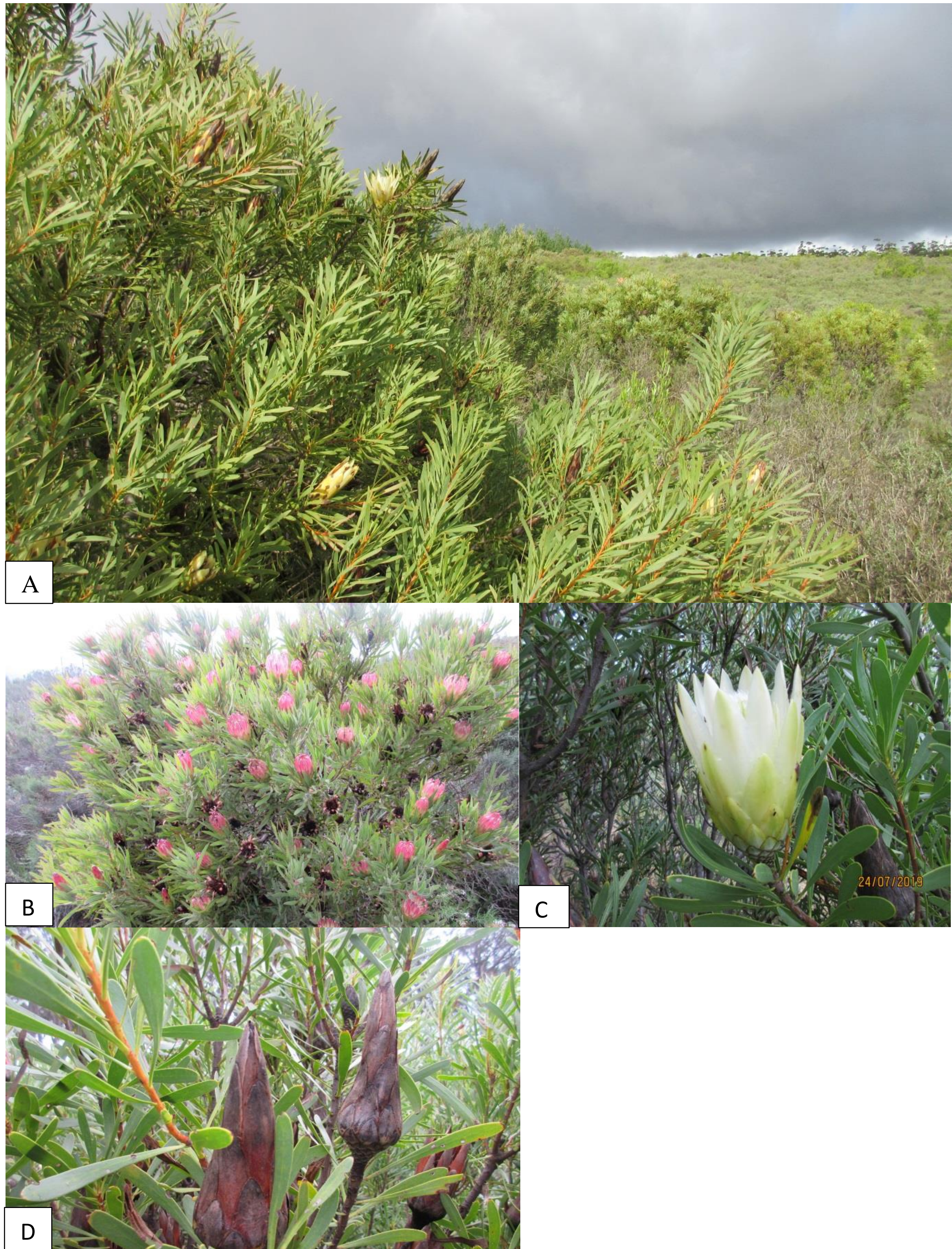

Figure 1: (A) - *Protea* population in Stellenbosch, Western Cape Province, South Africa. (B) - *Protea neriifolia* inflorescences. (C) - *Protea repens* inflorescence. (D) - *Protea repens* infructuscences

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Interactions between *Protea* and associated organisms

Interactions with other plants

Protea species are strong competitors against other plants in the fynbos. They are able to outcompete other species, including other Proteaceae, in terms of growth rate, reproduction, fire survival and seedling development (Yeaton & Bond 1991). *Protea* often dominates the overstory vegetation and provides ecosystem services such as provision of water, carbon, nutrient cycling and resources for pollinators and herbivores (Schurr *et al*. 2012). Overstory *Protea* plants often maintain fynbos communities, as they influence the growth of the understory plants, which contributes in the maintenance of plant species richness (Vlok & Yeaton 1999). Overstory *Protea* species can be vital for the protection of some understorey plants from fires (Vlok & Yeaton 1999). After fires, overstory *Protea* plants affect the regrowth of understory plants in terms of seed germination and seedling development (Vlok & Yeaton 2000). Conversely, they can also cause a decline in understory species richness, as they outcompete weaker competitors for resources such as sunlight (Bond & Ladd 2001; Yeaton & Bond 1991).

Unfortunately, *Protea* is also under considerable pressure from invasive alien plants in the fynbos (Holmes & Cowling 1997). Alien species suppress and eliminate natural elements and disrupt ecological systems (Richardson *et al*. 1990). Such alien invasive species cause a decline in fynbos communities, because of reductions in fynbos species richness, cover and frequency (Holmes & Cowling 1997; Van Wilgen & Richardson 1985). For example, alien pine trees reduce indigenous species cover and richness and can eventually eliminate Proteaceae species from a region (Holmes & Cowling 1997; Musil 1993; Witkowski 1991a). *Acacia saligna* (Labill.) Wendl. also often dominates in fynbos vegetation as it easily outgrows proteoid fynbos species (Witkowski 1991b).

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Interactions with vertebrates

Protea is visited by different animal pollinators, including birds, mammals and insects (Schmid *et al*. 2015). Arguably, one of the more interesting cases involves rodents as one of the main mammal pollinators of some *Protea* species in the CFR (Turner *et al*. 2011). About five *Protea* species, including *P. subulifolia* (Salisb. ex Knight) Rourke, *P. amplexicaulis* (Salisb.) R.Br., *P. humiflora* Andrews and *P. foliosa* Rourke (Biccard & Midgley 2009; Melidonis & Peter 2015; Rourke & Wies 1977), are adapted for exclusive rodent pollination (Rourke & Wies 1977). The main rodent pollinator of *Protea* species is the Cape striped field mouse (*Rhabdomys pumilio* Sparrman.). These *Protea* species produce large, open inflorescences that are typically held close to the substrate, are quite dull in colour and have a musky odour (Rebelo 1995). Rodents feed on nectar and on the involucral bracts of these inflorescences, and often create burrows close to these food sources (Rourke & Wies 1977). Therefore, typically the population size of the Cape striped field mouse is large near populations of these hosts. During feeding, the rodents accumulate pollen on their fur and transfer this between individual plants, enforcing pollination (van Tets 1997). Interestingly, *R. pumilio* is not *Protea*-species constant, but is known to also visit many other *Protea* species, including those seemingly adapted for pollination by other animals (Melidonis & Peter 2015). They also may be involved in the pollination of other Proteaceae taxa such as *Leucadendron arenarium* Rycroft and *L. modestrum* I. Williams (Rourke & Wies 1977). Other rodent species that may also be involved in *Protea* pollination include *Dendromus melanotis* Smith. (Climbing mouse), *Leggada minutoides* Smith. (Dwarf mouse), *Otomys irroratus* Brants. (Vlei otomys) and *Acomys subspinosus* Waterhouse. (Cape spiny mouse) (Melidonis & Peter 2015; Rourke & Wies 1977; Wies *et al.* 1983), *Myomyscus verreauxi* Smith. and *Aethomys namaquensis* Smith. (Biccard & Midgley 2009; Melidonis & Peter 2015) and *Mus minutoides* Smith. and *Praomys verreauxi* Smith. (Melidonis & Peter 2015).

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Rodents also feed on seeds and seedlings of *Protea* species, causing a low seed density and reduced germination success. This is especially evident in old fields (that have not burnt for a long time) as these house a high density of rodents compared to recently burnt areas (Bond 1984; Botha & Le Maitre 1992). It has been shown that rodents can disperse seeds far from mother plants, where they can later germinate in reduced competition (Botha & Pauw 2017). For example, the Cape spiny mouse can disperse seeds of *Leucadendron sessile* R. Br., *L. laureolum* (Lam). Fourc. and *L. conocarpodendron* (L.) H. Buek (Midgley *et al*. 2002; Rusch *et al*. 2013a). Rodents appear to actively select seeds with softer seed coats, which potentially drives the evolution of hard seed coats in some Proteaceae species (Rusch *et al*. 2013a, 2014).

Inflorescences of various Proteaceae are also often destroyed by baboons (*Chacma baboons* Kerr.). Omnivorous baboons have a wide dietary range and often feed on inflorescences of species such as *L. conocarpodendron* (L.) H.Buek., *L. hypophyllocarpodendron* (L.) Druce., *Mimetes fimbriifolius* Salisb. ex Knight., *Protea scolymocephala* Reichard, *P. humiflora* and *P. repens* L. They break the entire inflorescence open and drink the copious amounts of nectar produced, but may often also destroy inflorescence buds in search of insects (Botha & Pauw 2017). In addition, large spotted genets (*Genetta trigrina* Schreber.) and the Cape grey mangooses (*Galarella pulverulenta* Wagner.) have also been seen to visit *Protea* species (Steenhuisen *et al*. 2015). *Protea* may therefore present important carbohydrate and protein sources to these animals in this environment.

A large number of *Protea* species, including *P. lauriifolia* Thunberg, Carl Peter., *P. magnifica* Andrews, Henry Charles., *P. eximia* (Knight) Fourc., *P. compacta* R.Br., *P. repens*, *P. punctata* Meisn. and *P. longifolia* Andrews (Schmid *et al*. 2015), are mainly visited by nectar and sugar feeding birds. This despite the fact that the CFR is comparatively species poor in terms of bird fauna (Rebelo 1992). The main *Protea*-pollinating birds in the CFR are sunbirds (*Nectarinia famosa* L.) and Cape sugarbirds (*Promerops cafer* L.) (Rourke

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& Weis 1977; Weis *et al*. 1983). They visit flowers of many different Proteaceae species, but usually those that are more visible to them by displaying inflorescence colours of longer wavelength light at the top of fairly large bushes and trees (Weis *et al*. 1983 – unlike many insects birds can see red, orange and pink). Bird-pollinated *Protea* species are also characterised by producing long pollen presenters and copious amounts of nectar (Hargreaves *et al*. 2004). Bird pollination in the Proteaceae is not restricted to *Protea*, but also occurs in genera such as *Leucospermum* and *Mimetes* (Steenhuisen & Johnson 2012a). In addition to providing food, *Protea* often also provide nesting sites for these birds in a landscape devoid of larger trees.

Interactions with invertebrates

Protea species are associated with a variety of insects that visit them for different reasons. Most insects visit *Protea* species for food, and in the process may act as pollinators (Coetzee 1986; Gess 1968). Insect visitors can be categorized into three groups (a) flower visitors (b) leaf feeders and miners and (c) borers (stem and seed borers) (Coetzee & Latsky 1986). Most *Protea* species in the northern parts of South Africa, including *P. caffra* Meisn. *P. simplex* E. Phillips., *P. dracomontata* Beard. and *P. welwitschii* Engl., are primarily pollinated by insects. Beetles are their most common pollinators, and visit them in large numbers, leading to high abundance and massive pollen accumulation (Johnson & Nicolson 2001; Steenhuisen & Johnson 2012b). In the CFR, beetles are also commonly associated with *Protea* species. *Trichostetha fascicularis* Donovan. (Scarabaeidae: Cetoniinae: Cetoniini), the green *Protea* beetle, for example, is endemic to the CFR and is a well-known pollinator of Proteaceae in the southwestern Cape (Johnson & Nicolson 2001). Other beetles, such as monkey beetles (Scarabaeidae: Melolonthinae: Hopliini), leaf beetles (Chrysomelidae) and *Genuchus*

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hottentottus Fabricius. (Scarabaeidae: Cetoniinae), are also known to visit and pollinate members of the Proteaceae in the CFR. This diverse group of arthropods displays high levels of species diversity, and require more specific plant-association studies (Johnson & Nicolson 2001; Picker & Midgley 1996). In addition to beetles, honeybees (*Apis mellifera* L.) also frequent the flowers of some *Protea* species in large numbers. The high abundance of these bees visiting *P. repens* and *P. punctata* suggested that they may be one of the main pollinators of these taxa (Geerts & Pauw 2011; Johnson *et al*. 2012). Other pollinators such as nemestrinid flies *Prosoeca longipennis* Loew., *Prosoeca westermanni* Wiedemann. and the nymphalid butterfly *Aeropetes tulbaghia* L. are known to make regular contact with pollen and stigmas while feeding on nectar of *P. punctata* (Johnson *et al*. 2012).

Most insects known to feed on Proteaceae also cause tremendous damage to the plants they visit (Coetzee *et al*. 1997; Wright 2002; Wright & Saunderson 1993). Cape Proteaceae is highly infested by endophagous insects (Wright & Samways 2000). The larvae of endophagous insects such as those of Coleoptera and Lepidoptera are especially destructive to seeds in infructescences and may have a substantial influence on *Protea* population dynamics (Coetzee & Giliomee 1987a; Wright 1994). The larvae of *Genuchus hottentottus* (Scarabaeidae: Cetoninae), *Spenoptera* spp. (Coleoptera: Buprestidae), *Euderes lineicollis* Weid. (Coleoptera: Curculionidae), *Cryptolechia ammopleura* Meyrick. (Lepidoptera: Oecopboridae), *Argyroploce* sp. (Lepidoptera: Olethreutidae), *Tinea* sp. (Lepidoptera: Tineidae), *Bostra conspicualis* Warren. (Lepidoptera: Pyralidae), *Capys a1phaeus* Cramer. (Lepidoptera: Lycaeoidae) and *Resseliella proteae* Gagne. (Diptera: Cecidomyiidae) feed on different plant parts, including the seeds (Coetzee & Giliomee 1987b), which leads to reduced seed production (Myburgh *et al*. 1973, 1974; Myburgh & Rust 1975). Many insects also destroy leaf surfaces and growing apices, negatively impacting plant growth, and

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eventual flower and seed production (Coetzee 1986). Most of these larvae also pose a huge phytosanitory risk to the flower export market (Wright 2002; Wright & Saunderson 1993).

Interactions with microbes

Pathogens

Numerous pathogenic microbes have been reported to affect the flower industry negatively, especially in genera such as *Protea*, *Leucadendron* and *Leucospermum* (Taylor *et al*. 2001; Swart *et al*. 1998; 1999). They can affect different parts of Proteaceae plants, including leaves, stems and roots and create lesions in plants that cause foliage and bloom spoilage (Denman *et al*. 1999; Lombard *et al*. 2013; Table 1). Other common pathogen-induced diseases include damping-off, root and collar rot, seedling blight, cutting dieback and scab (Lombard *et al*. 2013). Below are just some of the few selected examples of the many pathogens that are known to affect *Protea* species.

Protea leaf pathogens include species such as *Coleroa senniana* (Sacc.) Arx., *Leptosphaeriae protearum* Syd. & Syd., *Mycosphaerella proteae* (Syd.) Arx., *Xenoteratosphaeria jonkershoekensis* (P.S. van Wyk, Marasas & Knox-Dav.) Quaedvl. & Crous., *Phyllachora protea* Wakef. *Teratospheria fibrillose* Syd. & Syd., *Teratospheria proteae-arborae* van Wyk, Marasas & Knox-Dav. and *Vizella interrupta* (Winter.) Hughes. (Crous & Palm 1999; Taylor & Crous 2000; van Wyk *et al*. 1975). They all produce spots on the leaf margins, followed by necrosis, and then dieback of the leaf (Swart *et al*. 1999; Van Wyk *et al*. 1975). Such leaves are later shed (Swart *et al.* 1999). Some pathogens are non-necrotic, and only change the colour of the leaves (Taylor *et al*. 1999). Leaf pathogens pose a significant phytosanitary concern for flower export markets (Wright 2002).

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Pathogens that infest the stem of the *Protea* plants typically cause cankers (ulcerous symptoms). For example, *Phomopsis saccharata* Kang., Mostert. & Crous. is known to cause significant cankers on *P. repens*, which may lead to dieback (Mostert *et al*. 2001). Various other, likely pathogenic fungal species, have been recorded from stem tissues, including *Schizophyllum commune* Fr., *Neofusicoccum ribis* (Slippers., Crous. & Wingf.) Crous., Slippers. & Phillips., *Botryosphaeria dotridea* (Moug.) Ces. & De Not., *Colletotrichum gloeosporiodes* (Penz.) Penz. & Sacc., *Pyrenophora dematioidea* (Bubák & Wróbl.) Rossman. & Hyde., *Elsione sp*. and *Epicoccum sorghinum* (Sacc.) Aveskamp., Gruyter. & Verkley. (Knox-Davies *et al*. 1987; Orffer & Knox-Davies 1989; Marincovitz *et al*. 2008). Soil borne fungal pathogens known to affect the roots of Proteaceae plants include *Botrytis cinerea* Pers., *Colletotrichum gloeosporioides* f. *gloeosporioides* (Penz.) Penz. & Sacc., *Fusarium oxysporum* Schltdl., *Phomopsis saccharata* Kang., Mostert. & Crous., *Epicoccum sorghinum* (Sacc.) Aveskamp., Gruyter. & Verkley., *Rhizoctonia solani* Kuhn. and *Phytophthora cinnamomi* Rands. (Knox-Davies *et al*. 1987). Most cause a disease called black foot rot, which includes root, collar, stem and crown rot, and it occurs mainly on *Protea* and *Leucospermum* species in South Africa (Lombard *et al*. 2013).

Table 1. Some important plant pathogenic fungi associated with the leaves, stems and roots of members of the Proteaceae.

Fungi	**Host**	**Leaf**	**Stem**	**Root**	**Reference**
Pseudoanthostomella conorum (Fuckel) Daranag., Camporesi & Hyde.	*P. neriifolia* R. Br.	*			Lee & Crous (2003)
Armillaria gallica Marxm. & Romagn.	*Leucadendron argenteum* (L). R.Br., *L. gandogeri* Schinz ex Gand., *L. grandiflorum* (Salisb.) R.Br., *P. longifolia* Andrews, *P.*			*	Coetzee *et al.* (2003)

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	eximia (Salisb. ex Knight) Fourc., *P. scolymocephala* (L.) Reichard				
Armillaria luteobubalina Watling & Kile	*P. repens* (L.) L., *P. cynaroides* (L.) L.			*	Falk & Parbery (1995)
Armillaria mellea (Vahl) Kumm.	*L. argenteum*, *L. gandogeri*, *L. grandiflorum, P. longifolia*, *P. eximia* (, *P. scolymocephala*			*	Coetzee *et al*. (2003)
Batcheloromyces leucadendri van Wyk, Marasas & Knox-Dav.	*L. gandogeri*	*			Taylor *et al.* (1999)
Batcheloromyces leucospermi Joanne E. Taylor & Crous.	*Leucospermum* sp.	*			Taylor *et al.* (1999)
Batcheloromyces proteae Marasas, P.S. van Wyk & Knox-Dav.	*P. cynaroides., P. grandiceps* Tratt., *P. magnifica* Link., *P. neriifolia* , *P. punctata* Meisn., *P. repens*	*			Taylor *et al.* (1999)
Peyronellaea obtusa (Fuckel) Aveskamp, Gruyter & Verkley.	*P. magnifica*		*		Denman *et al.* (2003)
Lasiodiplodia theobromae (pat.) Griffiths & Maubl.	*Banskia* L.f., *Grevillea* R.Br. ex Knight, *Leucospermum* R.Br., *Protea* L., *Telopea* (Sm.) R.Br.		*		Marincowitz *et al.* (2008)
Botrytis cinerea Pers.	*P. repens*	*	*		Serfontein & Knox-Davies (1990)
Ceratocystis albifundus Wingf., De Beer & Morris.	*P. cynaroides*, *P. grandiceps*, *P. gaguedi* J.F.Gmel.		*		Roux *et al.* (2007)
Colletotrichum acutatum Simmonds.	*Banskia*, *Grevillea*, *Hakea* Schrad. & J.C.Wendl., *Leucospermum*, *Leucadendron* R.Br., *Protea*, *Serruria* Burm. ex Salisb., *Telopea*		*		Lubbe *et al.* (2004)
Colletotrichum gloeosporioides f. *gloeosporioides* (Penz.) Penz. & Sacc.	*Banskia*, *Grevillea* , *Hakea*, *Leucospermum*, *Leucadendron*, *Protea*	*	*		Liu *et al.* (2013)
Fusarium oxysporum Schltdl.	*Protea aristata* E.Phillips, *P.*			*	Swart *et al.*

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	repens, *P. comptata* R.Br. *P. exima*, *P. magnifica*				(1999)
Harknessia protearum Crous & Lee.	*L. conocarpodendron* (L.) L., *Leucospermum oleifolium* R.Br., *Leucadendron* sp.			*	Lee *et al.* (2004)
Harknessia leucospermi Crous & Viljoen.	*Leucospermum praecox* Rourke, *P. lauriifolia* Thunb., *P. burchellii* Stapf, Otto., *Erica mammosa* L.	*			Lee *et al.* (2004)

Saprobes

Yeasts in nectar

Yeasts have been recorded in high abundance in plants and flowers of diverse plant species, including members of the Proteaceae (de Vega *et al*. 2009). They have also been isolated from pollinators associated with such plants (de Vega *et al*. 2014). The genus *Metchnikowia* T. Kamienski. is the most dominant nectar-associated yeast, and includes many different species (*M. gruessii* Gimenez-Jurado. and *M. koreensis* Hong., Chun., Oh & Bae., *M. reukaufii* Pitt. & Miller.) isolated from especially flowers that are associated with bees, butterflies and birds (de Vega *et al*. 2014). In *Protea*, yeasts such as *Candida* Berkh, *Corydalis* DC., *C. orthopsilosis, Hanseniaspora thailandica* Jindam., Ninomiya, Limtong, Kawas. & Nakase., *M. caudate* de Vega, Guzman & Lachance., *M. drakenbergensis* de Vega, Guzman & Lachance., *M. proteae* de Vega, Guzman & Lachance. and *Wickehamelia* sp. have been recorded from nectar of *P. dracomontata*, *P. roupelliae* Meisn., *P. subvestita* N.E. Br., *P. simplex* and *P. welwitschii* in KwaZulu-Natal, South Africa (de Vega *et al*. 2014). *Metchnikowia proteae* has also been isolated from *P. caffra* (de Vega *et al*. 2012) and from insects such as *Apis mellifera scutella* (Apidae) Lepeletier., *Atrichelaphinis trigrina* (Scarabaeidae) Olivier., *Cyrtothyrea marginalis* (Scarabaeidae) Rondani., *Drosophilid* flies (Drosopilidae) Rondani. and *Heterochelus* sp. (Scarabaeidae) Burmeister. that visit this

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Protea species. These yeasts often change nectar characteristics by utilising sugars (Herrera *et al*. 2008). In the Proteaceae, these yeasts further create volatile compounds that attract pollinators such as Centoniinae beetles to the flowers (Steenhuisen *et al*. 2010; 2012b). Yeasts therefore likely play a crucial role in *Protea* ecology as they can influence the pollination process of the plants.

Saprobic decay fungi

There is high diversity of saprobic microfungi associated with Proteaceae (Marais & Wingfield 1994; Lee *et al*. 2004; Table 2), recorded from dead twigs, leaves and from senescent inflorescences (Lee *et al*. 2004; Hyde *et al*. 2007). Saprobic fungi associated with *Protea* infructescences has been studied most extensively, and they have been found to be particularly rich in diversity and likely prerform decay functions (Marais & Wingfield 1994; Lee *et al*. 2004). There is also some overlap in taxa from different niches, for example, some saprobic fungi associated with members of the Proteaceae have also been recorded from the rhizosphere (Staffort *et al*. 2005).

Saprobic fungi are well-known for their decomposing functions and most species associated with dead leaves, twigs and material in the rhizosphere likely perform this main service (Ferrer & Gilbert 2003; Osono 2011). In the process of breaking down organic matter, they release key nutrients to the plants (Kumar *et al*. 2012). However, not all saprobic fungi are beneficial to plants. Some, e.g. decaying fungi, may be detrimental to living hosts when they colonise living plant tissues such as the wood (*e.g*. wood rot) and causes decay. *Penicillium* Link. is globally one of the most dominant and well-known fungal genera that decomposes organic matter in the soil (Schutte 1992; Visage *et al*. 2009), and this holds true in Fynbos vegetation as well (Visagie & Jacobs 2012). The following *Penicillium* species have been isolated from the senescent floral tissues in *Protea* infructescences: *P. cairnsense*

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Houbraken., Frisvad. & Samson., *P. citrium* Thom, C., *P. curticaule* Visagie. & Jacobs., *P. malacosphaerulum* Visagie. & Jacobs., *P. ortum* Visagie. & Jacobs., *P. oxalium* Currie, J.N.; Thom, C., *P. pancosmium* Houbraken., Frisvad. & Samson., *P. pasqualense* Houbraken., Frisvad. & Samson., *P. sanguifluum* (Sopp) Biourge., *P. sizovae* Baghd., *P. sumatrense* Svilv., *P. simplicissimum* (Oudem.) Thom. and *P. ubliquetum* Houbraken., Frisvad. & Samson. (Visagie *et al*. 2009, 2014, 2015). Their role in these structures is not yet understood.

Table 2. A collection of some saprobic fungi associated with different Proteaceae, with reference to the collection site on/near the host and references.

Fungus	**Host plant**	**Habitat**	**Reference**
Acremonium spp. Link.	*P. neriifolia*, *P. repens* , *P. lepidocarpendron* (L.) L.	Infructescence (Style)	Marais & Wingfield (1994)
Alternaria alternate (Fr.) Keissl.	*P. neriifolia*, *P. repens*	Infructescence (Style)	Marais & Wingfield (1994); Lee *et al.* (2005)
Aspiospora montagnei Sacc.	*Leucospermum parile* (Thunb.) Rourke	Soil (non-rhizosphere)	Allsop *et al.* (1987)
Aspergillus flavus Link	*L. parile*	Soil (non-rhizosphere)	Allsop *et al.* (1987)
Botryotrichum sp.	*L. parile*	Soil (non-rhizosphere)	Allsop *et al.* (1987)
Byssochlamys laguncularíae (C. Ram) Samson, Houbraken & Frisvad	*Hakea sericea* Schrad. & Wendl	Soil (rhizosphere)	Allsop *et al.* (1987)
Cephalotrichum stemonitis (Pers.) Nees	*P. repens*	Infructescence (Style)	Lee *et al.* (2005)
Chaetomium cochlioides Palliser	*H. sericea*	Soil (rhizosphere)	Allsop *et al.* (1987)
Cladosporium tenuissimum Cooke	*P. neriifolia*	Flowerhead (Style)	Lee *et al.* (2005)

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Cladosporium sphaerospermum Penz	*P. nitida* Mill.	Flowerhead (Style)	Lee *et al*. (2005)
Dichotomopilus indicus (Corda) Wei Wang & Samson	*P. repens*	Flowerhead (Style)	Marais & Wingfield (1994); Lee *et al*. (2005)
Dreshslera sp.	*L. parile*	Soil (non-rhizosphere)	Allsop *et al*. (1987)
Fusarium anthophilum (Braun) Wollenw.	*P. burchellii, P. nitida* *P. longifolia, P. repens* *P. magnifica, P. neriifolia*	Inflorescence (Style)	Lee *et al*. (2005)
Fusaruim spp. Link.	*L. parile*, *H. sericea*	Soil (non-rhizosphere) Soil (rhizosphere)	Allsop *et al*. (1987)
Harzia sp. Costantin.	*L. parile, H. sericea*	Inflorescence (Style)	Wingfield *et al*. (1988)
Helicoon sp.	*H. sericea*	Soil (rhizosphere)	Allsop *et al*. (1987)
Knoxdaviesia proteae (Wingf., van Wyk& Marasas) Marasas & Wingf.	*P. repens, P. neriifolia*	Inflorescence (Style)	Wingfield *et al*. (1988)
Knoxdaviesia capensis (Wingf., van Wyk & Wingf.	*P. amplexcaulis* (Salisb.) R.Br., *P. burchellii,* *P. lanceolate* E. Mey. ex Meisn., *P. lauriifolia,* *P. longifolia, P. lepidocadendron,* *P. magnifica*	Inflorescence (Style)	Wingfield & van Wyk (1993)
Penicillium canescens Sopp	*P. longifolia, P. neriifolia* *P. nitida*	Inflorescence (Style)	Lee *et al*. (2005)
Penicillium chrysogenum Thom	*P. repens*	Inflorescence (Style)	Lee *et al*. (2005)
Penicillium citreonigrum Dierckx	*H. sericea*	Soil (rhizosphere)	Allsop *et al*. (1987)
Penicillium noavae-zeelandiae Beyma	*L. parile*	Soil (non-rhizosphere)	Lee *et al*. (2005)
Penicillium raistrickii Sm	*L. parile*	Soil (non-rhizosphere)	Allsop *et al*. (1987)
Penicillium velutinum Beyma	*H. sericea*	Soil (rhizosphere)	Allsop *et al*. (1987)
Sporothrix africanum	*P. gaguedi*	Inflorescence (Style)	Marais & Wingfield

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Marais & Wingf.			(2001)
Sporothrix palmiculminatum Roets, de Beer & Wingf.	*P. repens*	Inflorescence (Style)	Roets *et al*. (2006a)
Sporothrix phasma Roets, de Beer & Wingf.	*P. lauriifolia, P. neriifolia*	Inflorescence (Style)	Roets *et al*. (2006a)
Talaromyces dendriticus (Pitt) Samson, Yilmaz, Frisvad & Seifert	*P. repens*	Inflorescence (Style)	Lee *et al*. (2005)

Saprobic ophiostomatoid fungi associated with infructescences

Protea infructescences is an important niche for saprobic fungi, but are seemingly dominated by ophiostomatoid fungi (Lee *et al*. 2005). Ophiostomatoid fungi include, amongst others, species in the genera *Ophiostoma* H. & P. Sydow and *Ceratocystis* Ellis & Halst. *Sensu lato*, and their anamorphs. They are morphologically similar in having ascospores produced in slimy masses at the apices of typically long-necked ascomata (Wingfield *et al*. 1993). They are, however, phylogenetically only distantly related (Wingfield *et al*. 1999). The first ophiostomatoid fungal species associated with *Protea* infructescences was discovered on *P. repens*, and was described as *Ceratocystiopsis proteae* Wingf, Van Wyk & Marasas. The generic demarcation of *Ceratocystiopsis* was later reconsidered due to strong morphological resemblances to genera such as *Ceratocystis* and *Ophiostoma* (Wingfield *et al*. 1988). *Ceratocystiopsis proteae* has sheathed, elongated ascospores, and anamorphs display apical wall building, both traits displayed by members of *Ophiostoma* (Minter *et al*. 1983). It is, however, sensitive to cycloheximide, which is typical for *Ceratocystis* and not of *Ophiostoma* (Hausner *et al*. 1993). Further surveys of fungi associated with *Protea* infructescence led to the description of another species with distinct *Knoxdaviesia* anamorphs similar to *C. proteae*, which was accommodated in the newly described genus *Gondwanamyces* (Wingfield & Van Wyk 1993). *Gondwanamyces capensis* Wingfield & Van Wyk. differs

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from *G. proteae* in having allointoid, non-sheathed ascospores, instead of elongate and sheathed ascospores. In addition, *G. capensis* has less distinct ostiolar hyphae than *G. proteae* (Wingfield *et al*. 1993).

Additional surveys from *Protea* infructescences revealed ophiostomatoid fungi with *Sporothrix* anamorphs that were accommodated in the genus *Ophiostoma* (*e.g. Ophiostoma splendens* G.J. Marais & M.J. Wingf.) (Marais & Wingfield 1994). *Ophiostoma* was subsequently been subdivided into many genera, and species associated with *Protea* infructescences are now all accommodated in the genus *Sporothrix* (De Beer *et al.* 2016). After adoption of the one fungus one name protocol (Crous *et al.* 2015), the genus *Gondwanamyces* was changed to *Knoxdaviesia* (De Beer *et al.* 2013). Currently fourteen ophiostomatoid species are known to occur in *Protea* infructescences (Table 3). Three have *Knoxdaviesia* anamorphs (*K. proteae, K. capensis* and *K. wingfieldii* (Roets & Dreyer) Z.W. de Beer & M.J. Wingf.) (Wingfield *et al.* 1988; Wingfield & Van Wyk 1993; Crous *et al.* 2012), while the other 11 *Protea*-associated ophiostomatoid species have *Sporothrix* anamorphs (*S. splendens*, *S. protearum* G.J. Marais & M.J. Wingf., *S. africanum* G.J. Marais & M.J. Wingf. 2001, *S. palmiculunum* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. phasma* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. gemella* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. variecibitus* Roets, Z.W. de Beer & Crous, *S. zambiensis* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. protea-sedis* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf., *S. nsini* N.P. Ngubane, L.L. Dreyer, K.C. Oberlander & F. Roets and *S. smangaliso* N.P. Ngubane, L.L. Dreyer, K.C. Oberlander & F. Roets) (Marais & Wingfield 1994, 1997, 2001; Ngubane *et al*. 2017; Roets *et al*. 2006a, 2008, 2010; Wingfield *et al*. 1988; Wingfield & van Wyk 1993).

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Table 3. A Summary of ophiostomatoid species with their associated host *Protea* species and the geographical distribution of the *Protea* hosts from which they have been reported.

Fungus	**Host plant**	**Distribution**	**References**
Knoxdaviesia proteae Wingf, Van Wyk & Marasas.	*P. repens*, *P. neriifolia*	Western and Eastern Cape Province, South Africa	Wingfield *et al.* (1988); Marincowitz *et al.* (2008)
K. capensis Wingfield & Van Wyk.	*P. lauriifolia*, *P. burchellii*, *P. coronata*, *P. lepidocarpodendron*, *P. longifolia*, *P. magnifica*, *P. neriifolia*, *P. repens*.	Western Cape Province, South Africa	Wingfield & van Wyk (1993); Aylward *et al.* (2015, 2017)
K. wingfieldii (Roets & Dreyer) de Beer & Wingf.	*P. caffra*	KwaZulu-Natal Province, South Africa	Crous *et al.* (2012)
Sporothrix splendens Marais & Wingf.	*P. repens*, *P. neriifolia*, *P. lauriifolia*, *P. lepidocarpodendron*, *P. longifolia*	Western Cape Province, South Africa	Marais & Wingfield (1994); Theron-de Bruin *et al.* (2018)
S. protearum Marais & Wingf.	*P. caffra*	Kwazulu-Natal Province, South Africa	Marais & Wingfield (1997)
S. africanum Marais & Wingf.	*P. gaguedi*		Marais & Wingfield (2001)
S. palmiculunum (Roets, de Beer & Wingf.) de Beer, Duong & Wingf.	*P. repens*	Western Cape Province, South Africa	Roets *et al.* (2006a)
S. phasma (Roets, de Beer & Wingf.) de Beer, Duong & Wingf.	*P. lauriifolia*, *P. neriifolia*, *P. longifolia*, *P. lepidocarpodendron*, *P. coronata*	Western Cape Province, South Africa	Roets *et al.* (2006a)
S. gemella (Roets, de Beer & Wingf.) de Beer, Duong & Wingf.	*P. caffra*	Gauteng Province, South Africa	Roets *et al.* (2008)
S. variecibitus Roets, de Beer & Crous	*P. repens*, *P. longifolia*	Western Cape Province, South Africa	Roets *et al.* (2008)

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S. zambiensis (Roets, de Beer & Wingf.) de Beer, Duong & M.J. Wingf.	*P. caffra*	Zambia	Roets *et al*. (2010)
S. protea-sedis (Roets, de Beer & Wingf.) de Beer, Duong & Wingf.	*P. caffra*	Zambia	Roets *et al*. (2010)
S. nsini Ngubane, Dreyer, Oberlander & Roets	*P. caffra*, *P. dracomontana*, *P. gaguedi*	Gauteng Province, South Africa; Kwazulu-Natal Province, South Africa; Mpumalanga Province, South Africa and North-West Province; South Africa	Ngubane *et al*. (2017)
S. smangaliso Ngubane, Dreyer, Oberlander & Roets	*P. dracomontana*, *P. gaguedi*	Kwazulu-Natal Province; South Africa	Ngubane *et al*. (2017)

Ophiostomatoid fungi produce sticky spores that are adapted to dispersal by arthropods (Roets *et al*. 2009). In the Northern Hemisphere, ophiostomatoid fungi are often associated with Scolytinae beetles (Klepzig & Six 2004; Six & Bentz 2003; Six 2012). Although no Scolytinae have been recorded in *Protea* (Coetzee 1986; Coetzee & Giliomee 1987(b)), proteas are visited by a wide variety of insects (Coetzee 1986; Coetzee & Giliomee 1987(a), (b); Gess 1968, Myburgh *et al*. 1973; Myburgh *et al*. 1974). Arthropods that visit *Protea* flowers and infructescences aid in the dispersal of ophiostomatoid fungi between hosts (Roets *et al*. 2007; 2008). Ophiostomatoid fungi in *Protea* infructescence are also very strongly associated with mites (Roets *et al.* 2009). Mites such as *Trichauropoda* Berlese, *Proctolaelaps vandengeri* Berlese and *Tarsonemus* Canestrini and Fonzago act as the primary vectors of ophiostomatoid fungi (Roets *et al*. 2007). There is a mutualistic association between the mites and these fungi, as the mites aid in fungal spore dispersal, while the fungi represent an important food source to the mites. Mites are able to complete their life cycle feeding exclusively on these fungi (Roets *et al*. 2007). For long-distance dispersal the mites

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are phoretic on pollinating beetles such as *Genuchus hottentottus* (Cetoniidae, Coleoptera) that visit *Protea* flowers, so the beetles act as secondary vectors of the fungal spores (Roets *et al*. 2006a; 2008; 2009).

The population genetic diversity of *K. proteae* was found to be extremely high in the Western Cape Province, suggesting very regular gene flow (Alward *et al*. 2014(b)). This is maintained over areas as far as 240 km apart. Beetles are not likely to cover such long distances, partly because of barriers such as mountains that separate these populations. It remained a riddle how these fungi could spread so easily over such vast distances (Alward *et al*. 2015). This mystery was clarified when Theron De-Bruin *et al*. (2018) showed that mites carrying ophiostomatoid fungal spores are also dispersed by birds over long distances. Mites climb onto the birds when they visit *Protea* flowers to feed on nectar (and in the process pollinate the plants), and once the birds fly off, these mites can be dispersed over very long distances. Sugarbirds and sunbirds have been reported to cover 160 km in short periods of time in search of food (Harrison *et al*. 1997).

The present study

Three *Knoxdaviesia* species (*Knoxdaviesia* capensis, *K. proteae* and *K. wingfieldii* (Wingfield *et al*. 1993; Marais & Wingfield 1994, 2001), and eleven *Sporothrix* species have been reported from *Protea* infructescences (Roets *et al*. 2010), but they differ dramatically in their host preference and specificity (Table 3). For example, *K. proteae* is specific to *P. repens*, but it has on one occasion been collected from *P. neriifolia* (Marincowitz *et al*. 2008). Thus it is dominant on *P. repens*, but may rarely also be seen on other hosts (defined as strongly host consistent). *Sporothrix splendens* is also commonly found on *P. repens* (host consistent), but it was also recently confirmed from *P. neriifolia* (Theron De-Bruin *et al*. 2018). *Knoxdaviesia*

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capensis is dominant on *P. neriifolia* (and similar proteas), but it is found on *P. repens* in low frequency (Aylward *et al*. 2015). *Sporothrix phasma* is only known from *P. neriifolia* and similar hosts, where it can be especially common. The reason for such strong host consistency in some *Protea*-associated ophiostomatoid fungi remains unknown, but it is expected that host related nutritional differences may play a role (Roets *et al*. 2012) as the vectors of these fungi are the same species of *Protea*-pollinating birds and insects (Alward *et al*. 2015; Theron De-Bruin *et al*. 2018). The first aim of this dissertation (Chapter 1) was thus to test the competitive abilities of fungi on normal and non-normal hosts to determine if specific fungal species can outcompete ecologically similar species from preferred *Protea* hosts.

The interaction between fungal species can be mutualistic, competitive or neutralistic (Rayner & Webber 1984). In mutualistic associations, both species benefit when they co-exist in the same host, while in neutralistic associations neither species will harm or benefit the other. In competitive associations, species that colonise the same host engage in competitive interactions and the stronger competitor may outcompete the weaker competitor (Klepzig *et al*. 2001). The competitive interactions of fungal species are further divided into primary resource capture (the ability to initially colonise and defend the area from competitors) and secondary resource capture (the ability to colonise an area already colonised by other fungal species) (Klepzig *et al*. 2001). I therefore set out to test the competitive interactions between *Sporothrix splendens*, *S. phasma*, *Knoxdaviesia proteae* and *K. capensis* on preferred and non-preferred hosts following the abovementioned definitions.

Interestingly, when ophiostomatoid fungi are present in a *Protea* infructescence, there appears to be an absence of non-ophiostomatoid fungi. The inverse is also true – when ophiostomatoid fungi are absent from *Protea* infructescences, such infructescences are densely colonized by non-ophiostomatoid fungi. Although never tested, this suggests that

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ophiostomatoid fungi appear to be dominant within infructescences and outcompete other fungi in this specialized niche (Roets *et al*. 2005). Aspects of the ecology of ophiostomatoid fungi are still unknown, but it has been shown that they initially utilise nectar sugars as carbon source, but can later switch to degrading cell wall components typical of saprobic fungi (Alward *et al*. 2017). They will therefore need to compete successfully against other saprobic fungi in the process. It has been speculated that the *Protea*-associated ophiostomatoid fungi may outcompete more antagonistic fungal species (*i.e.* protect seeds from pathogenic fungi and / or fungi that may lead to early release of seeds), but this has never been tested. In Chapter 2, I therefore set out to test this hypothesis by investigating the competitive abilities between ophiostomatoid fungi (*Sporothrix splendens*, *S. phasma*, *Knoxdaviesia proteae* and *K. capensis*) and various 'contaminant' fungi. I first identified the most common saprobic fungi in *Protea* infructescences in the absence of ophiostomatoid fungi and determined whether they may have a negative effect on seed viability or infructescence longevity. Hereafter I conducted competition studies as explained above to determine to what extent the ophiostomatoid fungi may outcompete possible detrimental fungi from these important plant structures.

Although the *Protea*-associated ophiostomatoid fungal species are associated with both inflorescences and infructescences, the highest diversity is found within closed infructescences (Roets *et al*. 2005). These fungi grow on various parts of these senescent infructescences including old styles, pollen presenters, perianth segments and involucral bracts (Lee *et al*. 2005). Some species seem to be restricted to one or only a few of these tissue types (Roets *et* al. 2013) and it is plausible that the maintenance of such a high diversity of ecologically similar species in such a restricted niche can be due to plant tissue specialization. In Chapter 3, I tested for competitive abilities between ophiostomatoid fungi (*Sporothrix splendens*, *S. phasma*, *Knoxdaviesia proteae* and *K. capensis*) on different tissue

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types from their preferred hosts. I first determined their growth rates on the different tissue types (bases, senescent pollen presenters, senescent unfertilised seeds and senescent bracts) and thereafter tested their competitive abilities on the different tissue types using a de Wit replacement series experiment.

Results from this study will lead to an enhanced understanding of the ecology of seemingly benign saprobic fungi that occupy this very unusual niche. The large diversity and perceived dominance of the ophiostomatoid fungi in this niche is intriguing and lends itself to testing hypotheses regarding the maintenance of diversity of ecologically similar saprobic fungi in a restricted space. The dominance of ophiostomatoid fungi in terms of competitive abilities against other harmful fungi will be beneficial to *Protea* plant communities, as they could mitigate the threat posed by 'contaminant' fungi and protect the seed viability of the plants.

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Chapter 1: Interplay between actions of spore-vectors and fungal differential competitive abilities may explain host exclusivity of saprobic ophiostomatoid fungi on *Protea* flowers.

Abstract

Protea flowers host numerous ophiostomatoid fungi from the genera *Knoxdaviesia* and *Sporothrix*. These fungi are primarily dispersed by mites that are phoretic on *Protea*-pollinating insects and birds. Flowers of different host species often contain sympatric populations of two or more different fungal species, even though their vectors are shared. For example, *P. repens* flowers are dominated by *S. splendens* and *K. proteae*, while flowers of *P. neriifolia* are dominated by *K. capensis* and *S. phasma*. Examples of cross-colonization by the fungi between different hosts are scant even though all can grow vigorously on alternative host material. Here we investigated the possible role of differential competitive abilities of the fungi on their usual and on alternative hosts to explain apparent host exclusivity observed in the field. In a de Wit replacement series experiment, *S. splendens* outcompeted all other fungal species on media prepared from its usual *P. repens* and alternative *P. neriifolia* hosts. It can also rapidly overgrow all other fungal species on both hosts. *Sporothrix phasma* could overgrow *K. proteae* on both hosts, but was not able to do so against *K. capensis*, the species with which it usually shares space within inflorescences of *P. neriifolia*. *Knoxdavesia proteae* may therefore only persist in its *P. repens* flower niche in the presence of *Sporothrix* if it colonizes a different area, or can complete its life cycle before it is overgrown. The restricted presence of *S. splendens* from hosts other than *P. repens* is more likely due to restricted movement of the spore vectors rather than weaker competitive abilities on alternative hosts. All other fungi had similar competitive abilities on both hosts, which may aid their co-existence in this niche. However, this excludes host-related differential competition as explanation for their host exclusivity. Apparent host specialization of *Protea*-associated ophiostomatoid fungi may therefore also be driven by the activities of spore vectors than only by changes in the competitive abilities of the fungi on different hosts.

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Introduction

Fungi that grow on living plant tissues are often specific towards their hosts, or a range of closely related hosts (Agrios 2005). Host specificity of pathogenic fungi may be enforced by closely co-evolved molecular recognition and detoxification systems that are often unique per interacting pair of host and fungus (Baldwin *et al*. 2006; Idnurm & Howlett 2001; Walton 1996, 2006; Zhao *et al.* 2013). This maintains boundaries between pathogenic fungal colonizers and may lead to speciation and subsequent increased regional biodiversity (Burdon & Silk 1997; McDermott & McDonald 1993). However, numerous saprobic fungal species also show strong preferences towards decaying material that originated from specific hosts. Rather than being host specific, these fungi are considered either as host-exclusive (growing on material that originated from a particular host or a restricted range of related hosts) or host-recurrent (growing predominantly on material originating from a particular host, but can also occur on material originating from other hosts in the same habitat) (Zhou & Hyde 2001). The maintenance of high levels of host-exclusivity and/or host-recurrence is more difficult to explain for saprobes than for pathogenic fungi, but are often linked to differences in substrate nutrient levels and/or physical structure (Mille-Lindblom *et al*. 2006; Osono 2011; Paulus *et al*. 2006; Tedersso *et al.* 2013; Wolfe & Pringle 2012). Such differences may lead to variability in the competitive abilities of saprobic fugal species when growing on different host material, which may explain the co-existence and diversification of a multitude of different fungal species on non-living host material (Kubicek *et al*. 2014; Zhao *et al*. 2013). In addition, succession of different fungal species on the same substrate, due to differences in competitive abilities, also leads to higher fungal diversity (Bleiker & Six 2009).

Host-exclusivity or recurrence of saprobic fungi has been extensively studied on leaf litter and other plant debris on forest floors (Ferrer & Gilbert 2003; Lodge 1997; Lodge & Cantrell 1995b). Plant-associated saprobic fungi are, however, known from many other niches. One very unusual niche is the flowers (inflorescences) and fruiting structures (infructescences) of members of the genus *Protea* (Proteaceae) in Africa. A high diversity of saprobic fungi has been documented from these structures (*e.g.* Lee *et al*. 2004, 2005; Marincowitz *et al.* 2008, Visagie *et al*. 2009), but of these, the ophiostomatoid fungi (Wingfield *et al*. 1993) have been the best studied. Ophiostomatoid fungi from *Protea* currently represents fourteen species in two genera; three species of *Knoxdavesia* in the Microascales (*K. proteae* M.J. Wingf., P.S. van Wyk & Marasas 1988, *K. capensis* M.J Wingf. & P.S. van Wyk 1993 and *K. wingfieldii*

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(Roets & Dreyer) Z.W. de Beer & M.J. Wingf. 2013) and eleven species of *Sporothrix* in the Ophiostomatales (*S. africana* G.J. Marais & M.J. Wingf. 2001, *S. palmiculminata* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. 2016, *S. phasma* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. 2016, *S. protearum* G.J. Marais &M.J. Wingf. 1997, *S. splendens* G.J. Marais & M.J. Wingf. 1994, *S. gemella* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. 2016, *S. variecibatus* Roets, Z.W. de Beer & Crous 2008, *S. zambiensis* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. 2016, *S. protea-sedis* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. 2016, *S. nsini* N.P. Ngubane, L.L. Dreyer, K.C. Oberlander & F. Roets and *S. smangaliso* N.P. Ngubane, L.L. Dreyer, K.C. Oberlander & F. Roets) (Wingfield *et al*. 1988; Wingfield & Van Wyk 1993; Marais & Wingfield 1994, 1997, 2001; Roets e*t al.* 2006a, 2008, 2010; Ngubane *et al.* 2017). Except for *S. variecibatus*, all species are confined to the inflorescences and/or infructescences of serotinous *Protea* species from Zambia in the north to the Western Cape Province of South Africa in the south. Individual *Protea* flowers are small and inconspicuous, but they are borne in large inflorescences surrounded by often very colourful involucral bracts, which aid in the attraction of insects or birds pollinators (Fig. 1A.) (Johnson & Nicolson 2001; Steenhuisen & Johnson 2012b). After seed formation, the inflorescences mature to closed, fire resistant cones (infructescences) that store seeds above-ground in serotinous *Protea* species. The cones comprise of the receptacle (base), hardened, brown and closed involucral bracts, and dead flower parts above the basal seed layer (Fig. 1B.). The flask-shaped sexual sporulating structures of ophiostomatoid fungi dominate fungal communities within these structures (Roets *et al*. 2005; Fig. 1C.).

Figure 1: (A) - inflorescence of *Protea repens*, (B) - infructescence of *Protea repens* (Roets *et al*. 2006), (C) - flask-shaped sexual sporulating structures of ophiostomatoid fungi.

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Protea-associated ophiostomatoid fungi are primarily dispersed by mites (Roets *et al.* 2007, 2008, 2011, Theron *et al.* 2018). Some of these mites have a mutualistic relationship with the fungi they carry, as they are able to complete their life cycle by feeding only on the fungus, without additional nutritional sources (Roets *et al.* 2007, Theron *et al.* 2018). The interaction between the mites and the fungi can be quite specific, as some taxa, for example *Tarsonemus* spp., have evolved spore-carrying structures to ensure dispersal of their fungal mutualists (Roets *et al*. 2007). For long-distance dispersal from the tightly enclosed infructescences, the mites rely on insects such as the *Protea*-pollinating *Genuchus hottentottus* (F.) (Cetoniidae, Coleoptera), whose larvae develop within infructescences (Roets *et al*. 2006, 2009). During the *Protea* flowering season, spore-carrying mites are also phoretic on other *Protea*-pollinating beetles (*e.g. Trichostetha* species) and pollinating sugarbirds and sunbirds (Roets *et al.* 2009b, Theron *et al.* 2018) that can disperse these over vast distances (Aylward *et al.* 2014a, b, 2015). The ophiostomatoid fungi can therefore colonise inflorescences very early in the flowering stage (as soon as the first flowers open) (Theron *et al.* 2018), which may lead to a competitive advantage over other fungal taxa in this nutritious niche.

Different *Protea*-associated ophiostomatoid fungi differ in their host exclusivity. For example, *K. proteae* has to date only been found within the infructescences and inflorescences of *P. repens* L. (Roets *et al.* 2009), while the closely related *K. capensis* is known from various *Protea* species namely *P. lauriifolia* Thunb., *P. burchellii* Stapf., *P. coronata* Curt., *P. lepidocarpodendron* L., *P. longifolia* Salisbury, *P. magnifica* Andrews and *P. neriifolia*R. Br. (Roets *et al.* 2009; Aylward *et al*. 2017). It has also been found on *P. repens*, but at very low frequency (Aylward *et al.* 2015). *Sporothrix phasma* is found within the infructescences of all aforementioned *Protea* species, accept *P. repens* (Roets *et al.* 2009). The closely related *S. splendens* is nearly exclusively found within *P. repens* infructescences, but has also been detected in *P. neriifolia* inflorescences at low frequency (Theron-de Bruin *et al*. 2018). These associations are maintained even though the mite, insect and bird vectors of these fungi are associated with all of the abovementioned *Protea* hosts, and the hosts often co-flower in sympatrically-growing populations (Nottebrock *et al.* 2016).

Different *Protea*-associated ophiostomatoid fungal species differ in their capabilities to degrade specific carbon sources, which may explain observed differences in their apparent host-exclusivity or recurrence (Aylward *et al.* 2017). For example, *Protea*-associated *Knoxdaviesia* and *Sporothrix* species grow best on tissues that originated from their usual hosts and host chemistry may therefore play a significant role in determining the level of host

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exclusivity of these fungi (Roets *et al.* 2012). Interestingly, most fungal species can also grow vigorously on tissue of *Protea* spp. other than the ones with which they usually associate (Roets *et al.* 2012). However, it is likely that the competitive abilities of these fungi would differ on substrates prepared from their preferred and non-preferred hosts, which may explain the maintenance of such a high diversity of ophiostomatoid fungi within a limited niche such as that provided within *Protea* infructescences.

The interaction between fungi can be classified into mutualistic, competitive and neutralistic (Rayner & Webber 1984). In mutualistic interactions, both fungal taxa would benefit from close association with one another, while in neutralistic interactions fungal taxa would not benefit nor be inhibited when interacting. However, when two fungal taxa colonise the same habitat, they often engage in competitive interactions for the limited resources (*e.g*. nutrients and space) with the superior competitor out-competing the weaker one (Klepzig *et al*. 2001; Wardle *et al.* 1993). The competitive abilities of competing fungi can further be divided into primary resource capture (the ability to initially colonize and defend resources from competitors) and secondary resource capture (the ability to colonize resources that were previously colonised by other fungi) (Klepzig *et al.* 2001). In the present study we focus on aspects pertaining to the competitive interactions between various ophiostomatoid fungi (*K. capensis*, *K. proteae*, *S. phasma* and *S. splendens*) in an effort to explain their observed host exclusivity. We first set out to determine whether there are detectable differences in the competitive abilities between these fungi on both their usual host *Protea* spp. and on host *Protea* spp. on which these species are not usually found. Thereafter we determined the primary resource capture capabilities of these species on usual and alternative hosts by testing their ability to initially capture and then maintain influence over a restricted resource such as the limited area within *Protea* infructescences (Roets *et al.* 2006b). For secondary resource capturing abilities we tested for the abilities of the various fungal species to capture space originally occupied by another species on usual and atypical host material. In these interactions, the most competitive species can either replace the less competitive species or the two species may simply co-exist when they have similar competitive abilities (Rayner & Webber 1984). We hypothesize that each fungal species would be a superior competitor on its own host when paired against fungi that are usually associated with other *Protea* hosts. As fungi that grow within flowers of the same *Protea* host are usually found growing sympatrically (Roets *et al.* 2005), we hypothesize that these species will have neutralistic competitive interactions. Any deviations from these hypotheses may point towards a larger

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role of fungal vectors in maintaining host exclusivity of the different ophiostomatoid fungal taxa on *Protea* than previously assumed (Roets *et al.* 2012).

Methods and Materials

Collection of ophiostomatoid fungi and preparation of experimental growth media

Isolates of ophiostomatoid fungi were collected during previous studies (Aylward *et al*. 2014, 2015) from the infructescences of *P. repens* (*K. proteae* and *S. splendens*) and *P. neriifolia* (*K. capensis* and *S. phasma*) in the Western Cape Province, South Africa. *Sporothrix phasma* was collected from Jonkershoek Nature Reserve (33˚59'24.5"S, 18˚57'25.2"E), *K. proteae* was collected from Stellenbosch mountain (-33.9466; 18.8805), *S. splendens* was collected from Betty's Bay (-34.3315; 18.9925) and *K. capensis* was collected from Betty's Bay (-34.35495; 18.90135). Five individuals, each originating from a different *Protea* infructescence, of each fungal species were isolated by transferring spores produced at the tips of ascomata to Petri dishes containing malt extract agar (MEA, Biolab, Midrand, South Africa) and subsequently purified via single spore isolation. All fungal cultures were maintained at 4˚C in the dark on malt extract agar (MEA, Biolab, Midrand, South Africa) until experimental use.

Growth media for experimental use was prepared from the two host species, *P. repens* and *P. neriifolia*, following methods detailed in Roets *et al*. (2012). In short, infructescences of both *Protea* species were collected from the Jonkershoek Nature Reserve, Stellenbosch. Dead floral parts were removed from within infructescences, dried and ground up into a fine powder. One litre of water-based growth medium contained 300 ml prepared *Protea* tissue (powder) and 1.5 % MEA. Media were autoclaved at 115˚C for 20 min and poured into 90 mm Petri dishes that acted as fungal competition arena

Differential competition between fungi on usual and alternative hosts

A de Wit replacement series experimental design (Adee *et al*. 1990; Klepzig 1998; Klepzig & Wilkens 1997; Wilson & Lindow 1994) was used to test the competitive abilities between all pair-wise combinations of the four fungal species (*K. capensis*, *S. phasma*, *S. splendens*, *K. proteae*) on media prepared from both *P. repens* and *P. neriifolia* host tissues. The modified

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de Wit replacement series entailed varying initial population ratios (as proportion of inoculum) of two interacting species. The outcome of these interactions (*e.g.* total area occupied by one fungus competing with another after a defined time) was expressed as a linear function of the initial proportion inoculum when both interacting species have similar competitive abilities. Significant deviation from linearity indicated differential competition with one species dominating over the other. Initial inoculum in Petri dishes consisted of aseptically removed disks (0.5 cm in diameter) of actively growing fungal colonies (*ca*. 2 weeks old, MEA, 25˚C) that were placed face-down on plates (9 cm in diameter) containing media prepared from the *Protea* species in a random block design (4 x 4 cm grid) following methods oulined by Klepzig & Wilkens (1997) . The total number of disks randomly placed within this grid was 16 (Fig. 2). We used the following inoculum ratios of interacting species A vs. interacting species B: 0:1 (16 disks species B), 0.25:0.75 (4 disks sp. A and 12 disks sp. B), 0.5:0.5 (1:1) (8 disks sp. A and 8 disks sp. B), 0.75:0.25 (12 disks sp. A and 4 disks sp. B), and 1:0 (16 disks species A). The procedure was repeated for all five ratios tested per pairwise species combination and the experiment was replicated five times per tested medium (each replicate using different fungal isolates). Plates were incubated at 25˚C in the dark for two weeks. The areas occupied by each fungus species after two weeks of interaction were determined using imageJ software (LOCI, University of Wisconsin). Deviations from linearity were calculated by performing analysis of variance (ANOVA) on log-transformed means of the area data (Wilson & Lindow 1994) in R (R Development Core Team 2013). In addition, Relative Crowding Coefficients (RCC) (De Wit 1960) were determined for all the pairwise combinations of fungi comparisons as [(mean area of species A at 1:1)/(mean area of species A at 1:0)] and [(mean area of species B at 1:1)/(mean area of species B at 1:0)]. The interacting species with a higher coefficient was regarded as dominant. If the product of the coefficients of the interacting fungal species was 1, then fungal competition was viewed as neutral. If the product of the coefficients of the interacting fungal species was less than 1, then the fungi were believed to negatively affect each other and if it was greater than 1 then the taxa benefited from growing together (Willey & Rao 1980).

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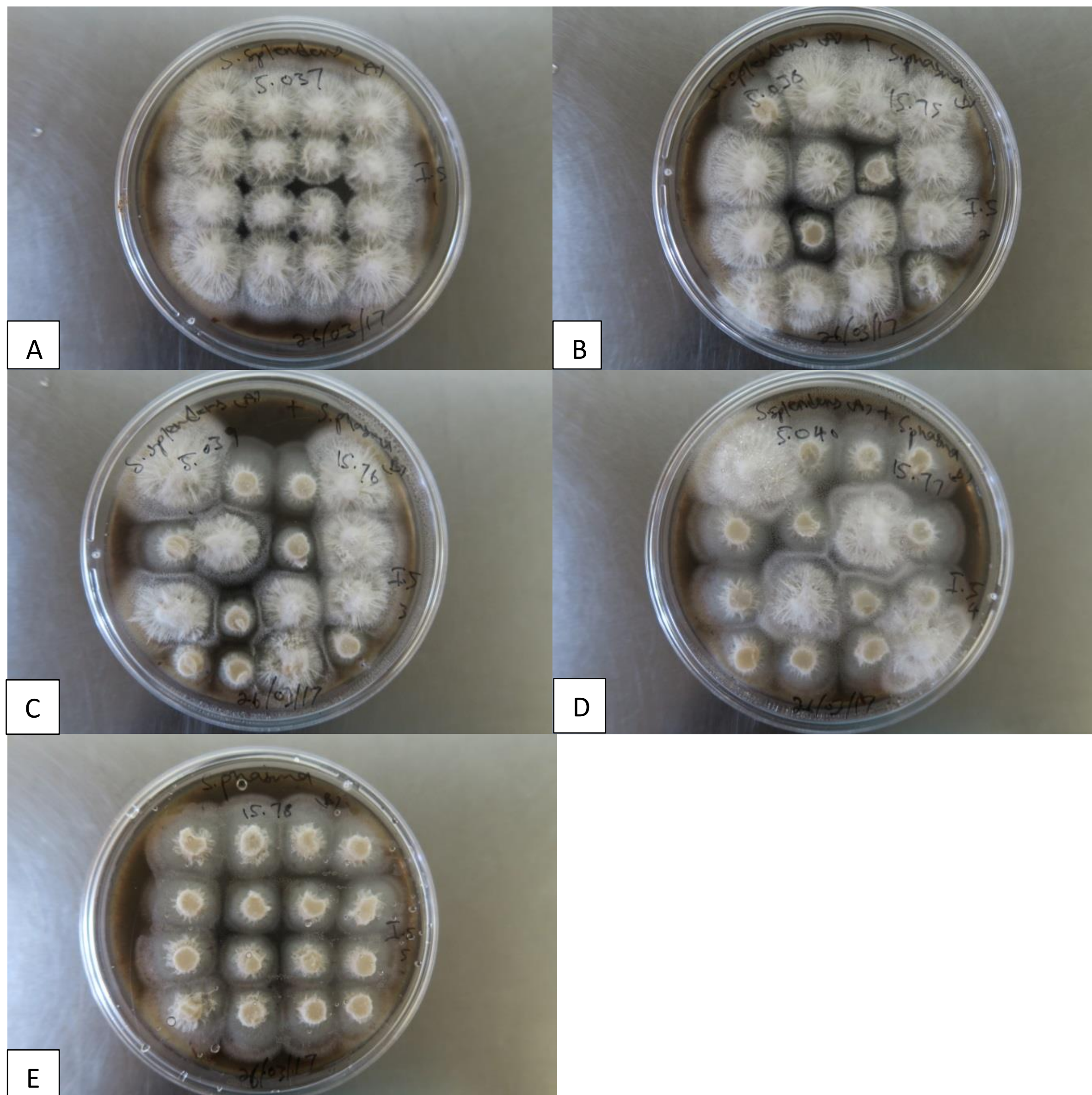

Figure 2: de Wit replacement treatment for testing competitive abilities between *S. splendens* and *S. phasma* on media prepared from *P. repens* host tissues. Inoculum ratios of interacting *S. phasma* vs. *S. splendens*: (A) - 0:1 (16 disks *S. splendens*), (B) - 0.25:0.75 (4 disks *S. phasma* and 12 disks *S. splendens*), (C) - 0.5:0.5 (1:1) (8 disks *S. phasma* and 8 disks *S. splendens* B), (D) - 0.75:0.25 (12 disks *S. phasma* and 4 disks *S. splendens*), and (E) - 1:0 (16 disks *S. phasma*) was used.

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Primary resource capture on usual and alternative hosts

Previously described techniques (Klepzig 1998; Klepzig & Wilkens 1997) were used to quantify the capabilities of specific ophiostomatoid species to initially capture an area within infructescences, and then defend it against other fungal species. We were interested in quantifying both primary resource capture without initial separation and primary resource capture with initial separation. In doing so, we were able to quantify any differences in primary resource capture abilities of the ophiostomatoid species when arriving at the same infructescence, either being separated in space (*e.g*. fungi are inoculated at different areas within an infructescence), or not (*e.g*. area within an infructescence is inoculated with two fungal species simultaneously). These experiments were performed on media prepared from both *P. repens* and *P. neriifolia* infructescences.

For spatial separation, two disks (0.5 mm in diameter) of fungal colonised MEA were aseptically removed from actively growing two-week-old colonies and placed face-down at opposite sides of a 9 cm diameter Petri dish (*ca*. 10 mm from the side) containing media prepared from the *Protea* hosts (Fig. 3A). This experiment was replicated five times per host medium type for each combination between the different fungal species, each time using a different isolate. Plates were incubated at 24° C in the dark. After 10 days, the areas occupied by each fungus was measured with imageJ software and means of the areas occupied by the fungi on the different media were compared using ANOVA's and post-hoc Tukey HSD tests in R (Keppel & Wickens 2004). For the experiment where there was no spatial separation between the interacting fungi, two fungus-covered disks of MEA were placed face-down in the middle of the petri dish, touching each other (Fig. 3B). All other procedures followed those outlined above.

Secondary resource capture on usual and alternative hosts

For secondary resource capture we determined the abilities of various fungal species to capture area that was already colonized by another species on media prepared from both hosts. A disk (0.5 mm in diameter) of fungal colonised MEA were aseptically removed from actively growing two-week-old colonies of the tested fungal species and placed face down in the centre of the dish (Fig. 3C). Plates inoculated like this were incubated at 24° C in the dark for 10 days after which these were used as base for the inoculation of the competing fungal

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species. The competing fungal species was introduced in the centre and on top of the species that has already colonised the plate and incubated for a further 10 days at 24° C in the dark. All possible combinations of different competing species were repeated on media prepared from each *Protea* species and the experiment was replicated five times (each replicate using different fungal isolates). After ten days, the area captured by the second species was determined using imageJ software and mean areas captured were compared using an ANOVA and post-hoc Tukey HSD test in R.

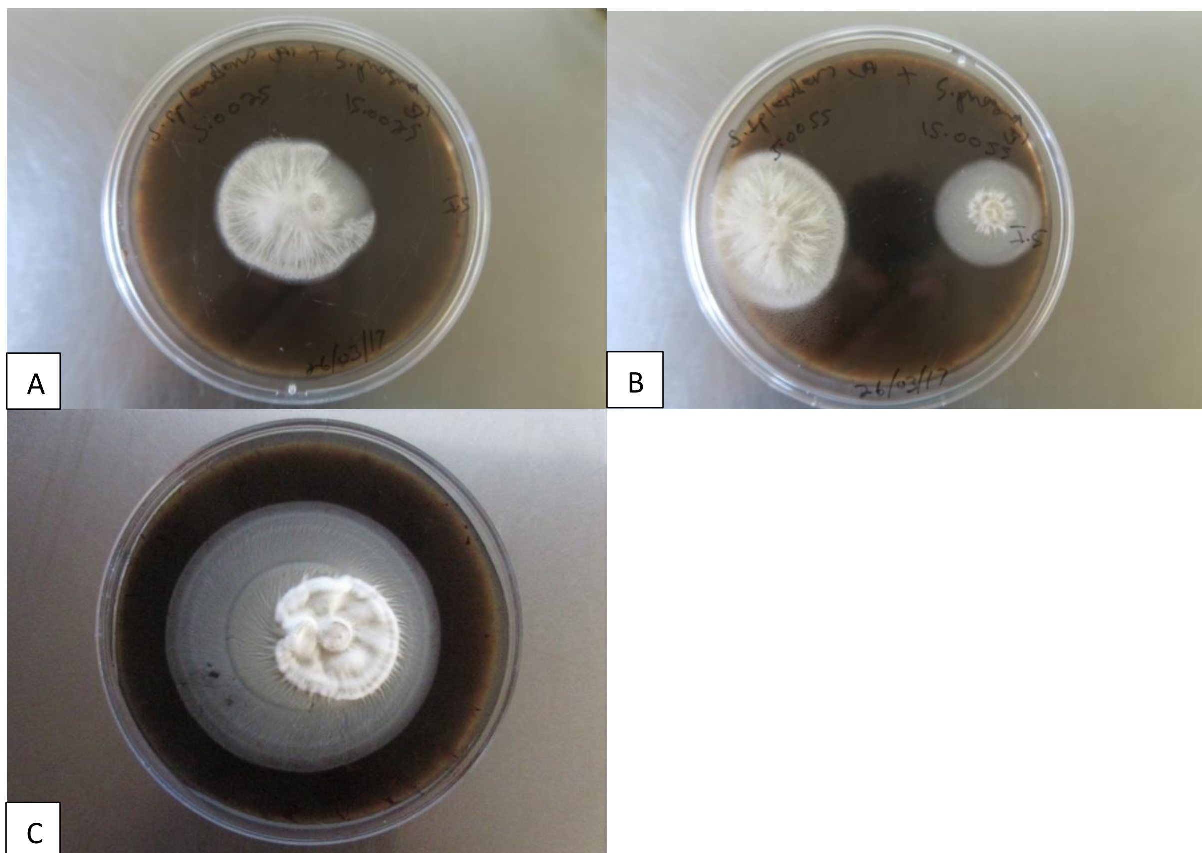

Figure 3: (A) - Primary resource capture without spatial between two fungal species *S. splendens* (left) and *S. phasma* (right) on media prepared from *P. repens*. (B) - Primary resource capture with spatial separation between two fungal species *S. splendens* (left) and *S. phasma* (right) on media prepared from *P. repens*. (C) - Secondary resource capture between two fungal species *S. splendens* (top colony) and *S. phasma* (bottom colony) on media prepared from *P. repens*.

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Results

Differential competition between fungi on usual and alternative hosts

In the de Wit replacement series experiment, only interactions between *S. splendens* and other fungi resulted in significant deviations from linearity for both competing fungal taxa (Table 1). When other significant deviations in linearity were observed, it was limited to one fungal species (always *K. proteae*) in the competing pair, and therefore competition did not occur between the two species in the pair. Based on the relative crowding coefficients, *Sporothrix splendens* was always the dominant competitor and was able to outcompete all three other fungal species on media prepared from both *Protea* species (Table 1). All fungal species were at a disadvantage when growing together as indicated by the combined crowding coefficients that were lower than 1 (Table 1). *Sporothrix phasma* and *K. capensis* had nearly completely neutralistic interaction when growing on media prepared from their preferred *P. neriifolia* host as indicated by their similar RCC scores.

Table 1: ANOVA statistics for tests in deviation from linearity in the relationships between different competing fungal species, in a de Wit replacement series, on media prepared from *P. repens* and *P. neriifolia* hosts. The competitive influence of each separate species in an interacting pair, or relative crowding coefficient (RCC), and the product of the RCC values of the interacting pairs (in brackets) are also provided. *df* = Degrees of freedom, SS = Sum of squares, MS = Mean square

Comparison	Source	*df*	SS	MS	F-value	P-value	RCC
On *P. repens* media							
S. splendens vs *K. proteae*							(0.257)
S. splendens area	Proportion	3	0.09159	0.030530	22.54	**<0.0001**	0.675
	Residual	11	0.01490	0.001355			
K. proteae area	Proportion	3	0.16457	0.05486	8.33	**0.0045**	0.381
	Residual	10	0.06584	0.00658			
S. splendens vs *K. capensis*							(0,214)
S. splendens area	Proportion	3	0.04	0.015	13.44	**<0.0001**	0.653

			709	696			
	Residual	11	0.01285	0.001168			
K. capensis area	Proportion	3	0.08613	0.028711	5.74	**0.0113**	0.328
	Residual	12	0.06004	0.005004			
S. splendens vs *S. phasma*							(0,238)
S. splendens area	Proportion	3	0.09706	0.03235	190.60	**<0.0001**	0.71
	Residual	15	0.00255	0.00017			
S. phasma area	Proportion	3	0.08317	0.02772	15.49	**<0.0001**	0.336
	Residual	15	0.02685	0.00179			
K. proteae vs *S. phasma*							(0,275)
K. proteae area	Proportion	3	0.03643	0.012143	13.54	**0.0003**	0.642
	Residual	13	0.01166	0.000897			
S. phasma area	Proportion	3	0.01104	0.003679	2.10	0.1580	0.428
	Residual	11	0.01924	0.001749			
K. proteae vs *K. capensis*							(0,272)
K. proteae area	Proportion	3	0.03208	0.010695	3.56	0.0604	0.542
	Residual	9	0.02703	0.003004			
K. capensis area	Proportion	3	0.00712	0.002372	0.52	0.6750	0.503
	Residual	10	0.04525	0.004525			
S. phasma vs *K. capensis*							(0,34)
S. phasma area	Proportion	3	0.00981	0.003271	1.24	0.3390	0.535
	Residual	12	0.03174	0.002645			
K. capensis area	Proportion	3	0.03855	0.012849	2.23	0.1540	0.636
	Residual	9	0.05191	0.005768			
On *P. neriifolia* media							

S. phasma vs *K. capensis*							(0.222)
S. phasma area	Proportion	1	0.00208	0.002085	0.35	0.5670	0.407
	Residual	12	0.07203	0.006003			
K. capensis area	Proportion	1	0.00021	0.000213	0.03	0.8640	0.547
	Residual	10	0.06942	0.006942			
S. phasma vs *K. proteae*							(0.273)
S. phasma area	Proportion	1	0.00017	0.0001709	0.09	0.7720	0.424
	Residual	17	0.03361	0.0019769			
K. proteae area	Proportion	1	0.04834	0.04834	66.45	**<0.0001**	0.646
	Residual	17	0.01237	0.00073			
S. phasma vs *S. splendens*							(0.220)
S. phasma area	Proportion	1	0.11978	0.11978	46.94	**<0.0001**	0.290
	Residual	16	0.04083	0.00255			
S. splendens area	Proportion	1	0.09868	0.09868	133.40	**<0.0001**	0.762
	Residual	13	0.00961	0.00074			
K. capensis vs *S. splendens*							(0.165)
K. capensis area	Proportion	1	0.3374	0.3374	47.35	**<0.0001**	0.203
	Residual	12	0.0855	0.0071			
S. splendens area	Proportion	1	0.15951	0.15951	491.60	**<0.0001**	0.812
	Residual	12	0.00389	0.00032			
K. capensis vs *K. proteae*							(0.193)
K. capensis area	Proportion	1	0.00435	0.004352	0.58	0.4600	0.362
	Residual	13	0.0974	0.007492			
K. proteae area	Proportion	1	0.00483	0.004828	1.05	0.3250	0.534
	Residual	12	0.05501	0.004584			

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S. splendens vs *K. proteae*							(0.216)
S. splendens area	Proportion	1	0.06952	0.06952	52.37	**<0.0001**	0.62
	Residual	13	0.01726	0.00133			
K. proteae area	Proportion	1	0.12746	0.12746	24.29	**<0.0001**	0.348
	Residual	13	0.06821	0.00525			

Primary resource capture on usual and alternative hosts

Fungal species varied significantly in their abilities to capture space on both *P. repens* (F=25.61, *df*=11, P<0.0001) and on *P. neriifolia* (F=38.78, *df*=11, P<0.0001) when their initial inocula were not spatially separated. On both hosts, *S. splendens* was able to capture space much faster than any other fungal species tested (Figs. 4 and 5). On its preferred *P. repens* host, *K. proteae* occupied similar-sized areas to *S. phasma*, but a significantly smaller area than *K. capensis*. Therefore, even though not on their preferred host, the latter two species could still compete for space against this species. On the alternative *P. repens* host, *S. phasma* and *K. capensis* occupied similar-sized areas, but when on their usual *P. neriifolia* host, *S. phasma* could occupy significantly more space than *K. capensis*. *Sporothrix phasma* was particularly good at capturing space when paired with *K. capensis* on both hosts, as this is when its colony size was largest when compared to all other fungal species (Figs. 4 and 5). It could not occupy more space than *K. proteae* on *P. neriifolia*, even though it is the alternative host of *K. proteae*. In contrast, *K. proteae* was able to capture significantly more space than *K. capensis* on this host.

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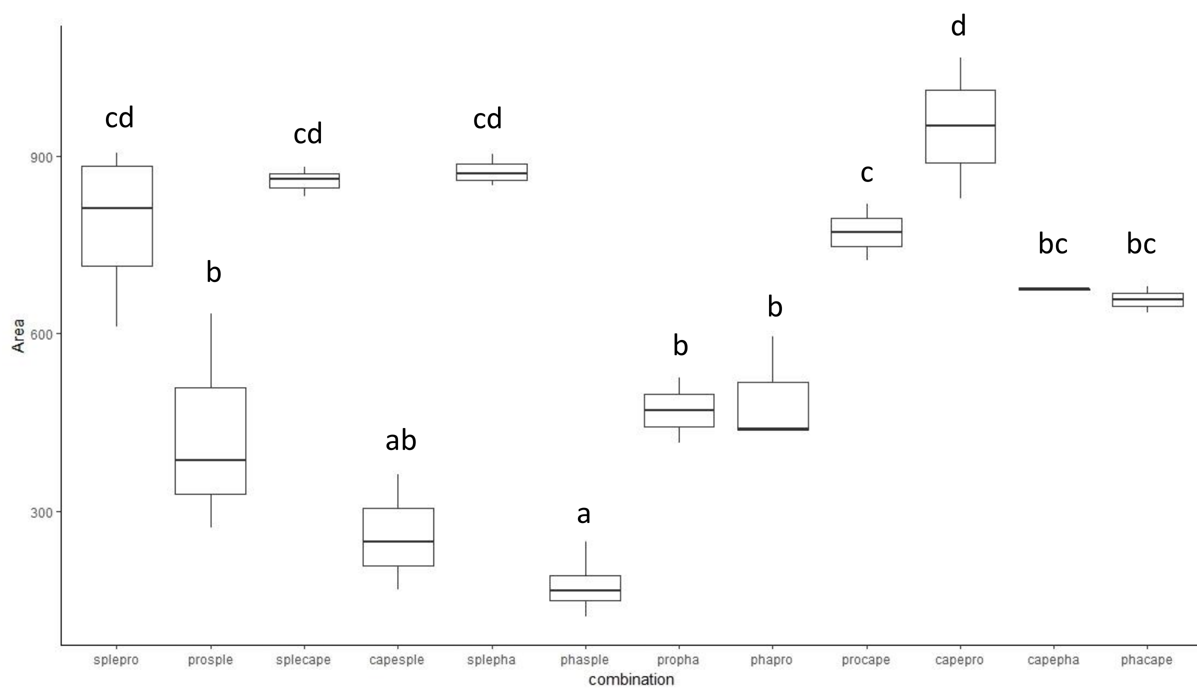

Figure 4: Median colony size (mm^2) after 10 days of growth as indication of primary resource capture capabilities without spatial separation between two competing fungal species on media prepared from *Protea repens* host tissues (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range, dots represent outliers). The first three letters of the labels on the x-axis indicate the area occupied by that particular species and the last three letters indicate the competitor species that it was paired with. pro = *K. proteae*, sple = *S. splendens*, cape = *K. capensis*, pha = *S. phasma*.

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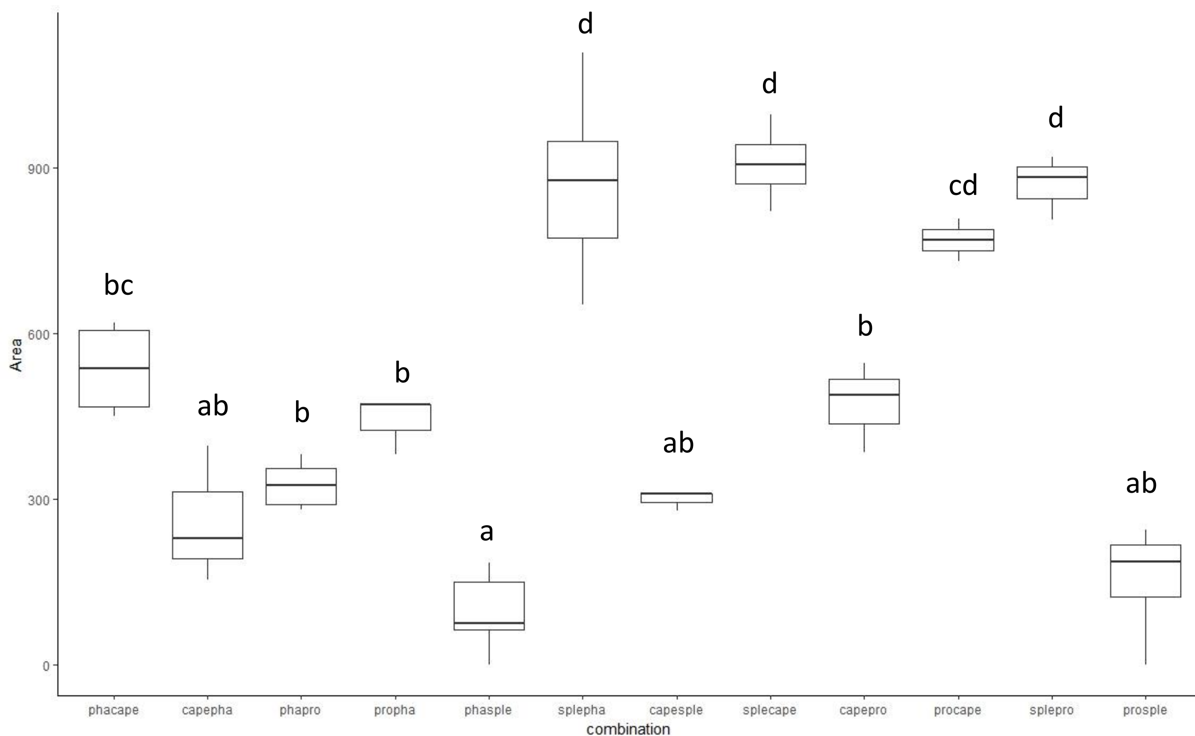

Figure 5: Median colony size (mm^2) after 10 days of growth as indication of primary resource capture capabilities without spatial separation between two competing fungal species on media prepared from *Protea neriifolia* host tissues (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range, dots represent outliers). The first three letters of the labels on the x-axis indicate the area occupied by that particular species and the last three letters indicate the competitor species that it was paired with. pro = *K. proteae*, sple = *S. splendens*, cape = *K. capensis*, pha = *S. phasma*.

Fungal species also varied significantly in their abilities to capture space on both *P. repens* (F=31.11, df=11, P<0.0001) and *P. neriifolia* (F=45.28, df=11, P<0.0001) when their initial inocula were spatially separated. On its preferred *P. repens* host, *S. splendens* was able to colonise more area than *K. proteae* and *S. phasma*, but not *K. capensis* (Fig. 6). On its alternative *P. neriifolia* host, *S. splendens* captured similar areas of space to all other species (Fig. 7). *Knoxdavesia proteae* captured similar-sized areas to *K. capensis* and *S. phasma* when growing on media prepared from its usual host, but it could capture more space than the latter on its alternative host. *Knoxdavesia capensis* was able to capture particularly large areas

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of space when paired with *S. phasma*, but only when on their preferred *P. neriifolia* host (Figs. 6 and 7).

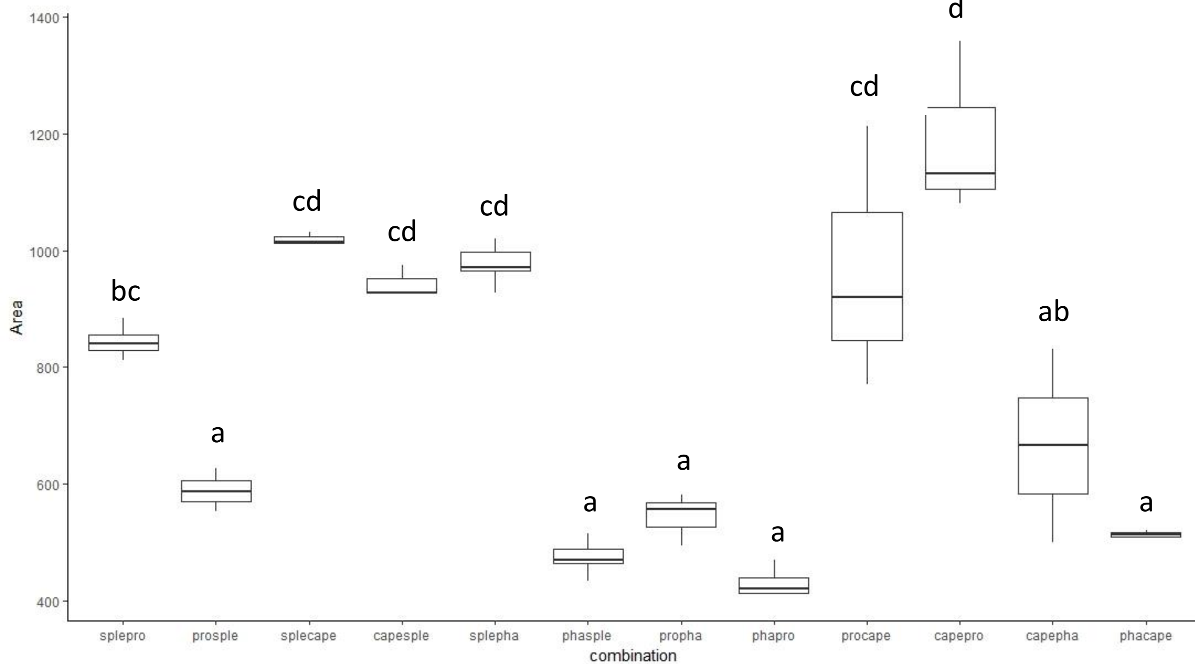

Figure 6: Median colony size (mm^2) after 10 days of growth as indication of primary resource capture capabilities with spatial separation between two competing fungal species on media prepared from *Protea repens* host tissues (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range, dots represent outliers). The first three letters of the labels on the x-axis indicate the area occupied by that particular species and the last three letters indicate the competitor species that it was paired with. pro = *K. proteae*, sple = *S. splendens*, cape = *K. capensis*, pha = *S. phasma*.

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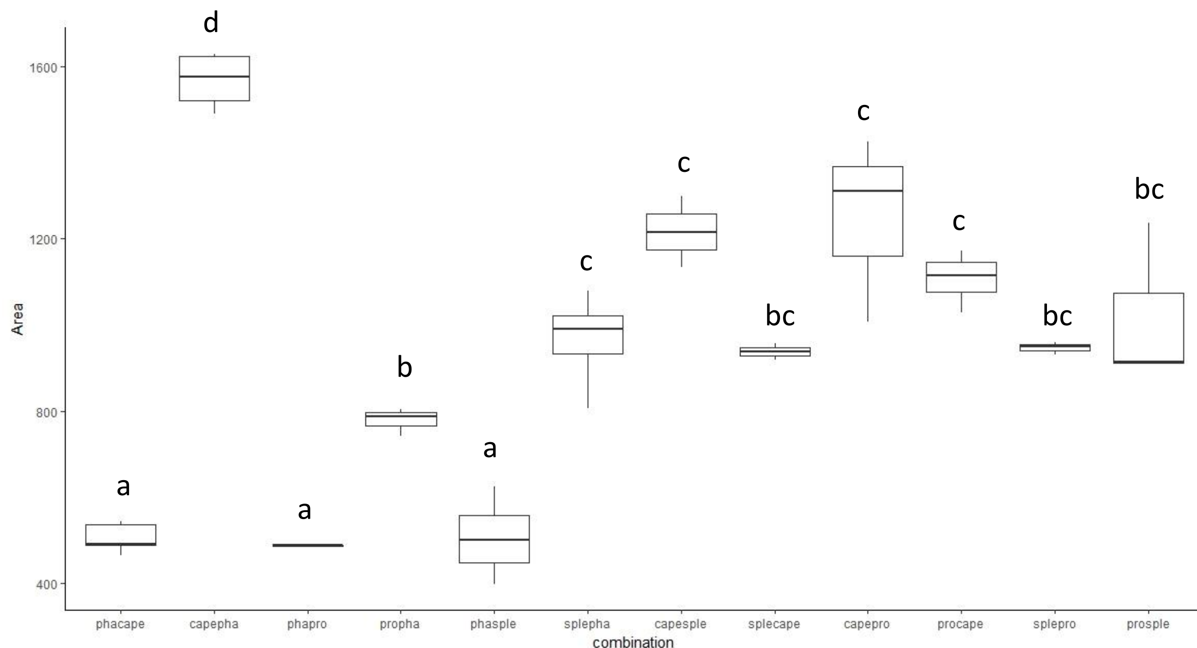

Figure 7: Median colony size (mm^2) after 10 days of growth as indication of primary resource capture capabilities with spatial separation between two competing fungal species on media prepared from *Protea neriifolia* host tissues (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range, dots represent outliers). The first three letters of the labels on the x-axis indicate the area occupied by that particular species and the last three letters indicate the competitor species that it was paired with. pro = *K. proteae*, sple = *S. splendens*, cape = *K. capensis*, pha = *S. phasma*.

Secondary resource capture on usual and alternative hosts

In terms of capturing space that was already occupied by another species, *S. splendens* was able to grow on all other species and on media prepared from both hosts, but none could grow on *S. splendens* ($F=25.03$, $df=5$, $P<0.0001$) (Fig. 8). When on its usual *P. repens* host, it was able to overgrow significantly larger areas that were previously occupied by *K. capensis* than on any other fungus on any other host. The areas of other fungi that were secondarily captured by *S. splendens* were statistically similar in size (Fig. 8). Consequently, *K. capensis* is a particularly weak competitor against *S. splendens* when it is growing on its alternative host. *Sporothrix phasma* could overgrow *K. proteae* (Fig. 9) at statistically similar rates on

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media prepared from both hosts (F=1.344, d =1, P=0.284), but it was not able to do so when placed on colonies of *K. capensis*.

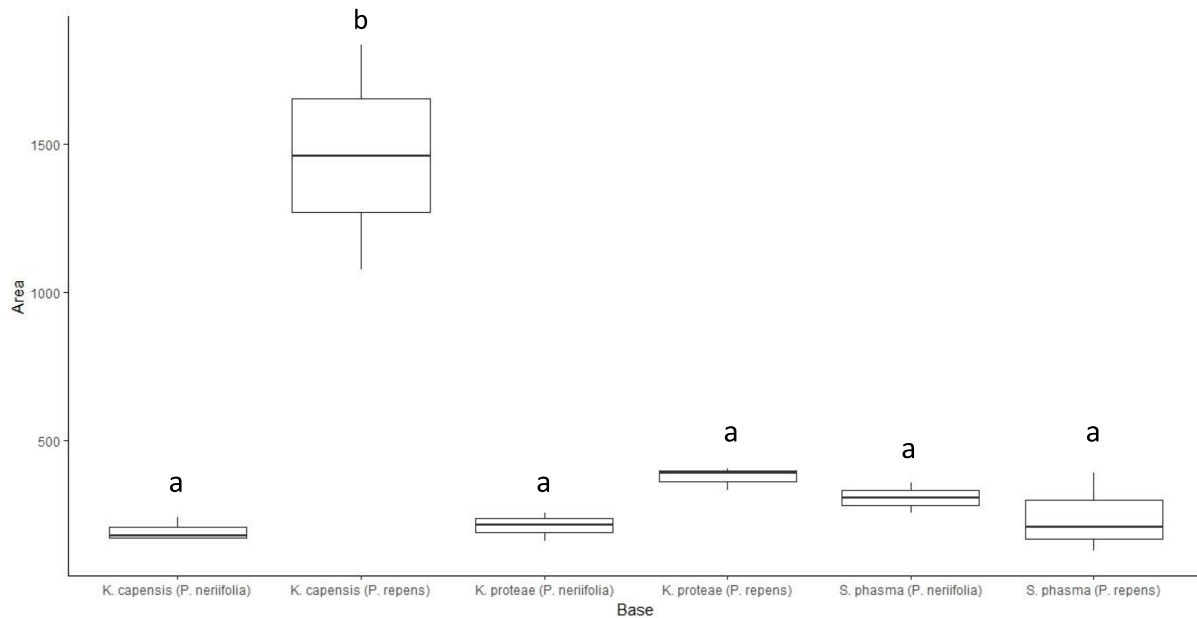

Figure 8: Median colony size (mm^2) of *S. splendens* after 10 days of growth on other fungal species as indication of its secondary resource capture capabilities on media prepared from *Protea repens* host tissues or *P. neriifolia* host tissues as indicated between brackets (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range).

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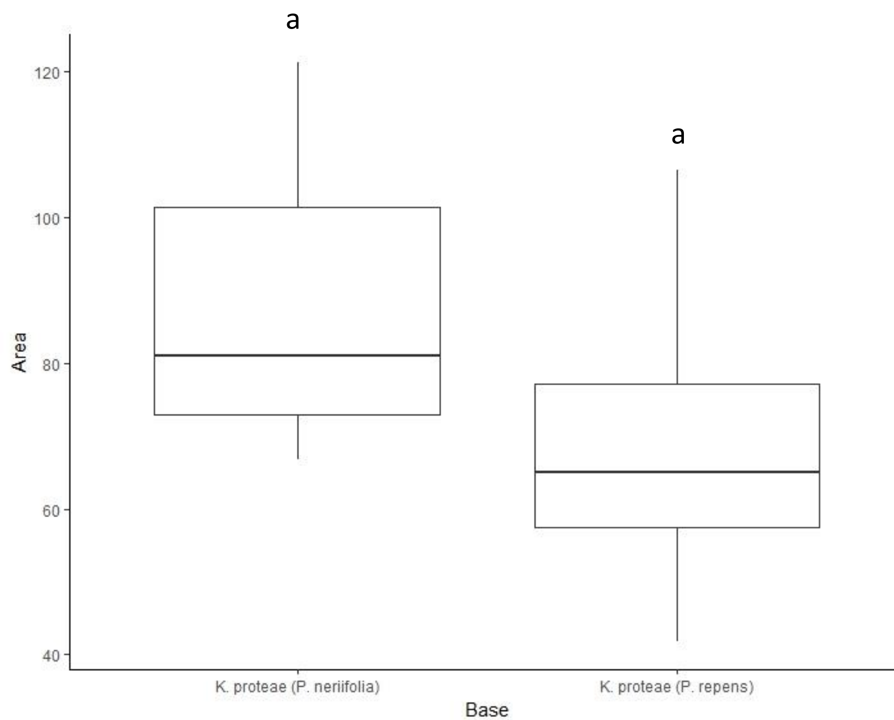

Figure 9: Median colony size (mm^2) of *S. phasma* after 10 days of growth on *K. proteae* as indication of its secondary resource capture capabilities on media prepared from *Protea repens* and *P. neriifolia* host tissues as indicated between brackets (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range).

Discussion

Here we set out to investigate the role of differential competition on enforcing host exclusivity of saprobic ophiostomatoid fungal species within the inflorescences and infructescences of two host *Protea* species. The fungi *K. proteae* and *S. splendens* are nearly exclusively associated with *P. repens*, while *K. capensis* and *S. phasma* are nearly exclusively associated with *P. neriifolia* (Roets *et al.* 2009b). We found that differential competitive abilities on different host species could only partly explain host exclusivity observed in the field. For example, *S. splendens* was by far the most successful competitor against all fungi and on both its usual and its alternative hosts. It was also able to capture more space on media prepared from host material, especially when on media prepared from its usual host. In addition, it was also able to capture space that was originally occupied by other species on both hosts. Given these results, it is unlikely that *S. splendens* is host

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exclusive on *P. repens* only due to strong competitive abilities. Rather, its general absence from the inflorescences and infructescences of *P. neriifolia* may be due to limitations posed in its dispersal.

Ophiostomatoid fungi associated with *Protea* species are primarily dispersed by fungus-feeding and pollen- and nectar-feeding mites (Roets *et al*. 2007; Theron de Bruin *et al*. 2018a and b). From within infructescences these mites are transported to other infructescences or to newly formed inflorescences by larger infructescence-dwelling arthropods such as beetles (Roets *et al.* 2009a). During the *Protea*-flowering stage, mites found within inflorescences are dispersed by pollinating beetles and birds (Roets *et al.* 2009a; Theron de Bruin *et al*. 2018a). Both the birds and the beetle species that vector these mites have often been recorded from both *P. repens* and *P. neriifolia* (Hargreaves *et al.* 2004; Steenhuisen & Johnson 2012b; Schmid *et al.* 2015). Therefore, differences in the identities of the spore vectors cannot explain host exclusivity of the fungi. These *Protea* species also have overlapping flowering seasons and they are often found growing in sympatry (Nottebrock *et al.* 2016). However, recent evidence suggests that there is a strong level of consistency and preference in the movement between different *Protea* species of individuals of the pollinating birds (Schmid *et al.* 2016). The same may hold true for the pollinating insects (A New, unpublished data). Therefore, even though the vector species are shared between *Protea* hosts, specific individuals may selectively forage on only one of the *Protea* species present at any given time in the population. This would restrict the movement of the spore-carrying mites that are phoretic on the pollinators to particular *Protea* species, with only occasional switching. This occasional switching may explain the infrequent colonization of *P. neriifolia* by *S. splendens* (Marais & Wingfield 1994; Roets *et al.* 2009b) and the infrequent colonization of *P. repens* by *K. capensis* (Aylward *et al*. 2015).

The persistence of the weaker competitor *K. proteae* within the same *P. repens* infructescences when in the presence of *S. splendens* (Roets *et al.* 2005) may also be explained by vector activities. Our results indicate that if both fungi arrive at the same time within an infructescence, *S. splendens* can occupy larger areas of space and thereafter proceed to overgrow *K. proteae*. For *K. proteae* to persist in this environment it needs to either arrive before *S. splendens*, or arrive in a different area within the infructescence, and then successfully produce its much larger sexual structures before being overgrown by *S. splendens* (that form relatively small sexual structures; Marais & Wingfield 1994; Winfield *et al*. 1988). Earlier arrival of *K. proteae* is unlikely given detection of *Sporothrix* at the onset of

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flowering (Theron-De Bruin *et al*. 2018), but it is not yet established whether *S. splendens* could also use *Protea* nectar sugars as carbon source, like what was shown for *K. proteae* (Aylward *et al*. 2017). Interestingly, initial survey results suggests that *K. proteae* usually occupy a different area within *P. repens* infructescences (the upper most regions within infructescences) than *S. splendens* (lower parts within infructescences) (Roets *et al.* 2013) and it may be possible that these two fungal species rely on different primary or secondary main spore vectors to gain access to these different areas. The mite vectors for S. splendens have been well established, but the vectors for Knoxdavesia species have not received much attention (Roets *et al*. 2007, 2008, 2009, 2011, Theron-deBruin *et al*. 2018). It should also be noted that tissue types are different between the two areas mainly colonised by *S. splendens* (primarily the seed coats and basal dead flower parts) and *K. proteae* (primarily the pollen presenters) within *P. repens* infructescences, which may play a role in their spatial separation.

Knoxdavesia capensis and *S. phasma* have neutral competitive abilities against each other, which explains their co-existence within this niche. Both species can effectively occupy space and defend it against the other species, although the abilities depend on whether they are in close association or not. For example, when arriving at the same time and place, *S. phasma* can occupy more space than *K. capensis*. When arriving at two different locations, *K. capensis* can occupy very large areas within infructescences. It is therefore likely that these two species, which often colonize the same areas within infructescences (Roets *et al.* 2009b), arrive at the same time and use the same spore vectors. As indicated by primary resource capture capabilities, *S. phasma* is a stronger competitor against *K. proteae* than against *K. capensis* when growing on its preferred *P. neriifolia* host. It is also able to overgrow this species as indicated in the secondary resource capture capabilities, particularly when growing on material from its usual host. In addition to selective dispersal by individual pollinators between different *Protea* species, *S. phasma* may therefore help maintain host exclusivity of *K. proteae*. Similarly, *S. splendens* will quickly outcompete *K. capensis* and *S. phasma* when these arrive within *P. repens* inflorescences.

In addition to host related differential competitive abilities and differential movement of spore vectors, other competing microorganisms may also help shape the observed host consistency in *Protea*-associated ophiostomatoid fungi. A vast number of other saprobic fungal species have been found within these structures (Lee *et al.* 2005; Roets *et al.* 2005), all of which may help shape ophiostomatoid communities. Given the specialised dispersal mechanism for the ophiostomatoid fungi in relation to most other fungi in this niche, these

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interactions may only come into play at later successional stages within infructescences, as the ophiostomatoid fungi are some of the very first colonizers (Theron-De Bruin *et al.* 2018). However, actinomycete bacteria have also been shown to colonize *Protea* inflorescences at the onset of flowering and my even share the same spore vectors (Human *et al.* 2016, 2018). Many of these bacterial species produce antifungal agents (Malloch & Blackwell 1993; Cassar & Blackwell 1996). The three dominant actinomycete taxa associated with *Protea* flowers produce varying concentrations of fungichromin and actiphenol that inhibit the growth of both ophiostomatoid fungi and other saprobes in this niche (Human *et al.* 2016). From that study it was clear that common saprotrophic fungi were very sensitive to some of the compounds produced by these bacteria, but that the ophiostomatoid fungi varied in their sensitivity. Even though no benefit to *Knoxdavesia* could be deduced from their results, it is possible that fungus-actinomycete interactions could shape and help maintain ophiostomatoid exclusivity on different hosts.

To conclude, host-related differential competitive abilities of the saprobic ophiostomatoid fungi studied here only partly explain their host exclusivity. The role of vectors in shaping these interactions may be much larger than previously assumed and should be studied in greater depth. The possible effect of differential competitive abilities based on specific tissues within infructesceces (*e.g.* seeds vs. pollen presenters vs. bracts) and the effect of other microorganisms (*e.g.* actinomycete bacteria) on shaping host exclusivity should not be ignored. Host consistency of *Protea*-associated ophiostomatoid fungi is therefore likely driven by a complex interplay between differential competitive abilities of the fungi on different hosts, the degree of consistency of spore vectors between flowers of co-flowering *Protea* species and competition from other microorganisms. The reason for the complete dominance of saprobic fungal communities by ophiostomatoid fungi within infructescences (Lee *et al.* 2005; Roets *et al.* 2005) should also be investigated in future studies.

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Chapter 2: Early colonization helps weaker competitors dominate saprobic fungal communities in *Protea* flowers, to the possible benefit of their hosts

Abstract

The flowers of *Protea* species in Africa are colonised by various species of ophiostomatoid fungi in the genera *Sporothrix* and *Knoxdaviesia*. These remain the dominant fungi in seed-storage structures (infructescences) of their hosts for at least a year after flowering, despite the availability of a nutrient rich environment conducive to the growth of other saprobes. When they are absent, however, potentially detrimental saprobes abound. Here we test the hypothesis that the ophiostomatoid fungi can competitively exclude potentially harmful saprobes from this environment to the benefit of the host plant. We tested the *in vitro* competitive abilities of these fungi against common 'contaminant' saprobes from *Protea* infructescences, and compared seed set and longevity of infructescence with and without ophiostomatoid fungi. Field-collected infructescences devoid of ophiostomatoid fungi were dominated by other saprobes such as *Penicillium* cf. *toxicarium*, *Cladosporium* cf. *cladosporoides* and *Fusarium* cf. *anthophilum*. Ophiostomatoid fungal presence had no effect on the number of viable seeds in infructescences, but seeds persisted in the infructescences longer than those colonised by 'environmentally acquired' species. In a de Wit replacement series experiment, all evaluated 'contaminant' species dominated *Protea*-associated ophiostomatoid fungi. Despite this, some ophiostomatoid species could capture large areas of un-colonised resources in the presence of other saprobes. No ophiostomatoid species was able to capture space already occupied by other saprobes and most 'contaminant' saprobes could capture only limited space occupied by ophiostomatoid fungi. The ophiostomatoid fungi were often better at capturing and maintaining area when growing on media prepared from their usual hosts. Our results therefore show that host consistency of the *Protea*-associated ophiostomatoid fungi may be enforced by stronger competitive abilities on preferred usual hosts. However, these fungi are comparatively weak competitors against common saprobes and need to colonise flowers very early (before colonization by other fungi) and rapidly (like when growing on their usual hosts) in order to persist in this environment. They can delay seed release of their hosts to when conditions are more optimal for germination and subsequent seedling development, which will be of mutual benefit to the fungi and the host plants.

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Introduction

Saprobic fungi perform extremely important ecosystem functions such as plant litter decomposition and nutrient cycling (Allegrucci *et al*. 2015; Kumar *et al*. 2012; McGuire & Treseder 2010). Saprobes may often also be detrimental to living hosts when colonizing structures intended to support living plant tissues such as the wood of trees (*e.g.* wood rot). However, the possible role of saprobes in the protection of living plant tissues has not received much attention. For example, the infructescences of serotinous *Protea* species in Africa, although comprised mostly of dead floral parts, function to protect *Protea* seeds during recurring fires and to release seeds in the relatively competition-free post fire environment (Bond 1985). Fungal communities within these structures are dominated by an atypical assemblage of saprobic *Knoxdaviesia* and *Sporothrix* species (ophiostomatoid fungi *sensu* Wingfield *et al*. 1993) for at least two years after flowering (Lee *et al*. 2003, 2005; Roets *et al*. 2005; Roets *et al*. 2010). Hereafter, and when no ophiostomatoid fungi are present, fungal communities become dominated by common 'environmentally acquired' taxa such as *Penicillium* Link and *Cladosporium* Link (Lee *et al*. 2003, 2004, 2005; Visagie & Jacobs, 2012; Visagie *et al*. 2009; Visagie *et al*. 2015; FR pers. observ.). These 'environmentally acquired' taxa rapidly break down organic matter (Aro *et al*. 2005; Thormann 2006) and may be detrimental to the *Protea* hosts if they have the ability to trigger premature seed release from infructescences, or if they interfere with seed viability (Haikal 2008; Roets *et al*. 2013). It has thus been suggested that the saprobic ophiostomatoid fungi from this environment (Aylward *et al*. 2017) may protect the *Protea* seeds from the actions of other, possibly detrimental, saprobic fungi, although this remains to be tested (Marais & Wingfield 2001; Roets *et al*. 2013).

To date, three species of *Knoxdaviesia* in the Microascales (Crous *et al*. 2012; Wingfield & van Wyk 1993; Wingfield *et al*. 1988) and 11 species of *Sporothrix* in the Ophiostomatales have been described from various *Protea* hosts (Marais & Wingfield 1994, 1997, 2001; Ngubane *et al*. 2017; Roets *et al*. 2006a, 2008, 2010). This large diversity of morphologically similar fungi in such a specialised niche has been ascribed to various factors, including isolated geographic distribution of some hosts (Roets *et al*. 2010) and strong host consistency of many taxa (Roets *et al*. 2013). Strong host consistency is thought to be enforced by differences in host chemistry, as all tested species grow best on tissues from their usual host species (Roets *et al*. 2012). Despite this, recent experimental studies indicated that differences in host chemistry only partly explains host consistency and that specificity in the

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movement of spore vectors may play a major role (Mukwevho *et al*. 2019, Chapter 1). Previous studies, however, only tested for the interactions between various ophiostomatoid fungi (*S. splendens, S. phasma, K. capensis* and *K. proteae*) from two different host species (*P. repens* and *P. neriifolia*) and ignored the possible effect of other saprobic fungi in the maintenance of host consistency. Here I hypothesise that the ophiostomatoid fungi may be stronger competitors against other saprobic fungi on tissues from their own hosts than on tissues of non-usual hosts, which may help explain both their host consistency, and their dominance in this niche.

Dominance of the ophiostomatoid fungi within *Protea* infructescences over other saprobes may also be explained by early colonization of their hosts. All *Knoxdaviesia* and *Sporothrix* species from this environment produce ascospores and conidia in sticky spore drops on elongated structures, morphological adaptations ascribed to vectored spore dispersal via arthropods (Malloch & Blackwell 1993). Mites that often have mutualistic associations with their fungal partners (Roets *et al*. 2007, 2009) are the primary dispersers of the *Protea*-associated ophiostomatoid fungi. These mites are phoretic on pollinating beetles (Roets *et al*. 2011) and, in an interesting case of mite hyperphoresy, also via pollinating birds (Theron-de Bruin *et al*. 2018). This vectored mode of dispersal of spores not only enables the *Protea*-associated ophiostomatoid fungi to colonise hosts over vast distances, but also ensure that they reach host tissues very early on in the development of mature flowers. For example, Aylward *et al*. (2015) showed that *Knoxdaviesia* species colonise *Protea* populations from unburned populations as soon as the very first post-fire individuals flower (*ca*. 3 years after a fire) and that populations separated by more than 100 km have regular gene flow (Aylward *et al*. 2014a, b). More impressively, Ngubane *et al*. (2017) showed that *Sporothrix* populations separated by more than 1000 km have regular gene flow, likely due to the association of the fungi with mites on birds (Theron-de Bruin *et al*. 2018). As these fungi are adapted to secondary transportation via *Protea* pollinators, they can colonise inflorescences as soon as the very first flowers open (Theron-de Bruin *et al*. 2018, Human *et al*. in press) and they remain dominant in infructescences well after these have matured (Roets *et al*. 2005, Human *et al*. in press). After this, they are often replaced by common decomposing fungal taxa (Human *et al*. in press).

Here I set out to determine whether *Protea*-associated ophiostomatoid fungi can competitively exclude other saprobes from infructescences using *in vitro* experiments (Klepzig *et al*. 2001, Mukwevho *et al*. 2019, Chapter 1). I first identified the most prominent

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saprobic fungi found in field-collected infructescences that were devoid of ophiostomatoid fungi, and tested interspecies competitive interactions using a de Wit replacement series (Klepzig & Wilkens 1997; Klepzig 1998). Hereafter I tested the ability of the various fungi to rapidly gain access to uncolonised area (primary resource capture) and their ability to colonise an area that had already been captured by other fungi (secondary resource capture) as defined by (Klepzig *et al*. (2001) and Mukwevho *et al*. (2019; Chapter 1). To determine whether host chemistry play a role in determining host consistency of *Protea*-associated ophiostomatoid fungi, I repeated these experiments on media prepared from both usual and non-usual host material (Mukwevho *et al*. 2019, Chapter 1). To determine if there is a potential positive relationship between *Protea* and the ophiostomatoid fungi that they host, I tested for a positive relationship between natural *Protea* seed set, infructescence longevity, and the presence of ophiostomatoid fungi.

Methods and Materials:

Possible host plant benefit

Infructescences of serotinous *Protea* species remain on trees for at least one, but often several years (Rebelo 1995). In the Franschoek area (South Africa), infructescences of *P. repens* (L.) L. often remain on the plants for more than three years (but generally not longer than 5 years), while those of *P. neriifolia* R.Br. rarely remain on the plants for more than three years (FR, *pers. observ*.). If these cones open too early, before a fire event, their seeds will be released into a landscape where rodent seed predators abound (Biccard & Midgley 2009; Rourke & Wies 1977), and where seedlings experience considerable competition by other vegetation (Yeaton & Bond 1991). Within at least the first two years after flowering, infructescences of these species should only open due to the death of the parental plant after a fire event (Bond 1985). However, infructescences may also open prematurely due to the activities of insects boring into the structures (Coetzee & Giliomee 1987), or, as hypothesised here, due to the actions of 'environmentally acquired' saprobic fungi.

To test whether the presence of ophiostomatoid fungi in infructescences may be associated with greater infructescence longevity and/or with increased numbers of viable seeds (expressed as the percentage of viable seeds per infructescence), I collected two-year-old infructescences of both *Protea repens* and *P. neriifolia* from the Franschoek Pass, Western

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Cape Province, South Africa (-33.90442; 19.156683) during December 2018 (mid summer). For each plant species, I collected 30 fully closed infructescences and 30 infructescences that had started to open (15-20% open) in pairs from 30 separate trees (sample sizes were limited by the availability of plants that had structures in both stages). Also, only cones with less than 5% internal insect damage as visually scored after opening were included in analyses. In the laboratory, all individual flowers of each infructescence were scanned for the presence of fresh or degraded ascomata of ophiostomatoid fungi. When any ascomata or their remnants were observed, the infructescence was scored as positive for ophiostomatoid fungal colonization. Hereafter the number of viable seeds per infructescence was determined as outlined by Theron-de Bruin e*t al.* (2019). The numbers of open vs. closed infructescences with and without ophiostomatoid fungal structures present were compared using a chi-square test in R (R Development Core Team 2013). The number of viable seeds contained in infructescences with or without ophiostomatoid fungi and from open vs. closed infructescences were compared using generalised linear models (GLMs) in R. These models followed the formulas: glmer (seed set ~ open vs. closed infructescence + infructescence with or without ophiostomatoid fungi, family = “binomial”) for the percentage data.

Identification of prominent ‘environmentally acquired’ saprobes

After assessment of seed set and the presence of ophiostomatoid fungi, the two-year-old infructescences collected above were assessed for prominent micro-fungi. In addition, further closed, *ca*. one-year-old infructescences of *Protea repens* and *P. neriifolia* were collected from Franschoek Pass and their micro-fungi present assessed. For each host species, 60 additional infructescences were assessed for prominent micro-fungi, 30 that contained sporulating structures of ophiostomatoid fungi, and 30 that contained no such structured (assessed in field with the aid of a 10x hand lens, later confirmed in the laboratory using a Olympus SZ stereo-microscope). In the laboratory, infructescences were quartered lengthwise and inspected for fungal colonization. The most prominent fungal taxon observed per infructescence was isolated by transferring spores or mycelia to malt extract agar plates (MEA, Midrand, South Africa) using a sterile needle. Prominence of fungal taxa was determined by visual scoring of the total surface area occupied by each fungus on the four quarters of the infructescence. Ophiostomatoid fungi were not isolated, as they could be identified based on morphology (Roets *et al*. 2013). Both *Protea* species always host a pair of

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ophiostomatoid fungal species (*S. splendens* and *K. proteae* on *P. repens*, and *S. phasma* and *K. capensis* on *P. neriifolia*) (Mukwevho *et al*. 2019; Chapter 1). In subsequent analyses, these species pairs were treated as a single taxon per host. All isolated 'environmentally acquired' taxa were allowed to proliferate on the MEA plates for 10 days in the dark, after which taxa were grouped according to morpho-species based on their culture characteristics. The three most dominant 'environmentally acquired' fungi present within infructescences (the ones isolated from most individual infructescences) per host were identified by sequencing the ITS regions of three representative cultures (using the same primers and protocols outlined in Aylward *et al*. (2014a, b)) and evaluation of their closest confident matches available on GenBank using the blast algorithm (www.ncbi.com). Five different isolates of these species were used in subsequent experiments.

Fungal cultures and preparation of competition media

Isolates of ophiostomatoid fungi used here were the same cultures used in a previous study that assessed competitive interactions between them (Mukwevho *et al*. 2019; Chapter 1). Taxa included *K. proteae* (Stellenbosch mountain (-33.9466; 18.8805)) and *S. splendens* (Betty's Bay (-34.3315 18.9925)) from *P. repens* and *K. capensis* (Betty's Bay (-34.35495; 18.90135)) and *S. phasma* (Jonkershoek Nature Reserve (33˚59'24.5"S, 18˚57'25.2"E)) from *P. neriifolia*. Five individual cultures of each of the four ophiostomatoid fungal species, and five individual cultures of each of the three most prominent 'environmentally acquired' fungal taxa were used in competition studies (each isolate originating from a different infructescence). All fungal isolates were maintained on MEA at 4˚C in the dark until further testing. Growth media for competition studies were prepared from pollen presenters of *P. repens* and *P. neriifolia* following previously described methods (Roets *et al*. 2012; Mukwevho *et al*. 2019; Chapter 1). One litre of water-based growth medium contained 300 ml of prepared *Protea* tissue (pollen presenter dust) and 1.5 % MEA. Media were autoclaved at 115˚C for 20 min and poured into 90 mm petri dishes, which served as the experimental arenas.

Differential competition

A de Wit replacement series experimental design (Klepzig & Wilkens 1997; Klepzig 1998) as modified by Mukwevho *et al*. (2019; Chapter 1) was used to test competitive abilities

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between the ophiostomatoid fungal species and the 'environmentally acquired' fungal species on both host *Protea* species. Two fungal species were introduced on plates in varying proportions of inoculum and left to compete for two weeks. Inoculation ratios that were used included: species A (ophiostomatoid species) vs. species B ('environmentally acquired' species); 0:1 (16 disks species B), 0.25:0.75 (4 disks sp. A and 12 disks sp. B), 0.5:0.5 (1:1) (8 disks sp. A and 8 disks sp. B), 0.75:0.25 (12 disks sp. A and 4 disks sp. B), and 1:0 (16 disks species A). Inoculum consisted of aseptically removed disks (0.5 mm in diameter) of actively growing fungal colonies on MEA that were placed face-down on plates (9 cm in diameter) containing MEA in a randomised block design (4 x 4 cm grid) as outlined in Mukwevho *et al*. (2019; Chapter 1). The procedure was repeated for the 5 different ratio's for each pairwise species combination (four ophiostomatoid fungal species vs. three 'environmentally acquired' taxa) and the entire experiment was replicated five times per tested medium (*P. repens* and *P. neriifolia*), each time using different fungal isolates. To determine if there was competition between the two fungi, the areas occupied by each fungus after two weeks were plotted against the initial proportion of inoculum introduced and the interaction was tested for deviation from linearity (Klepzig & Wilkens 1997; Klepzig 1998). No deviation from linearity for both fungal species indicated neutrality, but significant deviation from linearity in both species (one in a positive direction and the other in a negative direction) indicated that the one species was a superior competitor over the other on that particular medium (Klepzig & Wilkens 1997).

Some of the 'environmentally acquired' fungi identified in the present study produce copious amounts of aerial spores. In order to prevent contamination of plates in experiments, it was thus necessary to produce inoculum devoid of sporulating structures. In order to do so, I prepared inoculum on MEA plates from two-day-old cultures that originated from spore suspensions. Spore suspensions were made by adding 10 ml sterile water containing a drop of Tween 20 (Atlas Chemical Industries Inc, USA) to the surface of ten-day-old fungal colonies and gently shaking these by hand for 1 minute. Then 2.5 ml of this spore suspension was added to a fresh MEA plate, spread across the surface and incubated for two days in the dark at ambient room temperature. After two days of growth, plates were fully covered with fungal mycelia, but were still devoid of sporulating structures and could therefore be used as inoculum source in experiments without causing contamination beyond the point of inoculation.

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Experimental plates were sealed with parafilm and placed in an incubator at 25^{0}C in the dark for two weeks, after which the areas occupied by each fungus were measured using ImageJ software (LOCI, University of Wisconsin). Deviations from linearity were calculated by performing analyses of variance (ANOVA) on log transformed data (Wilson & Lindow 1994) in R (R Development Core Team 2013) as described in Mukwevho *et al*. (2019; Chapter 1). In addition, relative crowding coefficients (RCC, de Wit 1960) were calculated for all pairwise combinations of fungi as [(mean area of species A at 1:1)/(mean area of species A at 1:0)] and [(mean area of species B at 1:1)/(mean area of species B at 1:0)]. The species with the higher RCC value were considered dominant over the other species in the interacting pairs. If the product of the two RCC values per interacting pair was one, then the interaction between the two fungal species was viewed as neutral, if the RCC product was less than one, then it was viewed as competition between the fungal species, if the RCC product was greater than one, then both species were considered to benefit from growing together.

Primary resource capture

For primary resource capture capabilities of the various competing fungi (ability to initially capture uncolonised space), I again followed the experimental design used in Klepzig & Wilkens (1997) and Mukwevho *et al*. (2019; Chapter 1). This experiment was repeated to simulate both a situation where two fungal species were separated in space (*i.e.* when two fungi arrive in different areas within an infructescence) and a situation where they co-occured in close proximity (*i.e.* when two fungi arrive at the same point in the infructescence). These experiments were performed on media prepared from both *P. repens* and *P. neriifolia* and were replicated five times per ophiostomatoid fungus/'environmentally acquired' fungus combination, each time using different fungal isolates. For experiments investigating primary resource capture capabilities with spatial separation, two disks (0.5 mm in diameter) of fungal colonised MEA were aseptically removed from actively growing colonies and placed mycelia-side-down on opposite sides of a 90 mm plate (*ca*. 10 mm from the side of the plate). For experiments investigating the primary resource capture capabilities without special separation, experimental procedures followed that as described above, except that inoculum (fungus-colonised agar disks) of each competing taxon was placed in the middle of the plate (two discs in contact). Plates were sealed with parafilm, inverted and incubated at 24^0 C in the dark. After 10 days, the areas occupied by each fungus were measured using ImageJ

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software. The means of the areas occupied by the fungi on the different media were compared using ANOVA's and post-hoc Tukey HSD tests in R (Keppel & Wickens 2004).

Secondary resource capture:

I compared the abilities of ophiostomatoid species and 'environmentally acquired' taxa to capture area within infructescences already colonized by another taxon. In order to do so, a disk (0.5 mm in diameter) of fungal colonised MEA was aseptically removed from actively growing colonies and placed at the centre of a two-week-old colony of a different fungal taxon. The experiment was reversed for all the species combinations between ophiostomatoid and 'contaminant' fungal taxa and was repeated on both media types (derived from both *P. repens* and *P. neriifolia*). The experiment was replicated five times, each time using a different fungal isolate. The areas secondarily colonised by the second fungus placed in the plate were measured using ImageJ software and mean secondary area captured was compared between taxa and medium types using an ANOVA and post-hoc Tukey HSD test in R (Keppel & Wickens 2004).

Results:

Possible host plant benefit and dominant fungal taxa

Open and closed two-year-old infructescences did not differ in the percentage of viable seeds that they contained in either *P. repens* (AIC: 52.401, Z=0.446, p=0.656) or *P. neriifolia* (AIC=36.995, Z=0.006, p=0.995). Whether these were colonised by ophiostomatoid fungi also had no influence on seed set in either *P. repens* (AIC: 52.401, Z=0.843, p=0.399) or *P. neriifolia* (AIC=36.995, Z=0.936, p=0.349). However, compared to chance, significantly more of the closed infructescences contained ophiostomatoid fungi than those that had started to open in both *P. repens* (chi squared=11.736, df=1, p=0.001) and *P. neriifolia* (chi squared=9.697, df=1, p=0.002). Fungal communities in most of these closed, two-year-old infructescences that contained ophiostomatoid fungi were dominated by them, except for three infructescences of *P. neriifolia* that were dominated by other saprobes. It was not possible to determine dominant fungal taxa in open two-year-old infructescences, as these tended to quickly dry out, which prevented the observation of hyphomycete growth. In all

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cases where ophiostomatoid fungi was present in the closed one-year-old infructescences, two species dominated (*S. splendens* and *K. proteae* in *P. repens*, and *S. phasma* and *K. capensis* in *P. neriifolia*), and therefore no 'environmentally acquired' species were isolated from these infructescences. When no ophiostomatoid fungal species were present in closed one- and two-year-old infructescences, various other taxa dominated. However, three morphotypes were by far the most commonly encountered in both *P. neriifolia* and *P. repens*. Closest ITS matches on GenBank identified these taxa as *Penicillium toxicarium* (JX140943.1), *Cladosporium* cf. *cladosporoides* (JF340280.1), and *Fusarium* cf. *anthophilum* (LS422778.1). *Penicillium* cf. *toxicarium* was dominant in 26.47% of *P. repens* infructescences and in 22.5% of *P. neriifolia* infructescences. *Cladosporium* cf. *cladosporoides* was dominant in 26.47% of *P. repens* infructescences and in 30% of *P. neriifolia* infructescences. *Fusarium* cf. *anthophilum* was dominant in 29.41% of *P. repens* infructescences and in 17.5% of *P. neriifolia* infructescences. In all cases, sequences from isolates from the same fungal species obtained from the two host species were identical. Accession numbers for sequences of representative isolates available from GenBank include; for *Penicillium* cf. *toxicarium* (pending), for *Cladosporium* cf. *cladosporoides* (pending), and for *Fusarium* cf. *anthophilum* (pending).

Differential competition

In the de Wit replacement series experiment, based on the product of the relative crowding coefficients, all fungi were negatively influenced when growing in close proximity to one another (Table 1). In all cases and on both hosts, the ophiostomatoid fungi were always the weaker competitor when paired with any of the 'environmentally acquired' species based on this index. This was also evident from the ANOVA test for deviation from linearity in that significant deviations were observed for combination pairs of most fungal taxa tested (Table 1).

Table 1: ANOVA statistics for tests of deviation from linearity in relationships between the areas occupied by competing fungal species in a de Wit replacement series on media prepared from *P. repens* and *P. neriifolia* host tissues. The competitive influence of each separate species in an interacting pair, or relative crowding coefficient (RCC) and the product of the

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RCC values of the interacting pairs (in brackets) are also provided. *df* = Degrees of freedom, SS = Sum of squares, MS = Mean square

comparison	source	*df*	SS	MS	F-value	P-value	RCC
On *P. repens* media							
S. splendens vs *Cladosporium*							(0.142)
S. splendens area	proportion	1	0.01062	0.010622	3.363	**<0.001**	0.318
	Residual	13	0.04106	0.003159			
Cladosporium area	proportion	1	0.01595	0.015951	3.972	**0.063**	0.446
	Residual	17	0.06827	0.004016			
S. splendens vs *Penicillium*							(0.204)
S. splendens area	Proportion	1	0.07485	0.07485	29.62	**<0.001**	0.317
	Residual	16	0.04044	0.00253			
Penicillium area	Proportion	1	0.12672	0.12672	50.18	**<0.001**	0.645
	Residual	14	0.03535	0.00253			
S. splendens vs *Fusarium*							(0.185)
S. splendens area	Proportion	1	0.09748	0.09748	15.37	**<0.001**	0.227
	Residual	16	0.10148	0.00634			
Fusarium area	Proportion	1	0.3378	0.3378	556.8	**<0.001**	0.813
	Residual	16	0.0097	0.0006			
S. phasma vs *Cladosporium*							(0.148)
S. phasma area	Proportion	1	0.06978	0.06978	14.24	**0.002**	0.264
	Residual	15	0.07348	0.00490			
Cladosporium area	Proportion	1	0.05710	0.05710	28.06	**<0.001**	0.559
	Residual	15	0.03052	0.00203			

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S. phasma vs *Penicillium*							(0.085)
S. phasma area	Proportion	1	0.04619	0.4619	1.699	0.219	0.12
	Residual	11	0.29916	0.02720			
Penicillium area	Proportion	1	0.2965	0.29646	5.653	**0.037**	0.705
	Residual	11	0.5769	0.05244			

On *P. neriifolia* media

S. phasma vs *Cladosporium*							(0.256)
S. phasma area	Proportion	1	0.01390	0.013902	6.859	**0.024**	0.377
	Residual	11	0.02229	0.002027			
Cladosporium area	Proportion	1	0.0942	0.9420	50.01	**<0.001**	0.680
	Residual	12	0.0226	0.00188			
S. phasma vs *Penicillium*							(0.176)
S. phasma area	Proportion	1	0.11207	0.11207	55.08	**<0.001**	0.258
	Residual	14	0.02848	0.00203			
Penicillium area	Proportion	1	0.14469	0.14469	21.11	**<0.001**	0.684
	Residual	11	0.07538	0.00685			
S. splendens vs *Cladosporium*							(0.611)
S. splendens area	Proportion	1	0.04803	0.04803	58.57	**<0.001**	0.338
	Residual	15	0.01230	0.00082			
Cladosporium area	Proportion	1	0.06982	0.06982	21.39	**<0.001**	0.509
	Residual	15	0.04897	0.00326			
S. splendens vs *Penicillium*							(0.139)
S. splendens area	Proportion	1	0.11207	0.11207	55.08	**<0.001**	0.258
	Residual	14	0.028	0.0020			

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			48	3			
Penicillium area	Proportion	1	0.1061	0.10615	18.01	**<0.001**	0.540
	Residual	17	0.1002	0.00589			
S. splendens vs *Fusarium*							(0.125)
S. splendens area	Proportion	1	0.4225	0.4225	76.59	**<0.001**	0.151
	Residual	16	0.0883	0.005			
Fusarium area	Proportion	1	0.31275	0.31275	563	**<0.001**	0.831
	Residual	15	0.00833	0.00056			

Primary resource capture without spatial separation

We observed significant variation in the ability of fungal taxa to capture more space on media prepared from both *P. repens* (F-value = 434.6, *df* = 17, P-value = <0.001) and *P. neriifolia* (F-value = 83.28, *df* = 15, P-value = <0.001) when their initial inoculum was not separated in space, as would be expected when two fungal taxa arrive at the same point and at the same time (Figures 1 and 2). *Sporothrix splendens* could capture space at the same rate as *Cladosporium* and *Penicillium* on its usual host *P. repens* (Fig. 1), but struggled to capture more space than these two species on media prepared from its non-preferred host *P. neriifolia* (although not significantly so against *Penicillium*) (Fig. 2). The *Fusarium* species was a particularly fast grower on both types of media and rapidly colonised space when compared to any ophiostomatoid species. *Sporothrix phasma* grew and captured significantly less space when paired with *Cladosporium* and *Penicillium* on its non-usual *P. repens* host material (Fig. 1), but surprisingly, it was also a particularly weak competitor on media prepared from its usual *P. neriifolia* host against all 'contaminant' fungi when inocula were not spatially separated (Fig. 2). *Knoxdavesia proteae* and *K. capensis* failed to capture any space on media prepared from either hosts when not spatially separated from the competing 'environmentally acquired' fungi.

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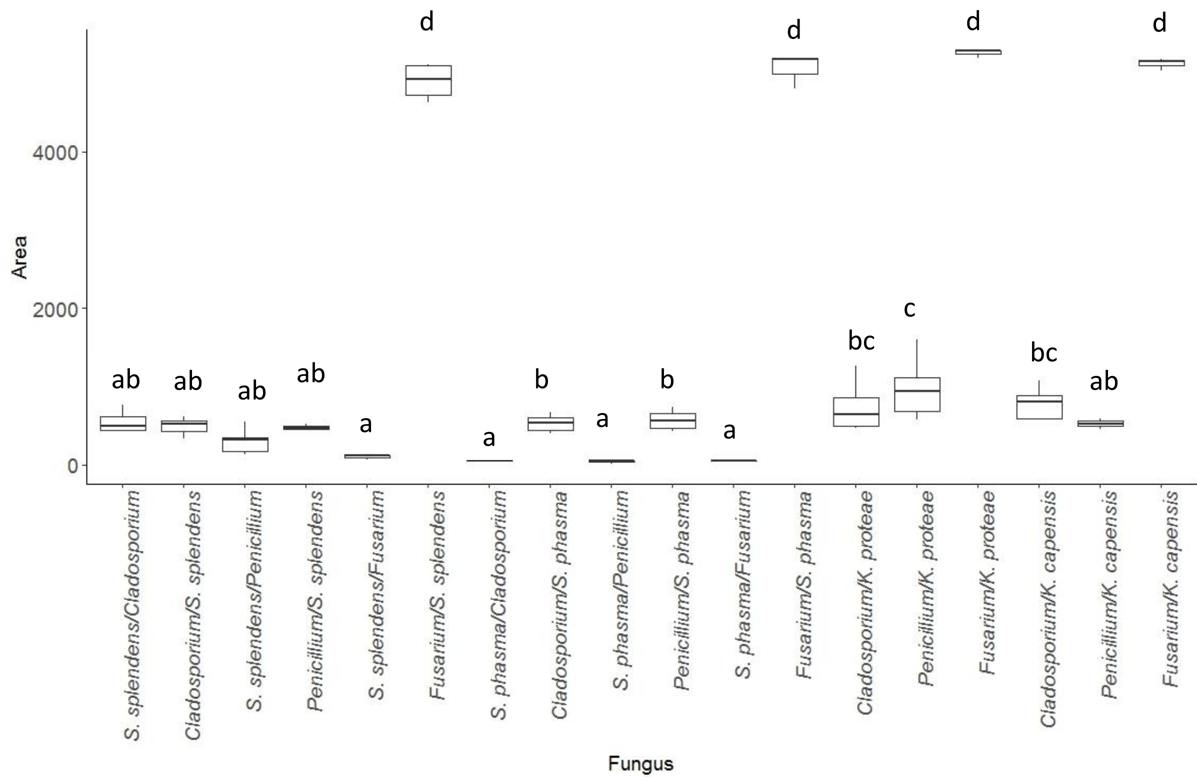

Figure 1: Median colony size (mm^2) after 10 days of growth as indication of primary resource capture capabilities without spatial separation between two competing fungal species on media prepared from *Protea repens* host tissues (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range). The first species on the label on the x-axis (to the left of the "/" separator) represents the area captured by that particular species when paired with the second species after the separator. In cases where zero area was captured (when the fungus was overgrown in all replicates), that particular taxon was omitted from analyses.

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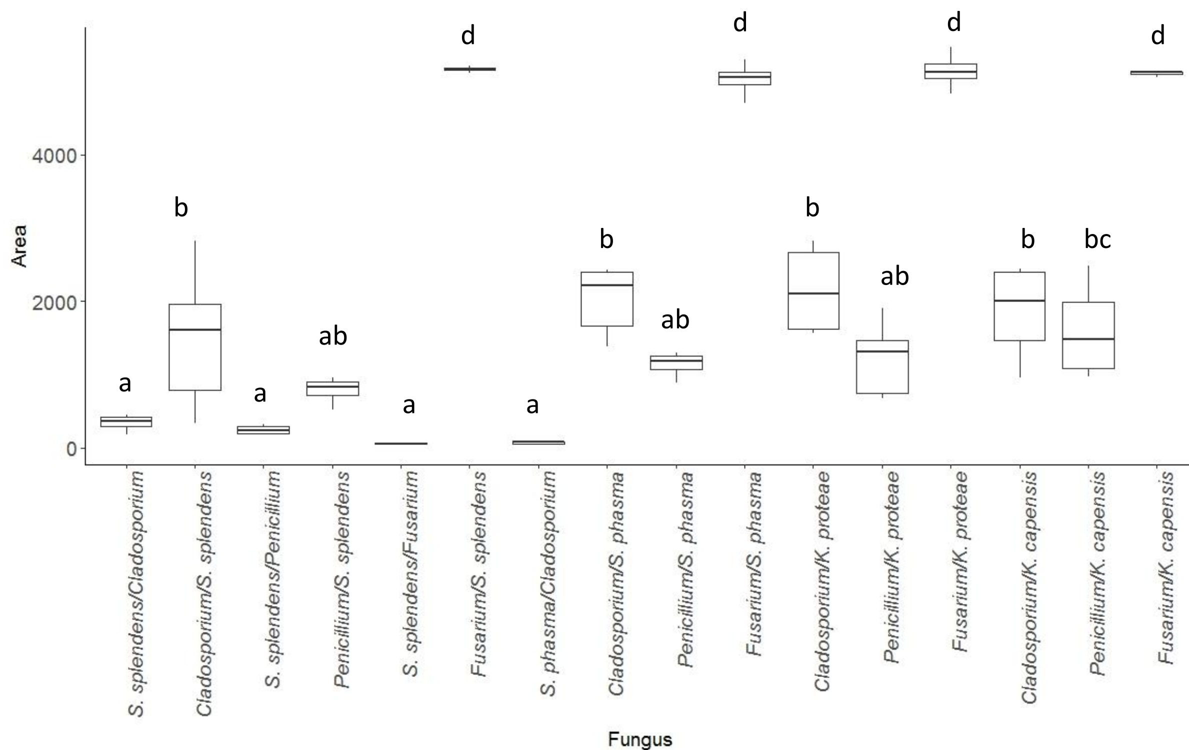

Figure 2: Median colony size (mm^2) after 10 days of growth as indication of primary resource capture capabilities without spatial separation between two competing fungal species on media prepared from *Protea neriifolia* host tissues (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range). The first species on the label on the x-axis (to the left of the "/" separator) represents the area captured by that particular species when paired with the second species after the separator. In cases where zero area was captured (when the fungus was overgrown in all replicates), that particular taxon was omitted from analyses.

Primary resource capture with spatial separation

Ophiostomatoid fungal species varied significantly in their abilities to capture area when paired with 'environmentall acquired' fungi on media prepared from both *P. repens* (F-value = 32.28, *df* = 23, P-value = <0.001) and *P. neriifolia* (F-value = 54.83, *df* = 23, P-value = <0.001) when inoculum was separated in space. All ophiostomatoid taxa were able to capture at least some space on the media when paired with 'environmentally acquired' species. However, none could capture more area than the 'contaminant' fungi on any host (Figs. 3 and 4). Area captured by *S. splendens* did not significantly differ from the area captured by *Cladosporium* or *Penicillium* on media prepared from both hosts. Area captured by *S.*

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phasma on *P. repens* (its non-usual host) did not differ significantly from the area captured by *Penicillium* or *Cladosporium*, but it captured significantly less space than *Cladosporium* on its usual *P. neriifolia* host material. *Fusarium* always captured significantly more space than the ophiostomatoid fungi on media prepared from both hosts. Area captured by *K. proteae* did not significantly differ from that captured by *Cladosporium* and *Penicillium* on both hosts (Figs. 3 and 4). Area captured by *K. capensis* was similar on both hosts when paired with *Penicillium*, but was significantly less when paired with *Cladosporium* (Figs. 3 and 4).

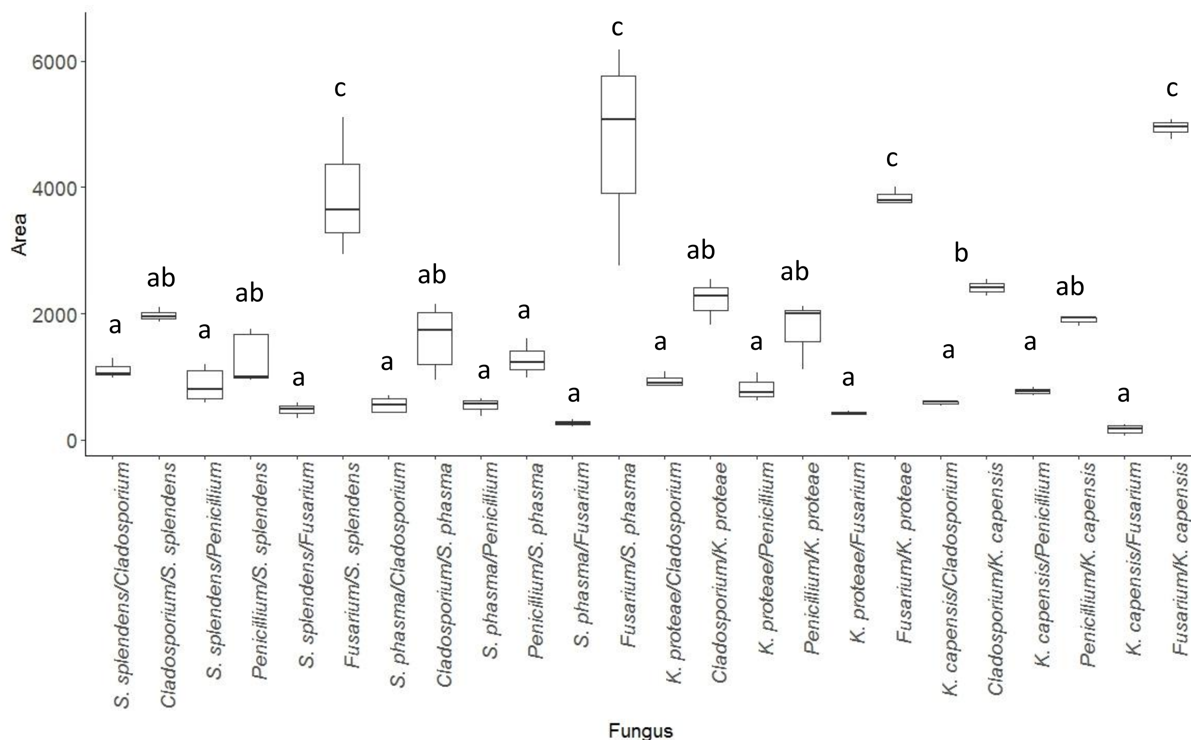

Figure 3: Median colony size (mm^2) after 10 days of growth as indication of primary resource capture capabilities with spatial separation between two competing fungal species on media prepared from *Protea repens* host tissues (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range). The first species on the label on the x-axis (to the left of the "/" separator) represents the area captured by that particular species when paired with the second species after the separator.

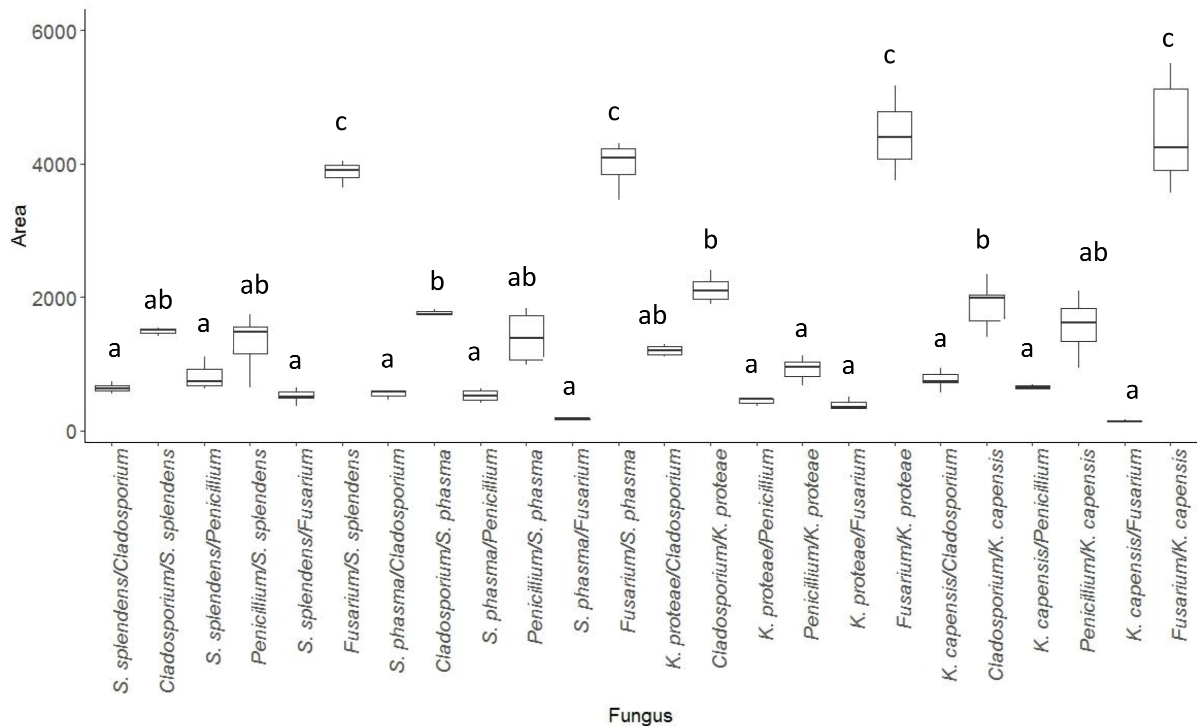

Figure 4: Median colony size (mm^2) after 10 days of growth as indication of primary resource capture capabilities with spatial separation between two competing fungal species on media prepared from *Protea neriifolia* host tissues (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range). The first species on the label on the x-axis (to the left of the "/" separator) represents the area captured by that particular species when paired with the second species after the separator.

Secondary resource capture

No ophiostomatoid fungi could grow on areas that were already captured by 'contaminant' fungi on media prepared from either host, but the 'environmentally acquired' fungi could often grow on the ophiostomatoid fungi. 'Environmentally acquired' species differed in their abilities to do so (F-value = 91.99, *df* = 20, P. value = <0.001), but the *Cladosporium*, *Fusarium* and the *Penicillium* species were only able to capture limited space on media already occupied by *S. splendens* and *S. phasma* (Fig. 5). Similarly, the *Cladosporium* species could capture only limited space already occupied by *K. capensis*, and the *Penicillium* species could only capture limited space already occupied by *K. capensis* on both hosts. The differences in the area captured did not differ significantly for any of these combinations.

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Interestingly, differences in capabilities of 'environmentally acquired' species to capture space on *K. capensis* and *K. proteae* were often host related (Fig. 5). For example, the *Cladosporium* was able to capture more space occupied by *K. proteae* on media prepared from *P. neriifolia* (non-preferred host) than that of *P. repens* (preferred host). Similarly, *K. capensis* was a significantly better competitor in terms of maintaining area originally captured on its preferred *P. neriifolia* host than on its non-usual *P. repens* host against both the *Cladosporium* and the *Fusarium* species. *Knoxdavesia proteae* was particularly weak at maintaining occupied area against the *Fusarium* species on media prepared from both hosts. The *Fusarium* species failed to capture space already occupied by *S. splendens* on both hosts and could only capture limited space already occupied by *S. phasma* and only on its preferred *P. neriifolia* host.

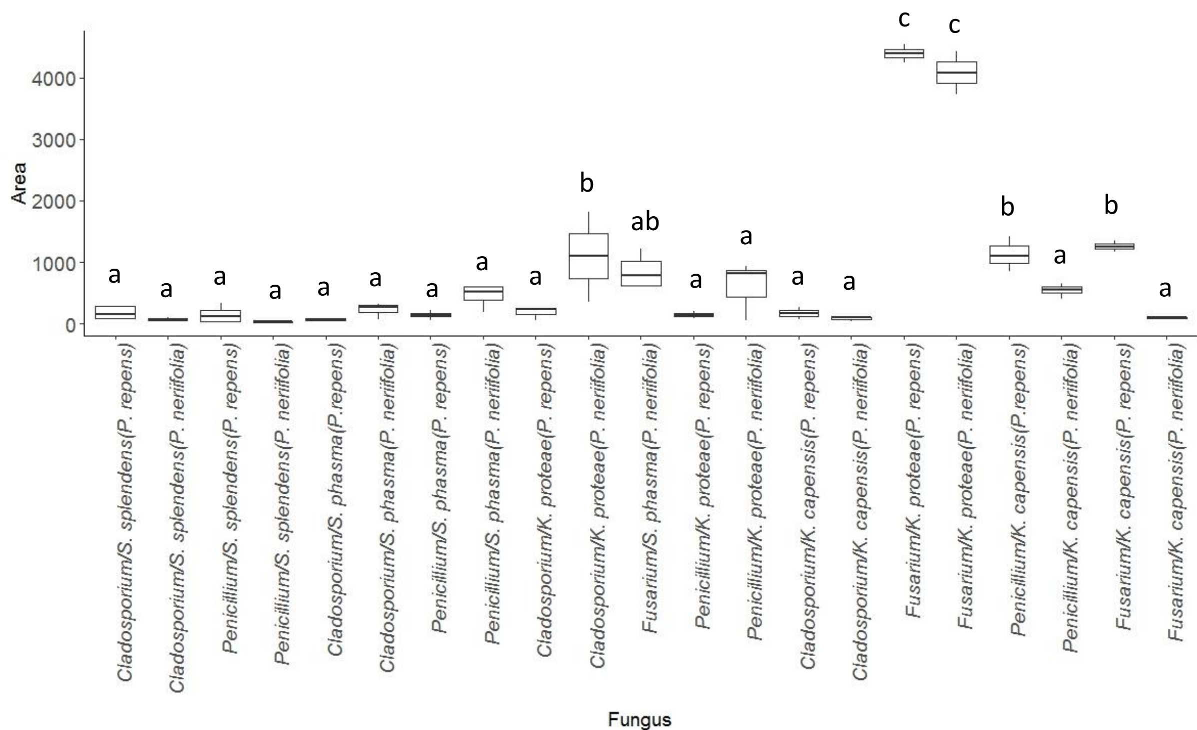

Figure 5: Median colony size (mm^2) of *Cladosporium* cf. *cladosporoides*, *Penicillium* cf. *toxicarium* and *Fusarium* cf. *anthophilum* after 10 days of growth on other fungal species as indication of its secondary resource capture capabilities on media prepared from *Protea repens* and *P. neriifolia* host tissues (box indicates 25–75% data range, whiskers indicate 1.5 times the interquartile range). The first species on the label on the x-axis (to the left of the "/" separator) represents the area captured by that particular species when paired with the second

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species after the separator. The host media used in each case are presented in parenthesis on these labels.

Discussion

I show that the *Protea*-associated ophiostomatoid fungi are comparatively weak when in direct competition with other common saprobes retrieved from their infructescence niche. Despite this, they remain the most dominant taxa for at least two years after flowering. It was not possible to include a negative control in this study (zero fungal colonization in infructescences) and it is therefore unknown whether colonization by ophiostomatoid fungi is beneficial or detrimental to the hosts. However, I show that even though both the ophiostomatoid fungi and the other fungi evaluated in this study are saprobes (Aylward *et al*. 2017), the ophiostomatoid fungi may be more beneficial to the host plant than other saprobes (or at least less detrimental). Structures that house ophiostomatoid fungi persist on the hosts for longer periods than those that contain only other saprobic fungi as more infructescences colonised by environmentally acquired fungi started to open. Excluding these 'environmentally acquired' fungi from mature infructescences therefore seems beneficial to the host as seed release is delayed when conditions are suboptimal for seedling recruitment (*e.g.* before a fire event (Biccard & Midgley 2009; Rourke & Wies 1977; Yeaton & Bond 1991)). Infructescences that persist for longer periods on hosts will also benefit the fungi they contain, as these will have enhanced prospects of successful dispersal and they will be protected from exposure to unfavourable conditions. In addition to previously described factors such as host plant death due to fire and the action of seed predators in infructescences (Coetzee & Giliomee 1987). I therefore describe another mechanism that could strongly influence *Protea* population dynamics, *i.e.* colonization by different communities of saprobic fungi.

The mechanism by which the ophiostomatoid fungi may be beneficial (or less detrimental) to their hosts over other saprobes is not yet known. When colonizing inflorescences (flowers) of *Protea*, these ophiostomatoid fungi utilise simple nectar sugars (Aylward *et al*. 2017). When floral parts in infructescences (cones) start to senesce, these fungi can switch to exploiting common cell-wall structural components such as cellulose and hemicellulose (Aylward *et al*. 2017). The other 'environmentally acquired' taxa studied here belong to genera that are well known for their superior abilities to utilize complex plant cell-wall components and are

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therefore often commercially exploited (*e.g.* Mohanram *et al*. 2013, Reina *et al*. 2018). Although not tested, we believe that the digestive actions of these infructescence-inhabiting 'environmentally acquired' fungi may be superior to those of the ophiostomatoid fungi. This will result in drier conditions within infructescences colonised by 'environmentally acquired' (less compact floral parts within infructescences) and that these drier conditions may cause their premature opening and seed release. However, all three of these hypotheses need verification through rigorous testing in future studies.

When competing for unoccupied space, species such as *S. splendens* were able to capture space at competitive rates against 'environmentally acquired' species. Even though weak competitors, all ophiostomatoid fungi negatively affected the growth rate of all other fungal species evaluated. Hereafter, most ophiostomatoid fungi were also able to defend occupied space fairly well. However, the ability to capture and defend primary space differed significantly according to the specific fungal combination and, given enough time, most 'environmentally acquired' fungi would eventually outgrow the ophiostomatoid fungi irrespective of whether they were initially separated in space or not. If the ophiostomatoid fungi and other saprobes arrive at an uncolonised infructescence at the same time, the ophiostomatoid fungi will therefore ultimately be outcompeted. For ophiostomatoid fungi to dominate in this highly competitive infructescence environment for at least two years, they will need a strong competitive advantage that may not be host related at all. This is accentuated by the fact that they have to spend more time on a resource to capture un-colonized space in the absence of other competitors before having to defend it.

Ophiostomatoid fungi characteristically produce sticky spores that are adapted to arthropod vectored dispersal (Malloch & Blackwell 1993). Most of the *Protea*-associated species have mutualistic associations with mites, and mites are the primary spore vectors for all ophiostomatoid species assessed to date (Roets *et al*. 2007, 2009; Theron-De Bruin *et al*. 2018). Importantly, all of these mites are phoretic, either directly or indirectly, on primary *Protea* pollinators such as beetles (Roets *et al*. 2011) and birds (Theron-De Bruin *et al*. 2018). This method of vectored dispersal ensures that the ophiostomatoid fungi disperse to newly formed inflorescences (flowering structures) at a very early stage, as soon as the first flowers open (Theron-De Bruin *et al*. 2018). In contrast, the 'environmentally acquired' species tested here are not known to have close associations with *Protea*-associated mites (Roets *et al*. 2007) and will likely depend on dispersal via wind as is usual for most representatives of these genera (although a few species may be vector dispersed *e.g.* Abdel-

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baky *et al*. 1998; Gracia-Garza *et al*. 1998; Visagie *et al.* 2014, 2016). Dispersal of fungal spores from infructescences via wind would be less effective than vectored spore dispersal, as only few spores can escape from these tightly closed structures and most spores carried by wind will not reach inflorescences as easily as targeted vectored dispersal (Malloch & Blackwell 1993). The ophiostomatoid fungi therefore have a competitive advantage over these 'environmentally acquired' taxa, since they have the evolved ability to colonise inflorescences much more efficiently and earlier. In addition, the ophiostomatoid fungi can disperse between different flowers over vast distances and from a very early stage (between-inflorescence (flower) dispersal can happen as soon as the first flowers open), which will help ensure dominance of this niche continuously throughout the flowering season of their *Protea* hosts (Theron-De Bruin *et al*. 2018).

Some ophiostomatoid species were better competitors in terms of capturing and defence of primary space when growing on material prepared from their usual host. The usual host for *S. splendens* and *K. proteae* is *P. repens* and the usual host for *S. phasma* and *K. capensis* is *P. neriifolia* (Mukwevho *et al*. 2019; Chapter 1). *Sporothrix splendens* is occasionally also collected from *P. neriifolia* and *K. capensis* is occasionally also collected from *P. neriifolia* (Aylward *et al.* 2015; Theron-de Bruin *et al*. 2018). I found that *S. splendens* could capture open space at similar rates to other competing saprobes on both hosts (except against the *Fusarium* species) and was able to defend captured space well against these on both hosts, which may explain its ability to persist on both hosts in natural conditions. As found in a previous study (Mukwevho *et al*. 2019; Chapter 1), the restricted presence of *S. splendens* on *P. neriifolia* is likely due to the restricted movement of its spore vectors rather than weaker competitive abilities on alternative hosts. *S. phasma* was a weaker competitor against other saprobes on its non-usual host (*P. repens*) than on its usual host (*P. neriifolia*). However, *S. phasma* often occupies the same areas as *K. capensis* in *P. neriifolia* infructescences (Mukwevho *et al*. 2019; Chapter 1). I demonstrated that both *S. phasma* and *K. capensis*, although weak competitors against other saprobes for available space, are effective at maintaining captured space against the 'environmentally acquired' fungi. *Knoxdavesia capensis* is generally better at doing so on its preferred host. These two species are unable to capture space occupied by the other fungi and can capture space at similar rates when growing in close proximity on *P. neriifolia* (Mukwevho *et al*. 2019; Chapter 1). *Knoxdaviesia capensis* may therefore help maintain area against other saprobes for the persistence of *S. phasma* in *P. neriifolia* infructescences, which would negate the need for *S. phasma* to be a

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strong competitor against 'contaminant' taxa. The absence of *S. phasma* on *P. repens* may be ascribed to the dominance of stronger competing species such as *S. splendens* (Mukwevho *et al*. 2019; Chapter 1) and the 'environmentally acquired' species tested here. The general absence of *K. capensis* from *P. repens* may be due to its decreased competitive abilities against both the 'environmentally acquired' species as described in this study and against *S. splendens* (Mukwevho *et al*. 2019; Chapter 1). *Knoxdavesia proteae* is a weaker competitor against some 'environmentally acquired' fungi on its alternate host compared to its preferred *P. repens* host, but its host exclusivity and persistence in this niche is likely due to a combination of weak competitive abilities against *S. phasma* and the actions of spore vectors (Mukwevho *et al*. 2019; Chapter 1).

Here I described an undocumented putative beneficial association between plants and saprobic fungi in a biodiversity hotspot (Goldblatt & Manning 2002). To the best of my knowledge, this is the first case of increased fitness of a host plant (increased storage time of seeds on plants) due to association with above-ground saprobic fungi, even though the precise mechanism remains unknown. Surprisingly, I found that the usually dominant, more beneficial saprobes are comparatively weak competitors against more detrimental saprobes and that the role of spore vectors are therefore probably central to the persistence of the ophiostomatoid fungi in this very unusual nice. Similarly, differential competitive abilities of the ophiostomatoid fungi against other saprobes and each other (Mukwevho *et al*. 2019; Chapter 1) partly explains host consistency observed in the field, but spore vectors may also play a central role in the co-existence of so many different species in this restricted niche. The host exclusivity of taxa such as *K. proteae* on a single host, usually in close proximity to superior and early colonizing competitors such as *S. splendens* is, however, difficult to explain and needs further assessment.

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Chapter 3: Co-occupancy of a restricted niche by ecologically similar fungi

Abstract

Knoxdaviesia and *Sporothrix* dominate fungal communities within *Protea* infructescences. Despite similar ecologies such as a saprobic habit and mite-mediated spore dispersal, these ophiostomatoid fungi show strong host recurrence and often occupy the same individual infructescences. Host chemistry and the actions of spore-vectors help maintain host recurrence patterns, but whether these factors also aid the co-occupancy of multiple species within individual infructescences is unknown. *Sporothrix splendens* and *K. proteae* often grow on different senescent tissue types within infructescences of their *P. repens* host, indicating that substrate-related differences may aid their spatial segregation. However, *S. phasma* and *K. capensis* usually grow on the same senescent tissues types of *P. neriifolia* and neutral competitive abilities may explain their infructescence co-occupancy. In the present study, I test the hypothesis that differences in host-tissue types dictate competitive abilities of different ophiostomatoid fungal species and explain the co-occupancy of multiple species in this spatially restricted niche. Growth media for fungal competition studies were prepared from senescent infructescence bases, bracts, seeds or pollen presenters of *P. neriifolia* and *P. repens*. As expected from field observations, *K. capensis* was unable to grow on *P. neriifolia* seeds and infructescence bases, but thrived on pollen presenters. Growth of *S. phasma* was strongly inhibited on infructescence bases, but it grew well on seeds and pollen presenters. These two fungal species had neutral competitive abilities on pollen presenters, explaining their co-occupancy of this resource. *Knoxdaviesia proteae* and *S. splendens* were unable to grow on *P. repens* infructescence bases. As expected, growth of *K. proteae* was strongly inhibited on bracts, but significantly enhanced on pollen presenters. In contrast, growth of *S. splendens* was only slightly inhibited on both bracts and pollen presenters. Despite this, *S. splendens* was a superior competitor on all tissue types. For *K. proteae* to co-occupy infructescences with *S. splendens* for extended periods, it likely needs to colonize pollen presenters before the arrival of *S. splendens* and may consequently depend on different spore vectors.

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Introduction

A high diversity of saprobic fungi colonize senescent plant materials such as leaf litter and wood (Kodsueb *et al.* 2008) and form integral parts of ecosystem processes such as decomposition and nutrient cycling (Kumar *et al.* 2012). Many factors can contribute to the maintenance of such high saprobe diversity on senescent plant parts, including differences in chemical composition and physical structure of different hosts (Lodge *et al.* 1997; Mille-Lindblom *et al.* 2006; Paulus *et al.* 2006; Hyde *et al.* 2007; Osono 2011; Wolfe & Pringle 2012; Tedersso *et al.* 2013). However, numerous saprobic fungal species can also colonise substrates that originate from a single host and often thrive in very close proximity. High numbers of fungal species on senescent parts of the same host may be maintained by differences in nutrient source usage, differences in colonising times related to differences in spore dispersal and differential competitive abilities, all of which may drive succession (Hyde *et al.* 2007; Bleiker & Six 2009; Zhao *et al.* 2013; Kubicek *et al.* 2014). In addition, plant structures usually contain many different tissue types that may each be exploited by different fungi (Hyde *et al.* 2007; Paulus *et al.* 2003a, b).

Protea L. (Proteaceae) infructescences represent a unique aerial niche for saprobic fungi and house communities that are strongly divergent from those on senescent *Protea* twigs and leaves (Lee *et al.* 2003, 2004; Marincowitz *et al*. 2008). These structures form on plants after a short flowering stage (Fig. 1), but can persist for several years as above-ground seed storage organs (Rebelo 1995). After pollination, the outer bracts enclose the old flowers in compact cone-like infructescences. Living tissues in these structures comprise only the disc-like bases and seeds (Fig. 1). The rest of these structures consist of dead material in the form of hundreds of infertile seeds, bracts and senescent flower parts (including tepals and pollen presenters). Infructescences provide a moist, protected environment (Roets *et al.* 2012) in which numerous micro-fungi (Marais & Wingfield 1994, 2001; Lee *et al.* 2003, 2005) and arthropods (Coetzee & Giliomee 1987a, b; Roets *et al.* 2006b) thrive.

Ophiostomatoid fungi in the genera *Knoxdaviesia* M.J. Wingf., Van Wyk & Marasas and *Sporothrix* M.J. Wingf., Van Wyk & Marasas dominate dead floral parts in *Protea* infructuscences (Marais & Wingfield 1994, 2001; Lee *et al.* 2005; Roets *et al.* 2005). Three species of *Knoxdaviesia* in the Microascales (Wingfield *et al*. 1988; Wingfield & Van Wyk 1993; Crous *et al.* 2012) and 11 species of *Sporothrix* in the Ophiostomatales (Marais & Wingfield 2001; Roets *et al.* 2006a, 2008, 2010; Marais & Wingfield 1994, 1997; Ngubane *et*

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al. 2017) have been described from this niche. Species show various degrees of host recurrence. For example, *K. proteae* M.J. Wingf., P.S. van Wyk & Marasas 1988 is exclusive to *P. repens* (Roets *et al.* 2009). In contrast, the closely related *K. capensis* M.J Wingf. & P.S. van Wyk 1993 is common on other hosts species such as *P. neriifolia* R. Br. and *P. lauriifolia* Thunb., and is only very rarely found on *P. repens*. infructescences (Roets *et al.* 2009; Aylward *et al*. 2015b). *Sporothrix splendens* G.J. Marais & M.J. Wingf. 1994 is nearly omnipresent within infructescences of *P. repens*, but has occasionally also been found on other hosts such as *P. neriifolia* R. Br. (Theron-De Bruin *et al.* 2018). In contrast, *S. phasma* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. 2016 is found on species such as *P. neriifolia* and *P. lauriifolia*, but never on *P. repens* (Roets *et al.* 2009). These strong host recurrence patterns are maintained even when the hosts grow sympatrically, and may be ascribed to differences in temperature and humidity within infructescences (Roets *et al.* 2012), differences in chemical composition of different *Protea* species (Roets *et al.* 2012), host-induced differences in competitive abilities (Mukwevho *et al.* 2019; Chapter 1) and the actions of their spore vectors (Roets *et al.* 2012, Mukwevho *et al.* 2019; Chapter 1).

Ophiostomatoid fungi produce sticky spores that are adapted to arthropod vectored dispersal (Malloch & Blackwell 1993). Various mites are the primary vectors of all *Protea*-associated species (Roets *et al.* 2007, 2009) and some may even have mutualistic associations with their fungal partners (Roets *et al.* 2007; Theron-De Bruin *et al.* 2018). For long-distance dispersal, the mites are phoretic on *Protea*-pollinating beetles (Roets *et al.* 2008) and birds (Theron-De Bruin *et al.* 2018). This vectored mode of dispersal not only ensures that these fungi can colonize new flowers over vast distances (Aylward *et al.* 2014, 2015a, 2016a; Ngubane *et al.* 2018), but can do so very early on, as soon as the very first flowers open within individual inflorescences (Theron-De Bruin *et al.* 2018). This likely gives these comparatively weak competitors an advantage over other saprobic fungi that compete for resources in the restricted inflorescence / infructescence environment (Mukwevho *et al.* 2019; Chapter 2).

Multiple ophiostomatoid fungal species often grow within the same individual infructescence and even sporulate concurrently (Roets *et al.* 2005, 2013). Within *P. neriifolia* infructescences, *S. phasma* grows on seeds near the base, but also towards the tips of old pollen presenters (Roets *et al.* 2006a; Theron-De Bruin *et al.* 2018; Fig. 1). *Knoxdaviesia capensis* is confined to pollen presenters (Aylward & Roets *pers. observ*.). No ophiostomatoid fungi have been found on the hard *P. neriifolia* infructescence bases (Roets *et*

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al. 2006; Fig. 1). Ophiostomatoid fungi in *P. repens* infructescences seem to be more segregated in space, as *S. palmiculminata* (Roets, Z.W. de Beer & M.J. Wingf.) Z.W. de Beer, T.A. Duong & M.J. Wingf. 2016 is only found in insect-damaged bases and *K. proteae* is only found on pollen presenters, while *S. splendens* is routinely found on the seeds (Roets *et al.* 2013). However, *S. splendens* has also been recovered from involucral bracts that enclose the other floral parts (Fig. 1) and pollen presenters of *P. repens* (Human, Ngubane & Roets, *pers. observ.*). This co-occurrence of ecologically similar fungi (in terms of saprobic life-style, host recurrence and spore dispersal agents) within the restricted area provided within a single *Protea* infructescence is intriguing. Possible explanations for this could include differences in position where the different fungi are initially inoculated on different tissues within infructescences, or that the different fungi may have different competitive abilities on the different tissue types. In terms of the former hypothesis it is possible that some fungal species are able to outcompete other species only on specific tissue types within infructescences. In terms of the latter hypothesis, inoculation on different tissue types within infructescences is only likely to happen when different fungal species have different main spore vectors.

It is known that the *Sporothrix* species are mainly dispersed by mites in the genera *Tarsonemus* Canestrini and Fonzago, *Glycyphagus* Hering and *Proctolaelaps* Ryke between open flowers and by *Trichaouropoda* Berlese mites between both infructescences and open flowers (Roets *et al.* 2007, 2009, Theron-De Bruin *et al.* 2018). Main spore vectors for the *Knoxdavesia* species in this system are not well-studied, but current evidence suggest that they are mainly dispersed by *Trichaouropoda* mites between infructescences and open flowers (Roets *et al.* 2011). Secondary vectors of the *Tarsonemus*, *Glycyphagus* and *Proctolaelaps* mites include various *Protea*-pollinating beetles and birds, while *Trichaouropoda* mites have only been collected from a single pollinating beetle species (Roets *et al.* 2011; Theron-De Bruin *et al.* 2018).

In the present study I set out to test the hypothesis that co-occupancy of individual *Protea* infructescences by ecologically similar ophiostomatoid fungi is related to differential competitive abilities on different tissue types within these structures. I tested the competitive abilities of *S. splendens* and *K. proteae* on media prepared from bases, senescent pollen presenters, unfertilised seeds and bracts of their usual *P. repens* host. I similarly tested the competitive abilities of *S. phasma* and *K. capensis* on media prepared from the bases, senescent pollen presenters and unfertilized seeds from their usual *P. neriifolia* host. Based

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on field observations, I expect that no fungi will be able to grow on media prepared from the bases of any host. On *P. neriifolia*, I expect that *S. phasma* can grow on seeds and pollen presenters. *Knoxdavesia capensis* can probably only grow on pollen presenters or, if it can also grow on seeds, it will be outcompeted by *S. phasma* on this tissue type. As both species often co-occur on pollen presenters, I expect that they will have similar competitive abilities on these structures. From observations on *P. repens*, I expect that *S. splendens* can grow on all structures (except infructescence bases) and that *K. proteae* can only grow on media prepared from pollen presenters. If *K. proteae* can grow on media prepared from seeds and bracts, I expect that *S. splendens* will be a superior competitor. On pollen presenters, I expect *K. proteae* to be a superior competitor. Deviations from these expectations may point towards a possible role of different vectors in the dispersal of the different fungal species.

Methods and Materials:

Collection of ophiostomatoid fungi and preparation of growth media

Ophiostomatoid fungi used in this study were the same species and isolates used in fungal competition studies (Mukwevho *et al.* 2019; Chapter 1; Chapter 2). *K. proteae* (Stellenbosch mountain (-33.9466; 18.8805)) and *S. splendens* (Betty's Bay (-34.3315 18.9925)) were collected from *P. repens* and *K. capensis* (Betty's Bay (-34.35495; 18.90135)) and *S. phasma* (Jonkershoek Nature Reserve (33°59'24.5"S, 18°57'25.2"E)) were collected from *P. neriifolia*. For growth media (following Roets *et al.* 2012 and Mukwevho *et al.* 2019; Chapter 1), two-year-old infructescences of *P. repens* and *P. neriifolia* were collected from the Jonkershoek Nature Reserve and air-dried in the laboratory until they opened *ca.* 3 weeks later. Hereafter infructescences were separated into the infructescence base (receptacle for bracts and florets), the bracts (for *P. repens* only, as the recurved bracts of *P. neriifolia* are not colonised by ophiostomatoid fungi), pollen presenters (including any remnants of tepals) and seeds. For media prepared from seeds, I removed all fertile seeds, identified by their larger size (Theron de-Bruin *et al.* 2018), as not to include possible antimicrobial compounds that they may contain into media. These separated dead floral parts were dried at 40˚C for 48 hours and ground into a fine powder using a milling machine (Monitoring and Control Laboratories (Pty) Ltd). One litre of water-based growth medium contained 300 ml prepared *Protea* tissue (powder) and 1.5 % MEA. Media was autoclaved at 115˚C for 20 min and poured into 90 mm petri dishes that acted as competition arenas.

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Fungal growth rates on different tissues

The growth of the ophiostomatoid fungi was tested on media prepared from the different tissues following methods described in Roets *et al.* (2012). In short, plates were centrally inoculated with 5 mm diameter agar discs containing actively growing, 2-week-old hyphae of one of five different isolates of each of the four fungal species tested (n = 5 per tested species on the different media). As I was interested in the growth of the fungi on their usual hosts, *S. splendens* and *K. protea* were grown on tissues that originated from *P. repens* and *S. phasma* and *K. capensis* were grown on media prepared from *P. neriifolia*. In addition, all isolates were also grown on plates containing only MEA as a control. All inoculated plates were inverted and incubated at 25˚C in the dark. The diameter of each fungal colony on the various media was determined after 10 d of growth by calculating the average of two perpendicular diameter measurements. Growth for each fungal species on each of the test media was determined by calculating the mean radial growth (±standard error) of the five representative isolates of each of the four fungi. A one-way analysis of variance (ANOVA) was used to analyse the normally distributed data in Statistica 9 (Statsoft Corporation, Tulsa, USA) software package with stigma-restricted parameterisation. A Fisher's Protected Least Significance Difference (LSD) *post hoc* test was performed to determine the significant differences between means. Differences between radial growths of the fungal species on each of the test media were considered significant when $P \leq 0.05$.

Differential competition between fungi on media prepared from different host tissues

A de Wit replacement series experimental design (Klepzig & Wilkens 1997; Klepzig 1998) was used to test the competition between *S. splendens* and *K. proteae* (on *P. repens* infructescence structures) and between *S. phasma* and *K. capensis* (on *P. neriifolia* infructescence structures) following a modified experimental procedure of Mukwevho *et al.* (2019; Chapter 1; Chapter 2). The two competing fungal species were introduced in a 90 mm diameter plate at different proportions of inoculum and left to compete for available space. Hereafter the total area occupied by each fungus was expressed as a log linear function of its initial proportion inoculum. If both interacting species had similar competitive abilities, there would be no deviation from linearity. However, significant deviation from linearity for both species, one positive and the other negative, would indicate differential competition with one species dominating over the other. Inoculum covered disks (0.5 mm in diameter) of

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ophiostomatoid fungi were aseptically removed from the edges of actively growing fungal colonies and introduced face-down onto plates in a randomised block design (4 x 4 cm grid) following Mukwevho *et al.* (2019; Chapter 1). Inoculation ratios used included: species A vs. species B: 0:1 (16 disks species B), 0.25:0.75 (4 disks sp. A and 12 disks sp. B), 0.5:0.5 (1:1) (8 disks sp. A and 8 disks sp. B), 0.75:0.25 (12 disks sp. A and 4 disks sp. B) and 1:0 (16 disks species A). The procedure was repeated for all five tests (5 different ratios) per pairwise species combination and replicated five times per tested medium type, each time using different isolates. Plates were incubated at 25^{0}C in dark for ten days. Hereafter the areas occupied by each fungus were measured using image J software (LOCI, University of Wisconsin). Deviations from linearity were calculated by performing an analysis of variance (ANOVA) on log-transformed means of the area data (Wilson & Lindow 1994) in R (R Development Core Team 2013). Relative crowding coefficients (RCC) were also calculated for all pairwise combinations as [(mean area of species A at 1:1)/(mean area of species A at 1:0)] and [(mean area of species B at 1:1)/(mean area of species B at 1:0)]. The interacting species with a higher coefficient was considered dominant. If the product of the coefficients was one, then fungal competition was considered to be neutral. If the product of the coefficients was less than one, then the fungi were viewed as negatively affecting each other and if it was greater than one the taxa were believed to benefit from growing together (Willey & Rao 1980).

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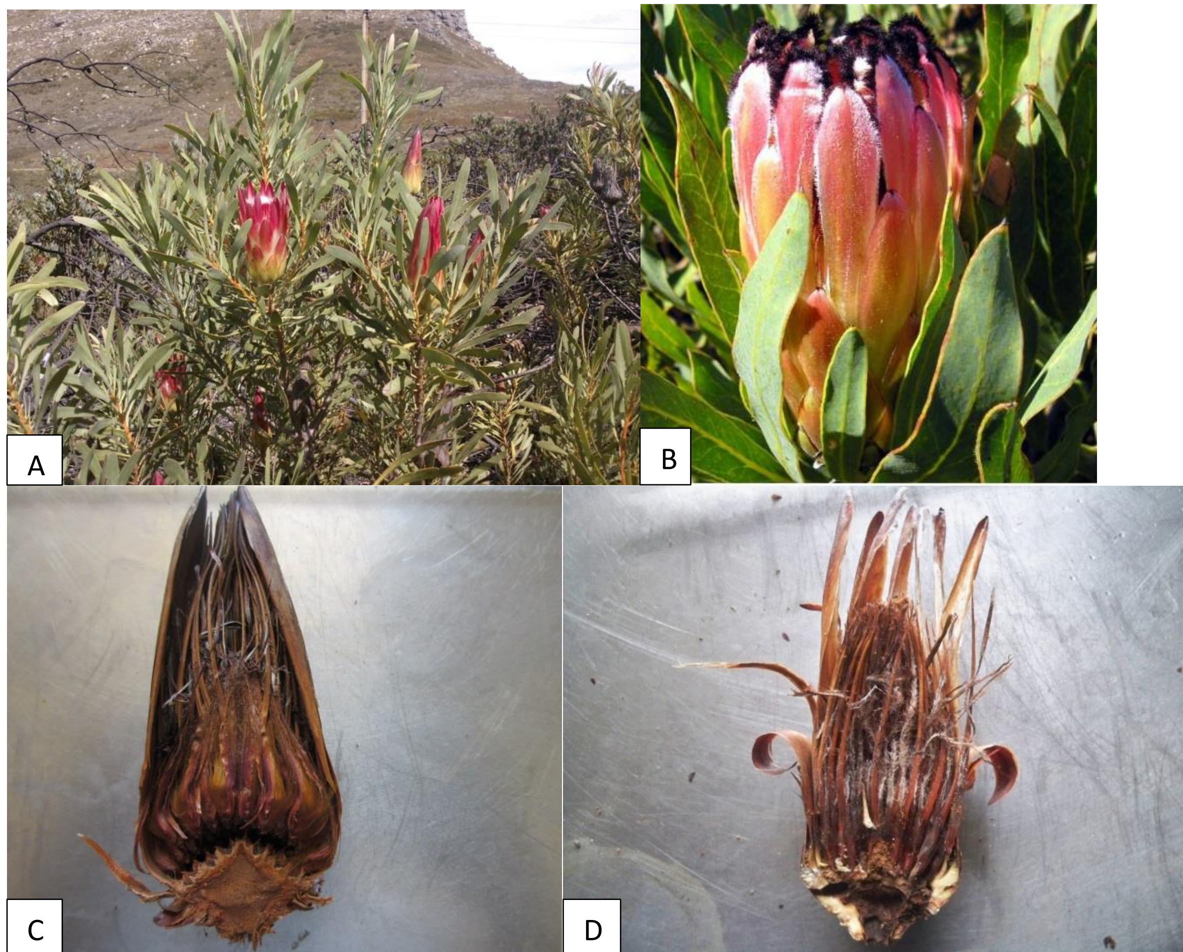

Figure 1: (A) - Inflorescence of *Protea repens*, (B) - inflorescence of *Protea neriifolia* (Theron-De Bruin et al. 2018), (C) - cross-section of *Protea repens* infructescence showing the hard base at the bottom with attached seeds, extended pollen presenters, and the surrounding bracts, (D) - cross-section of *P. neriifolia* infructuscence showing the hard base at the bottom with attached seeds, extended pollen presenters , and the surrounding bracts.

Results:

Fungal growth rates on different plant parts

Fungi grew at significantly different rates on media prepared from the different structures of both *P. repens* (*K. proteae* F = 40.373, *df* = 3, p = <0.001; *S. splendens* F = 12.735, *df* = 3, p = <0.001) and *P. neriifolia* (*K. capensis* F = 0.768, *df* = 1, p = 0.401; *S. phasma* F = 21.725, *df* = 3, p = <0.001). *Knoxdaviesia proteae* did not grow on media prepared from *P. repens* bases (Fig 2.). It also had a significantly reduced growth rate on media prepared from bracts of this species. It grew well on media prepared from seeds, but significantly better on media

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prepared from pollen presenters. As described in Roets *et al.* (2012), *K. proteae* produced denser hyphae when growing on media prepared from *P. repens* pollen presenters than on media prepared from the seeds and bracts. *Sporothrix splendens* also failed to grow on media prepared from *P. repens* infructescence bases and its growth rate was reduced on the other infructescence structures. It also grew best on media prepared from the pollen presenters. Similar to *K. proteae*, it produced denser hyphae on pollen presenter media than on media prepared from the seeds and bracts. *Knoxdaviesia capensis* could only grow on media prepared from pollen presenters of *P. neriifolia* (Fig 2.). The growth of *S. phasma* was significantly inhibited on media prepared from *P. neriifolia* infructescence bases. It grew well on seed media, but optimally on pollen presenter media, where it also had the most dense colony morphology. As was found by Roets *et al.* (2012), the two *Knoxdavesia* species grew significantly faster than the two *Sporothrix* species on pollen presenter media originating from their own hosts when not competing with other fungi (Fig. 2).

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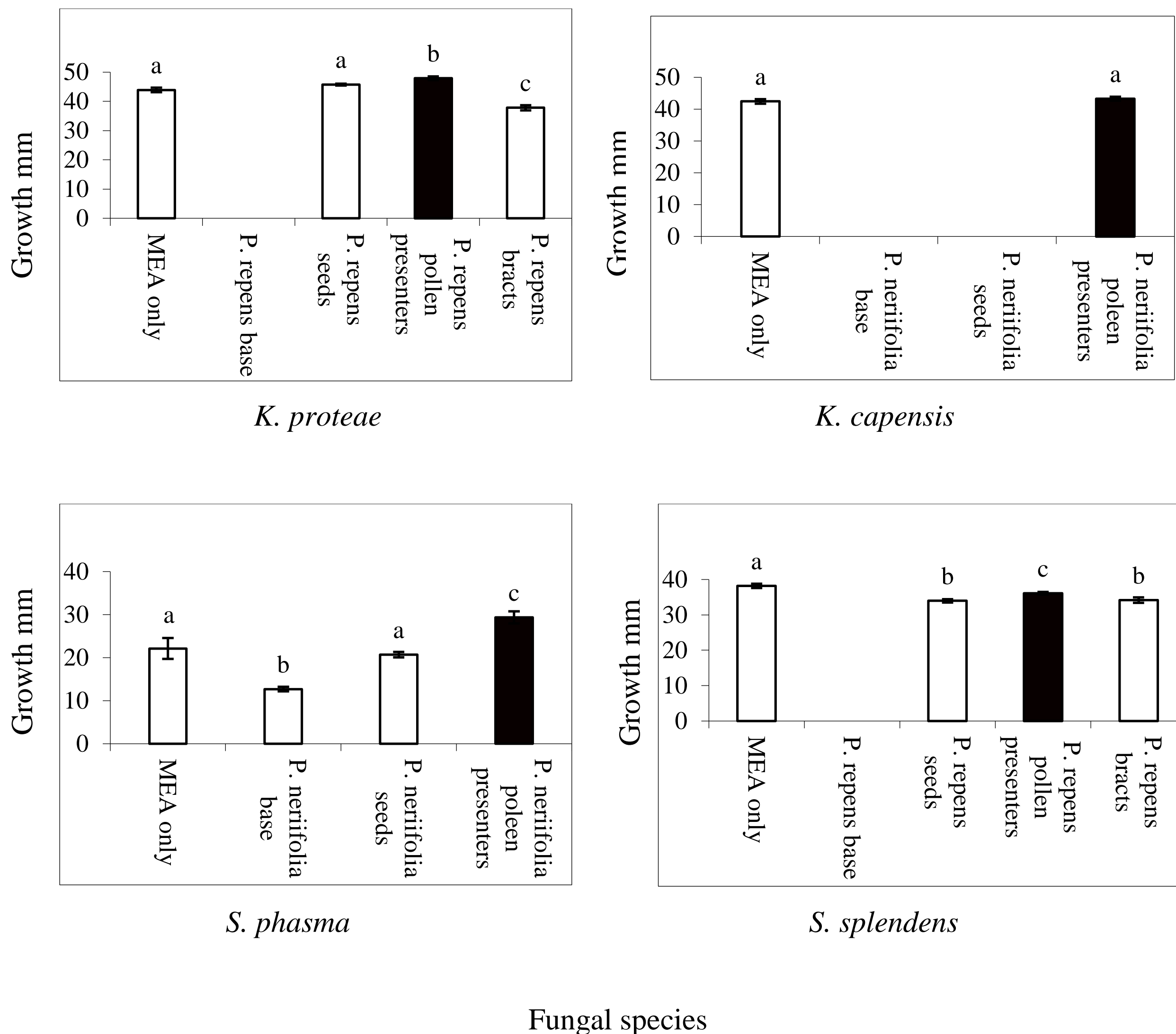

Figure 2: Mean radial growth (mm diameter after 10 d at 25°C) of *K. proteae*, *K. capensis*, *S. phasma* and *S. splendens* on media prepared from senescent structures from the infructescences of *P. repens*, *P. neriifolia* and malt extract agar (MEA). Error bars = standard error. Black columns indicate structures with the best fungal growth. Different letters indicate significant differences between mean radial growths per species.

Differential competition between fungi on media prepared from different host tissues

Differential competition was detected between *K. proteae* and *S. splendens* on *P. repens* pollen presenters, seeds and bracts (Table 1). *Sporothrix splendens* was always the strongest competitor, as was confirmed also by their relative crowding coefficients. Both fungal species were also always at a disadvantage when competing, as indicated by the product of their

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respective relative crowding coefficients. Neither *K. capensis*, nor *S. phasma*, was a superior competitor when growing on media prepared from *P. neriifolia* pollen presenters (Table 1). In addition, both species were at a disadvantage when competing on this medium.

Table 1: ANOVA statistics for tests of deviation from linearity in relationships between the areas occupied by competing fungal species in a de Wit replacement series on media prepared from senescent tissues within infructescences of *P. repens* and *P. neriifolia*. The competitive influence of each separate species in an interacting pair, or relative crowding coefficient (RCC) and the product of the RCC values of the interacting pairs (in brackets) are also provided. *df* = Degrees of freedom, SS = Sum of squares, MS = Mean square

Comparison	Source	*df*	SS	MS	F value	P value	RCC
On *P. repens* pollen presenters							
S. splendens vs K. proteae							(0.257)
S. splendens area	Proportion	3	0.09159	0.030530	22.54	<0.001	0.675
	Residual	11	0.01490	0.001355			
K. proteae area	Proportion	3	0.16457	0.05486	8.33	0.005	0.381
	Residual	10	0.06584	0.00658			
On *P. repens* bracts							
K. proteae vs *S. splendens*							(0.212)
K. proteae area	proportion	3	0.4996	0.16653	4.795	0.034	0.296
	Residual	8	0.2778	0.03473			
S. splendens area	proportion	3	1.030	0.3433	457.3	<0.001	0.717
	Residual	8	0.006	0.0008			
On *P. repens* seeds							
K. proteae vs *S. splendens*							(0.175)
K. proteae area	Proportion	3	0.6527	0.21758	17.32	<0.001	0.335
	Residual	10	0.1256	0.01256			

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S. splendens area	Proportion	3	1.5861	0.5287	177.6	<0.001	0.5230
	Residual	10	0.0298	0.0030			
On *P. neriifolia* pollen presenters							
S. phasma vs *K. capensis*							(0.222)
S. phasma area	Proportion	1	0.00208	0.002085	0.35	0.567	0.041
	Residual	12	0.07203	0.006003			
K. capensis area	Proportion	1	0.00021	0.000213	0.03	0.864	0.547
	Residual	10	0.06942	0.006942			

Discussion:

Here I provide evidence that factors related to differences in host infructescence structures help maintain co-occupancy of multiple fungal species with similar ecologies within individual *Protea* infructescences. This builds on previous data by showing that different senescent structures in plants may each be exploited separately by specific fungal species, leading to enhanced overall biodiversity levels (Paulus *et al.* 2003a, b; Hyde *et al.* 2007). However, differences in infructesence structures did not explain co-occupancy of all fungi tested, and the actions of spore-vectors may also have a significant influence on the persistence of comparatively weaker competitors within this restricted niche. The immense diversity of saprobes in general may therefore be explained by combinations of numerous factors that include host related differences (Hyde *et al.* 2007; Roets *et al.* 2012; Mukwevho *et al*. 2019; Chapter 1), differences in substrate colonisation times and differential competitive abilities (Hyde *et al.* 2007; Bleiker & Six 2009; Zhao *et al.* 2013; Kubicek *et al.* 2014).

Results of experimental studies presented here mostly reflected colonization patterns observed in the field. For example, the lack of growth of most fungi on media prepared from infructescence bases was expected from observational studies (Roets *et al.* 2006; 2013). *Sporothrix splendens* could grow on all parts of *P. repens* infructescences (except infructescence bases) and *K. capensis* was only able to grow on media prepared from pollen

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presenters of *P. neriifolia*. *S. phasma*, the species with which *K. capensis* mostly shares space within individual *P. neriifolia* infructescences, was able to grow on both the non-fertile seeds and the pollen presenters, confirming field observations (Roets *et al.* 2006; Theron-De Bruin *et al.* 2018). *S. phasma* and *K. capensis* therefore only compete for space on pollen presenters, where they have a neutral competitive interaction. Both species are also able to capture uncolonized space at similar rates when inoculated at the same point (*i.e.* when using the same spore vectors), but importantly, they can maintain this space, as they are not able to overgrow each other (Mukwevho *et al*. 2019; Chapter 1). These data thus neatly explain their co-existence on this *P. neriifolia* resource.

In contrast to the other species of ophiostomatoid fungi evaluated here, *K. proteae* was able to grow on media prepared from infructescence structures of *P. repens* with which it is not known to be associated in field-collected infructescences (*i.e.* non-viable seeds and bracts). As *S. splendens* can also grow on all structures, *K. proteae* will be in direct competition with *S. splendens* on this host. It is a significantly weaker competitor than *S. splendens* on all of these structures, thereby excluding differential competitive abilities as explanation for their co-existence in individual *P. repens* infructescences. Even though a previous study indicated that *K. capensis* can capture at least some space on pollen presenter media when in competition with *S. splendens*, *S. splendens* would likely eventually overgrow *K. proteae* colonies (Mukwevho *et al.* 2019; Chapter 1). For *K. proteae* to maintain area within *P. repens* infructescences for extended periods in the presence of *S. splendens*, it would need to exploit different initial available nutrient sources than *S. splendens*, or it would need to capture initial space rapidly, before colonization by *S. splendens*. *K. proteae* is known to colonise *P. repens* infructescences at least as early as *S. splendens* and can use *Protea* nectar sugars as main source when no senescent floral parts are available yet (Aylward *et al.* 2017). Nutrient sources for *S. splendens* are unknown, but likely include these nectar sugars (Rodriguez-Del Valle *et al.* 1983), excluding differential nutrient resource usage as explanation for co-occupancy. *K. protea* is a much faster coloniser of pollen presenter media than *S. splendens* in the absence of the latter (Roets *et al.* 2012), but to colonise pollen presenters sooner than *S. splendens* it would likely need to rely on a different main vector. *S. splendens* is mainly dispersed between inflorescences (flowers) by mites in the genera *Tarsonemus*, *Glycyphagus* and *Proctolaelaps* on *Protea* pollinating beetles and birds (Roets *et al*. 2007, 2009; Theron de-Bruin *et al.* 2018). They may be dispersed from infructescences to inflorescences on *Tarsonemus*, *Proctolaelaps* and a *Trichaouropoda* species vectored by a

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Protea pollinating beetle (*Genuchus hottentottus*) (Roets *et al*. 2007, 2009). Although significantly understudied compared to *Sporothrix* from this environment, the main vector for *K. proteae* is thought to be the same *Trichauropoda* mite, but it has also been detected on many other arthropod taxa in infructescences (Roets *et al.* 2011). Future studies may therefore need to re-examine the main vectors for *K. proteae* in light of the evidence presented here.

Interactions with other microbes may help shape the co-occurrence of ophiostomatoid fungi in individual infructescences. Most other fungal species likely arrive within infructescences after colonization by ophiostomatoid fungi, but these may have contrasting impacts on the persistence of the ophiostomatoid species at a later stage (Mukwevho *et al*. 2019; Chapter 2). Interactions of ophiostomatoid fungi have been evaluated with very few other fungal taxa to date and only on pollen presenter media. It is possible that an entire network of differential interactions is needed to help maintain the co-existence of multiple ophiostomatoid fungi in this niche. In addition to fungi, bacteria also abound within these structures and they can colonise infructescences at a very early stage (Human *et al.* 2016, 2018). Many *Protea* species produce antifungal agents such as fungichromin and actiphenol that inhibit the growth of both ophiostomatoid fungi and other saprobes (Human *et al.* 2016). It was shown that the *Protea*-associated ophiostomatoid fungi varied in their sensitivity towards these components (Human *et al.* 2016) and even though no benefit to *Knoxdavesia* could be deduced, it is possible that fungus-bacterial interactions help maintain co-occupancy of multiple ophiostomatoid fungi in individual *Protea* infructescences.

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Chapter 4: Concluding remarks

Protea is considered a keystone member of fynbos communities in the Cape Floristic Region, South Africa, as it is crucial to its ecological functioning (Cowling & Holmes 1992). It often structurally dominates plant communities and a large number of other organisms are directly and indirectly affected by its presence. Much of the previous work on the interactions of *Protea* with other organisms has been focussed on its pollinators (Rourke & Wies 1977; Johnson & Nicolson 2001) or detrimental organisms in agricultural or flower-export scenarios (Swart *et al*. 1999; Taylor *et al*. 2001; Wright 2002). Except for pathogenic microbes, no other study has investigated the effect of fungi on this plant genus or on the complex interactions that may exist between the different saprobes that it houses. In this regard the ophiostomatoid fungi from *Protea* inflorescences and infructescences present a particularly interesting subject for study due to their unique biology and ecology. In the present study I set out to test various hypotheses on the maintenance of high ophiostomatoid fungal diversity and dominance over other saprobes in this restricted environment, focusing on differences in their competitive abilities under various scenarios.

The host exclusivity of many *Protea*-associated ophiostomatoid species is well-documented (Roets *et al*. 2005), and it appears that they also occupy different structures within the infructuscences of *Protea* species (Roets *et al*. 2013). However, the ecological interactions between the various fungal taxa that underpin these patterns were not clearly understood. A previous study showed that host chemistry and association with particular spore vectors explain why certain *Protea* species can host ophiostomatoid fungi, while others can not (Roets *et al*. 2012). That study could, however, not detect any reason for the apparent host consistency of the ophiostomatoid fungi. In the present study, I hypothesised that host exclusivity of these ophiostomatoid fungi are due to differences in their competitive abilities when growing on different hosts. I therefore investigated the competitive abilities between ophiostomatoid fungi on media prepared from tissues originating from their usual and unusual hosts. I showed that host chemistry can only partially explain host exclusivity of a limited number of species. Even though not tested in this study, I therefore proposed the alternative hypothesis that differences in the movement of spore vectors between different *Protea* species are more important in maintaining host exclusivity for most species than differences in competitive abilities. *Protea*-associated ophiostomatoid fungi produce spores

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that are primarily vectored by mites (Roets *et al*. 2007, 2008) that use pollinating insects and birds as a means of long distance dispersal (Alward *et al*. 2015; Theron-De Bruin *et al.* 2018). The specific species of mites and pollinators are the same between all the investigated *Protea* species (Hargreaves *et al.* 2004; Steenhuisen & Johnson 2012b; Schmid *et al.* 2015), but the main pollinators show strong host consistency in flowering *Protea* populations (Schmid *et al.* 2016, New *et al*., unpublished). This restricted movement of spore vectors, with only occasional switching between different *Protea* species, likely enable ophiostomatoid fungi with similar or opposing competitive abilities to persist on different *Protea* hosts. Occasional switching between *Protea* species by pollinators also explain the infrequent colonization of specific *Protea* species with ophiostomatoid fungi that they do not normally associate with (Marais & Wingfield 1994; Roets *et al.* 2009b, Aylward *et al*. 2015). The effect of other microorganisms on host occupation can, however, not be ignored. Therefore, as an extension to these ideas, I also tested the competitive abilities of *Protea*-associated ophiostomatoid fungi to other saprobic fungi that are found in *Protea* infructescences. I demonstrated that some ophiostomatoid species are better at capturing initial space (primary resource capture) and defending this space from other saprobes (secondary resource capture), when growing on media prepared from their usual hosts. I also detected variability in the abilities of the different species to do so. I conclude that host consistency of the ophiostomatoid fungi studied here are likely driven by a complex interplay between differential competitive abilities on different hosts, the degree of consistency of their spore vectors between *Protea* species and competition with other microorganisms in this restricted niche.

Protea infructuscences house a particularly unique and high diversity of saprobic fungal species (Lee *et al*. 2005; Marincowitz *et al*. 2008), but the ophiostomatoid fungi are usually dominant (Lee *et al*. 2005). In the absence of ophiostomatoid fungi, other 'environmentally acquired' taxa such as *Penicillium* Link. and *Cladosporium* (Pers.) Link. dominate (Lee *et al.* 2003, 2004; Visagie & Jacobs 2012). These 'environmentally acquired' fungi are known to break down dead organic matter (Aro *et al*. 2005) and it was hypothesised that they may have a negative influence on infructescence longevity and/or seed viability in *Protea* infructuscences (Roets *et al*. 2013). I set out to test the hypotheses that the ophiostomatoid fungi are stronger competitors than these 'environmentally acquired' fungi and that the 'environmentally acquired' may be detrimental to *Protea* hosts even though they are saprobes. I found that infructescences without ophiostomatoid fungi were often colonised by

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Penicillium cf. *toxicarium* Miyake ex. Ramirez., *Cladosporium* cf. *cladosporoides* (Fresen.) de Vries. and *Fusarium* cf. *anthophilum* (Braun.) Wollenw. There was no difference in seed viability in infructescences dominated by either ophiostomatoid fungi or these 'environmentally acquired' saprobes, but infructescences colonised by ophiostomatoid fungi persist on plants longer than those dominated by 'environmentally acquired' species. Therefore, it is possible that the ophiostomatoid fungi in *Protea* infructuscences could prevent early release of *Protea* seeds. This is important, as many *Protea* species are serotinous and store seeds until after a fire event (Bond 1984). Premature release of *Protea* seeds in a pre-fire scenario will decrease chances of seedling survival and establishment due to increased predation and competition with other plants (Botha & Le Maitre 1992; Holmes & Cowling 1997). Nutrient recycling in the ashes of a post-fire fynbos environment is also known to boost germination and early seedling survival (Berg & Compton 2015). To the best of my knowledge this is therefore the first demonstration of a possible beneficial effect of a saprobic fungus growing on senescent tissues of a host plant.

I have demonstrated that the ophiostomatoid fungi are comparatively weak competitors against the other tested 'environmentally acquired' fungi in initial colonization of resources. However, many ophiostomatoid fungi could defend captured space fairly well against other saprobes, particularly when growing on media prepared from their usual hosts. Therefore if the ophiostomatoid fungi are to persist in this restricted environment, it is crucial that they arrive very early and colonise newly developing inflorescences before 'environmentally acquired' fungi arrive. It is not yet known whether the 'environmentally acquired' fungi can also be dispersed by vectors from one infructuscences to another, but spore morphology suggests that they are likely more reliant of wind dispersal (Visagie *et al.* 2009, 2014, 2016). It is expected that targeted dispersal, such as is provided by spore vectors of ophiostomatoid fungi, would result in earlier colonisation of the rather closed inflorescences than the more random colonization patterns provided by wind dispersal. At the early stages of colonization, the ophiostomatoid fungi can utilize the copious amounts of glucose, fructose and xylose produced in the *Protea* nectar (Nicolson & Van Wyk 1998) as carbon source (Aylward *et al.* 2017). Once all available nectar sugars have been depleted, and flowers senesce, these fungi can switch to degrading cell wall components such as arabinose, glucose, galactose, mannose and xylose (Aylward *et al.* 2017). The ability to initially use simple nectar sugars and then later switch to degradation of more complex polysaccharides in plant cell walls may also give the ophiostomatoid fungi a competitive advantage over other fungal taxa within this niche.

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Factors such as chemical composition and physical structure of particular host tissues can influence the diversity of saprobic fungi on senescent plant parts (Paulus *et al*. 2006; Osono 2011). High diversity of saprobic fungi on senescent parts of the same host may also be influenced by availability of nutrients, differences in colonising times, differences in spore dispersal and differential competitive abilities (Hyde *et al*. 2007; Kubicek *et al*. 2014). I therefore tested the hypothesis that differences in host infructescence parts lead to differences in the competitive abilities of different ophiostomatoid fungal species, which would explain the co-occupancy of multiple species within a single *Protea* infructescence. I demonstrated that the two fungal species that are known to colonise the same structures and grow in sympatry had neutral competitive abilities, explaining their co-existence within single *Protea* infructescences. However, differences in competitive abilities on different host structures could not explain the co-existence of a weak competitor in the presence of a strongly superior competitor. Here, again, it is seems likely that differences in colonization times, and therefore possibly the specific spore vectors, may be responsible for the co-occupancy of a single *Protea* infructescence by more than one ophiostomatoid fungal species.

In this study I demonstrated the use of fungal competition studies in investigations of host relations and dispersal ecology. Using these techniques, I have shown that host chemistry could only partially explain host exclusivity of ophiostomatoid fungi in *Protea* species and that their vectors may be important in terms of their dominance in this restricted niche. Early colonization by ophiostomatoid fungi, enforced by their strong associations with mites, not only ensures this dominance, but could also be of benefit to the host plant in terms of timely seed release, indicating a possible mutual benefit for the fungi and the hosts. However, a multitude of other microbes also colonise *Protea* infructescences and these may also help maintain diversity of the ophiostomatoid fungi. They include actinomycete bacteria that produce antifungal agents such as fungichromin and actiphenol (Human *et al*. 2016, 2018). All of these aspects need further investigation to truly understand the implications of the various biotic interactions in this atypical ecosystem, which may also direct studies in other systems containing multiple competing fungal species.

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