Biotic and abiotic constraints that facilitate host-exclusivity of *Gondwanamyces* and *Ophiostoma* on *Protea*

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Abstract

Estimations of global fungal diversity are hampered by a limited understanding of the forces that dictate host exclusivity in saprobic microfungi. To consider this problem for *Gondwanamyces* and *Ophiostoma* found in the flower heads of *Protea* in South Africa, we determined the role of various factors thought to influence their host exclusivity. Results showed that various biotic and abiotic factors influence the growth and survival of these fungi *in vitro*. Monitoring temperature and relative humidity fluctuations within infructescences *in vivo* revealed considerable microclimatic differences between different *Protea* spp. Fungal growth and survival at different relative humidity levels experienced in the field suggested that this factor does not play a major role in host exclusivity of these fungi. Maximum temperatures within infructescences and host preferences of the vectors of *Gondwanamyces* and *Ophiostoma* appear to play a substantial part in determining colonization of *Protea* in general. However, these factors did not explain host exclusivity of specific fungal species towards particular *Protea* hosts. In contrast, differential growth of fungal species on media containing macerated tissue of *Protea* showed that *Gondwanamyces* and *Ophiostoma* grow best on tissue from their natural hosts. Thus, host chemistry plays a role in host exclusivity of these fungi, although some species grew vigorously on tissue of *Protea* spp. with which they are not naturally associated. A combination of host chemistry and temperature partially explain host exclusivity, but the relationship for these factors on the tested saprobic microfungi and their hosts is clearly complex and most likely includes combinations of various biotic and abiotic factors including those emerging from this study.

Key words: *Ophiostoma*; *Gondwanamyces*; Spore-vector; Temperature; Relative humidity; Host chemistry

1. Introduction

It is well-recognized that most plant pathogenic fungi are relatively host specific (Agrios 2005; Daly 1979; Wood 1976). The term host-specificity is, however, reserved for species that derive their nutrition from live host plants. In their review of the topic, Zhou and Hyde (2001) suggested that saprobic species can be considered as either host-exclusive (i.e. species that occur on a particular host or on a restricted range of related host plants) or host-recurrent (species that predominantly occur on a particular host(s) and infrequently on other host plants in the same habitat. The factors dictating host-exclusive/recurrent relationships for saprobes are far less clear than for the host-specific relationships of pathogenic fungi (Hooper *et al.* 2000; Santana *et al.* 2005), but may be correlated to differences in the physical structure or nutrient levels provided by potential hosts (e.g. Boddy and Watkinson 1995; Lodge 1997; Mille-Lindblom *et al.* 2006; Paulus *et al.* 2006).

Saprobic fungi occurring on plants in the genus *Protea* (Proteaceae), a keystone member of the Cape Flora (Cowling *et al.* 1992) have been the subject of various studies e.g. in the *Proteaceae*; Lee *et al.* (2004, 2005) and Marincowitz *et al.* (2008). These studies have provided evidence that some saprobic microfungi associated with Proteaceae are host-exclusive and in some cases also restricted to specific tissue types. As an example, numerous saprobic species were exclusively isolated from either dead *Protea* twigs or the decaying fruiting structures of these plants (Lee *et al.* 2004; Marincowitz *et al.* 2008). Knowledge of such organ-exclusivity can facilitate investigations considering the basis for host-exclusivity of some saprobic microfungi. This makes it possible to focus studies on only those specific parts of the host that are of interest, for example the infructescenses of *Protea* and their associated *Gondwanamyces* and *Ophiostoma* spp.

The colourful inflorescences of *Protea* render them important plants in the ecotourism, horticulture and dried-flower industries of South Africa (Cowling *et al.* 1992). They also commonly dominate

plant communities in the world-renowned Fynbos Biome, where they sustain populations of numerous organisms including the birds and insects that pollinate them (Rebelo 1995). After pollination, the inflorescences of *Protea* mature into tightly-packed seed storage organs (infructescences). Infructescences of serotinous species persist on the plants for at least one, but often several years (Rebelo 1995). They consequently provide a moist, sheltered environment in which saprophytic fungi can thrive (Marincowitz *et al.* 2008). Interestingly, two fungal genera, *Gondwanamyces* (Microascales) and *Ophiostoma* (Ophiostomatales), dominate fungal communities within these infructescences (Roets *et al.* 2005).

Gondwanamyces and *Ophiostoma* are morphologically adapted to dispersal by arthropods. This relationship is closely linked to the fact that these fungi produce sticky spores at the tips of erect fruiting structures that are either asexual conidiophores or sexual ascocarps (Francke-Grosmann 1967; Malloch and Blackwell 1993; Münch 1907; Upadhyay 1981). The best-known vectors of these fungi are bark-beetles (Curculionidae: Scolytinae) that construct larval galleries in the phloem of coniferous hosts and that often have mutualistic associations with their fungal partners (Christiansen *et al.* 1987; Francke-Grosmann 1967; Kirisits 2004; Paine *et al.* 1997; Upadhyay 1981; Wingfield *et al*. 1993). In these interactions, the fungi benefit from being transported to otherwise inaccessible resources by the beetles, while the benefit to the insects is most likely very variable depending on the particular association (Klepzig *et al.* 2001a, 2001b; Six 2003) and could include nutrition (e.g. Bleiker and Six 2007; Six and Paine 1998) and the creation of more suitable environments for beetle development by killing trees. Recently however, Six and Wingfield (2011) argued that tree killing by these fungi may be more important for the fungi than the beetles as they mediate competitive interactions among fungi in living trees.

Recent studies have shown that *Protea*-associated *Gondwanamyces* and *Ophiostoma* are primarily dispersed by mites including *Proctolaelaps vandenbergi*, two *Tarsonemus* spp. and a

Trichouropoda sp. (Roets *et al.* 2007, 2008, 2011). Mites are also known as important vectors of *Ophiostoma* in the conifer-bark beetle systems (Bridges and Moser 1983, 1986; Moser 1997). The association between at least some of the vector mites and the fungi that they carry on *Protea* is considered mutualistic, as these mites exploit the fungi as food source (Roets *et al.* 2007). Long distance dispersal of the fungus-carrying mites is achieved by a phoretic association between the mites and the *Protea*-host specific beetles *Genuchus hottentottus, Trichostetha fascicularis,* and *T. capensis* (Roets *et al.* 2009a).

It is intriguing that the two species of *Gondwanamyces* and nine species of *Ophiostoma* described from *Protea* infructescences (Marais and Wingfield 1994, 1997, 2001; Roets *et al.* 2006, 2008, 2010; Wingfield *et al.* 1988; Wingfield and van Wyk 1993) show varying levels of host exclusivity on *Protea*. All but one of these species (*Spotothrix variecubatus*) has exclusively been isolated from the infructescences of *Protea*. Recent surveys have also indicated that these species are restricted to serotinous *Protea* spp. distributed from the southwestern tip of Africa to Zambia in the north (Roets *et al.* 2005, 2006, 2009b, 2010). Restriction to serotinous *Protea* spp. is most likely due to the evanescent nature of non-serotionous infructescences that dehisce shortly after pollination, resulting in an absence of suitable habitat for the fungi for the greater part of the year. However, not all serotinous and thus "ecologically suitable" *Protea* spp. have *Gondwanamyces* or *Ophiostoma* in their infructescences. This apparent exclusivity has been attributed to various biotic and abiotic factors (Roets *et al*. 2009b). For example, the absence of *Gondwanamyces* and *Ophiostoma* in *P. nitida* and species in the ''rodent sugarbush", the ''dwarf tufted sugar bushes", and the ''western ground proteas" (Rebelo 1995) may be attributed to the open morphology of their infructescences. These open infructescences may be prone to greater temperature fluctuations and thus not retain moisture as effectively as more closed structures. This could preclude the establishment of the relatively slow-growing *Gondwanamyces* and *Ophiostoma*. It is thus unsurprising that all known

hosts of *Protea*-associated *Gondwanamyces* and *Ophiostoma* form fairly tightly closed structures (e.g. *P. repens* and the "bearded sugarbushes" (Rebelo 1995; Roets *et al*. 2009b)).

In addition to postulated temperature and humidity constraints dictating the presence of *Gondwanamyces* and *Ophiostoma* in *Protea* infructescences, host chemistry may also play a role in host specificity of these fungi. For example, the hosts of *Gondwanamyces capensis* and *Ophiostoma phasma*, namely *P. burchellii*, *P. coronata*, *P. laurifolia*, *P. lepidocarpodendron*, *P. longifolia*, *P. lorifolia* and *P. neriifolia* (Roets *et al.* 2009b), reside in two closely allied clades based on DNA sequence data (Barraclough and Reeves 2005). It can thus be assumed that these species may be fairly similar in chemical composition. Organisms living on these *Protea* spp. could consequently easily jump from the one host to another. In contrast, *Gondwanamyces* and *Ophiostoma* associated with *P. repens*, such as *G. proteae* and *O. palmiculmiatum*, tend to be host species exclusive (Roets *et al.* 2009b). The high level of exclusivity of *Gondwanamyces* and *Ophiostoma* associated with *P. repens* may be ascribed to its phylogenetic uniqueness relative to all other ophiotomatoid-associated *Protea* spp., and the uniqueness of this hosts' chemistry (Barraclough and Reeves 2005). Despite previous morphology-based reports to the contrary (Lee *et al*. 2005; Marais and Wingfield 1994), DNA sequence comparisons suggest that *O. splendens* is also specific to *P. repens* and that previous confusion resulted from the very close similarity in the morphologies of the phylogenetically distantly related taxa *O. splendens* and *O. phasma* (Roets *et al.* 2009b).

Roets *et al.* (2009b) suggested that differences in the host-plant associations of *Gondwanamyces* and *Ophiostoma* in *Protea* infructescences are related to the vectors of these fungi. Thus, the apparent host-exclusivity observed for *Protea*-associated *Gondwanamyces* and *Ophiostoma* could be ascribed to specificity (and ecology) of their vectors rather than the specificity of the fungi. However, there is relatively little information available relating to the vectors of *Protea*-associated

Gondwanamyces and *Ophiostoma* and this precludes robust views relating to the role that vectors may play in shaping host ranges of these fungi.

In this study we consider various biotic and abiotic variables that may determine host-exclusivity of *Protea*-associated *Gondwanamyces* and *Ophiostoma*. The studies were focused on the *P. repens* exclusive species *G. proteae* and *O. splendes* and on the 'bearded' and 'spoon-bract' (Rebelo 1995) *Protea* host-exclusive taxa *G. capensis* and *O. phasma*. The basis of host-exclusivity in these species was investigated by testing the influence of host chemistry on fungal growth *in vitro*; determining whether there is a specific association between the fungal spore vectors and specific *Protea* spp. and by examining the influence of infructescence temperature and humidity on fungal growth and survival.

2. Materials and Methods

2.1. Fungal isolates

Isolates of *G. capensis* and *O. phasma* were obtained from colonized *P. neriifolia* infructescences collected from the Jonkershoek Forestry Reserve (JFR), Stellenbosch, South Africa during August 2009. Isolates of *G. proteae* and *O. splendens* were also collected at the same location from sympatric *P. repens* infructescences. In an effort to minimize the effect of fungus genotype on the repeatability of experimental results, each of the six isolates per tested fungal species was obtained from a different individual plant. Ascospores were removed from the apices of ascomatal necks from within infructescences using a small piece of agar attached to the tip of a dissecting needle and these were transferred to 1.5 % Malt Extract Agar (MEA; Biolab, Midrand, South Africa). Fungi were initially selected as being representative of the four species in terms of morphological appearance when grown on 1.5 % MEA and their distinctive sexual (teleomorph) characteristics.

Once purified, the identity of all cultures was verified by comparisons of DNA sequence data for the Internal-Transcribed-Spacer (ITS) and 5.8S regions, amplified following methods of Roets *et al.* (2010), and the type strains available from the NCBI's GenBank nucleotide database (http://www.ncbi.nlm.nih.gov). Cultures were maintained on Petri dishes containing 1.5 % MEA at 4 ºC in the dark until further experimentation.

2.2. Influence of host chemistry

The influence of host chemistry on fungal growth was tested in Petri dishes (90 mm diam.) containing growth media prepared from the pollen presenters and styles (hereafter referred to as pollen presenters) of *P. neriifolia*, *P. nitida* and *P. repens*. To prepare this medium, *Protea* infructescences were collected from JFR and dried in an oven at 50 ºC for 5 days. Pollen presenters were pulled from infructescence bases by hand and seeds were removed. Dried pollen presenters were ground into a fine powder using an electric mill and passing this through a screen with 2 mm perforations. Due to the small size of the pollen presenters in *P. nitida*, ground tissue was prepared from whole dried infructescences for this species. Depending on the specific medium being prepared, one liter of water-based growth medium contained 300ml prepared *Protea* tissue and either 1.5 % MEA or 1.5 % pure agar. The growth media were autoclaved at 115 ºC for 20 min. prior to dispensing 25 ml into Petri dishes. Plates containing only 1.5 % MEA or Agar without nutrients (Merck, Darmstadt, Germany) were used as controls.

Plates were inoculated at their centers with 2 mm diameter agar discs cut from the actively growing margins of two-week-old colonies of *G. capensis, G. proteae, O. phasma* and *O. splendens* grown on 1.5 % MEA. The growth of all collected isolates was tested on each medium type. Thus, there were four test fungal species (six isolates per fungal species) grown on media prepared from two Agar types (Agar only or MEA) infused with tissues of three *Protea* spp. (24 plates per tested

medium) and MEA or Agar only controls (192 plates in total). All inoculated plates were inverted and incubated at 25 ºC in the dark.

The diameter of each fungal colony on the various *Protea*-tissue media and the controls 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 were determined by calculating the mean radial growth (± standard error) of the 6 representative isolates of each of the four fungi. A one-way analysis of variance (ANOVA) was used to analyse the normally distributed data in the Statistica 9 (Statsoft Corporation, Tulsa, U.S.A.) software package with Sigma-restricted parameterisation. A Fisher's Protected LSD post hoc test was performed to determine significant differences between group means. Differences between the radial growth of the fungal species on each of the test media were considered significant when $P \leq 0.05$. At the time of making colony measurements, data were collected for colony morphology and the production of fungal reproductive structures.

2.3. Host association of fungus-vector

The associations between primary spore-vectoring mites (Roets *et al.* 2007, 2011) and various *Protea* spp. were assessed for collections across South Africa. Ten infructescences of 14 *Protea* spp., representing a wide morphological and taxonomic diversity, were collected from various localities between March and April (Autumn) 2009 (Table 1). This time-period was chosen as it is known to represent the peak sporulation time for *Protea*-associated *Gondwanamyces* and *Ophiostoma* (Roets *et al.* 2005) and thus the time when the spore-vectoring mites are likely to be present. Infructescences were opened with secateurs and all individuals of *P. vandenbergi*, *Tarsonemus* spp. and a *Trichouropoda* sp., the species identified by Roets *et al.* (2007, 2011) as

main vectors of *Protea*-associated *Gondwanamyces* and *Ophiostoma*, were collected and stored in 80 % ethanol.

Sørensen's coefficient of similarity (Southwood 1978) was used to determine the degree of similarity in the target mite species richness ($Cs = 2j / (a + b)$) and abundance ($Cn = 2jN / (aN + bN)$) between the different *Protea* spp., where j = number of mite species in common between two *Protea* spp., a and b respectively = total number of mite species present per *Protea* spp., a*N* and b*N* respectively = total number of individuals on each *Protea* spp., and j*N* = the sum of the smaller values (individual counts) for the mite species collected from both plants species.

In addition to considering host specificity of the vector mites, possible seasonal patterns in the abundance of the main *Gondwanamyces* and *Ophiostoma* spore carrying mites on *P. repens*, *P. neriifolia* and *P. nitida* were considered. Thus, 25 one-year-old infructescences of these species were collected in three areas where they occur sympatrically i.e. from JFR, Gordon's Bay and Franschoek Pass (Table 1). Infructescences were collected during September, December, March and June (2008 - 2009) and the target mites were extracted as previously described. Voucher specimens of all mite species collected are housed in the University of Stellenbosch Entomology Collection, Stellenbosch, South Africa. Mite abundance data were analyzed using a generalized linear model approach (GLZ) with Poisson distribution and the identity link function active in the software program SAS Enterprise Guide 4.1 (SAS Institute Inc., U.S.A.). Significance in differences between group means was determined using the least squares post hoc method.

2.4. Influence of environment within infructescences

In order to test abiotic constraints of *Protea*-associated *Gondwanamyces* and *Ophiostoma* growth, fluctuations in temperature and relative humidity within *Protea* infructescences was tested under

field conditions. iButtons (Maxim Integrated Products, U.S.A.) that simultaneously measure temperature and relative humidity (RH) were placed within infructescences of *P. neriifolia*, *P. nitida* and *P. repens* in the JFR during February 2009. Criteria for inclusion of experimental infructescences (one per plant) included: (1) similar height (*ca.* 1.5 m from the soil surface), (2) same age (ca. 5 months old), (3) similar orientation (infructescences on the southern side of plants), (4) similar micro-environmental conditions (individuals of all 3 *Protea* spp. were selected to have an interspacing distance of less than 1.5 meters). A control iButton was covered with fine gauze to eliminate artificial temperature peaks caused by exposure to direct sunlight and tied to the stem of one of the *Protea* plants in the shade. Selection for the placement of the control was similar to that for experimental infructescences. iButtons were set to record both temperature and RH at 15 min. intervals for 24 hours. Data were recorded for seven consecutive 24 hour cycles with each day representing a replicate.

Analysis of temperature and RH data was focused at the upper and lower ends of the recordings, respectively. This focus on high temperatures and low RH seemed appropriate, as these conditions are more likely to place constraints on fungal growth than the moderate temperatures and high RH recorded at the other end of the spectra. Means and standard deviations were calculated for the maximum daily temperature and minimum daily RH recorded per iButton (n = 7) and data were compared using ANOVA. A Fisher's Protected LSD post hoc test was performed to determine significant differences between group means. In addition, mean differences between the maximum temperature and maximum RH values within infructescences and their respective controls were compared. This was necessary because the maximum daily temperature and RH values of the ambient air fluctuated naturally. Standardising ambient conditions thus made it possible to determine whether the different *Protea* spp. reacted significantly differently to changes in ambient air temperature and RH.

2.5. In vitro growth at different temperatures

Based on temperature data recorded in the field, fungal growth at temperatures between 20 and 45 ºC (at 5 ºC intervals) were tested *in vitro*. Two mm diameter disks of mycelium-covered agar were excised from the leading edges of two-week-old colonies of *G. capensis*, *G. proteae*, *O. phasma* and *O. splendens* grown on 1.5 % MEA and placed mycelium-downwards on fresh plates, preparing six replicate plates per test fungus (six independent isolates for each of the four fungal species). Plates were inverted and incubated for two weeks in the dark at the different temperatures, after which the diameters of the fungal colonies were measured and compared as described in section 2.2.

After completion of the trial to determine growth at different temperatures, survival of the *Gondwanamyces* and *Ophiostoma* at the tested temperatures was determined. This was achieved by placing all previously incubated plates at the optimum growth temperature for each species. After two weeks, additional growth beyond the colony margins was determined. Once survival of the fungi at the various temperatures was determined *in vitro*, the length of time that field-selected infructescences spent above the upper limits of temperature for fungal growth and survival for each *Protea* spp. was determined. From the iButton data, it was possible to enumerate the number of datum points that were higher than the thermal limit for the growth and survival of each of the fungal species for the seven recorded 24 hour cycles.

2.6. In vitro growth at different relative humidities

Based on values of RH obtained from the field measurements, fungal growth was determined at a range of RH values between 0 and 100 % at 25 ºC *in vitro*. Different conditions of RH were established using saturated salt solutions (Winston and Bates 1960). Chambers at a range of different relative humidities at 25 ºC were established using silica gel (ca. 0 %), sodium hydroxide

(ca. 7 %), potassium acetate (ca. 22 %), magnesium chloride (ca. 32 %), potassium carbonate (ca. 40 %), magnesium nitrate hexahidrate (ca. 55 %), sodium nitrite (ca. 64 %), sodium chloride (ca. 75 %), potassium chloride (ca. 84 %), potassium nitrate (ca. 94 %) and pure water (ca. 100 %).

Equilibrium RH values were created in compartmentalised 90 mm diameter Petri dishes (two compartments). The one side contained 13 ml of prepared saturated salt solution (with a few added dry crystals) and the other side contained a piece of filter paper cut to fit the half of the Petri dish. Filter paper pieces were autoclaved and infused with warm (ca. 50 ºC) autoclaved 1.5 % MEA before placement in the Petri dishes. Plates containing the different salt solutions and media were sealed with parafilm and left for two days at 25 ºC to stabilise RH between the air, media on the one side of the plate and the salt solution on the other side of the plate. After two days, the growth media were inoculated in the centre of the filter paper pieces with the tested fungal strains and incubated at 25 ºC in the dark for an additional eight days.

Inoculation material was prepared from autoclaved filter paper disks (5 mm diam.) made using a paper punch. Autoclaved filter paper disks were infused with warm autoclaved MEA and placed on the surface of actively growing colonies of the tested fungal species. After *ca*. two weeks at 25 ºC in the dark, the paper disks (and the fungi that now covered them) were removed and placed on the centre of the filter paper within humidity chambers to inoculate these. Plates were again sealed with parafilm before incubation. This method allowed for the introduction of as little additional moisture as possible into the humidity chambers after stabilisation of the RH. The experiment was replicated six times (once for each isolate per tested fungal species) at each of the tested RH values. After eight days, diameters of colonies were determined and statistical comparisons were made as described in section 2.2.

Survival of *Gondwanamyces* and *Ophiostoma* at different RH values as hyphae or conidia was determined by removing the paper disks used to inoculate the compartmentalized dishes after eight days, placing them on fresh 1.5 % MEA plates and incubating these at 25 ºC (100 % RH). After two weeks of incubation, additional growth was assessed. Once survival of the fungi at the various RH values had been determined, the amount of time that field-based infructescences occupied below the lower limits for fungal growth and survival for each *Protea* spp. was determined as described in section 2.5.

3. Results

3.1. Influence of host chemistry

When grown on media containing nutrient-rich MEA, fungi produced denser hyphae and they gave rise to considerably more conidiophores per unit area (pers. obs.) than when they were grown on media containing nutrient-deficient WA (Fig 1). In addition to surface hyphae, most species also produced a dense mat of aerial hyphae on MEA-based media. On WA, *G. proteae* and *G. capensis* produced more abundant aerial hyphae when *P. repens* tissue was included in the medium than when either *P. neriifolia* or *P. nitida* tissues were added (Fig 1). In contrast, *O. phasma* and *O. splendens* produced abundant aerial hyphae only when *P. nitida* and *P. repens* tissue was added to the WA. *Ophiostoma splendens* produced relatively more abundant aerial hyphae when *P. repens* tissue was added to the WA than when tissues of any other *Protea* spp. was added (Fig 1).

Gondwanamyces and *Ophiostoma* grew at different rates on media amended with tissue of various *Protea* spp. (Fig 2). The ANOVA results indicated that there is a significant effect on fungal radial growth induced by the various media types for *G. proteae* ($F = 23.94$, d. f. = 7, $P < 0.001$), *G. capensis* ($F = 60.79$, d. f. = 7, $P < 0.001$), *O. splendens* ($F = 12.7$, d. f. = 7, $P < 0.001$) and *O. phasma* ($F = 6.67$, d. f. = 7, $P < 0.001$). When considering fungal growth on media prepared from

MEA and the infructescences of *P. repens* and *P. neriifolia* only, all species grew significantly better on media prepared from their natural hosts. *Ophiostoma phasma* was the only exception. Its radial growth was better on media prepared from its natural host, but this was not significantly better than when *P. repens* tissue was added to MEA (Fig 2). In contrast, radial growth of the tested *Gondwanamyces* and *Ophiostoma* species was significantly reduced on Water Agar amended with tissues from their natural hosts and in the absence of other nutrients. Again *O. phasma* was the only exception in that its radial growth was similar on Water Agar amended with *P. neriifolia* and Water Agar amended with *P. repens* tissues. No significant differences in either radial growth or culture morphology of any of the tested fungal species were observed between MEA media containing tissues from their natural hosts and MEA containing tissues prepared from *P. nitida* (Fig 2).

When the growth of *G. capensis* on MEA only (control) was compared to growth on prepared *Protea* media, radial growth of the fungus was slightly inhibited when it was grown on media prepared from its non-host (*P. repens*) and slightly enhanced on medium including *P. neriifolia* (host) tissues (Fig 2). Similarly, radial growth of *G. proteae* was significantly enhanced on MEA media containing *P. repens* (host) and unaffected by the addition of *P. neriifolia* (non-host) when compared to the MEA only control. *Ophiostoma splendens* was inhibited when material of either *P. repens* (host) or *P. neriifolia* (non-host) was added to the media when compared to the MEA only control. However, radial growth of this fungus was still significantly more rapid on media prepared from its natural host than when it was grown on media prepared from *P. neriifolia* (Fig 2). Compared to the MEA only control, radial growth of *O. phasma* was significantly enhanced by the addition of tissue from any of the tested *Protea* spp. Its radial growth was, however, slightly more rapid when *P. neriifolia* (host) tissue was added to the medium (Fig 2).

3.2. Host association of fungus-vector

A total of 278 individuals of the *Gondwanamyces* and *Ophiostoma* spore-carrying mites were collected from the various *Protea* spp. (Table 2). The infructescences of *Protea* spp. that are confirmed hosts of these fungi were all colonized with individuals of the target mite species. In addition *Protea obtusifolia* was identified as a host of *G. capensis* for the first time. For most of the *Gondwanamyces* and *Ophiostoma* -host *Protea* spp., the abundance of the target mites was also relatively high (e.g. *P. laurifolia, P. neriifolia* and *P. repens*). *Protea* spp., that are known not to have *Gondwanamyces* or *Ophiostoma* in their infructescences, e.g. *P. susannae, P. lanceolata* and *P. acaulos*, were also free of the target mites. However, some of the *Protea* spp. with no known association with *Gondwanamyces* or *Ophiostoma* also contained individuals of the target mite taxa, although these were never in high numbers (Table 2). Generally, the highest values for Sørensen's coefficients of similarity in abundance (Cn > 0.30) and species richness (Cs > 0.60) were obtained when target mite communities were compared between *Gondwanamyces* and *Ophiostoma* hosts (Table 2).

In total, 1574 individuals of the target mites were collected throughout the year from the three *Protea* spp. included in the seasonal study. GLZ results indicated that there were significant differences between mite numbers collected during the different seasons (d. f. = 11, Wald = 1999.1, $p < 0.0001$). These mites showed peak abundance during March (Autumn) when ca. 800 individuals were recorded from the three *Protea* spp. (Fig 3). This was mostly due to an increase in the numbers of the target mites collected from the infructescences of *P. neriifolia*. Seasonal mite data confirmed that the target mites are occasionally found in association with *P. nitida*, a species that has no known associations with *Gondwanamyces* or *Ophiostoma*.

3.3. Influence of environment within infructescences

There were significant differences in maximum temperatures reached within the infructescences of the three *Protea* spp. tested under field conditions (d. f. = 3, F = 3.08, P = 0.047) (Table 3). Maximum temperatures reached within *Protea* infructescences were always higher than the surrounding air temperature (Table 3). Differences in temperatures reached when compared to the ambient temperatures varied significantly between the three *Protea* spp. (d. f. = 2, F = 24.43, P < 0.01). Temperature differences between the infructescences of *P. repens* and the ambient air was less than in the other *Protea* spp. On average, maximum temperatures in *P. nitida* infructescences was *ca*. 7 ºC higher than the maximum ambient air temperature. In this species, temperatures of more than 45 ºC were reached, even when ambient air temperatures never rose above 39 ºC (Table 3). Although not quite as high, temperatures within the infructescences of *P. neriifolia* and *P. repens* reached *ca*. 41 ºC and 40 ºC, respectively.

Significant differences were found in minimum RH values within the infructescences of the three *Protea* spp. and the controls (d. f. = 3, F = 10.03, P < 0.001) (Table 3). Although not statistically significant, minimum RH values for *P. nitida* and *P. neriifolia* were always lower than the minimum RH reached in the ambient air. In contrast, minimum RH values for *P. repens* were always higher than that of the ambient air. Minimum relative humidity within the infructescences of *P. repens* was significantly higher than that of the ambient air and the other *Protea* spp. (d. f. = 2, F = 28.43, P < 0.001) (Table 3).

3.4. In vitro growth at different temperatures

Radial growth for *Gondwanamyces* and *Ophiostoma* (mm diam. after seven days) differed significantly at different temperatures (F = 600.45, d. f. = 11, P < 0.01). The radial growth for all species were the greatest at 25 ºC (Fig 4). No growth was observed for any of the tested fungi at 30 ºC and above (Fig 4).

Results of experiments to determine the survival of the various *Gondwanamyces* and *Ophiostoma* species after seven days at different temperatures showed that all fungal species kept at 30 ºC and 35 ºC for one week were still able to grow when placed at the optimal growth temperature. In contrast, isolates of all species kept at 40 ºC and 45 ºC for seven days were unable to grow when plates were returned to the optimal growth temperature.

3.5. In vitro growth at different relative humidities

Radial growth for *Gondwanamyces* and *Ophiostoma* (mm diam. after seven days at 25ºC) varied significantly at different relative humidities ($F = 402.06$, d. f. = 23, $P < 0.01$). The radial growth for all species declined with a decline in relative humidity (Fig 5). No growth was observed for the two *Gondwanamyces* spp. below a relative humidity of 75 % (Fig 5). Very slow radial growth was, however, still observed for the two *Ophiostoma* spp. when grown at a relative humidity of 64 %. None of the test fungi were able to grow below 64 % relative humidity (Fig 5). *Gondwanamyces proteae* showed a peak in radial growth at 94 % RH rather than at 100 % as was the case for the other fungal species tested. When filter paper disks were removed from the humidity chambers, plated onto MEA and kept at 25 ºC for a week, all isolates (from all RH values tested) were able to grow further.

4. Discussion

Results of this study elucidated various factors that directly influence the radial growth and survival of *Protea*-associated *Gondwanamyces* and *Ophiostoma*. Host chemistry, temperature and relative humidity all influenced the radial growth of the fungal species that were tested. However, not all of these factors may influence host associations. For example, the radial growth of all fungal species

was positively influenced by an increase in relative humidity and all were able to survive extremely low relative humidity levels for extended periods of time. All species also had fairly similar lower limits of relative humidity essential for fungal growth. Thus the observed differences in minimum relative humidity levels within the infructescences of different *Protea* spp. detected in the field are unlikely to dictate host association patterns of *Protea*-associated *Gondwanamyces* and *Ophiostoma*.

Factors that appeared to most strongly influence the ability of *Gondwanamyces* and *Ophiostoma* to colonise *Protea* included the association of vectors with specific *Protea* spp., as well as maximum infructescence temperature. For example, numerous non-host *Protea* spp. were found without individuals of the target mite species. Mite preferences for host plants could thus play a key role in host associations of these fungi. The absence of *Gondwanamyces* and *Ophiostoma* from infructescences of e.g. *P. acaulos*, *P. lanceolata* and *P. suzannae* may thus be due to ecological constraints acting on the vectors and not the specific fungi. Interestingly, numerous *Protea* spp. with no known associations with *Gondwanamyces* or *Ophiostoma* also housed individuals of the target mites, albeit at low abundance. A notable example was the association of all three vector mite species with *P. nitida* (data not shown). *Protea nitida* has no association with *Gondwanamyces* or *Ophiostoma* even though it often grows sympatrically with known host species such as *P. repens* and *P. neriifolia*. Associations between vector organisms and specific *Protea* spp. alone, can thus not explain the absence of *Gondwanamyces* and *Ophiostoma* from all non-hosts.

The abundance of *Gondwanamyces* and *Ophiostoma* -vectoring mites showed a marked increase during March (Autumn). This coincides with an increase in numbers of *Protea* infructescences with sexual fruiting structures of *Gondwanamyces* and *Ophiostoma* (Roets *et al.* 2005). It has been shown that mites have a mutualistic association with *Ophiostoma* spp. in this niche (Roets *et al.* 2007) and that the fungi are primarily dispersed by these mites (Roets *et al.* 2009a, 2011). The stability of this mutualistic association would be greatly enhanced by simultaneous peaks in vector

abundance and fungal sporulation. The timing of the increase in abundance of both organism groups during autumn coincides with the onset of the local rainy season. This suggests that sufficient moisture (and possibly also lower temperatures) is required to enable fungal growth and sporulation of the sexual stage within infructescences in the field.

The mite/fungi mutualism does not seem obligatory for the mites, because mites were present in infructescences of *Protea* spp. with no known *Gondwanamyces* or *Ophiostoma* associations. It is quite possible that the mites also predate/vector spores of other fungal genera. The association between *Gondwanamyces* and *Ophiostoma* and their spore vectors is, however, obligatory for the fungus, as other arthropods associated with *Protea* spp. rarely vector their spores (Roets *et al.* 2007, 2011). Although not obligatory for the mites, the presence of *Gondwanamyces* and *Ophiostoma* may greatly enhance mite numbers within host *Protea* spp. when compared to hosts without these fungi (Roets *et al.* 2007), further strengthening interactions between these two organism groups.

Temperature has been shown to play a significant role in determining the relative abundance of the mutualistic fungi associated with a bark beetle, *Dendroctonus ponderosae*, on conifers and that it determines which fungus is vectored (Six and Bentz 2007). Given the marked differences in maximum temperatures reached within infructescences of different *Protea* spp., it is reasonable to assume that differences in radial growth responses of the *Gondwanamyces* and *Ophiostoma* spp. towards these temperatures may influence host exclusivity. However, all species reacted similarly to growth at various tested temperatures: optimal radial growth at 25 ºC, discontinued growth at 30 ºC and death above 40 ºC. Temperature differences between *Protea* spp. infructescences thus cannot explain why these fungi are restricted to their particular hosts. Maximum *Protea* infructescence temperatures could, however, explain the general absence of *Gondwanamyces* and *Ophiostoma* from certain *Protea* spp. In the case of open-structured, dark-coloured infructescences such as those of *P. acaulos* and *P. nitida*, temperatures may often reach sub-optimal levels for the growth and

survival of *Gondwanamyces* and *Ophiostoma*. For example, when considering temperatures reached within infructescences in the field, temperatures within *P. nitida* infructescences reached lethal levels (above 40 ºC) more often and for longer periods than those of the host species *P. neriifolia* and *P. repens* (Table 3). If these temperatures are commonly reached and maintained, they will effectively lead to the elimination of *Gondwanamyces* and *Ophiostoma*. Interestingly, maximum temperatures within *P. nitida* infructescences were on average 7 ºC higher than the ambient air. Thus temperatures in the field need only reach ca. 33 ºC to effectively preclude the establishment and growth of *Gondwanamyces* and *Ophiostoma* within *P. nitida* infructescences. These temperatures are often exceeded in areas where *Protea* spp. grow. It is thus unlikely that these fungi would be able to colonize *P. nitida* and other species with open-structured infructescences under normal field conditions.

All *Gondwanamyces* and *Ophiostoma* considered in this study grew equally well on media prepared from *P. nitida* (a non-host species) compared to media prepared from their natural hosts. This suggests that host chemistry alone does not dictate which *Protea* sp. acts as host for these fungi in general. However, host chemistry appeared to influence host exclusivity between known hosts. Thus, the radial growth of all but one species was significantly higher on MEA media containing natural host tissue. The exception was *O. phasma*, for which there were no significant differences in radial growth between *P. repens* (non-host) and *P. neriifolia* (host). These *Protea* spp. often grow sympatrically and have similar vector mite communities. *Ophiostoma splendens* (naturally found on *P. repens*) and *O. phasma* (naturally found on *P. neriifolia*) also have very similar requirements regarding relative humidity and thermal tolerance. In theory, *O. phasma* should thus be able to colonise *P. repens*. However, despite repeated surveys (Roets *et al.* 2006, 2009b), *O. phasma* has never been observed within *P. repens* infructescences.

The absence of *O. phasma* from *P. repens* cannot be explained by a lack of association of the relevant mites with *P. neriifolia* and *P. repens*. The vector mites are the same on both *Protea* spp. (E. Uekermann pers. comm.) as are the beetles that vector the mites (Roets *et al.* 2009a). We believe that host exclusivity for *O. phasma* may be influenced by differential competitive abilities with other *Gondwanamyces* and *Ophiostoma* spp. Thus, if *O. phasma* is a weak competitor compared to *O. splendens* and / or *G. proteae* within *P. repens* infructescences it could be excluded from *P. repens*. Similarly, host exclusivity of other infructescence-colonising *Gondwanamyces* and *Ophiostoma* may be enhanced by differential competitive abilities when growing on different *Protea* hosts. Studies to elucidate interactions between these fungi on different *Protea* spp. are currently in progress.

In this study, we have sought to elucidate factors that influence host species exclusivity of *Gondwanamyces* and *Ophiostoma* from South African *Protea* spp. The results add to a growing database on microfungi associated with *Protea* spp. We have demonstrated that host exclusivity and probably also organ specificity may be enforced by various abiotic factors (dictated by host physical structure) and also host (substrate) chemistry. Future studies should include investigations into the effect of relative humidity and temperature on the suitability of infructescences of different *Protea* spp. for vector mite colonisation. In addition, studies on the role of interspecies competition of various fungi within the infructescences of different *Protea* spp. may help elucidate mechanisms for the maintenance of host exclusivity in this niche.

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Figure Captions:

Fig 1- Typical colony morphology of *Gondwanamyces proteae* (left column) and *Ophiostoma splendens* (right column) after seven days of growth at 25ºC in the dark on media prepared with MEA (a, b), MEA and *P. repens* tissues (c, d), WA and *P. repens* tissues (e, f) and WA and *P. neriifolia* tissues (g, h). Scale bars = 5 mm.

Fig 2- Mean radial growth (mm diam. after ten days at 25ºC) on growth media prepared from sawdust of various *Protea* spp. and either MEA (top graph) or Water agar (bottom graph). Error bars = standard error. White bars = *P. repens*, Light grey bars = *P. neriifolia*, Dark grey bars = *P. nitida*, Black bars = Agar only. Different letters indicate significant differences between radial growths. Bars with thickened borders indicate radial growth of a particular fungus on media prepared from its natural host.

Fig 3- Mean number of mites (± SE) that are known to vector *Gondwanamyces* and *Ophiostoma* collected from the infructescences of *P. neriifolia* (white bars), *P. repens* (grey bars) and *P. nitida* (black bars) over a one year period. Different letters indicate significant differences between least square means.

Fig 4- Mean radial growth on MEA (six isolates per tested species, ± standard error) of *G. proteae* (white bars), *G. capensis* (light grey bars), *O. phasma* (dark grey bars) and *O. splendens* (black bars) at a range of temperatures after 7 d in the dark. Different letters indicate significant differences between radial growths.

Fig 5- Mean radial growth on MEA (six isolates per tested species, ± standard error) of *G. proteae* (white bars), *G. capensis* (light grey bars), *O. phasma* (dark grey bars) and *O. splendens* (black

bars) after 7 d in the dark at a range of different relative humidities. Different letters indicate significant differences between radial growths.

Table 1 - Localities and morphological groups of the various *Protea* spp. collected in this study.

Protea Species	Collection site	Group[a]	Deg. South	Deg. East
P. acaulos	Bainskloof	Western Ground	34˚06"05.10'	19˚49"46.08'
P. aurea	George, Montaque Pass	White	33˚52"01.20'	22˚25"54.00'
P. burchellii[b]	Stellenbosch Mountain	Spoon-bract	33˚56"44.58'	13˚52"42.66'
P. caffra[b]	Pretoria	Grassland	25˚46"58.92'	28˚11"56.64'
P. eximia	Swartberg Pass	Spoon-bract	33˚21"59.10'	22˚05"46.44'
P. lanceolata	Albertinia	True	34˚04"58.80'	21˚15"20.52'
P. laurifolia[b]	Giftberg	Bearded	31˚45"46.38'	18˚47"17.64'
P. lorifolia	Swartberg Pass	Bearded	33˚22"11.22'	22˚06"33.90'
P. neriifolia[b]	Franschoek Pass	Bearded	33˚54"20.94'	19˚09"27.36'
P. neriifolia[b]	Gordon's Bay	Bearded	34˚04"58.80'	21˚15"20.52'
P. neriifolia[b]	Jonkershoek Reserve	Bearded	33˚59"14.58'	18˚57"15.30'
P. nitida	Franschoek Pass	Shaving-brush	33˚54"46.50'	19˚08"36.60'
P. nitida	Gordon's Bay	Shaving-brush	34˚04"58.80'	21˚15"20.52'
P. nitida	Jonkershoek Reserve	Shaving-brush	33˚59"48.30'	18˚56"26.88'
P. obtusifolia[b]	Cape Agaulas	Spoon-bract	34˚48"49.32'	20˚01"15.00'
P. punctata	Swartberg Pass	White	33˚21"48.24'	22˚03"50.04'
P. repens[b]	Franschoek Pass	True	33˚55"13.86'	19˚09"40.74'
P. repens[b]	Gordon's Bay	True	34˚04"58.80'	21˚15"20.52'
P. repens[b]	Jonkershoek Reserve	True	33˚58"40.02'	18˚56"39.36'
P. susannae	Struisbaai	Spoon-bract	34˚45"02.94'	19˚58"48.60'

[a] Morphological groupings follow Rebelo (1995)
[b] Species with confirmed *Gondwanamyces* and *Ophiostoma* relationships

Table 2 - Abundance and Sørensen's coefficients of similarity for abundance (Cn) and species richness (Cs) for *Gondwanamyces* and *Ophiostoma* spore-carrying mite communities collected from the infructescences of 14 *Protea* spp. (n = 10). Species indicated in bold typeface are known hosts of *Gondwanamyces* and/or *Ophiostoma*. - = not applicable.

Abundance

	Protea species[a]													
Mite species	**P. caf**	P. nit	**P. bur**	P. exi	**P. obt**	P. suz	P. lor	**P. lau**	**P. ner**	P. lan	**P. rep**	P. pun	P. aur	P. aca
P. vanderbergi	2	1	0	0	1	0	0	0	31	0	3	0	0	0
Tarsonemus sp.	0	0	0	2	6	0	0	84	0	0	0	1	0	0
Tricauropoda sp.	1	0	1	0	22	0	5	89	20	0	8	0	1	0
Total abundance	3	1	1	2	29	0	5	173	51	0	11	1	1	0

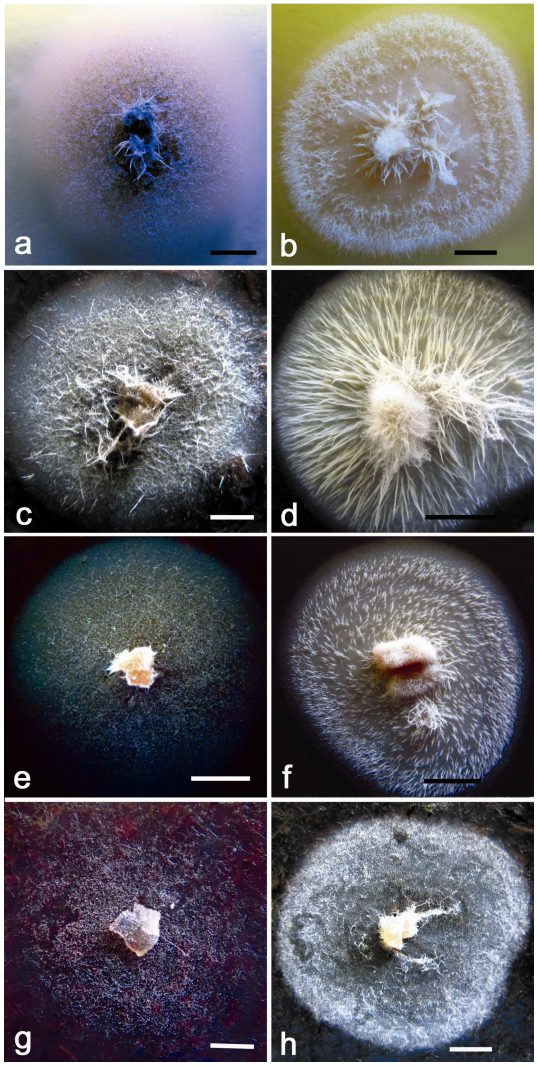
Sørensen's coefficients of similarity in abundance (Cn, bottom of diagonal) and species richness (Cs, top of diagonal)

P. caf		0.67	0.67	0.00	0.80	-	0.67	0.50	1.00	-	1.00	0.00	0.67	-
P. nit	0.50		0.00	0.00	0.50	-	0.00	0.00	0.67	-	0.67	0.00	0.00	-
P. bur	0.50	0.00		0.00	0.50	-	1.00	0.67	0.67	-	0.67	0.00	1.00	-
P. exi	0.00	0.00	0.00		0.50	-	0.00	0.67	0.00	-	0.00	1.00	0.00	-
P. obt	0.13	0.07	0.07	0.13		-	0.50	0.80	0.80	-	0.80	0.50	0.50	-
P. lor	0.25	0.00	0.33	0.00	0.29	-		0.67	0.67	-	0.67	0.00	1.00	-
P. lau	0.01	0.00	0.11	0.02	0.28	-	0.60		0.50	-	0.50	0.67	0.67	-
P. ner	0.11	0.38	0.38	0.00	0.53	-	0.18	0.18		-	1.00	0.00	0.67	-
P. lan	-	-	-	-	-	-	-	-	-		-	-	-	-
P. rep	0.43	0.17	0.17	0.00	0.45	-	0.62	0.09	0.35	-		0.00	0.67	-
P. pun	0.00	0.00	0.00	0.33	0.07	-	0.00	0.01	0.00	-	0.00		0.00	-
P. aur	0.50	0.00	1.00	0.00	0.07	-	0.33	0.01	0.04	-	0.17	0.00		-
P. aca	0.00	0.00	0.00	0.00	0.00	-	0.00	0.00	0.00	-	0.00	0.00	0.00	

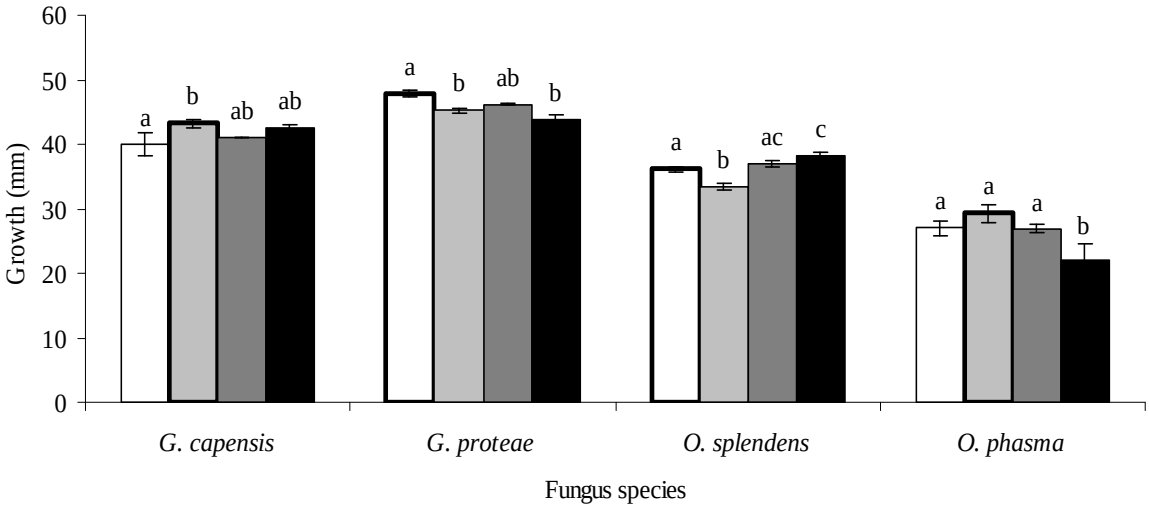
[a] P. caf = *P. caffra*, P. nit = *P. nitida*, P. bur = *P. burchellii*, P. exi = *P. eximii*, P. obt = *P. obtusifolia*, P. suz = *P. suzannae*, P. lor = *P. lorifolia*, P. laur = *P. laurifolia*, P. ner = *P. nerifolia*, P. lan = *P. lanceoloata*, P. rep = *P. repens*, P. pun = *P. punctata*, P. aur = *P. aurea*, P. aca = *P. acaulos*

Table 3 - Temperature and relative humidity within the infructescences of three *Protea* spp. Different superscript letters indicate significant differences ($P < 0.05$, $n = 7$). SE = Standard error.

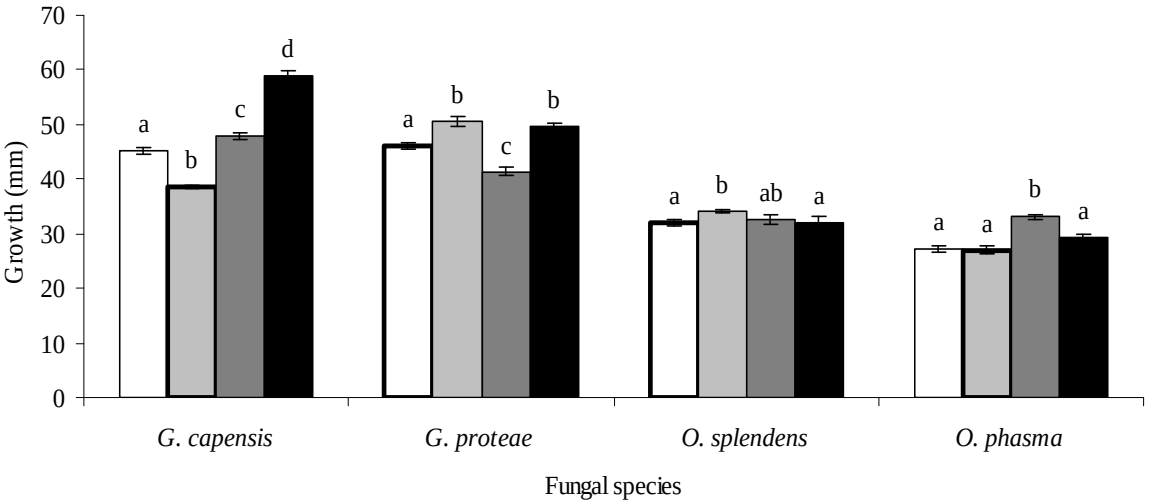
	Control	*P. repens*	*P. neriifolia*	*P. nitida*
Temperature (ºC)				
Mean maximum (SE)	32.24 (1.89)^{a}	33.37 (2.09)^{a}	35.89 (1.65)^{ab}	39.54 (1.71)^{b}
Mean difference between maximum and control (SE)	-	1.14 (0.28)^{a}	3.67 (0.47)^{b}	7.31 (0.94)^{c}
Absolute maximum	38.66	40.64	41.09	45.67
Absolute minimum	13.16	13.16	13.10	13.19
Events over 35	23	64	79	80
Time over 35 (h)	6.75	16	19.75	20
Events over 40	0	9	23	31
Time over 40 (h)	0	2.25	5.75	7.75
Relative humidity (%)				
Mean minimum (SE)	32.34 (4.90)^{a}	55.24 (3.25)^{b}	28.82 (4.04)^{a}	29.36 (3.55)^{a}
Mean difference between minimum and control (SE)	-	22.90 (4.27)^{a}	-3.42 (1.55)^{b}	-2.98 (1.86)^{b}
Absolute minimum	16.42	42.44	17.31	17.00
Absolute maximum	96.11	83.36	82.01	85.01
Events under 50	270	194	313	22
Time under 50 (h)	67.50	5.50	78.75	48.50
Events under 30	88	0	95	65.00
Time under 30 (h)	22.00	0	23.75	16.25

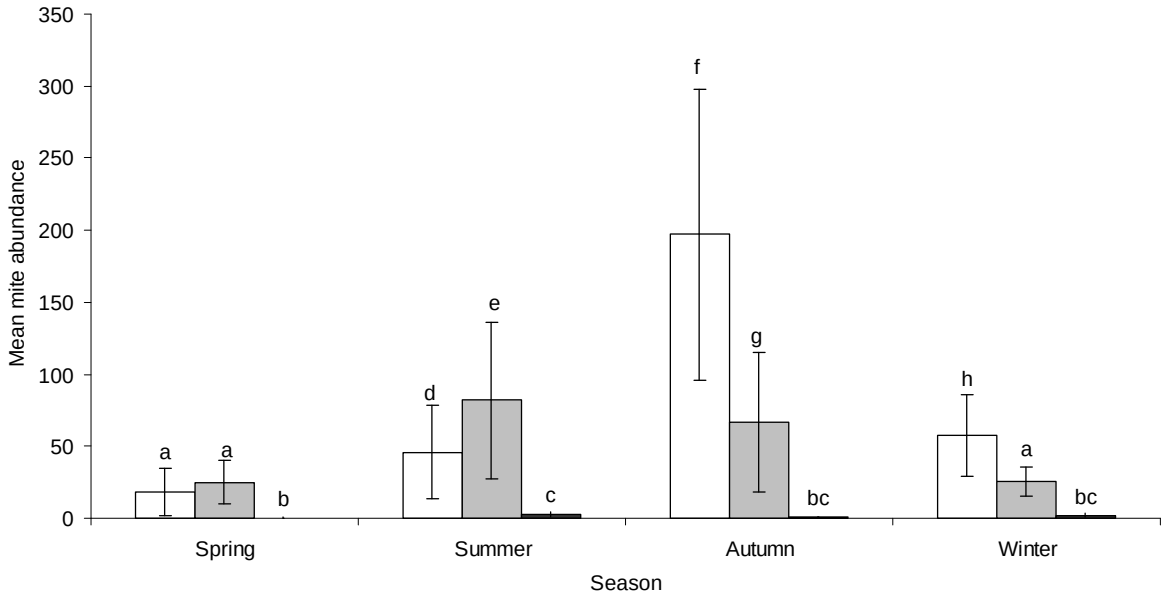

a
b
c
d
e
f
g
h

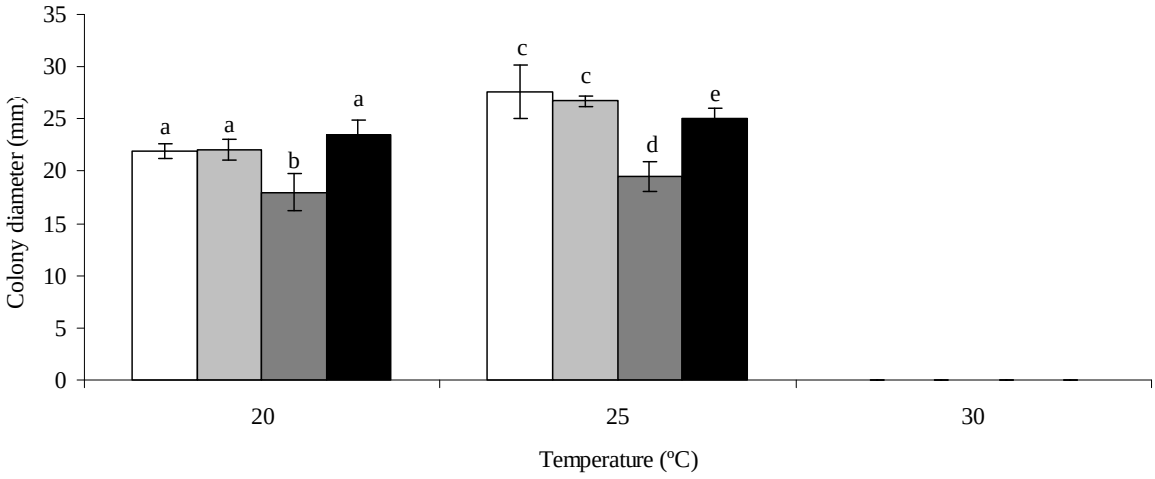
Malt Extract Agar

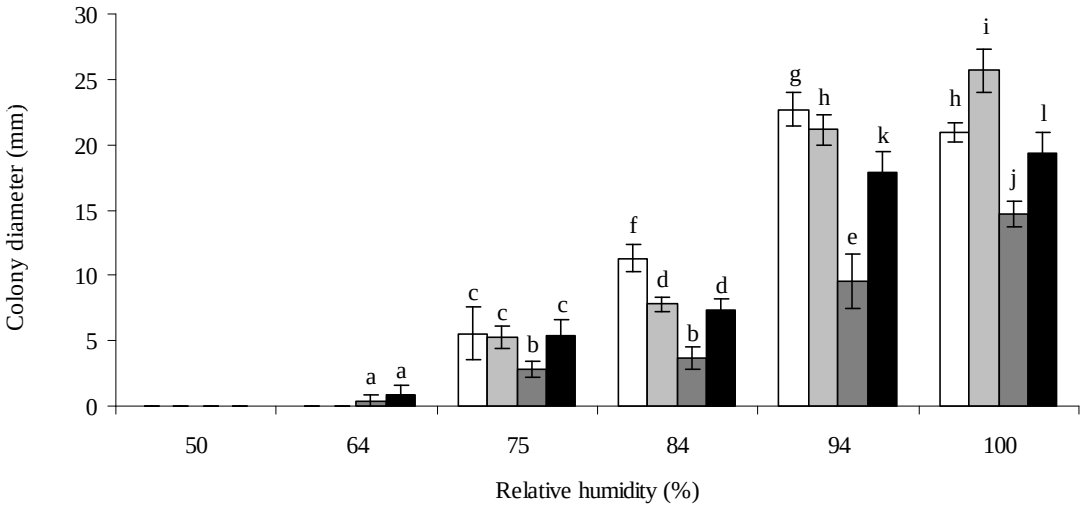


Water Agar




Mean mite abundance
350
300
250
200
150
100
50
0
a
a
b
d
e
c
f
g
bc
h
a
bc
Spring
Summer
Autumn
Winter
Season


35
30
25
20
15
10
5
0
Colony diameter (mm)
a
a
b
a
c
c
d
e
20
25
30
Temperature (ºC)


Colony diameter (mm)
30
25
20
15
10
5
0
a a
c c c
b
f d d
b
g h k
e
h i j l
50
64
75
84
94
100
Relative humidity (%)