Mite-Mediated Hyperphoretic Dispersal of *Ophiostoma* spp. from the Infructescences of South African *Protea* spp.

F. ROETS,[1,2,3] P. W. CROUS,[4] M. J. WINGFIELD,[2] AND L. L. DREYER[1]

Environ. Entomol. 38(1): 143–152 (2009)

ABSTRACT Ophiostomatoid fungi are well known as economically important pathogens and agents of timber degradation. A unique assemblage of these arthropod-associated organisms including species of *Gondwanamyces* G. J. Marais and M. J. Wingf., and *Ophiostoma* Syd. and P. Syd. occur in the floral heads (infructescences) of *Protea* L. species in South Africa. It has recently been discovered that *Ophiostoma* found in *Protea* flower-heads are vectored by mites (Acarina) including species of: *Tarsonemus* Canestrini and Fonzago, *Proctolaelaps* Berlese, and *Trichouropoda* Berlese. It is, however, not known how the mites carry the fungi between host plants. In this study, we consider two possible modes of mite dispersal. These include self-dispersal between infructescences and dispersal through insect vectors. Results showed that, as infructescences desiccate, mites self-disperse to fresh moist infructescences. Long-range dispersal is achieved through a phoretic association with three beetle species: *Genuchus hottentottus* (F.), *Trichostetha fascicularis* L., and *T. capensis* L. The long-range, hyperphoretic dispersal of *O. splendens* G. J. Marais and M. J. Wingf. and *O. phasma* Roets et al. seemed effective, because their hosts were colonized during the first flowering season 3–4 yr after fire.

KEY WORDS fungal transmission, ophiostomatoid fungi, phoresy, vector, *Tarsonemus*

Ophiostomatoid fungi (Wingfield et al. 1993) are best known as associates of bark beetles that infest trees, especially conifers. These fungi include some of the most important plant pathogens, particularly of trees, where they also act as important agents of sap-stain in lumber (Sinclair et al. 1987, Wingfield et al. 1993, Jacobs and Wingfield 2001). One of the most unusual assemblages of the ophiostomatoid fungi occurs in the infructescences (floral heads) of *Protea* L. species (Proteaceae) that grow in the unique Fynbos Biome (Acocks 1953) in the Western Cape province of South Africa.

Ophiostomatoid fungi provide a remarkable example of fungi that have evolved novel morphological structures to accomplish their dispersal by vector arthropods. The group includes species in well-known genera such as *Ophiostoma* Syd. and P. Syd. and *Ceratocystis* Ellis and Halst., which are characterized by the production of spores in sticky masses, usually at the apices of elongated structures (Upadhyay 1981, Wingfield et al. 1993). This architecture facilitates dispersal through vector arthropods to which sticky spores typically attach (Münch 1907, 1908, Francke-Grosmann 1967).

The interactions between scolytine bark beetles and their phoretic fungal partners, including *Ophiostoma*, have been relatively well studied (Malloch and Blackwell 1993, Paine et al. 1997, Kirisits 2004, Harrington 2005), with some relationships believed to be mutualistic (Paine et al. 1997, Six and Paine 1998, Ayres et al. 2000). In addition to bark beetles, mites may also play a significant role in the dispersal of ophiostomatoid fungi (Bridges and Moser 1983, Moser 1985, Klepzig et al. 2001a, b, Lombardero et al. 2003). The relationship between these mites and their phoretic fungi may also be mutualistic (Bridges and Moser 1983, Moser 1985, Hofstetter et al. 2006). These mite species are phoretic on bark beetles (Moser and Roton 1971, Klepzig et al. 2001a, b, Lombardero et al. 2003). For example, it has been shown that *Ophiostoma minus* (Hedgc.) H. and P. Sydow, a mutualistic associate of *Tarsonemus* Canestrini and Fonzago species, limits the reproductive success of the bark beetle *Dendroctonus frontalis* Zimmermann on which these mites are phoretic (Lombardero et al. 2003, Hofstetter et al. 2006).

Scolytine bark beetle–ophiostomatoid fungus–mite systems are confined to the Northern Hemisphere. However, similar interactions may also exist in the Southern Hemisphere albeit with different insect vectors. *Ophiostoma* species are known to be associated with indigenous *Protea* hosts, which often dominate plant communities in the Fynbos Biome (Linder 2003). However, it is not known how the fungi achieve short- or long-distance dispersal in these systems.

[1] Department of Botany and Zoology, Stellenbosch University, Stellenbosch, South Africa.
[2] Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.
[3] Corresponding author, e-mail: fr@sun.ac.za.
[4] Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands.

The inflorescences of *Protea* species mature into tightly packed infructescences that open to release their seeds only when their water supply is severed, such as when the plants die after fire (Bond 1985). Infructescences may thus persist on the plants for several years, during which time they are colonized by various arthropods and fungi (Coetzee and Giliomee 1987, Lee et al. 2005, Roets et al. 2006c). *Ophiostoma* often dominate the fungal community within these infructescences and may be fundamental to the ecology of these plants (Marais and Wingfield 2001, Roets et al. 2005).

Five species of *Ophiostoma* have been described from *Protea* hosts in South Africa (Marais and Wingfield 1994, 1997, 2001, Roets et al. 2006a). Three of these (*O. splendens* G. J. Marais and M. J. Wingf., *O. phasma* Roets et al., and *O. palmiculminatum* Roets et al.) have recently been shown to be phoretic on four mite species (Roets et al. 2006b). The remaining two species, *O. protearum* G. J. Marais and M. J. Wingf. and *O. africanum* G. J. Marais and M. J. Wingf., are most likely transported in a similar way. Two of the *Ophiostoma*-vectoring mite species, *Tarsonemus* sp. A and *Trichouropoda* Berlese, possess seemingly specialized spore-carrying structures (sporothecae; Bridges and Moser 1983, Lombardero et al. 2003) that frequently contain spores of *Ophiostoma* (Roets et al. 2006b). In addition, the *Trichouropoda* sp. is able to feed and reproduce on a diet consisting exclusively of its phoretic fungi (Roets et al. 2006b). The interaction between this *Trichouropoda* sp. and *Ophiostoma* is, therefore, likely to be mutualistic.

It is not known how the mites that vector the *Protea*-associated *Ophiostoma* disperse from one infructescence to another. Because other species of *Tarsonemus*, *Proctolaelaps*, and *Trichouropoda* (among others) from the Northern Hemisphere are known to be carried phoretically on scolytine bark beetles (Bridges and Moser 1983, Lindquist 1969, Moser and Roton 1971, Blackwell et al. 1986), we assume that the mites carrying *Protea*-associated *Ophiostoma* may also be transported in a similar way by arthropods that coexist with *Protea.* Mites may also move from one *Protea* infructescence to another by physically migrating between aging and fresh infructescences on individual host plants.

The aim of this study was to explore how the *Protea*-associated, *Ophiostoma*-vectoring mites move from one infected infructescence to another. We considered whether mites self-dispersed over short distances between infructescences in reaction to desiccating conditions. In addition, we studied the ability of *Ophiostoma*-carrying mites to use insect vectors as long-distance dispersal agents. We determined the timing of initial colonization (time since fire) and the timing of seasonal colonization of host infructescences by *O. splendens* and *O. phasma.* To establish whether mites inoculated their hosts in an active or passive manor, we tested for the transfer of fungal spores from these to both artificial media and natural host substrate. From the results, the life cycles of *O. phasma* and *O. splendens* are constructed and compared with the conifer-based systems.

Materials and Methods

Within-Plant Dispersal of Mites and Fungi. To study whether the movement of mites occurs between infructescences in response to changes in moisture (e.g., from older drying flowers to moist fresh flowers), we compared numbers of mites moving from drying infructescences to moist or dry toweling placed within artificially constructed infructescences. The artificially manufactured "infructescences: ($n = 52$) consisted of small glass containers (30-ml wide-neck bottles) filled with shredded filter paper. The filter paper in one half of these bottles ($n = 26$) was dry, whereas the other half was slightly moistened with 3 ml dH_2O. In an effort to simulate conditions within natural infructescences, one half of the bottles ($n = 13$) from each of the two treatments were placed within larger containers and covered with black plastic bags to eliminate light, whereas the remaining bottles were left uncovered.

The apices of 52 *Protea repens* L. shoots (~60 cm long), collected from the Jonkershoek Forestry Reserve, Stellenbosch, South Africa, in December 2005, were covered with these upturned containers. The shoots chosen contained a single ~4-mo-old infructescence colonized by *O. splendens* and individuals of *Tarsonemus*, *Proctolaelaps vandenbergi*, and *Trichouropoda.* Morphologically similar shoots in which the infructescences were situated a third of the way down from the tip of the main branch were chosen. After the side branches and leaves had been removed from the main branches, the shoots were placed in empty plastic containers to maintain them in an upright position. Because of the unavailability of water, the infructescences dried out rapidly, which caused the involucral bracts to open and release the enclosed seeds. The absence of water below the opening infructescences also allowed free upward or downward movement by the mites. Mites were collected from these containers at 6-d intervals over a period of 1 mo and stored at 4°C.

Comparisons were made between the total numbers of mites accumulating within the artificial infructescences of the different treatments. Data were analyzed using a *t*-test for independent samples within the Statistica 7 (StafSoft, Tulsa, OK) software package.

Between-Plant Dispersal. To test whether long distance dispersal of mites and fungi occurs through phoresy on insects, 56 *Ophiostoma*-colonized *P. repens* and *P. neriifolia* R. Br. 3- to 12-mo-old infructescences ($n = 56$ for each *Protea* spp.) were collected from the Jonkershoek Forestry Reserve between May 2004 and July 2005. Infructescences of the two *Protea* species were kept separate and placed in specially designed emergence cages as described by Roets et al. (2006b), and all arthropods that emerged over a 3-mo period were collected. In addition, various larger insects (≥5 mm) were haphazardly collected from the open flower heads of these two plant species in the field

during August 2005. All arthropods were classified into morpho-species and inspected for the presence of phoretic mites using a Nikon SMZ800 (Nikon, Tokyo, Japan) dissecting microscope. Light microscopy photographs were taken with a Nikon DXM1200 digital camera.

When present, individual mites were removed with a fine dissecting needle and stored at 4°C until further study. A selected set of arthropod specimens was also studied with a Leo 1430 VP7 scanning electron microscope (SEM). For these studies, the arthropods were frozen at −20°C overnight and dried for 3 d at 50°C. Specimens were mounted onto stubs using double-sided carbon tape, sputter coated with gold-palladium, and studied using standard SEM methods. Voucher specimens of all arthropods collected in this study are housed in the insect collection (USEC), Department of Conservation Ecology and Entomology, Stellenbosch University, Stellenbosch, South Africa.

Phoretic Mites and Hyperphoretic *Ophiostoma* spp. All mites collected in this study were identified to morpho-species, crushed, mixed with 1 ml ddH_2O, and plated onto 2% malt extract agar plates (MEA; Biolab, Midrand, South Africa) amended with the antibiotics streptomycin sulfate (0.04 g/liter) and cycloheximide (0.05 g/liter) to restrict the growth of fungal contaminants and bacteria. Plates were periodically inspected for the presence of the *Sporothrix* Hekt. and C.F. Perkins asexual states of the *Protea*-associated *Ophiostoma* spp., all of which were identified based on their morphological characters.

Timing of Seasonal Colonization. The floral development of both *P. repens* and *P. neriifolia* was studied, and flowering was divided into six stages. These were (1) young bud stage (~3 mo before the inflorescence opens), (2) late bud stage just before the opening of the inflorescence, (3) early flowering stage where 30–50% of the individual flowers within the inflorescences were open, (4) late flowering stage where >70% of flowers within the inflorescence were open, (5) after flowering where all of the flowers had opened and the involucral bracts had started to close, and (6) 1 mo after flowering. A total of 20 flower heads per flowering stage of *P. repens* and *P. neriifolia* were covered with fine gauze to exclude insect visits to flower heads at different stages of floral maturity. This was done to determine the time when *Ophiostoma* species are vectored to the infructescences. Study sites included the Jonkershoek Forestry Reserve, Franschoek Pass, Franschoek, and the Riviersonderend mountains, Riviersonderend.

The stems beneath the infructescences (~10 cm) were smeared with petroleum jelly to prevent mites and other small arthropods from migrating up the stems to the infructescences after they had been containerized. This experiment was repeated during the main *P. repens* and *P. neriifolia* flowering season from May to August 2003, 2004, and 2005. All infructescences were inspected for the presence of *Ophiostoma* ascomata and their asexual states 2–3 mo after flowering.

A univariate test of significance (analysis of variance [ANOVA]) was performed on the data within the Statistica version 7 (StafSoft) software package with Sigma-restricted parameterization. A significance of $P = 0.05$ was used as minimum value for reports of significance.

Artificial Transfer of *Ophiostoma* to Uncolonized Tissue by Mites. Mites identified as *Trichouropoda* sp. were collected from *O. splendens*–colonized *P. repens* infructescences from the Jan S. Marais Park, Stellenbosch. They were placed in 40-ml specimen vials (20 individuals per vial) containing double autoclaved *P. repens* floral parts that had been collected from flower heads at flowering stage 4. The experiment was replicated 20 times and included two additional negative controls containing only autoclaved floral parts. Vials were kept at 24°C in the dark for 3 mo to simulate the natural state within infructescences. Floral parts were removed from the vials, cleared of mites, and agitated using a vortex mixer in 10 ml ddH_2O under sterile conditions to loosen fungal spores deposited or produced on the plant material. The suspension was transferred to 10 MEA plates (1 ml/plate) that had been amended with streptomycin sulfate (0.04 g/liter) and cycloheximide (0.05 g/liter). Plates were regularly inspected for the presence of *Sporothrix* colonies.

In a separate experiment, individuals of the *Trichouropoda* sp. collected from *O. splendens*–colonized *P. repens* infructescences were allowed to move freely on MEA plates containing streptomycin sulfate (0.04 g/liter) and cycloheximide (0.05 g/liter). Here, one mite was placed on the surface of each plate ($n = 80$), and plates were observed regularly for the presence of *Sporothrix* colonies.

Timing of Initial Colonization (Time Since Last Fire). Three sites containing populations of *P. repens* and *P. neriifolia* at 3–4, 9–11, and 14–17 yr after flowering were selected in the Jonkershoek Forestry Reserve during November 2005. Plants were chosen at random ($n = 10$ per *Protea* species per site), and all the infructescences of a chosen plant were inspected with a hand lens for the presence of *Ophiostoma* ascomata. When no ascomata were found, infructescences ($n \approx 10$) were collected and inspected with a dissecting microscope for the presence of the asexual states of these fungi. Plants were counted as positive for colonization by *Ophiostoma* if any of the collected infructescences contained ascomata or asexual states of these fungi.

Results

Within-Plant Dispersal. A total of 779 mites were collected from the false infructescences throughout the duration of the experiment. Most of these belonged to one of the three species known to carry spores of *Ophiostoma* (Roets et al. 2006b). These included *Tarsonemus* sp. A ($n = 688$), *P. vandenbergi* ($n = 19$), and *Trichouropoda* sp. ($n = 54$). Only two mites, both individuals of *P. vandenbergi* were observed within the bottles before the opening of the infructescences ~2 wk after the infructescences were

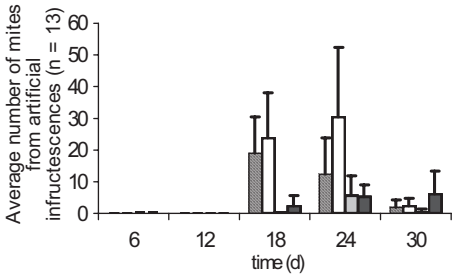

Fig. 1. Average number of mites (±SD) collected from artificial infructescences over a 30-d period: Bars with diagonal stripes, mites collected from uncovered artificial infructescences containing moist filter paper shreds; white bars, mites collected from covered artificial infructescences containing moist filter paper shreds; gray bars, mites collected from uncovered artificial infructescences containing dry filter paper shreds; black bars, mites collected from covered artificial infructescences containing dry filter paper shreds.

picked. A significant increase in mite numbers was, however, observed at the time when the infructescences started to open from days 12–18 ($T = 4.20$, df = 102, $P < 0.0001$; Fig. 1). A significant decrease in mite numbers was observed from day 24 to day 30 ($T = 2.94$, df = 102, $P = 0.004$; Fig. 1). Furthermore, significantly more mites were collected from containers with moist filter paper than those without moisture ($T = 2.42$, df = 24, $P = 0.02$ for open and $T = 2.47$, df = 24, $P = 0.02$ for closed containers). No significant difference was found between the numbers of mites that were collected in the open containers versus containers covered with black plastic bags, except when the closed containers contained moistened filter paper ($T = 2.94$, df = 24, $P = 0.01$). Filter papers were observed not to dry out over the experimental period. After the experiment was terminated, a small number of mites continued to accumulate in the containers left on the plants.

Between-Plant Dispersal. Thirteen insect morphospecies (168 individuals) were collected from the emergence cages (Table 1). These belonged to various families, all of which had been reported on these *Protea* spp. in a previous study (Roets et al. 2006c). Mites were, however, observed only on the surface of *G. hottentottus* F. (Coleoptera: Scarabeidae) individuals that emerged from these infructescences. Although four species of mite were collected from the surface of this beetle (Table 2), only three species were commonly observed. They were *Tarsonemus* sp. A, *P. vandenbergi*, and the *Trichouropoda* sp. (Fig. 2, A–D). The fourth mite species on this beetle was represented by only two hypopi (a deutonymph stage) of a species of *Caloglyphus* Berlese. In one case, all four mite species were found to co-occur on the same *G. hottentottus* individual.

The numbers of mites carried by an individual *G. hottentottus* beetle varied considerably. Most beetles carried individuals of *Tarsonemus* sp. A and the *Trichouropoda* sp. When present, *Tarsonemus* sp. A was particularly numerous, with up to 108 individuals collected from a single beetle. This same beetle also carried three individuals of the *Trichouropoda* sp. and one individual of *P. vandenbergi*.

Table 1. Insects collected from the infructescences of *P. repens* and *P. neriifolia* during May 2004 to July 2006 ($n = 56$ for each *Protea* spp.) in emergence cages and searched for the presence of phoretic mites

Insect taxa	Ref. no.	*P. repens*	*P. neriifolia*
Argyroploce Hbner sp. (Tortricidae)	68	2	1
Braconidae	52	1	1
Crematogaster Lund sp. (Formicidae)	15	35	12
Diptera	5	5	0
Euderes lineicolis Wiedemann (Curculionidae)	33	2	0
Genuchus hottentottus **(F.) (Scarabaeidae)**	70	9	2
Gyponyx Gorham sp. (Cleridae)	55	3	0
Miridae	20	4	0
Oxycarenus maculates Stal. (Lygaeidae)	7	18	24
Pentatomidae	24	0	1
Psocoptera (sp. 3)	13	15	29
Sphenoptera Solier sp. (Buprestidae)	49	2	0
Thysanoptera	34	1	1

Arthropod taxa indicated in bold were found to vector mites. Reference numbers indicate collection no. of reference specimen housed in insect collection (USEC), Department of Conservation Ecology and Entomology, University of Stellenbosch, Stellenbosch, South Africa.

Mites were observed on the ventral sides of the beetles only (Fig. 2, A–D). Here they were usually found in groups associated with the front legs and heads of the beetles. A smaller number of mites were also observed at the bases of the middle pair of legs. The *Tarsonemus* sp. A appeared to be gregarious, because numerous individuals usually occupied the space between the head and the front legs of the beetles.

Various insects were collected in the field from the flower heads of *P. neriifolia* and *P. repens*, including bees, flies, and beetles. The target mites were, however, observed from only two beetle species, namely

Table 2. Average no. (±SD) of phoretic mites collected from the surface of three beetles (*G. hottentottus*, *T. capensis*, and *T. fascicularis*) associated with *P. repens* and *P. neriifolia* in the Jonkershoek Forestry Reserve, Stellenbosch, South Africa, during May 2004 to August 2005

Mite taxa	*Protea neriifolia*		
	G. hottentottus	*T. capensis*	*T. fascicularis*
Tarsonemus sp. A	7 (9.9)	9.3 (24.6)	0
Trichouropoda sp.	0	0	0
Proctolaelaps vandenbergi	0	135.9 (131.7)	39
Caloglyphus sp.	0	0	0
	Protea repens		
Tarsonemus sp. A	31.2 (39.3)	15.5 (6.4)	89 (106.2)
Trichouropoda sp.	4.7 (6.5)	0	0
Proctolaelaps vandenbergi	1.7 (3.2)	80 (72.1)	321 (264.9)
Caloglyphus sp.	0.2 (0.4)	0	0

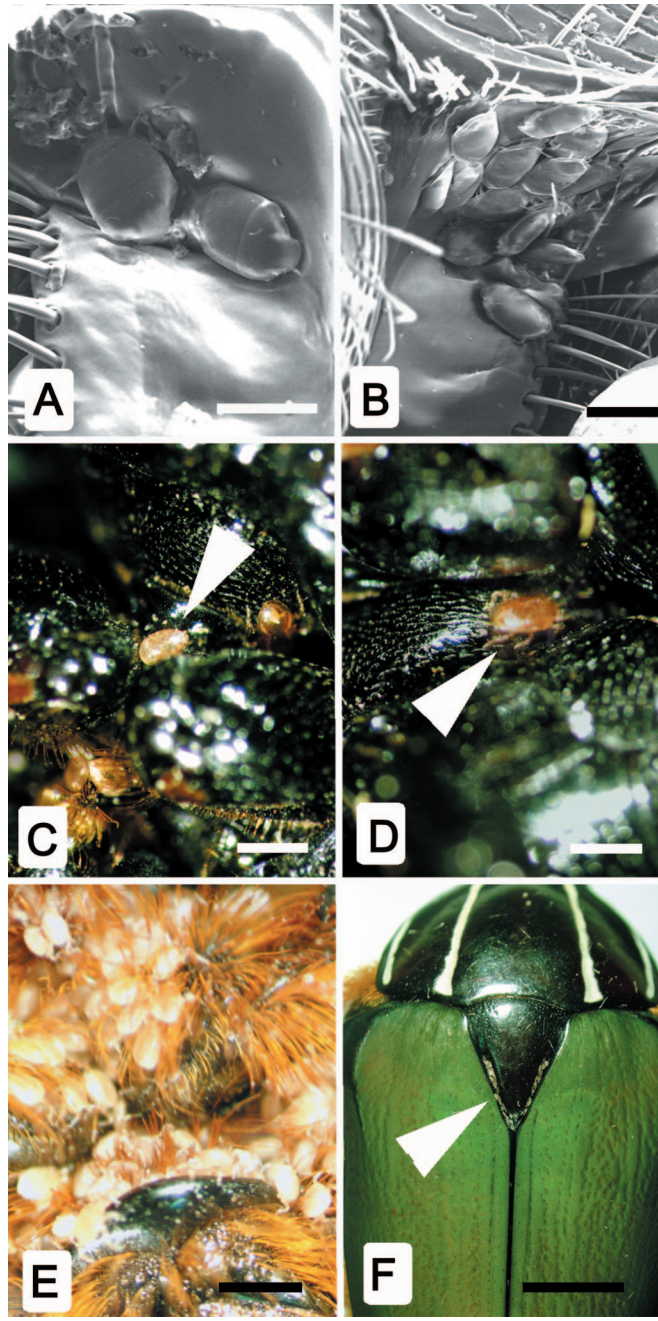

Fig. 2. Mites phoretic on *Protea*-associated beetles. (A and B) Scanning electron micrographs of *Tarsonemus* sp. A on ventral surface of *G. hottentottus.* (C) Light micrograph of *Trichouropoda* sp. on ventral surface of *G. hottentottus.* (D) Light micrograph of *P. vandenbergi* on ventral surface of *G. hottentottus.* (E) Light micrograph of *P. vandenbergi* on ventral side of *T. fascicularis.* (F) Light micrograph of *Tarsonemus* sp. A in mesoscutellular groove of *T. fascicularis.* Scale bars: A and B = 100 μm; C and D = 400 μm; E = 800 μm; F = 5 mm.

Trichostetha capensis L. and *T. fascicularis* L. (Scarabeidae: Cetoniinae). Four individuals of *T. fascicularis* were collected from *P. repens*, whereas only one of these beetles was collected from *P. neriifolia.* Of the nine *T. capensis* individuals collected, seven were collected from *P. neriifolia* and two from *P. repens.*

Individuals of the mite *P. vandenbergi* were common (usually >100 individuals) on all five *Trichostetha capensis* and nine *T. fascicularis* individuals. They were located on the hairy ventral surface of the beetles (Fig. 2E). Individuals of *Tarsonemus* sp. A were also observed on all *T. fascicularis* and four of the *T. capensis*

Table 3. Isolates of *Ophiostoma* spp. obtained from mites phoretic on *G. hottentottus* and collected in false infructescences

Fungal species	Isolate no.	Phoretic mite/false infructescence	Host plant
Ophiostoma phasma	CMW23061	*Tarsonemus* sp. A	*P. neriifolia*
O. splendens	OSP101	*Trichouropoda* sp.	*P. repens*
O. splendens	CMW23062	*Trichouropoda* sp.	*P. repens*
O. splendens	CMW23063	*Trichouropoda* sp.	*P. repens*
O. splendens	OSP104	*Trichouropoda* sp.	*P. repens*
O. splendens	CMW23064	*Trichouropoda* sp.	*P. repens*
O. splendens	OSP106	*Tarsonemus* sp. A	*P. repens*
O. splendens	CMW23065	*Tarsonemus* sp. A	*P. repens*
O. splendens	CMW23066	False infructescence	*P. repens*
O. splendens	CMW23067	False infructescence	*P. repens*

Collections were made from *P. neriifolia* and *P. repens* from the Jonkershoek Forestry Reserve, Stellenbosch, South Africa.

CMW, reference no. in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; OSP, reference no. in the personal culture collection of F. Roets, Stellenbosch University, Stellenbosch, South Africa.

individuals (Table 2). Instead of being ventrally carried as was the case with *P. vandenbergi*, individuals of *Tarsonemus* sp. A were more common in the grooves on the dorsal beetle surfaces, with numbers exceeding 50 (Fig. 2F). Individuals of *Tarsonemus* sp. A were also occasionally observed underneath the elytra of both beetle species.

Phoretic Mites and Hyperphoretic *Ophiostoma* spp. Many different fungal species were isolated from mites collected using the various trapping methods included in this study. Of these, a total of 10 isolates of *Ophiostoma* spp. were obtained from mites (Table 3). *O. splendens* was isolated nine times; twice from *Trichouropoda* sp. collected from the artificial "infructescences," twice from *Tarsonemus* sp. A collected on the surface of a *G. hottentottus* individual that emerged from a *P. repens* infructescence, and five times from *Trichouropoda* sp. also from emerging *G. hottentottus* individuals from *P. repens* infructescences. The single isolate of *O. phasma* was obtained from a *Tarsonemus* sp. A that was carried on a *G. hottentottus* individual that emerged from *P. neriifolia* infructescences. Humidity problems were encountered during the storage of *T. capensis* and *T. fascicularis* specimens that were collected from the flower heads of the two *Protea* species. Mites stuck to the sides of the collection vials containing the beetles because of moisture released by respiration of the insects. It was, therefore, not possible to isolate fungi from these mites.

Timing of Sesonal Colonization. Ascomata of *Ophiostoma* and their asexual states were never observed from *P. neriifolia* and *P. repens* inflorescences that were covered with the gauze bags at the first two bud stages (Fig. 3). This confirmed that the exclusion method followed in this study was effective in preventing arthropods carrying spores of *Ophiostoma* to come into contact with the flower heads. Species of *Ophiostoma* were initially observed within ~2-mo-old infructescences. There was a significant increase in the numbers of infructescences colonized by these fungi with an increase in inflorescence age for both *P. neriifolia* ($F = 3.5$; df = 1,5; $P = 0.0350$) and *P. repens* ($F = 8.5$; df = 1,5; $P = 0.001$; Fig. 3). No significant increase in the numbers of infructescences colonized by *Ophiostoma* was observed between flowering stages 5 (late flowering stage) and 6 (1 mo after flowering).

Artificial Transfer of *Ophiostoma* to Uncolonized Tissue by Mites. Various fungal species were isolated from the autoclaved floral parts artificially colonized by the *Trichouropoda* sp. mites, whereas no fungi were isolated from the negative controls. Colonies of *O. splendens* (in the *Sporothrix* asexual state) were observed from suspensions made from four of the vials containing floral parts colonized by the *Trichouropoda* sp. No colonies of *Ophiostoma* were initiated from mites placed directly onto agar plates.

Timing of Initial Colonization (Time Since Last Fire). Colonies of *O. splendens* and *O. phasma* were observed in the infructescences of *P. repens* and *P. neriifolia* formed after their first flowering season after a fire (~3–4 yr old). Seventy percent of *P. repens* plants contained colonies of *O. splendens*, whereas 50% of *P. neriifolia* plants were colonized by *O. phasma*. When these numbers are compared with the numbers of infructescences colonized by *Ophiostoma* in the two older vegetation sites, it is apparent that there was a slight increase in numbers with an increase in plant population age. *O. splendens* was found to colonize 80 and 70% of *P. repens* plants at the 9- to 11- and 14- to 16-yr-old populations, respectively, whereas the colonization percentage for *O. phasma* on *P. neriifolia* was 70% at both of the older vegetation sites.

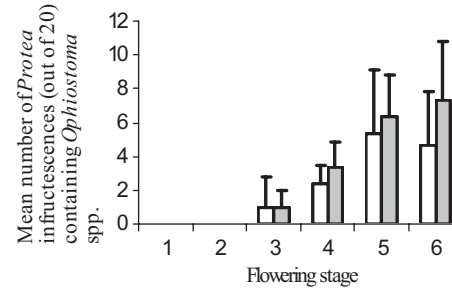

Fig. 3. Mean percentage of infructescences (±SD; $n = 20$) containing *Ophiostoma* spp. at various flowering stages. White bars, *P. neriifolia*; gray bars, *P. repens*.

Discussion

Results of this study provide the first case of mite-mediated hyperphoretic dispersal of *Ophiostoma* associated with plant hosts native to the Southern Hemisphere. The three mite species, *Trichouropoda* sp., *P. vandenbergi*, and *Tarsonemus* sp. A, that have been shown to vector *Ophiostoma* (Roets et al. 2006b) were found to be phoretic on the beetles *G. hottentotus*, *T. fascicularis*, and *T. capensis*. In turn, mites phoretic on *G. hottentottus* were shown to carry isolates of *O. splendens* and *O. phasma*. This is the first time *O. splendens* was isolated from *Tarsonemus* sp. A.

The three beetles identified in this study seem to provide an important means of long-range dispersal of three species of *Protea*-associated mites and the *Ophiostoma* species that they carry. Although the frequency of phoretic mites carrying spores of *Ophiostoma* was low, beetles were found to carry numerous mite individuals. The number of mites vectored by the beetles varied greatly, but mite loads upward of 200 individuals were common on both species of *Trichostetha*. *Genuchus hottentottus* beetles generally carried fewer mites, although one insect was found carrying 112 mite individuals. A limited number of beetles would thus still be able to transport many *Ophiostoma*-carrying mite individuals to a new host plant. This explains why successful *Ophiostoma* spore transfer to new substrates seems to be highly effective, despite the relatively low percentage of spore-carrying mites observed on the beetles.

Colonization of *P. repens* and *P. neriifolia* infructescences by *O. splendens* and *O. phasma* took place mainly during the late flowering and postflowering stages. This coincides with the peak in activity for the secondary *Ophiostoma*-vectoring arthropods (*G. hottentottus*, *T. fascicularis*, and *T. capensis*) because these feed on nectar and pollen of *Protea* (Coetzee and Giliomee 1985, Holm and Marais 1992). The main *Protea* flowering period in the Jonkershoek Forestry Reserve (May–August) also coincides with a peak in numbers of *Ophiostoma* within *Protea* infructescences (Roets et al. 2005). Although not tested, the primary vectors of these fungi (mites) are also expected to be more active and/or more numerous during the flowering season of the host plants. These observations suggest that the timing of *Ophiostoma* spore deposition on the host plant coincides closely with periods of activity of both the primary (mites) and secondary (beetles) *Ophiostoma* vectors.

The beetle-mediated phoretic dispersal of mites and hyperphoretic dispersal of *Ophiostoma* species is evidently very effective. This is evident because the first *P. repens* and *P. neriifolia* infructescences that formed after the first flowering season (3–4 yr after a fire) had already been respectively colonized by *O. splendens* and *O. phasma*. The natural fire cycle ranges between 5 and 50 yr (Van Wilgen 1981, 1987) and reseeding *Protea* species takes at least 3 yr to mature and to start flowering (le Maitre and Midgley 1992). It is thus essential that the mites and the fungi that they vector are able to move over large distances to ensure successful recolonisation of regenerating postfire *Protea* populations.

Based on the results of this study, we are able to propose a life history for *O. splendens* and *O. phasma* (Fig. 4). Sporulating ascomata of *O. splendens* and *O. phasma* are present in *P. repens* and *P. neriifolia* infructescences within 3–4 mo after flowering (Roets et al. 2005). During this time, mites acquire the fungal spores while they feed. Some mite species, notably *Trichouropoda* sp., feed directly on certain *Ophiostoma* spp. (Roets et al. 2006b), which would certainly aid in spore acquisition. We have shown that mites that carry spores of *Ophiostoma* migrate along the main stem of the plant, from the open, desiccating infructescences to closed artificial infructescences, which provide a sheltered, moist environment analogous to the environment within intact infructescences and/or inflorescences. These desiccating conditions occur naturally in the field during the warm summer months when many of the infructescences of *Protea* open to release their seeds (Bond 1985). The small size of the mites and their need for moist, sheltered habitats would probably limit their movement between infructescences to the same plant or only closely associated neighboring plants.

Long-range dispersal of the mites associated with *Protea* infructescences is probably limited to phoresy on the beetle genera *Trichostetha* and *Genuchus*. We propose that the mites crawl onto the young adult *G. hottentottus* (Fig. 4, 2B) beetles as they emerge from the infructescences within which they have developed (Coetzee and Giliomee 1987) or onto the *Trichostetha* spp. (Fig. 4, 2A) that visit *Protea* flowers. They are transported between flower heads by the beetles in their quest for food. The beetles are strong fliers and are capable of covering large distances in search of food. In vitro experiments showed that the mites would be able to inoculate uncolonized plant material with *Ophiostoma* species, at least in the case of *O. splendens* vectored by *Trichouropoda* sp. mites.

During the *Protea* nonflowering season, the eggs of *G. hottentottus* (Fig. 4, 3A) laid during the flowering season develop into c-shaped larvae (Fig. 4, 3B) that bore into the seeds and involucral receptacle while feeding (Coetzee and Giliomee 1987). These form pupae within ovoid structures constructed from frass and plant debris by the larvae (Fig. 4, 3C). Mature beetles emerge during the following *Protea* flowering season and leave the infructescences in search of nectar and pollen (Fig. 4, 3D).

Our results show that the *Protea*-associated *Ophiostoma* seem to be primarily dispersed by mites, with beetles playing a secondary role. Although this has been tested for only a small number of species, *Ophiostoma* spp. associated with bark beetles that infest conifers also seem to have a close relationship with phoretic mites (Klepzig et al. 2001a, b). With *Ophiostoma*–bark beetle associations in conifers, most individual beetles carry the fungal associates found in beetle galleries. However, Hofstetter et al. (2006) showed that *Tarsonemus* mites, and not beetles, drive the abundance of *O. minus* within southern pine beetle infested trees. In contrast, very low numbers of

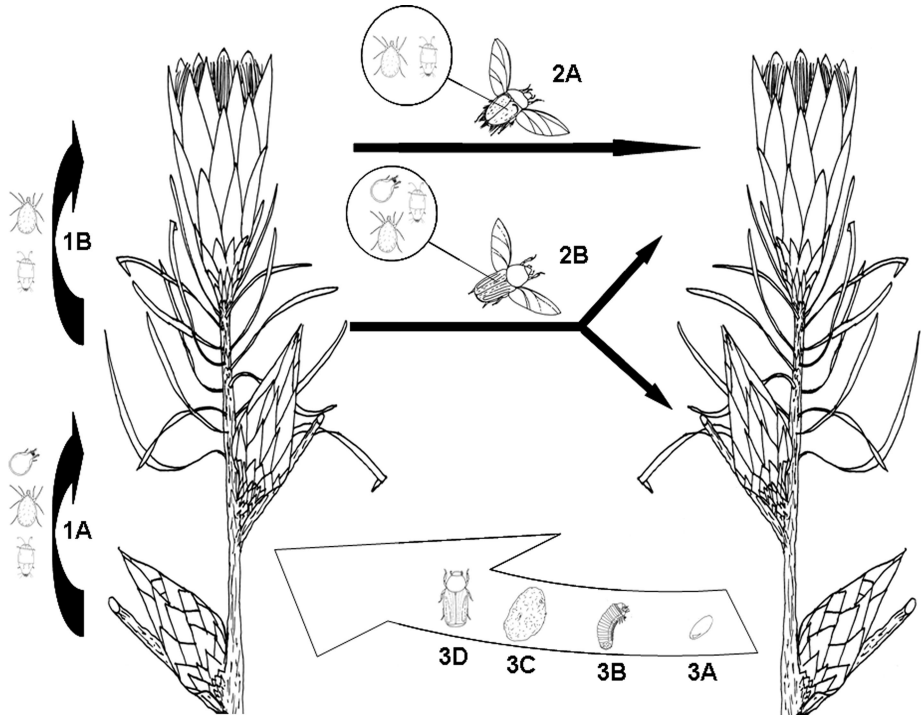

Fig. 4. Schematic drawing of the proposed life history and dispersal of *O. phasma* and *O. splendens* on *Protea* spp. (not to scale). Mites acquire spores when moving and feeding within *Ophiostoma* spp. colonized infructescences. (1A) From top to bottom: *Trichouropoda* sp., *P. vandenbergi*, and *Tarsonemus* sp. A move between infructescences by climbing among the branches. (1B) *P. vandenbergi* and *Tarsonemus* sp. A also move from infructescences to open flowers. (2A) *P. vandenbergi* and *Tarsonemus* sp. A hitching a ride on *Trichostetha* spp. to open flowers as the beetles search for *Protea* pollen and nectar. (2B) *Trichouropoda* sp., *P. vandenbergi* and *Tarsonemus* sp. A hitching a ride on *G. hottentottus* to open flowers as the beetles search for *Protea* pollen and nectar or to infructescences where the beetles lay their eggs. (3A–3D) Development of *G. hottentottus* from egg to adult where the adults will emerge during the next *Protea* flowering season to complete the cycle.

Protea infructescences house beetles such as *G. hottentottus* (Roets et al. 2006c), even though most *Protea* infructescences are infected with *Ophiostoma* (Roets et al. 2005). In contrast to *Ophiostoma* associated with bark beetles, *G. hottentottus* does not live in direct contact with *Ophiostoma* or with the mites this beetle carries from one infructescence to another (Lombardero et al. 2003). The abundance of mites within almost all infructescences and on most *G. hottentottus, T. fascicularis*, and *T. capensis* individuals supports the notion that these mites represent the primary vectors of *Protea*-associated *Ophiostoma* species.

Interesting parallels can be drawn between the conifer-associated and *Protea*-associated *Ophiostoma.* In both systems, the host plants are drought tolerant woody plants of ancient origin (Bowe et al. 2000, Reeves 2001). Likewise, the beetles involved in both systems live within plant tissues (excluding *Trichostetha* spp.) that are strongly associated with and dependant on their host plants. Various mites belonging to the genus *Tarsonemus* have been implicated as associates of *Ophiostoma* on both conifers (Bridges and Moser 1983, Moser 1985, Klepzig et al. 2001a, b, Lombardero et al. 2003) and *Protea.* In both systems, these *Tarsonemus* mites possess sporothecae (specialized spore-carrying structures) formed by tergite one (Moser 1985, Roets et al. 2006b). These parallels not only suggest similar origins of the two systems, but also predict that they have been maintained over a very long period of time, which may even predate the Gondwanan break-up 140 m.y.a. (Goldblatt and Manning 2000, Farrell et al. 2001). Coevolution seems to have occurred between *Tarsonemus* and species of *Ophiostoma* and the existence of this relationship in association with *Protea* in South Africa suggests that it deserves further study.

Acknowledgments

We thank E. Ueckermann and J. Błoszyk for identification of the various mite species collected in this study; two anonymous reviewers for valuable comments made on the manuscript; the National Research Foundation and the NRF/DST Centre of Excellence in Tree Health Biotechnology (CTHB) for financial support; and the Western Cape Nature Conservation Board for permission to work on conserved land. The experimental procedures followed in this manuscript comply with current South African laws.

References Cited

Acocks, J.P.H. 1953. Veld types of South Africa. Mem. Bot. Sur. South Afr. 28: 1–192.

Ayres, M. P., R. T. Wilkens, J. J. Ruel, and E. Vallery. 2000. Fungal relationships and the nitrogen budget of phloem-feeding bark beetles (Coleoptera: Scolytidae). Ecology 81: 2198–2210.

Blackwell, M., J. R. Bridges, J. C. Moser, and T. J. Perry. 1986. Hyperphoretic dispersal of a *Pyxidiophora* anamorph. Science 232: 993–995.

Bond, W. J. 1985. Canopy-stored seed reserves (serotiny) in Cape Proteaceae. South Afr. J. Bot. 51: 181–186.

Bowe, L. M., G. Coat, and C. W. dePamphilis. 2000. Phylogeny of seedplants based on all three genomic compartments: Extant gymnosperms are monophyletic and Gnetales' closest relatives are conifers. Proc. Natl. Acad. Sci. U.S.A. 97: 4083–4091.

Bridges, J. R., and J. C. Moser. 1983. Role of two phoretic mites in transmission of bluestain fungus, *Ceratocystis minor.* Ecol. Entomol. 8: 9–12.

Coetzee, J. H., and J. H. Giliomee. 1985. Insects in association with the inflorescence of *Protea repens* (Proteaceae) and their role in pollination. J. Entomol. Soc. South Afr. 48: 303–314.

Coetzee, J. H., and J. H. Giliomee. 1987. Borers and other inhabitants of the inflorescences and infructescences of *Protea repens* in the western Cape. Phytophylactica 19: 1–6.

Farrell, B. D., A. S. Sequeira, B. C. O'Meara, B. B. Normark, J. H. Chung, and B. H. Jordal. 2001. The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). Evolution 55: 2011–2027.

Francke-Grosmann, H. 1967. Ectosymbiosis in wood-inhabiting insects, pp. 171–180. *In* S. M. Henry (ed.), Symbiosis, vol. 2. Academic, New York.

Goldblatt, P., and J. Manning. 2000. Cape plants: a conspectus of the Cape Flora of South Africa, Strelitzia 9. National Botanical Institute of South Africa, Pretoria, South Africa.

Harrington, T. C. 2005. Ecology and evolution of mycophagous bark beetles and their fungal partners, pp. 257–291. *In* F. E. Vega and M. Blackwell (eds.), Ecological and evolutionary advances in insect-fungal associations. Oxford University Press, Oxford, United Kingdom.

Hofstetter, R. W., J. Cronin, K. D. Klepzig, J. C. Moser, and M. P. Ayres. 2006. Antagonisms, mutualisms and commensalisms affect outbreak dynamics of the southern pine beetle. Oecologia (Berl.) 147: 679–691.

Holm, E., and E. Marais. 1992. Fruit chafers of Southern Africa. Sigma Press, Pretoria, South Africa.

Jacobs, K., and M. J. Wingfield. 2001. *Leptographium* species: tree pathogens, insect associates and agents of bluestain. APS Press, St. Paul, MN.

Kirisits, T. 2004. Fungal associates of European bark beetles with special emphasis on the ophiostomatoid fungi, pp. 1–55. *In* F. Lieutier, K. R. Day, A. Battisti, J. C. Grégoire, and H. Evans (eds.), Bark and wood boring insects in living trees in Europe, a synthesis. Kluwer Academic, Dordrecht, The Netherlands.

Klepzig, K. D., J. C. Moser, F. J. Lombardero, M. P. Ayres, R. W. Hofstetter, and C. J. Walkinshaw. 2001a. Mutualism and antagonism: ecological interactions among bark beetles, mites and fungi, pp. 237–267. *In* M. J. Jeger and N. J. Spence (eds.), Biotic interactions in plant-pathogen associations. CAB International, New York.

Klepzig, K. D., J. C. Moser, F. J. Lombardero, R. W. Hofstetter, and M. P. Ayres. 2001b. Symbiosis and competition: complex interactions among beetles fungi and mites. Symbiosis 30: 83–96.

Lee, S., F. Roets, and P. W. Crous. 2005. Biodiversity of saprobic microfungi associated with the infructescences of *Protea* species in South Africa. Fungal Divers. 19: 69–78.

le Maitre, D. C., and J. J. Midgley. 1992. Plant reproductive ecology, pp. 135–174. *In* R. M. Cowling (ed.), Fynbos, fire and diversity. Oxford University Press, Cape Town, South Africa.

Linder, H. P. 2003. The radiation of the Cape flora, southern Africa. Biol. Rev. 78: 597–638.

Lindquist, E. 1969. New species of *Tarsonemus* (Acarina: Tarsonemidae) associated with bark beetles. Can. Entomol. 101: 1291–1314.

Lombardero, M. J., M. P. Ayres, M. W. Hofstetter, M. C. Moser, and K. D. Klepzig. 2003. Strong indirect interactions of *Tarsonemus* mites (Acarina: Tarsonemidae) and *Dendroctonus frontalis* (Coleoptera: Scolytidae). Oikos 102: 243–252.

Malloch, D., and M. Blackwell. 1993. Dispersal biology of the ophiostomatoid fungi, pp. 195–206. *In* M. J. Wingfield, K. A. Seifert, and J. F. Webber (eds.), *Ceratocystes* and *Ophiostoma:* taxonomy, ecology and pathogenicity. APS Press, St. Paul, MN.

Marais, G. J., and M. J. Wingfield. 1994. Fungi associated with infructescences of *Protea* species in South Africa including a new species of *Ophiostoma.* Mycol Res. 98: 396–374.

Marais, G. J., and M. J. Wingfield. 1997. *Ophiostoma proteae-rum* sp. nov. associated with *Protea caffra* infructescences. Can. J. Botany 75: 362–367.

Marais, G. J., and M. J. Wingfield. 2001. *Ophiostoma africanum* sp. nov. and a key to ophiostomatoid species from *Protea* infructescences. Mycol. Res. 105: 240–246.

Moser, J. C. 1985. Use of sporothecae by phoretic *Tarsonemus* mites to transport ascospores of coniferous bluestain fungi. Trans. Br. Mycol. Soc. 84: 750–753.

Moser, J. C., and L. M. Roton. 1971. Mites associated with southern pine bark beetles in Allen Parish Lousiana. Can. Entomol. 103: 1775–1798.

Münch, E. 1907. Die Blaufäule des Nadelholzes. I–II. Naturwissenschaftliche Zeitschrifr Land Forstwitschaft 5: 531–573.

Münch, E. 1908. Die Blaufäule des Nadelholzes. III–IV. Naturwissenschaftliche Zeitschrifr Land Forstwitschaft 6: 297–323.

Paine, T. D., K. F. Raffa, and T. C. Harrington. 1997. Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. Annu. Rev. Entomol. 42: 179–206.

Reeves, G. 2001. Radiation and macro-evolutionary ecology of the African genus *Protea* L. PhD thesis, Imperial College of Science, Technology and Medicine and NERC Centre for Population Biology, University of London, London, United Kingdom.

Roets, F., P. W. Crous, and L. L. Dreyer. 2005. Seasonal trends in colonization of *Protea* infructescences by *Gondwanamyces* and *Ophiostoma* spp. South Afr. J. Bot. 71: 307–311.

Roets, F., Z. W. de Beer, L. L. Dreyer, R. Zipfel, P. W. Crous, and M. J. Wingfield. 2006a. Multigene phylogeny for *Ophiostoma* spp. reveals two new species from *Protea* infructescences. Stud. Mycol. 55: 199–212.

Roets, F., P. W. Crous, M. J. Wingfield, and L. L. Dreyer. 2006b. Discovery of fungus-mite-mutualism within a unique niche of the Cape Floral Kingdom: Environ. Entomol. 36: 1226–1237.

Roets, F., L. L. Dreyer, H. G. Geertsema, and P. W. Crous. 2006c. Arthropod communities in *Proteaceae* infructescences: seasonal variation and the influence of infructescence phenology. Afr. Entomol. 14: 257–265.

Sinclair, W. A., H. Lyon, and W. T. Johnson. 1987. Diseases of trees and shrubs. Cornell University Press, Ithaca, NY.

Six, D. L., and T. D. Paine. 1998. Effects of mycangial fungi on host tree species progeny survival and emergence of

Dendroctonus ponderosae (Coleoptera: Scolytidae). Environ. Entomol. 27: 1393–1401.

Upadhyay, H. P. 1981. A monograph of *Ceratocystis* and *Ceratocystiopsis.* University of Georgia Press, Athens, GA.

Van Wilgen, B. W. 1981. Some effects of fire frequency on Fynbos plant community composition and structure at Jonkershoek, Stellenbosch. South Afr. J. Bot. 118: 42–55.

Van Wilgen, B. W. 1987. Fire regimes in the Fynbos biome. Disturbance and dynamics of Fynbos biome communities, pp. 6–14. *In* R. M. Cowling, C. D. le Maitre, B. McKenzie, R. P. Prys-Jones, and B. W. van Wilgen (eds.), South African national scientific programmes report no. 135. CSIR, Pretoria, South Africa.

Wingfield, M. J., K. A. Seifert, and J. F. Weber. 1993. *Ceratocystis* and *Ophiostoma:* taxonomy, ecology, and pathogenicity. APS Press, St. Paul, MN.

Received 2 November 2007; accepted 27 March 2008.