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Infection of the cones and seeds of *Welwitschia mirabilis* by *Aspergillus niger* var. *phoenicis* in the Namib-Naukluft Park

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Abstract

Welwitschia mirabilis Hook. fil. is a unique and rare dioecious desert gymnosperm endemic to the Namib Desert. The female plants bear 90–100 megasporophylls, of which 50–60% may be fertile, but up to 80% of those fertile seeds may be infected by *Aspergillus niger* var. *phoenicis*. This contamination results in seed and seedling death, potentially negatively affecting recruitment of plants into the population.

The pattern of infection of the cones and seeds in the field was studied over a period of eight months at the Hope Mine in the Namib-Naukluft Park. Infection of the cones was found to peak coincident with the appearance of the pollination drops, and with high temperatures, winds and significant rainfall. *W. mirabilis* cones were microscopically examined and spores of *A. niger* var. *phoenicis* were found embedded in the dehydrating pollination drop at the tip of the micropyle, suggesting that this was one of the routes by which the seeds were infected. The heteropteran, *Probergrothius sexpunctatis*, was also implicated in the infection of the seeds, since it was found to be carrying spores of *A. niger* var. *phoenicis* and was observed feeding on mature seeds and immature cones. While most post-harvest treatments have proved ineffective in eradicating *A. niger* var. *phoenicis* from the seeds, promising results are presently reported using a fungicide containing tebuconazole as the active ingredient.

Keywords: *Aspergillus niger* var. *phoenicis*; Cones; Infection; Seeds; *Welwitschia mirabilis*

1. Introduction

Welwitschia mirabilis Hook. fil. is a unique and rare plant indigenous to the Namib Desert. It is categorised among the Gymnospermae (order Gnetales), but displays many characteristics intermediate between gymnosperms and angiosperms. It is dioecious, bearing either micro- or megastrobili. The morphology of the microsporophyll and microsporangia has led to the term, anthers, for these structures as they resemble those found in angiosperm flowers, whereas the reproductive structures of the female plant are more comparable to the cones of conifers (Bornman, 1978). The plant bears only two leaves each of which is continually regenerated from a basal meristem (Bierhorst, 1971), and which become longitudinally dissected by uneven growth of the truncated stem (Bornman et al., 1972), giving the plant the appearance of having many long ribbon-like leaves.

Although studies have been undertaken on the ecophysiology of the plant, very little is known about the seed biology of *W. mirabilis*. Studies alluding to only limited aspects of the seed biology in particular were published in the 1970s (Butler et al., 1973; Bornman, 1978; Bornman et al., 1979a). More recently, it has been established that the seeds of *W. mirabilis* are highly orthodox and thus should be storable by conventional means and are also amenable to cryostorage (Whitaker et al., 2004). According to Bornman et al. (1972), 50–60% of the 90–100 megasporophylls borne by the female plant may be fertile, while the fungus *Aspergillus niger* infects up to 80% of the seeds and causes seedling death.

Seed-associated fungi could pose a serious problem in terms of propagation of the species, both in its natural habitat, and in planting programmes. Survival of *W. mirabilis* could become jeopardised by lack of seedling recruitment in the field as well as by the inability to produce vigorous young plants for regeneration programmes. It is apparent that the course of infection of the seeds by the fungus, originally suspected and subsequently confirmed to be *A. niger* var. *phoenicis* (Corda) Al-Musallam

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(Cooper-Driver et al., 2000; Whitaker et al., 2004 respectively), requires investigation. Testing of an accession of seeds provided by the National Plant Genetic Resources Centre (NPGRC) of Namibia revealed 100% of the seeds to be infected by *A. niger* var. *phoenicis*; the seeds were discoloured and shrunken, and were essentially non-viable, (only some 7% of the accession was germinable, but none of the seedlings survived (Whitaker et al., 2004)). One of the questions asked was whether the seeds become infected during development on the parent plant, and, if so, whether that infection results in the degeneration and subsequent death of the seed. Therefore the stage of development of the female cones at which infection occurs was investigated, as was the possible route to the seeds of the infection. These studies have the potential to lead to recommendations for timeous fungicidal treatment of at-risk individuals or seeds.

To this end, the population of *W. mirabilis* plants at Hope Mine (23°34′17.4″S; 15°15′31.4″E), near the Gobabeb Training and Research Centre (GTRC) in the Namib-Naukluft Park was selected for a study of the course of infection of the cones over a single season (November 2004 to April 2005). In addition, the effects of the fungicide tebuconazole (Orius 200EW, Makhteshim-Agan SA Pty [Ltd]) on infection and germination were investigated in two accessions of heavily infected seeds provided by the NPGRC.

2. Materials and methods

2.1. Collection and fungal status of cones

Forty female plants were randomly selected. Single cones were collected from these plants every two weeks by cutting the stalk at the base with scissors. The scissors and cut stalk area were sprayed with 90% ethanol before and after cutting. Each cone was placed in a separate Ziploc® bag and stored in a refrigerator (4 °C, but some fluctuation due to equipment breakdown) until processed. At the initial sampling and at one month intervals thereafter, 10 extra cones were collected and placed in phosphate-buffered 2.5% glutaraldehyde fixative and stored in the refrigerator for later processing for fluorescence and scanning electron microscopy. The cones to be plated at the GTRC for fungal isolation were surface sterilised in separate glass vials or in the Ziploc bag in 1% NaOCl for 10 min and rinsed thrice with sterile distilled water. Vials were closed and sealed with Parafilm until the cones were plated. Cones were cut in half down the long axis using a sterilised scalpel and plated cut side down on 90 mm diameter dishes of Malt Extract Agar (MEA, Scharlau, Barcelona, Spain). Two cones were placed on each plate. The plates were incubated in the dark at ambient temperature and monitored periodically for the presence of fungi.

Cones could not be sampled from each individual at every sampling trip. Some plants did not produce cones in the 2004/2005 season, while others had too few to sample at every trip. On average, however, 25 cones were sampled each time.

Upon return to the University of KwaZulu-Natal in Durban, selected seed and cone samples stored in phosphate-buffered 2.5% glutaraldehyde were processed for fluorescence and scanning electron microscopy (SEM) and viewed.

The staining procedure for fluorescence microscopy was modified from Rohringer et al. (1977).

Specimens were:

1. cleared and fixed in ethanol:dichloromethane (3:1 v/v)+ 0.15% trichloroacetic acid for at least 18–24 h
2. washed 2× for 15 min in 50% ethanol
3. washed 2× for 15 min with 0.05 M NaOH
4. rinsed 3× with water
5. soaked in Tris/HCl buffer, pH 5.8 for 30 min
6. stained for 5 min in 0.1% Uvitex (Ciba) in buffer
7. rinsed thoroughly 4× in water
8. washed with 25% aqueous glycerol for 30 min
9. stored in 50% glycerol+trace lactophenol (to prevent deterioration of fungi and drying of material)

Specimens were viewed using a Nikon Eclipse E400 Microscope equipped with a Nikon Y-fl Epi-fluorescence attachment, and a Nikon Super High Pressure Mercury Lamp power supply. The filter combination V-2A (excitation filter 380–420 nm and barrier filter 450 nm) was used to visualise the fungi. Images were digitally captured.

Specimens were processed routinely for SEM and viewed with a LEO1450 scanning electron microscope; images were digitally captured.

2.2. Presence and distribution of *A. niger* var. *phoenicis* colony-forming units (CFU)

Ten female *W. mirabilis* plants were randomly selected. Soil cores 100 × 100 × 20 mm, were removed from points 0, 5, 10, 15 and 20 m away from each plant to the north, south, east and west. The soil was passed through a 1000 μm mesh and samples collected in plastic vials. A 1:10 000 dilution series of a 1 g soil suspension in 10 mL sterile distilled water was made up by serial dilution and samples were plated to evaluate the colony-forming unit (CFU) load. All test tubes were shaken by hand for 1 min prior to each dilution; aliquots (3× 100 μL) were withdrawn from the final tube and dispensed onto separate 90 mm plates of MEA. The liquid was distributed using a flamed bent glass rod. The MEA plates were incubated in the dark at ambient temperatures and monitored periodically for colony growth. The number of colonies present was recorded after 7 days.

One gram of soil from each sample was dried in an oven at 105 °C for 72 h to determine the water content. The number of CFU per gram of soil was then calculated from the equation: $N = a*d*100/(100-X)$, where N = number of CFU per gram dry soil, a = average number of colonies/CFU per plate, d = dilution factor and X = soil moisture percent, wet weight basis (Christensen, Date unknown). The data yielded an estimate of the *A. niger* var. *phoenicis* CFU load of the soil at 5 m intervals up to 20 m away from the plant in four directions.

This procedure was performed once.

2.3. Insects

Insects found associated with *W. mirabilis* were collected. Specimens were preserved in formalin/acetic acid/alcohol (5:1:25; FAA), while single live insects were placed on MEA for 5 min and the plate then sealed with Parafilm and incubated (after removal of the insects) for 5 days to assess their vector status for fungal inoculum.

Two adult and one juvenile *Probergrothius sexpunctatis*, and two small and one large ant collected and stored in FAA were gold-coated and viewed using the SEM.

2.4. Air testing

Plates of MEA were exposed for 10 min on the crowns of 10 randomly selected female *W. mirabilis* plants from the group selected for monitoring of fungal infection. The plates were sealed with Parafilm, incubated in the dark at ambient temperatures and monitored periodically for fungal colony growth for up to 7 days.

Air testing was performed twice, once each in November 2004 and April 2005.

2.5. Fungal isolates from surrounding vegetation

Samples were cut from randomly selected *Adenolobus pechuelii* plants and various grasses in the vicinity of the *W. mirabilis* plants and sealed in marked Ziploc bags. The samples were surface sterilised in 1% NaOCl for 10 min, rinsed thrice with sterile distilled water, cut into pieces and plated on MEA. The plates were incubated at ambient temperatures in the dark and monitored periodically over 7 days.

Culturing was performed twice, once each in January and April 2005.

2.6. Collection of seeds for use in fungicide testing

Accession HK1603 was collected in 2005 in the Kunene region (18°11′18″S 12°44′16″E); accession HK2031 was collected in 2006 near the Messum Crater (21°23′46.3″S 14°10′13.3″E). Seeds were collected in the field by staff of the NPGRC (courtesy of Herta Kolberg). Seeds were transported from the field in cloth bags to the laboratories of the NPGRC from where they were dispatched to Durban by overnight courier.

2.7. In vitro testing of selected fungicides against seeds of W. mirabilis

Malt extract agar containing the fungicide, Orius® was prepared in 90 mm Petri dishes by adding quantities of fungicide to autoclaved malt extract agar to achieve 0.1, 0.01 and 0.005 g/L of the active ingredient, tebuconazole.

Seeds ($n = 140$) from accession HK1603 (heavily infected) were prepared by removal of the outer coverings. Twenty seeds were allocated to each treatment set and two individual seeds were aseptically plated on the surface of the medium in each Petri dish. The dishes were incubated under ambient light and temperature conditions, and checked daily for germination, emergence of the cotyledons, and fungal proliferation. Control seeds were plated on plain malt extract agar. The results after 14 days monitoring are presented here.

2.8. Soaking of W. mirabilis seeds in a tebuconazole-containing solution

Seeds ($n = 160$) from accession HK2031 (heavily infected) were prepared by removal of the outer seed coverings. A solution of 200 mL sterile distilled water containing 0.1 g/L tebuconazole was made up in a glass beaker. The seeds to be treated ($n = 140$) were immersed in the tebuconazole solution, which was then covered with foil and placed on an orbital shaker operating at 115 rpm. Forty seeds were removed from the solution after 1, 2 and 3 h and blotted on sterile filter paper before plating. Twenty seeds were plated on 1% water agar (five seeds per plate) in order to assess viability, and the remaining twenty plated on malt extract agar (five seeds per plate) to assess fungal contamination. A control set of 40 seeds not treated with fungicide was similarly plated. Seeds on water agar were incubated in a high light intensity growth room (26 °C, 16/8 h light cycle), and those on malt extract agar were incubated at ambient temperatures in the dark. Cultures were monitored daily for germination and fungal contamination/infection, and results after 14 days are presented here.

2.9. Combined soaking and in vitro treatment of W. mirabilis seeds in a solution and on media containing 0.1 g/L tebuconazole

Plates were prepared in 90 mm diameter Petri dishes by adding the Orius fungicide to autoclaved 1% water agar and malt extract agar to achieve a concentration of 0.1 g/L tebuconazole.

Seeds ($n = 100$) from accession HK2031 (heavily infected) were prepared by removal of the outer seed coverings. The seeds were treated in a 0.1 g/L solution of tebuconazole, as described above. All seeds were removed from the solution after 3 h and blotted on sterile laboratory paper in a laminar air flow. Fifty seeds (two per plate) were plated onto 1% water agar plates containing 0.1 g/L tebuconazole, and the remaining 50 on MEA plates (two seeds per plate), also containing 0.1 g/L tebuconazole, for assessment of fungal infection.

Seeds on water agar were incubated in a high light intensity growth room (26 °C, 16/8 h light cycle), and those on malt extract agar were incubated at ambient temperatures in the dark. Cultures were monitored daily for germination and fungal contamination/infection, and results after 14 days are presented here.

2.10. Data analysis

Spatial distribution of CFU was analysed using the one-way ANOVA procedure of SPSS 11.5 for Windows. Fungicidal

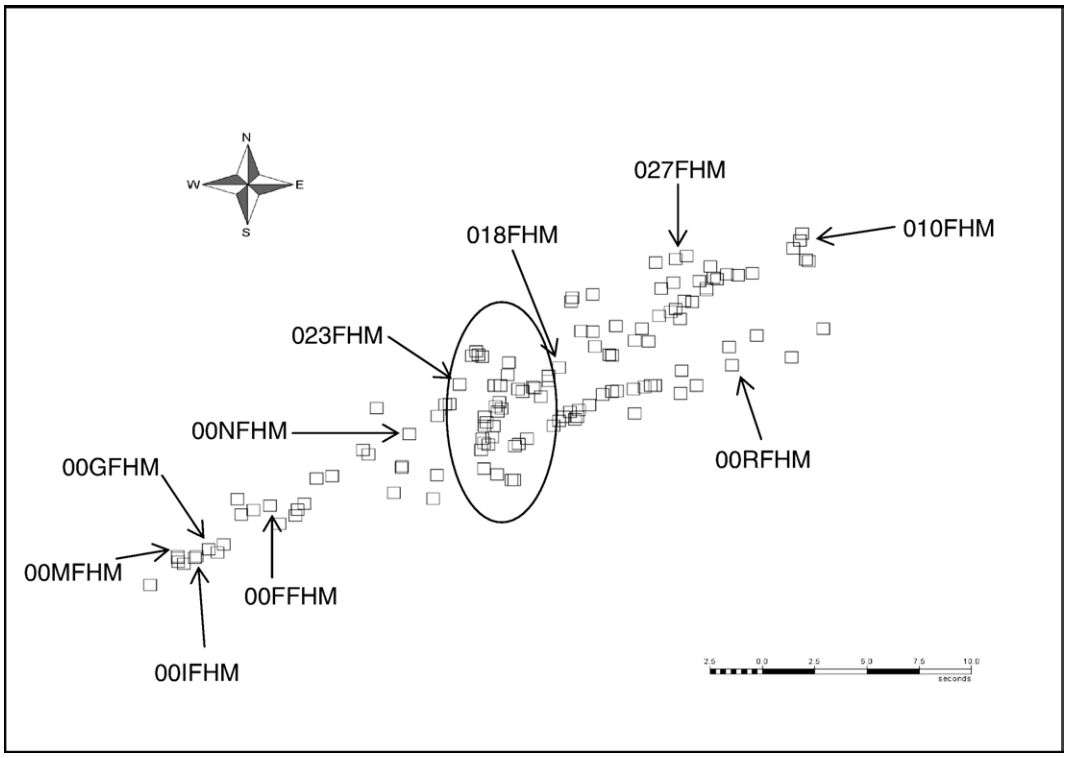

Fig. 1. Map of the population of *W. mirabilis* plants at Hope Mine. The oval indicates the main wash area. Some female plants are identified by the codes ending in FHM, and are arrowed.

trial data were analysed using the Chi Square Goodness-of-Fit procedure.

3. Results

A total of 131 individuals (62 females and 69 males) were recorded at Hope Mine (Fig. 1). The population was distributed along a north-east to south-west axis. The highest concentration and the largest plants were found in the 'main wash', a declivity running from north to south. Most plants were established in the sandy bottom of the washes although a few occurred on the rocky slopes of the wash.

Forty of the 62 female plants were randomly selected for the monitoring of fungal status over time, while a separate group of ten were randomly selected for the study of the presence and distribution of *A. niger* var. *phoenicis* CFUs.

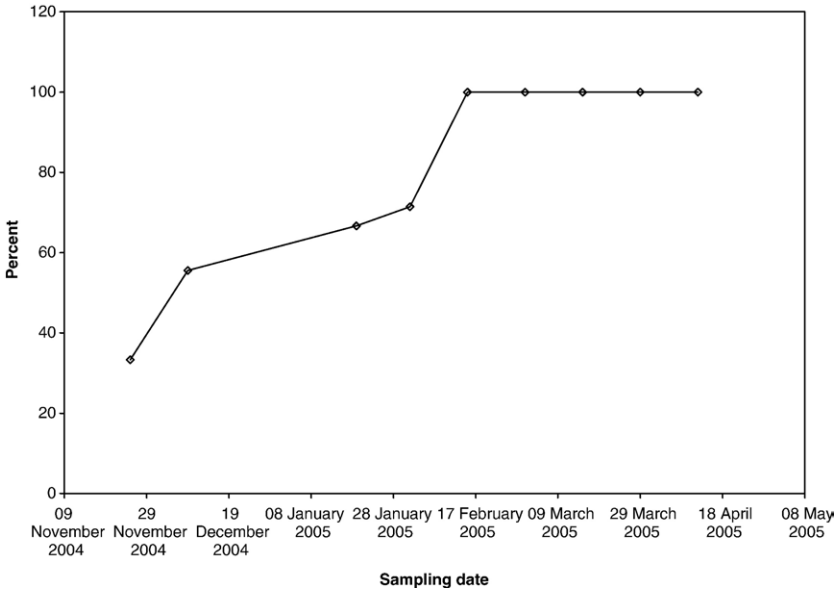

Fig. 2. Percentage of cones infected by *A. niger* var. *phoenicis* over time (sample size varied between datum points because of differences in number of cones produced by each plant).

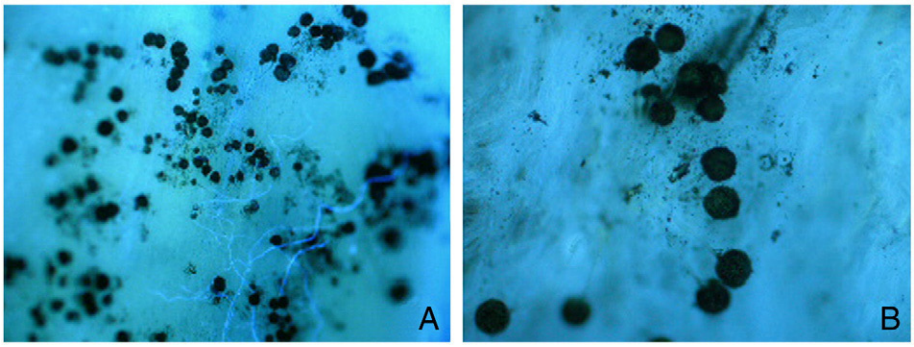

Fig. 3. (A) Hyphae and sporulating conidiophores on the outer seed covering (×40); (B) Conidiophores on the wing of a seed (×100).

3.1. Collection and fungal status of cones

Infection of *W. mirabilis* cones reached a peak in February, coincident with the appearance of the pollination drops and with weather conditions conducive to fungal growth and propagule dissemination (Fig. 2). A record high temperature of 45.5 °C for the GTRC primary weather station (25 km from Hope Mine) was recorded on the 5th of February 2005, while heavy falls of rain at Hope Mine occurred on the 14th of February and at the beginning of April.

Infection of the cones by *A. niger* var. *phoenicis* was not microscopically evident until January 2005, after which hyphae and conidiophores became apparent (Fig. 3A and B). It was found that 66% of cones were contaminated by *A. niger* var. *phoenicis* at the 19th of January sampling (Fig. 2). However, the infection levels surged to 100% of cones sampled on the 15th of February, some of which showed extensive degradation associated with *A. niger* var. *phoenicis*, with macro- and microscopically visible fungal structures (Fig. 3A and B). Seed quality was extremely poor at this stage; the seed itself was crumbly and degraded, and a large space was evident between the inner tissues and the testa (Fig. 4A and B), indicating that the inner tissues had shrunk in volume. This is apparent as illustrated in Fig. 4C and D, which show the structural differences between an infected, degraded seed, and an uninfected seed from an earlier collection in a previous year from a different location.

Seeds and cones sampled in the early stages of development (December 2004) and after the production of the pollination drops (February 2005 and later) were prepared for SEM. These sampling times, before, during and after pollination drop production, were chosen to ascertain whether *A. niger* var.

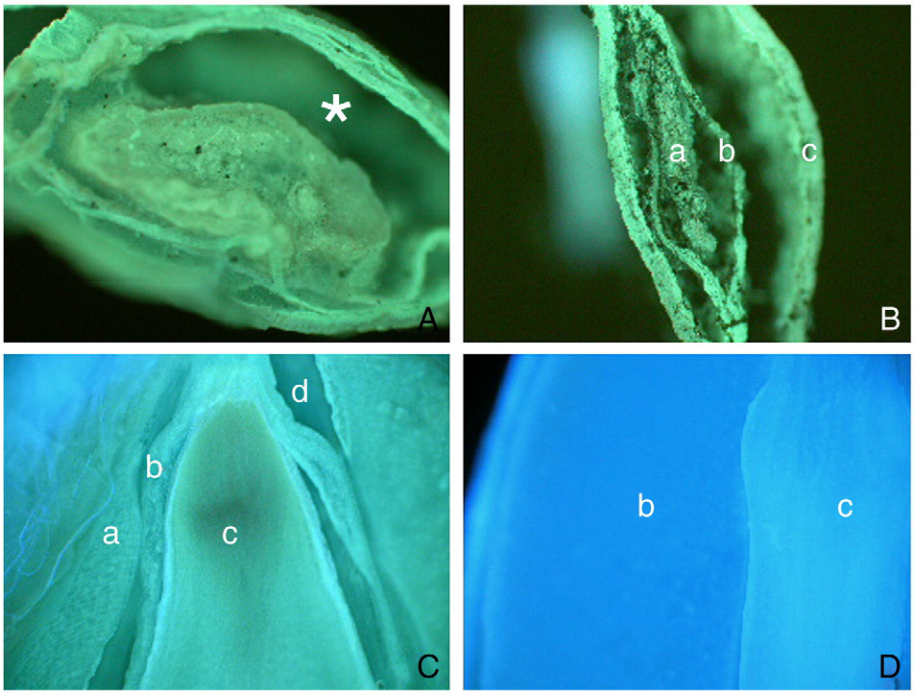

Fig. 4. (A) Cross-section of an infected seed; space between rest of seed and testa indicated with an asterisk (×40); (B) Cross-section of a heavily infected seed in which an abundance of spores occurred; a, remains of embryo and gametophyte; b, testa; c, outer seed coverings (×40); (C) The micropylar end of a degraded seed; a, the outer seed covering; b, degraded gametophyte remains; c, embryo and d, space between gametophyte and outer seed covering (×40); (D) An uninfected seed harvested from a different location (outer seed coverings removed); b, gametophyte; c, embryo (×40).

phoenicis spores gained access to the developing ovule via the secretion, since infection increased greatly during this period (Fig. 2).

Fig. 5A shows the micropyle of a mature ovule at the mid-February sampling, prior to pollination drop production. The apparent lack of any fungal structures at this stage suggested that ovule infection was still to occur, as all cones were later revealed to be infected. Fig. 5B shows the micropyle of a seed sampled in mid-March when the lumen of the micropyle was almost completely obstructed by dehydrating pollination drop. Fig. 5C is a magnified image of part of the micropyle shown in Fig. 5B, and illustrates *A. niger* var. *phoenicis* spores (arrowed) embedded in the dehydrating pollination drop. At this stage, spores were found clustered around the entrance to the micropyle, adhering to the outer surface and in the lumen. By mid-March hyphae and conidiophores with sporulating heads were observed on the exterior of the testa of many seeds. These appeared to have originated from the mycelium within the seed tissues, having erupted externally (Fig. 5D).

3.2. Presence and distribution of A. niger var. phoenicis CFUs

The spatial distribution of the spores around the plants was determined and assessed using one-way ANOVA tests of CFU against distance and CFU against direction. No significant effect of distance or direction on CFU was noted ($p > 0.05$). A univariate analysis of variance indicated no interaction effects ($p > 0.05$). Colony-forming unit counts ranged from zero to over 460 000 CFU/g dry soil at individual sampling points, and it appears that a directly proportional (although not statistically significant) relationship exists between the size of the plant and the average number of CFU per gram dry soil within a 40 m diameter circle surrounding it. This phenomenon is presumed to be related to the presence of greater amounts of decaying plant material (leaves and shed cones) beneath larger plants which provide the substratum for *A. niger* var. *phoenicis* proliferation. It has previously been established that soils directly beneath *W. mirabilis* plants have significantly higher nutrient levels than soils between plants (Abrams et al., 1997), but correlations between levels of nutrients and plant size were not presented in that study. It does not follow that smaller plants were at lower risk of infection as all plants were contaminated/infected during the 2004/2005 season. Larger sample sizes may reveal a possible pattern. The *A. niger* var. *phoenicis* CFUs were distributed throughout the greater local environment which was associated with widespread infection of developing cones.

3.3. Insects

P. sexpunctatis, ants, termites, fluted scale insects (*Icerya* sp. [Family Margarodidae]), spiders (Family Thomisidae), and flies were collected from the *W. mirabilis* plants.

P. sexpunctatis were observed feeding on both mature shed seeds and on the stalks of immature cones. Four live *P. sexpunctatis* were placed on separate MEA plates under sterile conditions. The insects were removed after 10 min and the plates were sealed and incubated. *A. niger* var. *phoenicis* colonies were noted in all cases after incubation of the plates. Scanning electron microscopy was carried out on two adult and one juvenile *P. sexpunctatis*. Spores, presumed to be of *A. niger* var. *phoenicis*, were visualised on all three individuals. The spores were particularly concentrated in the inverted genitalic capsule found on the lower underside of the abdomen of the adult insects.

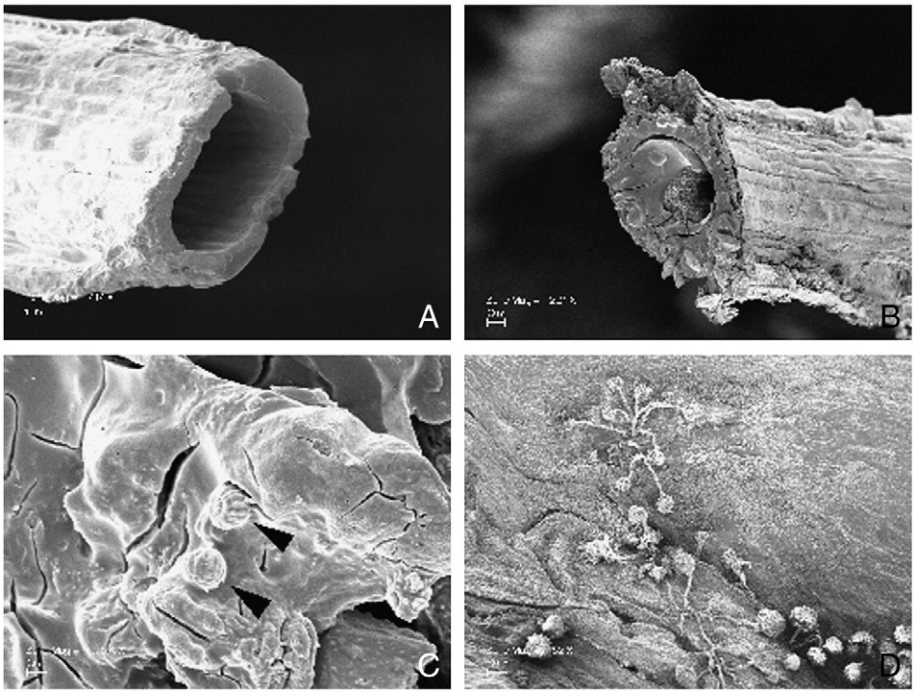

Fig. 5. (A) Micropyle (×700); (B) Micropyle showing dehydrating pollination drop (×220); (C) *Aspergillus niger* var. *phoenicis* spores (arrowed) embedded in the dehydrating pollination drop (×2700); (D) Conidiophores with sporulating heads which appear to have erupted through the testa from the inner seed tissues (×50).

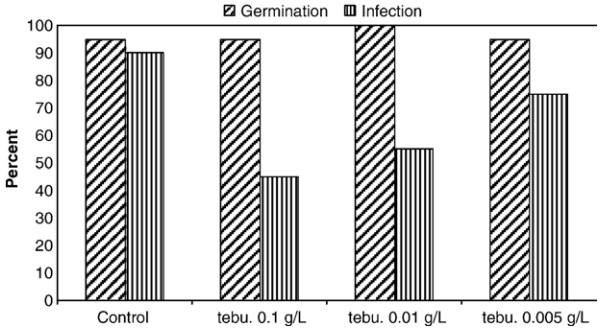

Fig. 6. Germination and infection by *A. niger* var. *phoenicis* of *W. mirabilis* seeds on media containing various concentrations of tebuconazole ($n=20$).

The close association of *P. sexpunctatis* with the plants, combined with the observed incidence of *A. niger* var. *phoenicis* spores on their bodies, implicates them as vectors in the infection and spread of infection of the cones and seeds.

Five ants collected from *W. mirabilis* plants were also placed on MEA. *A. niger* var. *phoenicis* and another *Aspergillus* species were noted after the plates had been incubated. Scanning electron microscopy of two small and one large ant did not reveal any *A. niger* var. *phoenicis* spores. However, some debris were visualised on the legs and body segments of the ants, suggesting that spores could be carried in a similar way by these insects.

3.4. Air testing

Air testing was performed twice. In the first instance (November 2004) 100% of plates exposed showed one or more colonies of *A. niger* var. *phoenicis*. Other fungi and bacteria were also noted. On repeat testing (April 2005), *A. niger* var. *phoenicis* colonies developed on 70% of the plates exposed, with the remaining 30% showing other fungal species.

3.5. Fungal isolates from surrounding vegetation

Both *A. pechuelii* and the grass samples (thought to be *Stipagrostis* spp.) were found to harbour several readily distinguishable types of fungi including *A. niger* (subspecies not identified). *A. niger* (subspecies not identified) was also noted on the seeds of *Acacia erioloba* (during the course of research undertaken by P. Moser[1] at the GTRC).

3.6. In vitro testing of selected fungicides against seeds of W. mirabilis

There were no significant negative effects of either of the fungicides, at the concentrations used, on germination of the seeds ($\chi^2=0.195$, $df=3$, $p>0.05$).

Fungal proliferation was halved from 90% of seeds (control set) to 45% in the 0.1 g/L tebuconazole treatment set, and the inhibitory effect of this compound on the fungi declined as the concentration decreased (Fig. 6). The fungicide exerted a significant effect on infection of seeds ($\chi^2=18.39$, $df=3$, $p<0.05$).

Tebuconazole incorporated in the medium at 0.1 g/L was the most effective treatment as infection was reduced and the fungicide did not adversely affect germination of the seeds (Fig. 6). The potential of tebuconazole to reduce infection further was assessed by soaking the seeds in a solution of the fungicide.

3.7. Soaking of W. mirabilis seeds in a tebuconazole-containing solution

Soaking of the seeds from accession HK2031 in a 0.1 g/L solution of tebuconazole for 1 h improved germinability from 80% to 100% (Fig. 7). Concomitantly, infection of the seeds was reduced by nearly half, from 100% of seeds to 55% after 1 h of treatment. Germination was reduced essentially to the level of untreated seeds after longer soaking periods (75% after 2 h), while isolatable infection by *A. niger* var. *phoenicis* was reduced to 20% of seeds after 3 h soaking. Germination was comparable with the control at that stage (80% for both the control and 3 h soaked seeds). Germination was not significantly affected by the soaking treatment ($\chi^2=4.402$, $df=3$, $p>0.05$), while the effects on infection were found to be highly significant ($\chi^2=56.086$, $df=3$, $p<0.05$).

3.8. Combined soaking and in vitro treatment of W. mirabilis seeds in a solution and on medium containing 0.1 g/L tebuconazole

The soaking of seeds from accession HK2031 for 3 h in a 0.1 g/L solution of tebuconazole, followed by plating of the seeds on a medium containing the same concentration of the fungicide, resulted in a decrease in fungal proliferation from 100% in the control group to 8% of seeds. Fig. 8 shows the comparison of results obtained for various modes of treatment using 0.1 g/L tebuconazole.

It should be noted that infection in all cases other than the combined treatment was manifested exclusively by *A. niger* var. *phoenicis*. However, infection following the combined treatment was exclusively a species tentatively identified as

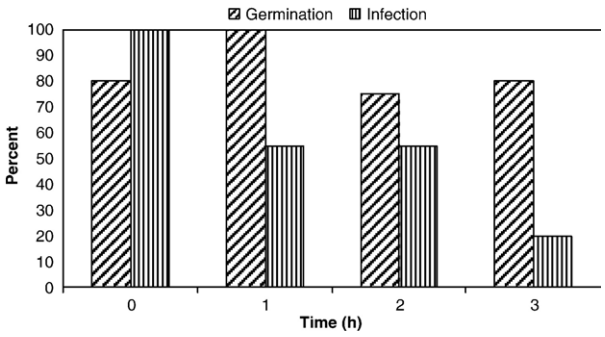

Fig. 7. Germination and infection by *A. niger* var. *phoenicis* of *W. mirabilis* seeds on 1% water agar and MEA respectively, following soaking in a 0.1 g/L tebuconazole solution for 0, 1, 2 or 3 h ($n=20$).

[1] Petra Moser, Center for Development Research, University of Bonn, Walter-Flex Strabe 3, D-53113, Bonn, Germany.

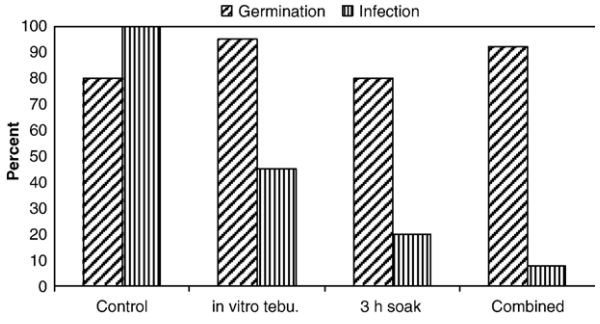

Fig. 8. Germination and infection of *W. mirabilis* seeds on 1% water agar and MEA respectively, following various treatments with 0.1 g/L tebuconazole ($n=20$, except for combined treatment, where $n=50$). *Note that seeds utilised in the *in vitro* trial were from accession HK1603, all others from accession HK2031.

A. flavus, but not *A. niger* var. *phoenicis*. The appearance and proliferation of *A. flavus* was probably the consequence of the elimination of inter-specific competition between the fungal species. The effects of treatments on infection were found to be highly significant ($\chi^2=115.763$, $df=3$, $p<0.05$).

Germinability of seeds was essentially unaffected by any of the treatments when compared with the control ($\chi^2=2.152$, $df=3$, $p>0.05$).

4. Discussion

Spores of *A. niger* var. *phoenicis* were present in the air and soil surrounding the *W. mirabilis* plants as evidenced by the results of air and soil testing. The cones were infected early during development and percentage infection increased to 100% by the end of February 2005, a sharp increase occurring after appearance of the pollination (micropylar) drops. In this regard, it has been found that the gymnosperm, *Juniperus oxycedrus*, displays a lack of specificity in the recognition of pollen, and may take into the ovule inorganic particles and non-viable pollen (Barbour et al., 2004). Wetschnig and Depisch (1999) observed that pollination drop production in *W. mirabilis* occurred with a diurnal rhythm, with production of drops at noon, and re-sorption at 17h00, while Carafa et al. (1992) noted that hand pollination of the drops did not result in their withdrawal. It is suggested that no 'recognition mechanism' exists in *W. mirabilis*, and that the pollination drop is resorbed each day, regardless of whether pollen has been trapped or not. It is proposed that ungerminated *A. niger* var. *phoenicis* spores gain access to the interior of the *W. mirabilis* seed through the pollination drop, as well as via inoculum introduced by associated insects. Infection via the micropyle is borne out by microscopical evidence (Fig. 5A, B and C), which shows *A. niger* var. *phoenicis* spores embedded in the dehydrating pollination drop (Fig. 5C, arrowed). Spores gaining access to the interior of the seed would provide the inoculum from which hyphal proliferation is probably facilitated in the sporophyte and nutrient-rich (Butler et al., 1979a) gametophyte tissues.

Sporulation was observed on the exterior of the seed surface, from conidiophores that appeared to have erupted from the interior (Fig. 5D). A similar disease process characterises *Tilletia caries* – the stinking smut of wheat – which proliferates in the interior of the caryopsis, while leaving the pericarp apparently intact (Ingold, 1971) (consider Fig. 4A–D). Macroscopically-visible *A. niger* var. *phoenicis* infection was first noted on the 1st of February 2005, while browning and discolouration of the ovules were recorded earlier, on the 19th of January 2005. This browning is assumed to be associated with the infection of the seeds and the consequent deterioration in the gametophyte and embryo tissue (Fig. 4A and B) caused by fungal activity. The browning was most concentrated in the collar zone of the embryo. The "feeder" which grows from the collar has been proposed to function in the uptake or transfer of nutrients and water from the gametophyte to the embryo (Bornman et al., 1979a; Butler et al., 1979c) and cells in this region are rich in starch (Bornman et al., 1979b), which is utilised during early germination (Butler et al., 1979b,c). It is possible that spores which gain access to the interior of the seed through the micropyle germinate and proliferate most readily in this area of the embryo as a consequence of the presence of the nutrient reserves. It seems possible that the embryo may be destroyed by fungal action first, following which, the fungus exhausts the gametophyte tissues (which are particularly atrophied in infected seeds — Fig. 4A–D).

The sharp increase in infection levels during February was concurrent with the onset of the rainy season, during which temperatures up to 45.5 °C and high winds were recorded. The experiments by Hayden and Maude (1992) showed that *A. niger* affected more onion seedlings when seeds were germinated at 30 °C, than at 20 °C, and that the fungus failed to proliferate at temperatures of 13 and 15 °C. In the desert environment it appears likely that windy conditions and temperatures as high as 45.5 °C would favour the spread and growth of *A. niger* var. *phoenicis* on *W. mirabilis* cones. Wind is the major factor in the spread of parasitic fungi (Deverall, 1981) and is known to aid in the liberation of spores from the conidiophore after being shaken (Ingold, 1971). It was observed that rain caused the surrounding soil to splash upwards onto the cones, providing an additional means of inoculation (i.e. additional to insect and wind-borne inoculum). Raindrops pick up spores from surfaces they strike, which are then deposited as rebounding droplets strike another surface (Ingold, 1971; Deverall, 1981). Wind and rain-splash dispersal contribute greatly to the spread of parasitic fungi (Deverall, 1981). Aspergilli (including *A. niger*) have been found to be the most prevalent fungal group in airborne inoculum during hot, dry, dusty summer weather in Khartoum (Abdalla, 1988), and *A. niger* specifically, is a common saprophyte and soil inhabitant (Griffin, 1972; Anon, 1997). Pekarek et al. (2006) have recently concluded that *A. niger* var. *phoenicis* spores are transmitted by wind between individual *W. mirabilis* plants and distant populations. Those authors suggested that *A. niger* var. *phoenicis* is harboured by "an as of yet undocumented broader host range of the fungus on other desert vegetation". In the present study *A. niger* was found to be harboured by *Adenolobus pechuelli*, putative *Stipagrostis* spp. and *Acacia erioloba* in addition to *W. mirabilis*. It is suggested that the reservoir of spores or hyphae is to be found in the soil and seed remnants (under the canopy) surrounding *W. mirabilis*

plants, and that individual plants become periodically contaminated/infected in a rhythm dependent on growth stage and environmental conditions. Infection would be further facilitated by the movements and feeding behaviour of *P. sexpunctatis*. Cooper-Driver et al. (2000) concluded from spatial analysis of observed clustering of *A. niger* var. *phoenicis* infection that transmission via *P. sexpunctatis* was highly likely.

Spores of *A. niger* have been found to withstand progressive drying to extremely low water contents (Walters et al., 2005), suggesting that those of *A. niger* var. *phoenicis* would probably be well adapted to the extreme dryness of the Namib Desert. Members of the Aspergilli, while ubiquitous, are also particularly noted as desert soil inhabitants (Klich, 2002), and *A. niger* has been found to be more prevalent in onion seeds from desert locations, compared with those from temperate and tropical areas (Hayden and Maude, 1992). Evidence exists which suggests that the dark brown or black pigment of the conidia of *A. niger* enables them to resist high UV intensities (Imshenetsky et al., 1979) similar to those encountered in the desert environment.

It is unlikely that infection of the seeds can be effectively prevented in areas where *A. niger* var. *phoenicis* inoculum is present at a high level in the local environment. However, post-shedding disinfection may be possible after harvest of seeds infected in the late stages of development, when infection of mature dry seeds may not result in the destruction of tissues since less water is available for fungal proliferation at that developmental stage.

Tebuconazole has been found to satisfactorily control or prevent *Fusarium* infection of wheat grain (Homdork et al., 2000), and *Puccinia striiformis* infection in wheat leaves (Han et al., 2006), and has been shown here to eliminate a substantial proportion of the infection from *W. mirabilis* seeds, when applied at a rate of 0.1 g/L as a combined treatment. Infection was reduced from 90% to 45% by inclusion of 0.1 g/L tebuconazole in the medium (Fig. 6), without affecting germination. While germination was essentially unaffected by the length of soaking time (increasing from 80% to 100% after 1 h and thereafter decreasing to 75% [2 h] and 80% [3 h]), infection was reduced from 100% to 20% after 3 h soaking (Fig. 7). It has been noted for orthodox seeds that the effectiveness of treatment with fungicides is improved by soaking the seed in a fungicide solution rather than simply by dressing the seeds (Nakagawa and Yamaguchi, 1989). A further highly significant decrease in seed infection (from 100% to 8%) was achieved by the combination of a 3 h soak in 0.1 g/L tebuconazole, followed by germination/incubation of seeds on a medium containing the same concentration of fungicide (Fig. 8). This treatment eradicated *A. niger* var. *phoenicis* from the seeds, but appeared to result in the proliferation of *A. flavus*, probably as a consequence of the removal of inter-specific competition. Although the seeds incubated on MEA were assessed for fungal infection and those on 1% water agar for germination, it was noted that 4% of seeds on water agar were infected with *A. flavus*, and that those infected seeds did not germinate. This suggests an effect of tebuconazole in improving the innate responses of live seeds to suppress fungi, an effect also noted by Han et al. (2006) for wheat leaves. The combined treatment described here is recommended as standard practice for the germination of *W. mirabilis* seeds. Since no adverse effects on germination were noted, the treatment may be applied as a precautionary measure, whether the seeds show evidence of contamination/infection or not.

In this case, treatment of the infected *W. mirabilis* seeds was post-harvest; however, Homdork et al. (2000) found that pre-infectional application of the fungicide as a spray provided more satisfactory results than post-infectional application by the same method. Similar results were found by Holb and Schnabel (2007) for tebuconazole treatment of *Monilinia fructicola* infection of peaches. The effects of pre-infectional application could not be tested in the context of the present study; however, an experiment of this nature on a persistently contaminated population of *W. mirabilis* would provide valuable data on the effectiveness of such treatment. *W. mirabilis* cones become infected in the field during development, and fungicidal treatment, however practically challenging, could be important in prevention of infection. Tebuconazole was also found to control the synthesis of deoxynivalenol, a mycotoxin (Homdork et al., 2000); this could be of importance in the context of infection by *A. niger* varieties since this species is known to produce mycotoxins (malformins) which induce deformities in seedlings (Curtis et al., 1974). It is possible that *A. niger* var. *phoenicis* not only degrades the *W. mirabilis* seed during development on the parent plant, but that mycotoxins could compromise seedling development post-shedding.

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