STUDIES ON THE BIOLOGY, FUNGAL CONTAMINATION, AND CONSERVATION OF SEEDS OF *Welwitschia mirabilis* Hook. fil.

by

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For Mom and Dad: without you, none of this would have been possible.

Abstract

Welwitschia mirabilis is a unique and rare desert gymnosperm endemic to the Kaokoveld Centre of southern Angola and Namibia. The adult plants possess only two leaves which grow continuously from the basal meristem, and which become longitudinally dissected by uneven growth of the stem. The species is dioecious. The female plants bear 90-100 megasporophylls, of which 50-60% may be fertile, and up to 80% of those fertile seeds may be infected by *Aspergillus niger* var. *phoenicis*. This contamination of the seeds results in seed and seedling death, potentially negatively affecting recruitment of plants into the adult population.

The physiology, storage behaviour, ecology and disinfection of the seeds were investigated. The dry outer coverings of the seeds were shown to impose a constraint on germination. Removal of these structures without any other treatment enabled rapid germination, whereas soaking the seeds for 9 h either with the coverings intact or removed, resulted in poor germination, indicating that the seed coverings, rather than an inhibitor, impose the constraint. It was found that *W. mirabilis* seeds, at the shedding water content, may be cryopreserved in liquid nitrogen, and were unaffected by hydration followed by dehydration, thus establishing their highly orthodox nature. A medium-term storage experiment established that the viability of the seeds was not significantly affected by storage at -20, 5.5 and 16°C over a period of two years, whether the seeds had been dried by treatment at 80°C for 48 h prior to storage, or not.

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 Desert. Infection of the cones was found to peak coincident with the appearance of the pollination drops, and with high temperatures, winds and significant rainfall. *Welwitschia mirabilis* cones were microscopically examined and spores of *Aspergillus 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.

The potential of microwave irradiation, dry heat thermotherapy, hot water thermotherapy and chemical treatment (including conventional surface sterilants and fungicidal compounds) to eradicate *A. niger* var. *phoenicis* from the seeds was assessed. The most successful treatment was found to be soaking of the seeds for 3 h in a fungicide solution containing tebuconazole as the active ingredient, followed by germination on a medium also containing tebuconazole. This approach eliminated a substantial proportion of the fungal infection, and is thus likely to enhance maintenance of seed viability.

The present study establishes the foundations of means by which the seeds of *Welwitschia mirabilis* Hook. fil. may be conserved, thus ensuring healthy planting material for seedling establishment in nursery practice.

Preface

The experimental work described in this thesis was carried out in the School of Biological and Conservation Sciences, Howard College, University of KwaZulu-Natal, Durban and at the Gobabeb Training and Research Centre, Namibia between June 2002 and December 2006, under the supervision of Professors P. Berjak and N. W. Pammenter.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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CHAPTER 1

INTRODUCTION TO *Welwitschia mirabilis* Hook. fil.

Welwitschia mirabilis Hook. fil., the lone member of the Welwitschiaceae, is recorded as being 'discovered' by Friedrich Welwitsch in 1859 – and the very plants he described may still be extant, as some living specimens are estimated to be 1500 – 2000 years old (Bornman *et al.*, 1972). Although a gymnosperm classified among the Gnetales, *W. mirabilis* shows several characters that are angiosperm-like, leading to the one-time opinion among botanists that the immediate ancestor of the angiosperms had been discovered (reviewed by Takhtajan, 1969). However, many characteristics of *W. mirabilis* are too specialised for this to have been the case, and the fact that the plants are dioecious is one of the major factors eliminating them from the direct line of ancestry (Takhtajan, 1969). Recent genetic analyses tentatively indicate that the Gnetales arose from within the conifers, and that morphological similarities between angiosperms and Gnetales are separately derived (Chaw *et al.*, 2000). Supporting fossil evidence (*Palaeognetaleana auspicia* cones) from the Upper Permian has been reported in North China – the earliest known evidence of the Gnetales (Wang, 2004). The ancestors of the extant gymnosperm orders – Gnetales, Coniferales, Cycadales and Ginkgoales – arose during the Late Paleozoic, and became dominant components of the Late Permian and Mesozoic flora (Wang, 2004). Fossil evidence indicates that members of the Welwitschiaceae, specifically, were present in South America and possibly over a much wider area, during the Lower Cretaceous (Mesozoic era) (Dilcher *et al.*, 2005). Jacobson and Lester (2003) posit that these early habitats of Welwitschiaceae were more mesic than the present desert conditions, and that the current, somewhat fragmented and isolated, distributions can be attributed to aridification during the Tertiary and Quaternary, which restricted the plants to locales providing sufficient water for their needs.

The current distribution of *Welwitschia mirabilis*, which is endemic to the Kaokoveld Centre (van Wyk and Smith, 2001), extends from the Nicolau River in southern Angola to the Kuiseb River in Namibia (Kers, 1967), encompassing the northern and central

Namib and mopane savanna (Giess, 1971). Populations of as few as two individuals to as many as 1000 (Henschel and Seely, 2000) are found in, and surrounding, ephemeral water courses (Jacobson and Lester, 2003), up to 100 km inland of the ocean (Bornman, 1978). In the Namib-Naukluft Park, populations are geographically discrete, while further north between the Ugab and Hoanib Rivers (Fig. 1.1), a more contiguous population is found (Jacobson and Lester, 2003). This area is amongst the most arid world-wide, with the coast recorded as having almost zero rainfall, while less than 100 mm fall annually below the escarpment, mainly from February to April, in the late summer (van Wyk and Smith, 2001). While *W. mirabilis* is established in regions receiving less than 25 mm rainfall, this is supplemented throughout the area by an equivalent of ~50 mm provided by the coastal fog (Bornman *et al.*, 1972). Precipitation provided by fog and rain is, however, insufficient to provide for the daily water needs of the plant, and it is thought that the restriction of populations to ephemeral water courses indicates a dependence on ground water (Henschel and Seely, 2000).

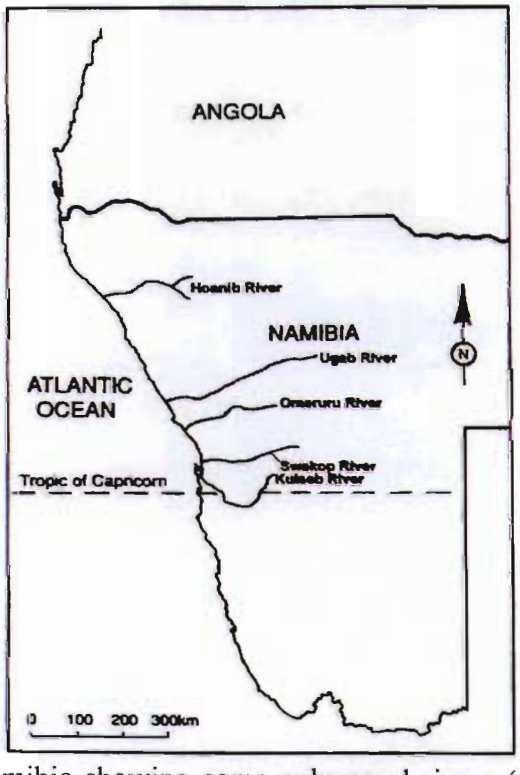

Figure 1.1. Map of Namibia showing some ephemeral rivers (adapted from Jacobson and Lester, 2003).

Welwitschia mirabilis plants retain their first- and only-formed leaves, the tissue of each being continually regenerated from a deep-set meristem, throughout their lifespan, with growth rates varying seasonally, and in response to rainfall events (Henschel and Seely, 2000). The tips of the leaves die off as a result of extensive water loss induced by climatic factors and age (Eller *et al.*, 1983). There are only two of these leaves, although they become so longitudinally dissected by uneven growth of the truncated stem (Fig. 1.2) that the basal meristem becomes segmented (Bornman *et al.*, 1972), giving the plant the appearance of having many long, ribbon-like leaves, with micro- or mega-strobili appearing between the bases. In form, the plant has been described as resembling a "woody carrot" or "turnip" with an elongated but still relatively shallow root system and a woody, fibrous unbranched stem (Bornman *et al.*, 1972; Bornman, 1972). Some of the largest specimens are no more than 1.5 m tall above ground, but the circumference at the base of the leaves can exceed 8 m (Bornman *et al.*, 1972). Physiologically, *W. mirabilis* has now been classified as a CAM plant, after some initially contradictory and confusing results (Eller *et al.*, 1983; von Willert *et al.*, 2005).

Figure 1.2. A mature female *Welwitschia mirabilis* plant.

Scale (white object) = 300 mm

Individual simple microstrobili have six basally-fused microsporangiophores, each bearing three fused sporangia (Bierhorst, 1971) which have also been termed anthers, as they appear morphologically similar to these structures in angiosperm flowers. On the female plants, each megastrobilus (Fig. 1.3) comprises 90-100 megasporophylls, of which Bornman *et al.* (1972) found only 50-60% to be fertile. Ovules are bitegmented; the outer integument, formed from a pair of fused bracts, also gives rise to the wings of the seed, while the inner originates as a complete ring of tissue, extending apically as the micropylar tube in the mature ovule (Chamberlain, 1966; Bierhorst, 1971). The agent of pollination has been the subject of some debate, with anemophily (Bornman, 1972; Bornman, 1978; Erickson and Buchmann, 1983) and entomophily (Pearson, 1906; Pearson, 1909; Bristow, 1988; Wetschnig and Depsich, 1999) favoured at various stages. Local pollen dispersal (mainly by flies, but also by bees and wasps) has been supported by direct observation (Wetschnig and Depisch, 1999) and genetic analysis of the relationships between mature plants, which indicated that minimal gene flow (ie. limited exchange of pollen or seeds) occurred between populations separated by 18 km (Jacobson and Lester, 2003). Wetschnig and Depisch (1999) showed through the use of pollen traps that the wind was responsible for distribution of pollen only up to 6 m away from a male plant, and concluded that distribution to nearby females was effected through the agency of flies, bees and wasps. Although the heteropteran *Probergrothius sexpunctatis* is closely associated with the female cones, it is not thought to be involved in pollination (Wetschnig and Depisch, 1999). During fertilisation, cells of the megagametophyte produce tubes, which grow upwards into the nucellus; these tubes have been suggested to fuse at the apices with pollen tubes growing downwards in the micropyle. The female (egg) nucleus moves up the "embryo sac tube" and encounters the male gametes, whereupon fertilisation takes place. Subsequently, the zygote is retracted down the embryo sac tube to the megagametophyte and development of the embryo ensues (Bierhorst, 1971).

Figure 1.3. Three *W. mirabilis* megastrobili.

Seed development is recorded as being complete in less than four months (Chamberlain, 1966). While the seeds may be wind-blown along the surface of the sand (Bornman, 1978; Endress, 1996), they are considered to be too heavy to become airborne, but during episodic heavy rains (Pearson, 1906), the wings may function for buoyancy in run-off water, thus facilitating seed dispersal. Local seed dispersal is confirmed by the minimal gene flow found between adjacent populations – some separated by as little as 18 km (Jacobson and Lester, 2003). The dry seed (*c.* 7 x 5 x 2-3 mm; average mass 120 mg) contains a centrally located embryo, often separated by a narrow space from the surrounding gametophyte tissue, as described by Bornman *et al.* (1979). The seeds contain ~35% oil (Bornman, 1978), making them attractive as food for various desert animals.

When separated from the megasporophyll, each seed is enveloped by tenacious dry bracts, thus constituting a "seed unit" (Fig. 1.4). Although the term, "perianth" has been used to describe the structures enclosing the *W. mirabilis* seed (Pearson, 1906; Bornman *et al.*, 1972), these are termed seed coverings in the present work. From earlier investigations, it has been proposed that some component of the seed unit contains an inhibitor, which has to be leached before germination proceeds (Bornman *et al.*, 1972).

This led to the proposal by those authors that seeds would germinate naturally only if there had been sufficient rainfall to leach out the putative inhibitor and facilitate its diffusion away from the developing root.

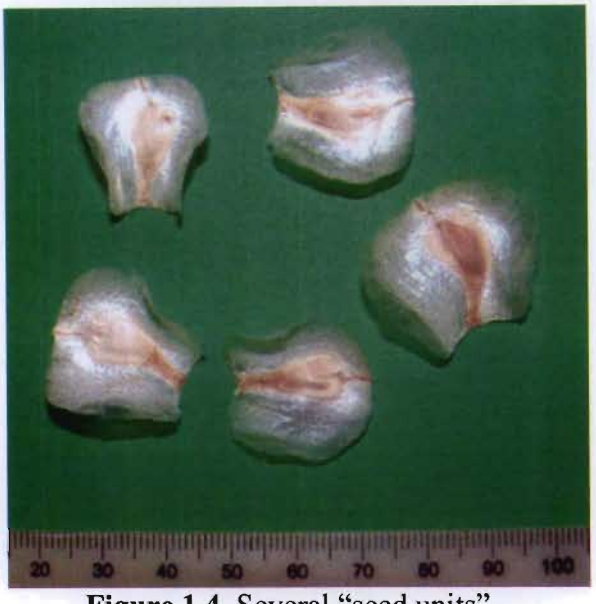

Figure 1.4. Several "seed units".

Bornman *et al.* (1972) record, that under laboratory conditions, germination will occur within 48 h after seeds have been supplied with the equivalent of 25 mm rain, and that root growth is extremely rapid - up to 1.5 mm per hour. The shoot apex, with its paired cotyledons, appears to be withdrawn from the seed as the hypocotyl elongates (Bierhorst, 1971). The attenuated cotyledons, which become photosynthetic, persist for a matter of two to three years (Chamberlain, 1966), even when the two later-developing true leaves have become considerably longer than they are (Bornman *et al.*, 1972). According to Chamberlain (1966), a continuous plate that covers and arrests development of the stem apex, is formed from two laterally-expanded buds, each arising in the axil of one of the cotyledons. The arrested axial vegetative growth is 'compensated' for by the continued radial growth of the stem, the extensive growth of the leaves, and development of the reproductive branches (Martens, 1977).

Although there are many mature *W. mirabilis* plants, the long-recorded scarcity of young plants (Pearson, 1906) has now become a matter of concern (Cooper-Driver *et al.*, 2000).

As the dissected leaf mass provides a microhabitat for a variety of animals including cryptic mammals and various insects (Bornman, 1978), many seeds may be wholly or partially consumed. Fungal proliferation is also likely to be an aggravating factor in seed/seedling mortality (Fig. 1.5). Fungus is commonly associated with seeds of *W. mirabilis*: Bornman *et al.* (1972) recorded that 80% of the 10,000 seeds they collected, were infected by *Aspergillus niger* – and the dominance of this fungus could well have masked the presence of others (M^{c}Lean and Berjak, 1987). The work of Cooper-Driver *et al.* (2000) has shown that while *Aspergillus niger* was associated with the seeds from a variety of locations offering somewhat differing habitats for *W. mirabilis*, infection was significantly higher in particular localities. Seed-associated fungi could pose a serious problem in terms of propagation of the species in its natural habitat, as well as via planting programmes, as quiescent inoculum is likely to survive within the dry seeds, but could proliferate when water is provided. This could either preclude seed germination or, if germination does occur, overcome the developing seedlings (Kolberg, pers. comm.[1]). Thus survival of this unique species could become jeopardised by lack of seedling recruitment in the field, leading to the inability to provide vigorous young plants for regeneration programmes.

Aside from its intrinsic value as an endemic species, *W. mirabilis* plants provide refuge, shade, food and water (from intercepted fog) for a variety of desert animals. As an example, large numbers of the heteropteran, *Probergrothius sexpunctatis*, can be found associated with most female *W. mirabilis* plants, where they feed on the sap (Lovegrove, 1999). High frequencies of *A. niger* spores associated with the megastrobili, have been correlated with the presence of these insects (Cooper-Driver *et al.*, 2000), which exhibit a preference for female over male cones (Bornman *et al.*, 1972). The spatial distribution of plants with which fungal spores are associated, supports the hypothesis that the vector is a non-flying insect – such as *P. sexpunctatis* (Cooper-Driver *et al.*, 2000).

[1] Dr H. Kolberg, National Bontanical Research Institute, Windhoek, Namibia.

Figure 1.5. A seedling in culture contaminated by *Aspergillus niger* var. *phoenicis*.

The present study aimed to elucidate the biology and storage behaviour of the seeds of *Welwitschia mirabilis*, as well as to investigate their potential for long-term storage. The relationship between *Aspergillus niger* var. *phoenicis* and *W. mirabilis* was investigated in the field, and in the laboratory, and methods to eradicate inoculum from the seeds were sought. The combined results and findings are aimed to provide the basis for conservation of the seeds.

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CHAPTER 2
GENERAL MATERIALS AND METHODS

Seeds

Seeds were collected in the field by staff of the National Plant Genetic Resources Centre (NPGRC) of Namibia. Seeds were transported from the field in cloth bags to the laboratories of the NPGRC. Seeds from accessions HK1249 and HK1250 were de-winged and dusted with a Benomyl-containing fungicide before being sealed into laminated foil bags. Seeds from accession HK1322 were not de-winged before being dusted and sealed into the bags. Accession HK1603 was not de-winged or dusted with fungicide, and seeds were sent to Durban in the cloth bags. Seeds were dispatched by overnight courier to the laboratory in Durban, where they were stored in the foil bags at 5 ± 2°C until used. Seeds from accession HK1603 were de-winged and the outer seed coverings removed before being sealed into laminated foil bags and then stored at 5 ± 2°C in the laboratory in Durban (no fungicides were applied at this stage). Details of the location of collection points for each accession are shown in Table 2.1.

Table 2.1. Location of collection of accessions utilised during the present study.

Accession	Location, altitude, date of collection	Description
HK1249	21°20'48"S 14°09'12"E; 400m a.s.l, 25/05/2002	Banks of the Messum River
HK1250	21°24'18"S 14°06'27"E; 340m a.s.l, 25/05/2002	Tributary of Messum River, near Messum Crater
HK1322	21°24'23"S 14°07'50"E; 315m a.s.l, 28/06/2003	North-western side of Messum Crater
HK1603	18°11'18"S 12°44'16"E; 900m a.s.l, 2005	Kunene region; Opuwo District
HK2031	21°23'46.3"S 14°10'13.3"E; elevation unknown, 2006	Near Messum Crater

Material was also collected from Hope Mine (23°34'17.4"S; 15°15'31.4"E) in the Namib-Naukluft Park by the author during the course of the fieldwork aspect of the investigation (between September 2004 and April 2005; collection methods are described in detail in Chapter 5). Figure 2.1 shows a view of Namibia with each collection point marked, while a qualitative description of the seeds supplied by the NPGRC can be found in Table 2.2. Obviously degraded seeds were removed from accessions HK1603 and HK2031 prior to assessment of germination. A comparison of seeds from accessions HK1250 and HK1322 is displayed in Figure 2.2, illustrating the discoloured and contaminated state of seeds from the latter accession.

Figure 2.1. A view of Namibia showing the relative locations of collection points.

Table 2.2. A description of the seeds from the various accessions utilised during the course of study; viability was assessed by radicle emergence.

Accession	Viability on receipt (%)	Contamination by *A. niger* (%)	Water concentration on receipt ($g.g^{-1}$ dry mass)	Description/Notes
HK1249	83	0	0.06	Plump, clean seeds
HK1250	84	0	0.06	Plump, clean seeds
HK1322	0	100	0.049	Discoloured, brittle, malformed seeds
HK1603	98	100	0.05	Plump seeds, some spores visible on outer coverings
HK2031	31-80 (variable)	100	0.046	Plump seeds, some spores visible on outer coverings

Figure 2.2. A) Seeds from accessions HK1250 (left) and HK1322 (right) with outer coverings intact. Note the macroscopically visible contamination of seeds from accession HK1322. B and C) Seeds from accessions HK1250 and 1322, respectively, with outer coverings removed (both in 90 mm diameter Petri dishes).

Determination of water concentration

Water concentration was determined gravimetrically using a Mettler MT5 six-place balance subsequent to drying at 80°C for 48 h in a forced air oven. Water concentrations are reported as g water per g dry mass ($g.g^{-1}$), unless otherwise stated.

Germination assessment

Seeds were maintained on 1% water agar in Petri dishes sealed with Parafilm® in a growth room at 24°C with a 16 h photoperiod (light intensity 50-90 $\mu mol.m^{-2}.s^{-1}$), unless otherwise stated.

Fungal identification

Axenic cultures on Czapek Dox Agar or Malt Extract Agar (MEA, Scharlau, Barcelona, Spain) prepared from the most commonly isolated seed-associated fungi, were identified by the Plant Protection Research Institute, Pretoria, South Africa.

Scanning electron microscopy (SEM)

Specimens were processed as stated in each section (and as outlined in Chapter 9), then mounted on aluminium stubs using graphite tape and sputter-coated with gold in a Polaron E5300 SEM coating unit. The specimens were viewed with a LEO1450 scanning electron microscope and the images captured digitally.

Fluorescence microscopy

Specimens were processed according to the protocol outlined in Chapter 9, and 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 380-420nm and barrier filter 450nm) was used to visualise the fungi. Images were digitally captured.

CHAPTER 3
SEED BIOLOGY AND RESPONSES TO CRYOSTORAGE

Although seed post-harvest behaviour probably constitutes a continuum (Pammenter and Berjak, 1999), seeds are presently broadly categorised into three types – recalcitrant, intermediate or orthodox (Roberts, 1973; Ellis *et al.*, 1990). Orthodox seeds progress through a series of stages of development usually culminating in maturation drying while still attached to the parent plant. During the initial developmental progress and maturation of the seed, patterns of embryo formation, differentiation, and growth are highly regulated; large amounts of carbohydrate, lipid and/or protein reserves are laid down, and the seed ultimately becomes quiescent. At this point, the seed has acquired the ability to withstand the loss of water that ends this phase of its development (Black, 2000). Such seeds will dehydrate to a water content which is in equilibrium with the relative humidity of the atmosphere (Sutherland *et al.*, 2002) and, depending on storage conditions, are capable of surviving in this state of dehydration for many years, renewing metabolic activity and growth when rehydrated (Black, 2000). If of initial good quality, suitably dry orthodox seeds may be relatively easily maintained in cold storage or sub-zero temperatures for predictable periods, based on initial seed properties and storage parameters (Roberts, 1973; Ellis and Roberts, 1980).

Seeds described as recalcitrant undergo no (or limited) maturation drying during the final phase of development (Berjak *et al.*, 1989). Most plants that produce recalcitrant seeds grow in habitats that favour rapid seedling establishment, such as humid tropical forests (Roberts and King, 1980). In such moist environments, there is little advantage to be gained from the process of maturation drying. Besides not drying down during development, recalcitrant seeds are intolerant of post-shedding desiccation and many are chilling-sensitive (Chin and Roberts, 1980). As such, these seeds are not storable by any of the methods used for orthodox seeds. An additional problem is posed by the active metabolic status of recalcitrant seeds (Berjak *et al.*, 1989; Pammenter and Berjak, 1999) many of which will degenerate rapidly in short-term hydrated storage, usually after they have progressed into germinative metabolism. Stored recalcitrant seeds display an

increase in desiccation-sensitivity as germinative metabolism reaches the stage when mitosis and vacuolation occur – both processes requiring external water sources (Farrant *et al.*, 1986; 1989; Berjak *et al.*, 1989). The additional water required is not available in the storage environment, probably leading to unbalanced metabolism in the seed tissues and consequently the uncontrolled production of free radicals (Fu and Song, 1994; Leprince *et al.*, 1994). Free radicals are thought to be particularly damaging to a variety of macromolecules including those of membranes (Hendry, 1993) hence leading to extensive cellular degradation (Pammenter *et al.*, 1994). Evidence exists suggesting that anti-oxidant systems become ineffective during dehydration of recalcitrant seeds, resulting in an inability to repair the damage (upon rehydration) and consequent death of the seed (Hendry *et al.*, 1992; Côme and Corbineau, 1996; Berjak and Pammenter, 2004).

Many of the processes accompanying acquisition of desiccation tolerance in orthodox seeds are incomplete or absent in recalcitrant seeds (Pammenter and Berjak, 1999). Orthodox seeds are able to withstand the reduction in cell volume which accompanies dehydration; this implies the orderly dissociation of the cyto- and nucleoskeleton, and plasticity of cell walls. Dehydration in orthodox seeds is preceded/accompanied by a controlled shut-down of metabolic activity and dismantling of structures involved in metabolism. Recalcitrant seeds do not display these processes which prepare orthodox seeds for dehydration (Farrant *et al.*, 1997; Berjak and Pammenter, 2004). Orthodox seeds accumulate protective molecules such as sugars, late embryogenic abundant (LEAs) and small heat shock proteins (sHSP), as well as amphipathic substances during maturation; while these substances have also been shown to occur sporadically among recalcitrant seed species, it is thought that they have a particular role to play in the acquisition of desiccation tolerance in orthodox seeds (Farrant *et al.*, 1996; Wehmeyer and Vierling, 2000; Berjak and Pammenter, 2004).

Mature dry orthodox seeds enter a resting state at the end of development, which continues until external conditions create an environment which influences the seed to germinate (Kozlowski and Gunn, 1972). Such a seed is quiescent, but not necessarily dormant, which may be defined as the failure of an intact viable seed to proceed to, and

complete, germination under seemingly favourable conditions (Bewley, 1997), caused by a block to germination within the imbibed seed (Murdoch and Ellis, 2000; Baskin and Baskin, 2001). This is referred to as organic dormancy (Baskin and Baskin, 2001), which may be divided into endogenous (embryo dependant), exogenous (endosperm, seed coat or fruit wall dependant) and combined dormancy (Nikolaeva, 1977, 2001). Some change within the seed must occur in order to facilitate the breaking of dormancy and the germination of the seed (Baskin and Baskin, 2001). The consequence of delaying germination through dormancy is to enhance the survival of plant species in habitats with alternating favourable/unfavourable environmental conditions; for example, seed germination in hot dry regions is restricted to the short wet periods of the year (Koslowski and Gunn, 1972), as would be expected for *W. mirabilis*.

The various types of organic dormancy (endogenous, exogenous and combined), may be further divided according to the scheme of Nikolaeva (1977, 2001). Endogenous dormancy includes morphological dormancy (MD), physiological dormancy (PD), and a combination of the two (morphophysiological dormancy, MPD). Morphological dormancy is a result of underdevelopment of the embryo at the time of seed shedding, and may be broken by stratification. In cases of physiological dormancy, the embryo is not held to be dormant itself, but properties of the embryo coverings prevent radicle emergence (Nikolaeva, 1977, 2001; Baskin and Baskin, 2001). Physiological dormancy is further divided into deep, intermediate, and non-deep PD. In morphophysiological dormancy, underdevelopment of the embryo is combined with the restrictive nature of the embryo coverings. Thus development of the embryo and breaking of physiological dormancy must occur before germination can occur (Baskin and Baskin, 2001).

Exogenous dormancy includes physical, physical plus physiological, chemical and mechanical dormancy (Nikolaeva, 1977, 2001; Baskin and Baskin, 2001). Physical dormancy is associated with the presence of a seed coat which is impermeable to water, and which usually has one or more layers of impermeable palisade cells (Baskin and Baskin, 2001). Physical plus physiological dormancy results from the presence of an impermeable seed coat in conjunction with a physiologically dormant seed. Chemical

dormancy is held to be a consequence of the presence of inhibitors in the pericarp which must be removed or leached in order for germination to take place (Nikolaeva, 1977). Mechanical dormancy arises from the presence of a hard, lignified fruit wall (Nikolaeva, 1969 quoted by Baskin and Baskin, 2001), which may or may not be water permeable and which restricts radicle emergence. In cases in which the fruit wall is water impermeable, the seeds are considered to be physically dormant, while in instances in which the endocarp is water permeable, germination does not occur until a dormancy-breaking treatment is applied (Baskin and Baskin, 2001). A recent modification of the dormancy classification system by Baskin and Baskin (2004) excludes mechanical and chemical dormancy as true types of dormancy, categorising mechanical dormancy as a type of physiological dormancy, and excluding chemical dormancy from the classification scheme altogether. Those authors postulated that what may appear to be mechanical dormancy arising from restrictive seed coverings, in fact arises from the low growth potential of the embryo within the intact seed to break out of those coverings. The dormancy is therefore a function of the physiological state of the embryo, and should be categorised as such (Baskin and Baskin, 2004). Chemical dormancy is excluded from the classification scheme on the basis of a lack of unequivocal evidence that the presence of chemical compounds in the embryo covering layers or fruit walls has an effect on dormancy and/or germination (Baskin and Baskin, 2004).

Seed dormancy and subsequent germination are generally agreed to involve abscisic acid (ABA) and gibberellins (GA) (Baskin and Baskin, 2004). ABA appears to be involved in the induction of dormancy during seed development, but its role in dormancy maintenance is not clear (Bewley, 1997). GA is involved in the promotion and maintenance of germination, and therefore acts after the influence of ABA has decreased (Bewley, 1997; Baskin and Baskin, 2004 and references therein). Skriver and Mundy (1990) postulated that ABA and GA acted in a similar manner to auxin and cytokinins, in that the maintenance and breaking of dormancy might be controlled by the relative levels of each hormone, rather than absolute levels of either.

Whilst it would be highly unusual for a desert plant such as *Welwitschia mirabilis* to produce recalcitrant seeds, its non-xeromorphic nature has led to speculation that the plant evolved under tropical conditions and that it adapted to the changing climatic conditions of the Namib coast (Bornman, 1972). Thus the unlikely possibility that it could produce recalcitrant seeds had to be ruled out. Some evidence supporting the general view that such a plant would produce orthodox seeds is provided by Pearson (1910), who stated that "it is generally assumed that the seeds of *Welwitschia* retain their power of germinating for a long period". Pearson maintained a number of seeds collected during 1907 in his laboratory in Cape Town (South Africa) until 1910, when four were sown. Two germinated, but one of those succumbed to damping off. He concluded that seeds would be capable of "lying dormant" for much longer periods. Baskin and Baskin (2001) concluded from this evidence that *W. mirabilis* seeds display physiological dormancy, while Bornman *et al.* (1972) held that an inhibitor was present in the seed unit, which had to be leached before germination could proceed, categorising them as chemically dormant.

Determination of the seed biology and storage behaviour of *Welwitschia mirabilis*, required that the seeds be manipulated in various ways. Pre-germination manipulations have previously been outlined by Bornman *et al.* (1972), and included some soaking of the seeds in order to leach away a putative inhibitor prior to germination. Currently, tolerance of seeds to hydration and subsequent dehydration, and to cryostorage was tested in order to assess the seed behaviour (whether orthodox or recalcitrant), and to assess potential methods of seed conservation. Seed cryostorage usually refers to the maintenance of seeds in liquid nitrogen at -196°C or in the vapour phase of liquid nitrogen at *c.* -150°C (although storage at -80°C is also understood to be cryostorage) and will be tolerated only by orthodox seeds.

Materials and methods

In all cases, seeds from accession HK1249 were utilised.

Determination of water concentration

After removal of the seed coverings, water concentration was determined gravimetrically after drying at 80°C for 48 h. In all but the first instance, water concentration was determined for whole seeds (i.e. embryo plus gametophyte tissue), as it proved too difficult to separate the component parts. Water concentrations determined for 10 seeds individually, are reported as g water per g dry mass ($g.g^{-1}$).

Pre-germination manipulation

Seeds in six samples of 25 each were subjected to one of the following treatments (Table 3.1) before being plated on water agar with the coverings in the condition indicated.

Table 3.1. Pre-germination manipulation and condition of *W. mirabilis* seeds when plated. (n = 25).

Pre-germination	Seed coverings when plated
no soak	intact
no soak	removed
soaked 9 h; coverings intact	intact
soaked 9 h; coverings intact	removed
soaked 9 h; coverings removed	
no soak	upper & lower surface incised

Hydration and dehydration

A plastic bucket, suspending ring and mesh were washed with hot soapy water, swabbed with concentrated Jik® (3.5% NaOCl m/v) and sprayed with 100% ethanol. One hundred and forty seeds (outer coverings removed) were lightly sprayed with 100% ethanol and suspended on the mesh over sterile distilled water; the bucket was sealed with masking tape and stored at room temperature for 7 d, to allow the seeds to equilibrate to 100% RH.

The seeds were then divided into two batches. One batch was placed in a Ziploc bag with 300 ml of silica gel. The second batch was placed in a clean bucket (prepared in the same manner as the first) over a saturated KOH solution made up in sterile distilled water that facilitated an RH of 8% (Walters *et al.*, 1998). Both batches were maintained at room temperature. Seeds were sampled for water concentration (n=10) and germination (n=25) before and after equilibration and from both batches after 2, 6, 8 and 10 d of exposure to the drying regime.

Cryopreservation trials

Randomly selected seed samples were briefly frozen in liquid nitrogen (LN, -196°C). Two sets of 25 seeds each, with intact coverings and a further 25 seeds from which the coverings had been removed, were used. For LN immersion, seeds were placed in 2 ml cryotubes (three or four seeds per tube), which were immersed in the cryogen for 5 min, and then rapidly warmed within the tubes in a water bath at 40°C. After removal of the coverings from one of the two sets of seeds frozen intact in LN, all were plated on water agar for germination assessment.

Scanning electron microscopy (SEM)

Specimens were mounted on aluminium stubs using graphite tape and sputter-coated with gold in a Polaron E5300 SEM coating unit. The specimens were viewed with a LEO1450 scanning electron microscope and the images captured digitally.

Results

Determination of water concentration

Assessment of the individual seed components (Fig. 3.1) indicated that the water concentration of the embryo was surprisingly high – 0.147 ± 0.068 g g^{-1} – while that of the gametophyte tissue was 0.060 ± 0.007 g g^{-1}. However, as the unimbibed seeds were extremely brittle, it was difficult to prevent them shattering when dissected to separate the components: thus subsequent assessments of water concentration were carried out on whole seeds after removal of the coverings. Whole seed water concentration was 0.063 ± 0.010 g g^{-1}, indicating that, even in these very dry seeds, water concentration of the embryo is masked by that of the storage tissue. A comparison of the water concentrations of seeds with and without the coverings indicated that the latter contributed negligibly, the value for the seed units being 0.065 ± 0.003 g g^{-1}.

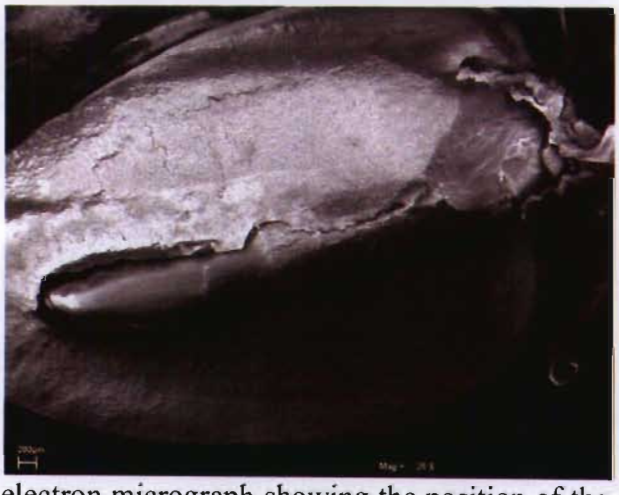

Figure 3.1. Scanning electron micrograph showing the position of the embryo within the surrounding gametophyte tissue in a dry, longitudinally-split *W. mirabilis* seed (x25).

Pre-germination manipulation

Germination trials were conducted simultaneously to test the effects of soaking and the presence or absence of the seed coverings (Table 3.2). By far the best germination performance over 21 d, assessed by radicle growth and usually also cotyledon extension, occurred when seeds were not soaked, but placed on water agar immediately after the coverings had been removed. In contrast, seeds that were soaked with the coverings intact and set to germinate in this state, showed the worst germination performance. The situation was not much improved when the coverings were removed after the 9 h soaking period when germination totality (assessed by root growth) was essentially similar to that achieved by seeds that had not been soaked, but set to germinate with the coverings intact.

Table 3.2. Germination totalities achieved by *W. mirabilis* seeds in the context of pre-germination manipulation, and condition when plated. (n = 25).

Pre-germination	Seed coverings when plated	Germination totality (%)	
		Root emergence	Cotyledons
no soak	intact	48	40
no soak	removed	83	78
soaked 9 h; coverings intact	intact	40	16
soaked 9 h; coverings intact	removed	46	36
soaked 9 h; coverings removed		56	56
no soak	upper & lower surface incised	64	56

Hydration and dehydration

The water concentration of seeds equilibrated for 7 d with 100% RH returned from a mean of 0.34 $g.g^{-1}$ to a value comparable with that of untreated seeds after only 2 days of drying in silica gel (Fig. 3.2). Water concentrations of seeds dried over KOH were slightly higher at all stages of sampling. Germination of seeds at all stages of hydration and dehydration was essentially unaffected by the treatment (Fig. 3.3).

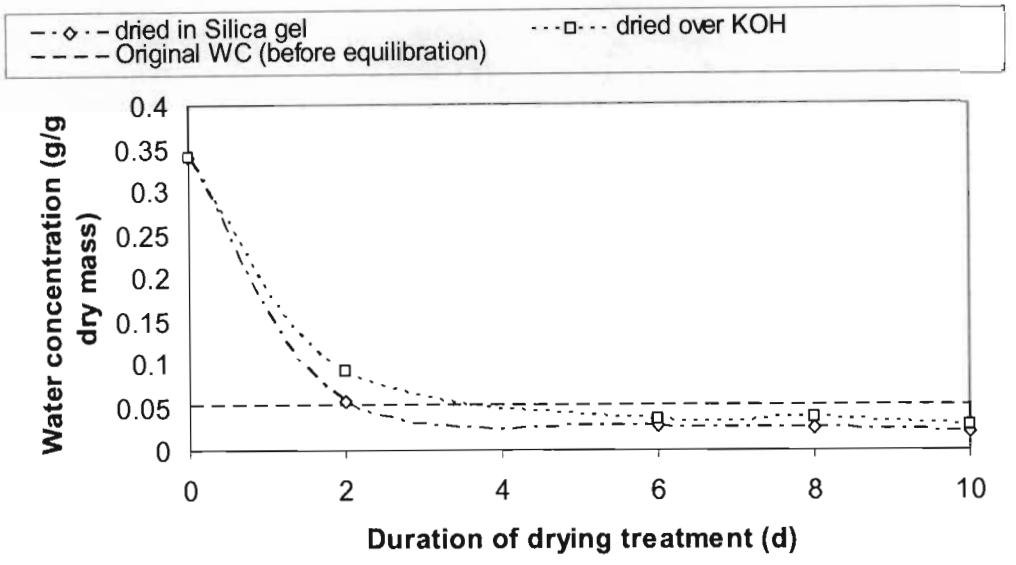

Figure 3.2. The effect of hydration and subsequent dehydration on water concentration of seeds of *W. mirabilis*.

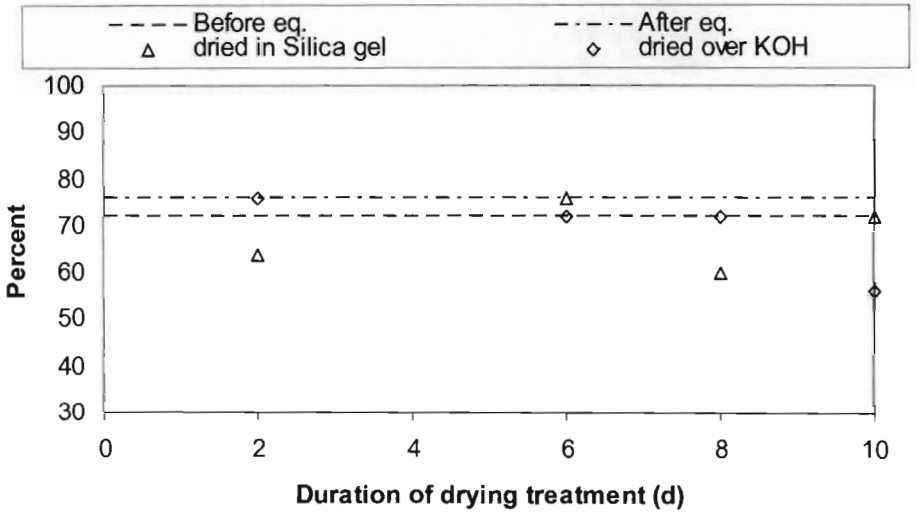

Figure 3.3. The effect of hydration and subsequent dehydration on germination of seeds of *W. mirabilis*.

Cryopreservation trials

Randomly selected seeds of *W. mirabilis* (water concentration 0.063 ± 0.010 g g^{-1}) were briefly immersed in liquid nitrogen with the coverings either intact or removed (Table 3.3). As reported above (Table 3.2), the presence of the seed coverings during treatment had an adverse effect on germination, even when these were removed prior to the seeds being plated, and the same effect was apparent after LN immersion. In contrast, when the coverings were removed prior to the seeds being immersed in liquid nitrogen, germination capacity was undiminished (Table 3.3).

Table 3.3. Effects of manipulation of randomly-selected seeds and immersion for 5 min in liquid nitrogen (LN) at -196°C on germination totality of *W. mirabilis* seeds. (n = 25).

Pre-germination	Seed coverings when plated	Germination totality (%)	
		Root emergence	Cotyledons
not frozen (control)	removed	83	78
frozen in LN, coverings intact	removed	60	48
coverings removed		88	84
coverings intact	intact	44	44

Immersion of *W. mirabilis* seeds in liquid nitrogen after removal of the coverings had a second relevant outcome: maximum germination occurred in 3 d – half the time taken for the non-frozen control group to achieve this. Scanning electron microscopy of the outer surface of the testa revealed that while only occasional, superficial cracks occurred before immersion in liquid nitrogen (Fig. 3.4A, B), after retrieval from the cryogen there were deeper, randomly-orientated fissures interrupting the continuity of the coat, which also appeared comparatively contracted (Fig. 3.4C, D).

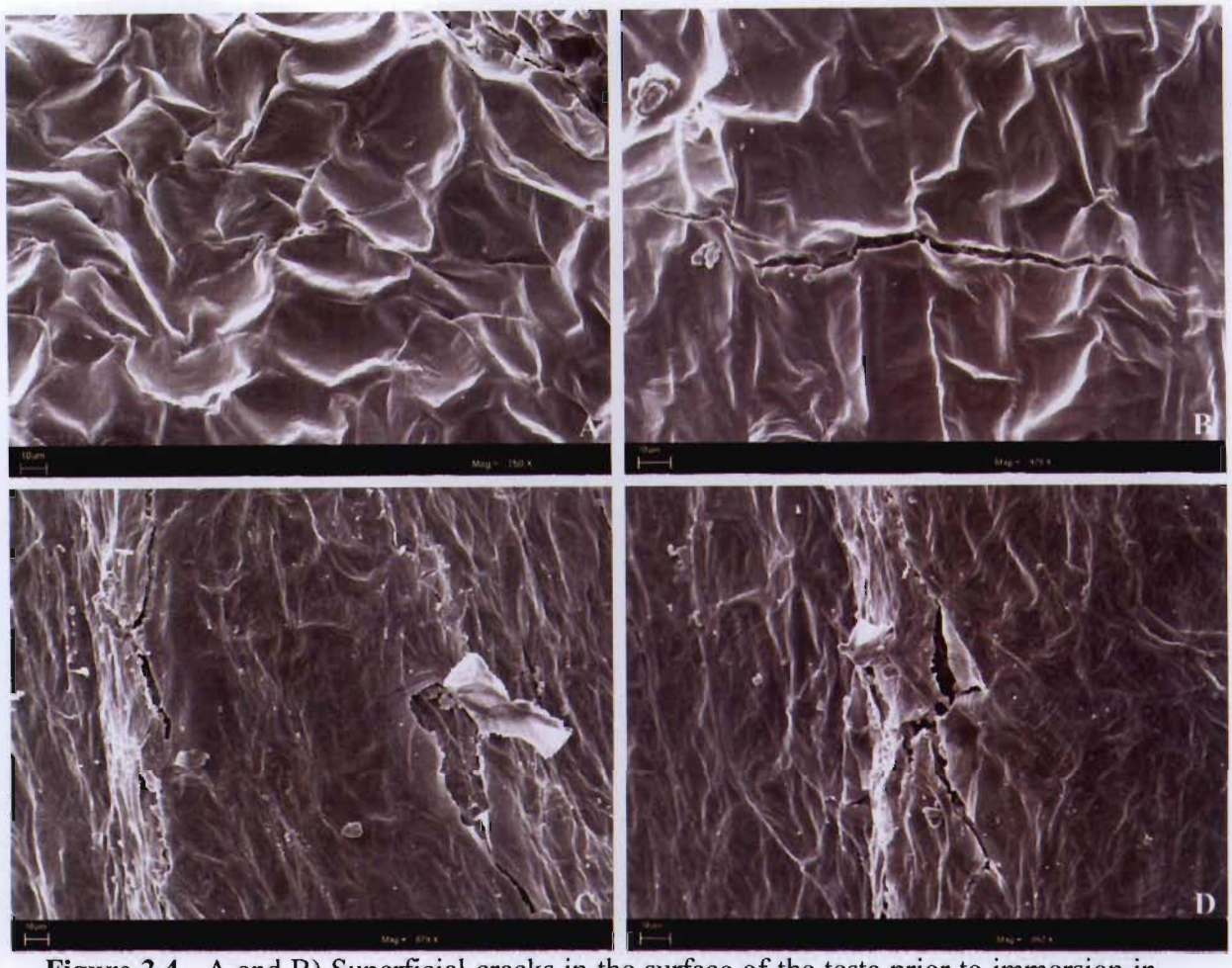

Figure 3.4. A and B) Superficial cracks in the surface of the testa prior to immersion in LN (x750 and x930 respectively). C and D) Deeper fissures and apparent contraction of the surface subsequent to freezing at -196°C are illustrated (x680 and x860 respectively).

Discussion

The present results confirmed that the mature seeds of *W. mirabilis* were very dry. Whole seed water concentration was 0.063 ± 0.010 g g^{-1}, revealing that, even in these very dry seeds, water concentration of the embryo (0.147 ± 0.068 g g^{-1}) is masked by that of the storage tissue (0.060 ± 0.007 g g^{-1}), as seems to be generally the case for embryonic axes in comparison with cotyledons or endosperm in angiosperm seeds (Isaacs and Mycock, 1999).

The data presented in Table 3.2 indicate that it is the presence of the seed coverings themselves, rather than a leachable inhibitor (as suggested by Bornman *et al.*, 1972), that curtails germination of *W. mirabilis* seeds. This view was supported by the germination performance of those seeds that were not soaked, but were plated on to water agar after incision of the upper and lower surfaces of the seed coverings. It seems, therefore, that the dormancy recorded for seeds of *W. mirabilis* is either physical, or non-deep physiological, but not chemical as previously suggested (Bornman *et al.*, 1972; Baskin and Baskin, 2001). Baskin and Baskin (2004) report that scarification of seeds with non-deep physiological dormancy will promote germination, and that it may thus appear that the seeds display physical dormancy. Dormancy break by scarification in non-deep physiologically dormant seeds is correlated with the weakening of the embryo covering layer, which lowers resistance to radicle penetration (Baskin and Baskin, 2004). If the dormancy in *W. mirabilis* was physical, this implies that the seed coverings completely exclude water. To the contrary, the outer seed coverings are known to be hygroscopic, and intact seeds are able to more than double their initial weight within seven hours upon imbibition, the outer "husk" displaying a greater capacity for water uptake than the seed itself (Bornman *et al.*, 1972). *Welwitschia mirabilis* seeds should therefore be categorised as non-deep physiologically dormant; further, Baskin and Baskin (2001) state that there is no evidence to suggest that seeds of any gymnosperms have physical dormancy.

The results also suggest that soaking seeds by immersion is, in itself, deleterious, especially when the coverings are intact. This might have been the outcome of the build-

up of anoxic conditions during the 9 h period presently used. As a result of these germination trials, in subsequent experiments unsoaked seeds were set to germinate after removal of the coverings, and this procedure is recommended by the author for nursery practice.

Seeds of *W. mirabilis* tolerate equilibration to 100% RH, followed by subsequent dehydration, with no marked effect on germination (Fig. 3.3). Water concentration of the seeds rose from 0.05 $g.g^{-1}$ (untreated seeds), to 0.34 $g.g^{-1}$ after equilibration (Fig. 3.2), representing a seven-fold increase. After 2 d dehydration, water concentrations had reached 0.056 $g.g^{-1}$ (silica gel) and 0.09 $g.g^{-1}$ (KOH), indicating that drying was achieved more rapidly by silica gel, than by storage of the seeds over a saturated KOH solution. After 6 d, water concentrations of seeds dehydrated in both silica gel and over KOH had dropped somewhat below that of untreated seeds (0.027 and 0.035 $g.g^{-1}$, respectively). No marked change in water concentration of seeds in silica gel or over KOH between 6 and 10 d was noted, indicating that a limit to dehydration using these methods had been reached. Berjak and Pammenter (2003) state that all orthodox seeds can withstand drying to approximately 0.05 $g.g^{-1}$, and that any seed that does not, is not orthodox. Given, then, that *W. mirabilis* seeds are shed from the parent plant at a comparable water concentration, and tolerate further dehydration, it is concluded that they are orthodox. The unchanging germinability of the seeds irrespective of the duration of dehydration for up to 10 d confirmed their categorisation as orthodox, since recalcitrant seeds are intolerant of the loss of water (Chin and Roberts, 1980), particularly when such drying is slow (Berjak and Pammenter, 2004), as here.

However, no matter how low a water concentration seeds will survive, there is no substitute for low-temperature storage as the rate of inherent deterioration is directly related to temperature (Walters and Engels, 1998). While it might be thought that such a brief immersion (5 min) in liquid nitrogen would not be a measure of the capacity of the seeds to be cryostored in the long term, this is unlikely to be the case. The critical stages at which seed survival is likely to be adversely affected are during cooling in, and upon warming after retrieval from, liquid nitrogen (Wesley-Smith *et al.*, 2004). It is considered

that if these two steps in the procedure are non-injurious and other manipulations are optimised, then survival of seeds (or any other form of plant germplasm) in liquid nitrogen should theoretically be almost indefinite.

The present results suggest that rupture of the testa (Fig. 3.4) in places consequent upon immersion in, and/or retrieval from, liquid nitrogen, facilitated more rapid uptake of water from the medium, or perhaps removed a measure of coat-imposed constraint from the expanding embryo, such that germination rate was enhanced. At the very least, cryostorage should be non-injurious to *W. mirabilis* seeds, and is suggested to enhance germination rate.

The present results illustrate the extreme orthodox nature of the seeds of *Welwitschia mirabilis*, which are shed from the parent plant in a very dry state. Seeds at the shedding water concentration tolerate hydration and subsequent dehydration with no attendant loss of viability and were found to be excellent candidates for cryostorage, with germination even improving after such treatment.

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CHAPTER 4

MEDIUM-TERM STORAGE OF THE SEEDS OF *Welwitschia mirabilis* Hook. fil.

The data presented in Chapter 3 indicate that seeds of *Welwitschia mirabilis* display highly orthodox behaviour, withstanding immersion, and thus should be able to be successfully cryopreserved, in liquid nitrogen, thereby ensuring long-term conservation. However, even modest cryopreservation facilities using LN may not be available in areas where storage of the seeds is required. Thus a medium-term storage experiment was devised to investigate parameters which would facilitate successful storage under less stringent genebanking conditions. Typically, orthodox seeds are stored under conditions of low relative humidity and temperature (Roberts, 1973; Ellis and Roberts, 1980), with temperatures approaching -20°C considered more favourable for long-term storage than those around 5°C (Genebank Standards, 1994). Ambient temperatures are considered the least favourable for long-term storage, unless seeds are stored ultra-dry (Zheng *et al.*, 1998), and even then, longevity is ultimately likely to be negatively affected (Walters and Engels, 1998).

The general axiom of seed storage is that the drier the seed, the longer it will survive in storage (Vertucci and Roos, 1990; Walters and Engels, 1998). This is held to be a consequence of the decrease in seed metabolism associated with a decline in water concentration, and associated declines in the production of toxic products of metabolism and depletion of food reserves (Vertucci and Roos, 1990). This lead to the logical conclusion that seed life-spans could be extended indefinitely by reducing their water concentration to a figure approaching 0% (Walters and Engels, 1998). Water concentrations below 5% were termed "ultra-dry", but this was later redefined as the water concentration achieved by seeds dried to 10-12% RH at 20°C (Genebank Standards, 1994; Walters and Engels, 1998). However, a "low-water-content" limit to the increase in longevity experienced by drying seeds was discovered; drying to less than this critical level resulted in more rapid accumulation of age-related damage in seeds (reviewed by Walters and Engels, 1998). Thus there exists, for each species, an optimum

water concentration for storage at which longevity is maximised (termed wc_1) and a water concentration below which aging rates increase (wc_2), both of which may be influenced by storage temperature (Walters and Engels, 1998). The optimum water concentrations for effective storage are proposed to vary with storage temperature – the higher the temperature, the lower the water concentration, and vice versa; ie. as storage temperature decreases, optimum water concentration increases (Walters, 1998). Walters and Engels (1998) summarised the effects of ultra-dry storage thus: ultra-dry seeds at less than 5% water concentration are likely to be at the optimum moisture level for storage at 65 or 40-45°C; if the seeds are stored at 18-26°C, they *may* be at the optimum level, depending on their lipid content; while ultra-dry seeds in equilibrium with 10% RH at 20°C will be at the optimum water concentration for storage at temperatures between 25 and 35°C. A codicil was applied by Walters, who stated that seeds in the ultra-dry state should not be stored at less than 20°C, as this is likely to result in decreased longevity for most species (Walters and Engels, 1998). However, ten years of ultra-dry storage of seeds of carrot, groundnut, lettuce, oilseed rape and onion at -20°C did not result in any deterioration in viability (except for groundnut and lettuce) (Hong *et al.*, 2005), indicating either that no deterioration occurs during ultra-dry storage at sub-zero temperatures, or, more likely, that much longer time-spans are necessary in order to detect any resultant damage.

It was previously noted that *W. mirabilis* seeds subjected to the drying necessary to carry out a water concentration determination (80°C for 48 h), retained an undiminished capacity for germination (data not shown). It was decided to include such a treatment here in order to investigate the short- to medium-term effects of such extreme drying on the viability of the seeds, both immediately, and after two years of storage at three temperatures. Whilst this particular treatment combination is unlikely to contribute further information on the longevity of ultra-dry seeds at low temperatures, since the time-span of the study was relatively short, the possibility of such further experiments could be indicated by the success or failure of the present investigation. As noted by Walters (1998), ultra-dry storage experiments at temperatures lower than 20-25°C are almost non-existent. As such, the present investigation could provide a valuable first step in this direction.

Materials and methods

The coverings were removed from all seeds (accession HK1249) used prior to heat treatment or germination (in the case of untreated controls). Batches of seeds were treated in open 90 mm glass Petri dishes (on a sheet of aluminium foil over a layer of silica gel) at 80±2°C for 48 h by placing in one of four ovens. The Petri dishes were closed and cooled for 10 min under laminar air flow before the batches of treated seeds to be stored were transferred into foil laminate bags and randomly assigned to storage temperature categories (three replicate bags of seed per temperature). Three replicate bags of untreated (control) seeds were stored along with the treated seeds at each temperature. Seeds were stored at 16±2°C in an air-conditioned store, 4±2°C in a cold room and at -20°C in a domestic chest freezer.

Samples were taken immediately after heat treatment and after one and two years of storage. Three replicates of 10 seeds (one set of 10 from each bag in storage) were sampled for water concentration from each treatment and storage set, at every sampling interval. Water concentration was determined gravimetrically by drying in a forced air oven at 80°C for 48 h (over and above the 80°C/48 h treatment imposed on the treated seeds). Similarly, three replicates of 50 seeds were sampled for germination, although the number of seeds available after two years required that the samples size be reduced to three replicates of 25. Seeds were germinated on 1% water agar in 90 mm Petri dishes, under a 16 h photoperiod at 24°C. The effects of treatment, storage temperature and length of storage time on water concentration and germination were assessed using the univariate analysis of variance function of SPSS 11.5 for Windows. Germination results in percentage form were converted using the arcsin procedure prior to analysis.

Results

The results for replicate samples were pooled for the purpose of analysis.

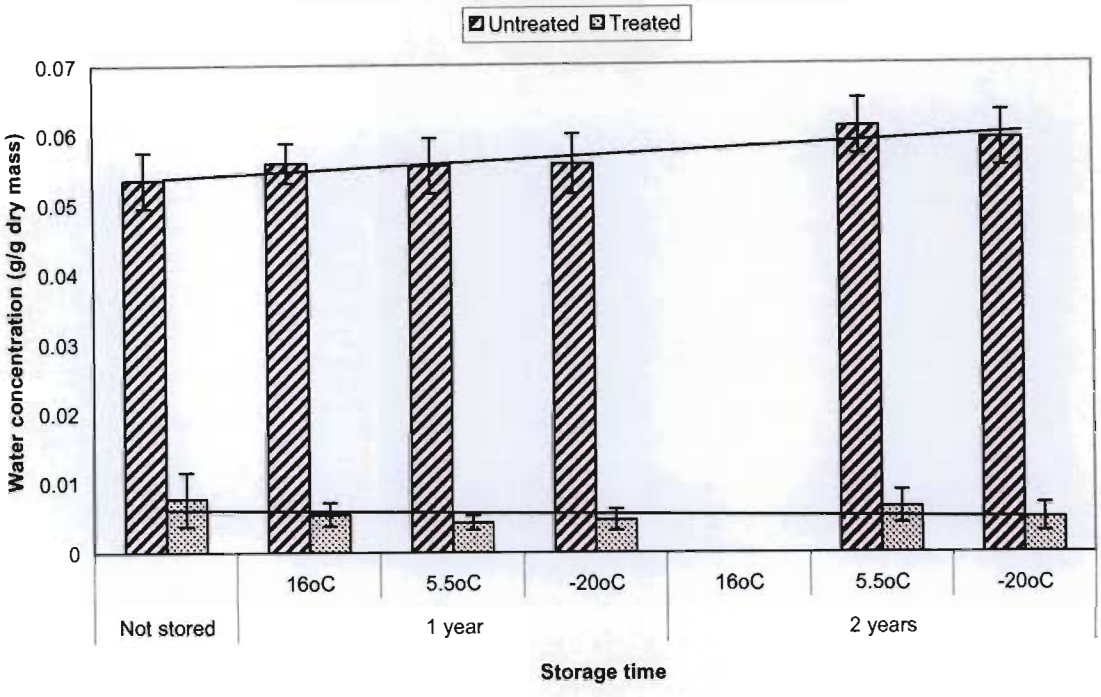

Figure 4.1. Water concentration before and after treatment and storage at three temperatures.

*Error bars represent standard deviation

*Trendlines fitted by Excel

The water concentration of seeds was not significantly affected by storage temperature ($p>0.05$ in all cases), and was only slightly significantly affected by storage time after two years ($p = 0.000$). However, treatment of the seeds at 80°C had a significant effect on the water concentration (Fig. 4.1) ($p<0.5$), lowering it from 0.054 ± 0.004 $g.g^{-1}$ to 0.008 ± 0.004 $g.g^{-1}$ for seeds before storage. Significant interaction effects were noted for treatment*time ($p = 0.000$) and temperature*time ($p = 0.018$), the effect being attributable to a slight significant effect of storage time on water concentration after two years ($p = 0.000$), and the significant drying effect of treating the seeds at 80°C for 48 h.

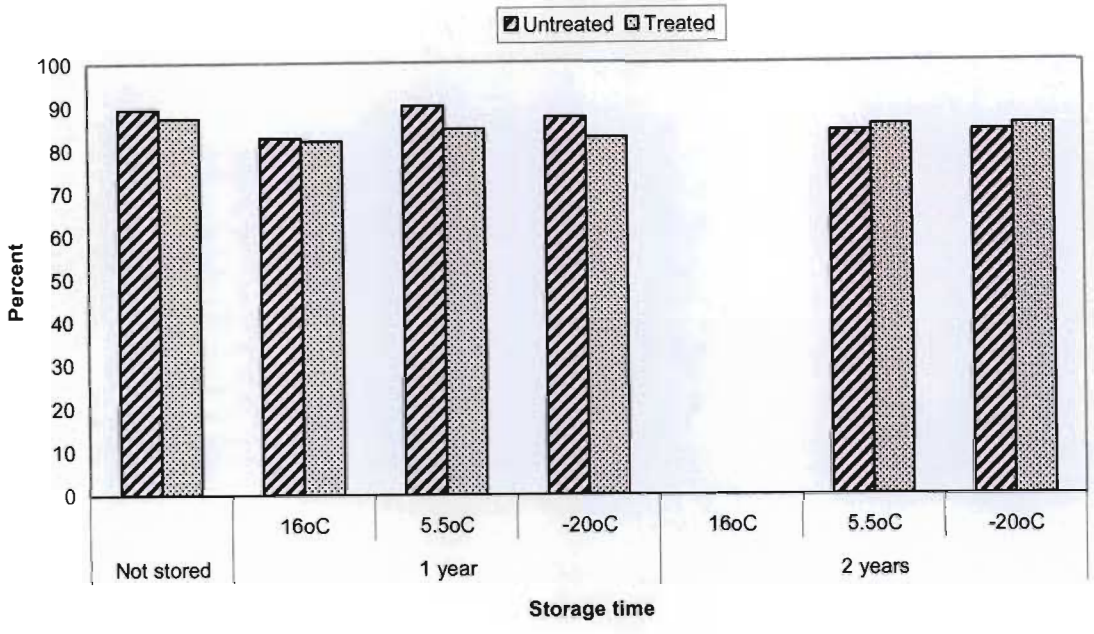

Figure 4.2. Germination of *W. mirabilis* seeds after 14 days on 1% water agar

Germination was essentially unaffected by treatment, storage temperature or length of time in storage (Fig. 4.2), no significant effect of any parameter being noted ($p>0.05$).

Discussion

Untreated seeds appeared to gain water more readily during storage than treated seeds (see trend lines in Fig. 4.1), and it is possible that the uptake of water could have detrimental effects on the seeds over much longer time periods than investigated here. It is therefore essential that the air surrounding the seeds in the packaging material be kept as dry as possible, by evacuating the air and including a desiccant in the package.

High quality seeds of *W. mirabilis* may be successfully stored at temperatures between -20 and 16°C, although the results obtained after 1 year of storage at 16°C indicate a possible deleterious temperature effect (Fig. 4.2). Unfortunately the samples scheduled to be assessed after two years at 16°C were lost when the seed store was re-organised while the author was conducting field trials over eight months in Namibia.

It is recommended that *W. mirabilis* seeds be stored at the lowest temperature practically possible, under conditions allowing no increase in water concentration. Water concentration was significantly lowered by drying seeds at 80°C for 48 h (Fig. 4.1); however, this did not affect germination following storage for up to two years (at any temperature). Such dry heat treatment has previously been used as a thermotherapy for the elimination of pathogens on seed (Clear *et al.*, 2002; Thomas and Adcock, 2004); however, no mention was made of medium- or long-term storage thereafter. Conventionally, it is recommended that seeds be stored between 3 and 7% seed water concentration (wet mass basis [wmb]) and at -18°C or lower (Genebank standards, 1994). These conditions may easily be met for storage of uncontaminated *W. mirabilis* seeds, since they are shed from the parent plant at *c.* 5% (wmb) seed water concentration and temperature requirements may be provided by a domestic freezer (-20°C), as demonstrated here.

The conditions investigated in this experiment provide limited information on the feasibility of long-term ultra-dry storage for *W. mirabilis* seeds. However, given the resilience of the seeds to the harsh drying method used here, and the low water concentrations reached, it may be concluded that ultra-dry storage experiments using *W. mirabilis* seeds would be possible and could contribute valuable data to the current body of knowledge on such methods of storage. Unfortunately, the equilibrium RH of seeds in this instance was not determined, thus limiting comparisons with published literature. Further studies could contribute information about desert seed responses and vigour and viability retention. It appears that no damage was caused to the seeds by drying them at 80°C (or at least, none was apparent within the storage timeframe available). However, seeds may also be dried using silica gel, although not to such low water concentrations (0.008 $g.g^{-1}$ by oven-drying versus 0.027 $g.g^{-1}$; see Chapter 3). Walters and Engels (1998) comment that storage at such low water concentrations as achieved here, for long periods at low temperature, would not necessarily be useful in extending the life span of the seeds, and could be damaging (Hu *et al.*, 1998). Results of ultra-dry storage of peanuts and rice at water concentrations of 1% and 2% (fmb) at 0°C (Hu *et al.*, 1998), showed a significant decline in germination after 30 months of storage. The water

concentration of the peanuts, in that instance, was roughly comparable to the water concentration of the ultra-dry *W. mirabilis* seeds here (0.008 $g.g^{-1}$), and it is interesting that no significant decline in the viability of these seeds (*W. mirabilis*) was experienced at a slightly higher temperature (5.5°C) over a time period only six months shorter (two years). This suggests that *W. mirabilis* seeds may respond differently to ultra-dry storage, and that much longer time periods would be required in order to obtain evidence of seed deterioration, if such deterioration occurred at all. Similarly, no deterioration was evident in ultra-dried seeds of Chinese cabbage, two varieties of chive, green Chinese onion, cucumber and pepper stored over three years at -20 or 4°C (Shen and Qi, 1998), or in carrot, oilseed rape and onion stored at -20°C for ten years (Hong *et al.*, 2005).

Unfortunately, time constraints prohibited a longer-term experiment being carried out in this instance, and conclusions given here for effects of storage on dried seeds cannot be extrapolated for longer storage times. However, it can be concluded that successful storage of seeds at the shedding water concentration can be achieved at temperatures of 5.5 and -20°C, and that such storage is non-injurious in the medium- to long-term.

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CHAPTER 5

FIELD OBSERVATIONS OF THE INFECTION OF THE CONES AND SEEDS OF *Welwitschia mirabilis* Hook. fil. BY *Aspergillus niger* var. *phoenicis* IN THE NAMIB-NAUKLUFT PARK

The majority of the 100 000 known fungus species are saprophytes living on dead organic matter. Although only about 50 species are capable of causing disease in humans, in excess of 8000 species can cause disease in plants (Agarwal and Sinclair, 1987; Agrios, 1988). Plant pathogenic fungi may be host-specific, or may attack a variety of species. Those fungi that require a close association with their host plant throughout their lives are known as obligate parasites or biotrophs, whilst species that require a host plant for only part of their life cycle are termed non-obligate parasites (Agrios, 1988). Fungi may be further divided into three groups: (1) those disseminated by spores but otherwise associated with the host throughout their lifecycles. In these species the spores are released, but remain inactive until they encounter a host plant to which they have been transported by wind, water or other agents; (2) those that pass part of the lifecycle on the host as a parasite and part saprophytically on dead tissues (Agrios, 1988). This group remains constantly associated with host tissues, even after the death of the host. The third group of fungi grow parasitically on their hosts, live on the dead tissues of the host, and may also move into the soil or to the dead tissues of another plant or plant species in the soil. These are the soil pathogens. They have a wide host range and are able to survive in the soil for many years in the absence of their hosts (Agrios, 1988). Seeds are considered the most important means by which plant-pathogenic fungi are perpetuated (Agarwal and Sinclair, 1987).

The success and ability of most plant pathogenic fungi to thrive is dependent on the temperature and moisture conditions of the environment. Free mycelium survives within the temperature range of -5° C to 45°C and in contact with moist surfaces. However, fungal spores are able to survive much broader ranges of both temperature and moisture, although they do require favourable temperatures and moisture in order to germinate (Agrios, 1988).

Seeds naturally harbour a wide range of pathogens, many of which cause disease in the seedling or growing plant. For example, soybean seeds are known to carry spores of such fungal genera as *Alternaria*, *Phytophthora* and *Rhizoctonia*. The international trade in seeds by humans has provided a means for pathogens to be imported into, and established in, countries where conditions may be more conducive to disease development than their countries of origin (Dickinson and Lucas, 1977).

A broad division is usually made between "field fungi", which grow on/in seeds while still on the plant before harvest, and "storage fungi", which become apparent during storage (Christensen and Kauffman, 1969). The storage fungi consist predominantly of xerotolerant members of the anamorph genera *Penicillium* and *Aspergillus* but others such as *Wallemia sebi*, *Scopulariopsis* spp., *Paecilomyces* spp. and *Absidia* spp. have also been noted (Ayerst, 1986), whereas the spectrum of field fungi includes such species as *Fusarium, Alternaria*, *Helminthosporium* and *Cladosporium* (Hudson, 1980; Halloin, 1986). The typical xerotolerant storage fungi proliferate under conditions of low RH and concomitantly low seed water activity in air-dried stored orthodox seeds (seed moisture contents in the range of 12-18%; Berjak, 1996).

M^{c}Lean and Berjak (1987) found that the prevalence of different species of fungus changed with increasing storage time in orthodox maize seeds. It was concluded that inoculum of the fungi found at later stages was present in the seeds at the time of harvest but that their presence was not detectable as their growth was suppressed by the species most suited to the intra-seminal conditions at the time. The succession of fungal species was suggested to occur as the activity of one fungal species modified the intra-seminal micro-environment making it conducive to proliferation of its successor.

Dispersal of fungi may occur in the aerial or soil environment or by vectors (Ingold, 1971; Dickinson and Lucas, 1977). Atmospheric dispersal involves three phases: liberation of the propagules, dispersion, and subsequent deposition. Many fungi have developed specialised methods of propagule liberation which enable the spores to pass through the boundary layer of air into the turbulent atmosphere beyond (Dickinson and

Lucas, 1977). Some fungi make use of "external dispersal agencies" such as rain-drops which splash or tap spores from surfaces (Ingold, 1971; Dickinson and Lucas, 1977; Deverall, 1981). In the case of dry-spored fungi such as *Aspergillus* and *Penicillium*, the morphology of the conidiophore raises the sporulating head above the substratum, increasing the chance of spore dispersal by occasional eddies of air breaking into the laminar layer of air close to the surface (Ingold, 1971). Any agitation of the substratum (live or dead plant matter), such as by gusts of wind, heavy rain, or passing animals, is likely to be effective in the liberation of dry spores (Ingold, 1971), and a combination of wind and rain-splash dispersal accounts for most of the spread of parasitic fungi (Deverall, 1981). Following liberation, the spores are dispersed, generally rapidly, and only over short distances. In other cases, dispersal may be accomplished through transport in the upper atmosphere over very great distances (such as across continents) (Dickinson and Lucas, 1977). Deposition of spores is thought to occur largely under the influence of gravity, or through the action of rain – depositing these on plant surfaces (Dickinson and Lucas, 1977; Deverall, 1981). Ingold (1971) contended that dispersal of fungi is primarily anemophilous, but provided some examples of specialized dispersal by insects such as *Melanostoma mellina*, night-flying moths, bees, fig-wasps, bark-beetles and hemipterous insects.

Soil does not provide the same dispersal opportunities as the aerial environment, which is compensated for, in part, by providing a suitable medium for fungal growth. Dispersal occurs through hyphal growth, although some fungi such as *Gaeumannomyces*, can also produce an annual crop of air-borne spores (Dickinson and Lucas, 1977; Deverall, 1981). Most fungi in soil usually exist in the form of spores, which germinate upon provision of a suitable nutrient source (Robinson, 1967). The local distribution of fungi within a soil profile is likely to be influenced by the soil depth and type, and levels of moisture and organic matter (fungal nutrients) (M^{c}Donald, 1997). A wide variety of animals may be exploited as vectors by fungi. Most commonly, the pathogen contaminates the vector internally or externally and achieves dispersion as the vector moves about and feeds (Dickinson and Lucas, 1977).

Fungal propagules are suggested to gain access to the seed tissues at any time from flowering to the post-shedding phase. Besides post-harvest contamination or infection, seeds may be infected by systemic transmission from the parent plant during development (Sutherland *et al.*, 2002) or through the stigma-style continuum (or pollen chamber in gymnosperms). Microflora may also become established on seed surfaces, from where they are able to enter through cracks, pores, wounds, or by direct penetration through thin seed coats (Halloin, 1986). Infection during seed development allows the fungal mycelium to become established in the internal tissues of the seed, from where it is very difficult to eradicate. Insects and mites also contribute to the infection of the seeds; invasive fungal structures readily gain access to the seed tissues via lesions caused by these spore-carrying vectors. In addition, seeds may be infected after shedding while on the ground. The resultant superficial contamination of seeds may be successfully eliminated if they are collected soon after shedding (Sutherland *et al.*, 2002). Agarwal and Sinclair (1987) make a clear distinction between infection and contamination, in that infection implies that the fungus (or other pathogen) is carried internally, surrounded by the seed tissues, while contamination refers to the passive carrying of a pathogen on the seed surface.

The course of infection of the seeds of *W. mirabilis* by the fungus *A. niger* var. *phoenicis* in the field clearly required investigation. Testing of seeds from accession HK1322 (provided by the National Plant Genetic Resources Centre of Namibia) showed that all were 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. One of the questions posed was whether the seeds become infected during development on the parent plant, and that infection results in the malformation of the seed and death. Therefore the stage of development of the female cones at which infection might occur was investigated, as was the possible route of the infection to the seeds. These studies were seen as having the potential to lead to recommendations for timeous fungicidal treatment of at-risk individuals or populations.

In addition to consideration of the infection of seeds on the parent plant, a small-scale exploratory investigation of the persistence and dispersal of seeds in the local environment was conducted.

Thompson (2000) considered that no simple relationship exists between dormancy and seed persistence in the soil, noting that many seeds persist in the soil for years or decades in a non-dormant state. Also, longevity in dry storage is not necessarily a good indicator of persistence in soil, the conditions experienced being very different in each case (Thompson, 2000). Therefore, the knowledge that seeds of *W. mirabilis* possess non-deep physiological dormancy, and tolerate medium-term dry storage very well (Chapters 3 and 4), cannot lead to the assumption that a large reservoir of persistent seeds exists in the environment. Such a reservoir of persistent seeds is termed a seed bank, which may be defined as an aggregation of ungerminated seeds which could potentially replace adult plants, should they die (in the case of perennials) (Baker, 1989). An effective seed bank must be located in the environment in such a way as to position the seeds to take advantage of opportunities for germination – seeds must not be buried too deeply in the soil, nor should they be removed by foraging animals (Baker, 1989). Seeds in desert soils are distributed very near the surface (Kemp, 1989), and often are not dispersed far from the parent plants, since the likelihood of a seed landing in a patch favourable for germination away from its parents is low (Kemp, 1989; Fenner and Thompson, 2005). Seeds that achieve some degree of dispersal tend to collect in depressions (which could potentially accumulate water) or against obstructions (Kemp, 1989). Most research on seed banks in hot deserts has focussed on annual species (see Kemp, 1989), presumably because desert perennials depend minimally on seed banks for regeneration and protection against climatic variation, the source of seeds during favourable years being the previous season's crop (Kemp, 1989). The perennial nature of plants significantly reduces the impact of environmental uncertainty, and trades off against seed persistence (Thompson, 2000).

The population of *W. mirabilis* plants at Hope Mine (23°34'12"S, 15°15'0"E), near the Gobabeb Training and Research Centre in the Namib-Naukluft Park was selected for a

study of the course of infection of the cones over a single season, and other aspects of the seed ecology.

Materials and methods

Mapping the population

The positions of all individual plants (male and female) were recorded using a Garmin III Plus GPS handset, and mapped using Map Maker 3. A total of 131 individuals (62 females and 69 males) was recorded.

Seed persistence and dispersal in the surrounding environment

Ten female *W. mirabilis* plants were randomly selected. Soil cores 100x100x20 mm, were removed from points 0, 5, 10, 15 and 20 m away from the plant to the north, south, east and west. The soil was passed through a 1000 µm mesh and the seeds separated were collected in Ziploc bags. Soil samples from the same points were collected in plastic vials.

Seeds were removed from the cone axis and sporophylls (if present), counted, and the condition of some examples photographically recorded. The outer seed covering was then removed from all seeds and the number of intact individuals recorded. Seeds were placed on 1% water agar and MEA (if sufficient numbers were collected) to assess viability and fungal infection, respectively. The number of colony forming units (cfu) per gram of soil was assessed using the soil dilution plate technique described below.

Presence and distribution of *Aspergillus niger* var. *phoenicis* colony forming units

1:10 000 dilutions of a 1 g soil suspension in 10 ml sterile distilled water were made up by serial dilution and samples were plated to evaluate the colony forming unit load. All test tubes were shaken by hand for 1 min prior to each dilution; aliquots (3x100 µl) were withdrawn from the final test 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 d.

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 colony forming units per gram of soil was then calculated from the equation: N=a*d*100/(100-X), where N=number of organisms (or colony forming units) per gram dry soil, a=average number of organisms 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.

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 d 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.

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 occurred) 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 (see Table 5.1 for sampling dates). The cones to be plated 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 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 were not 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.

Table 5.1. Sampling dates.

Sample Date	Cones sampled for culturing?	Cones sampled for fixation?
11 November 2004	Yes	Yes
25 November 2004	Yes	No
9 December 2004	Yes	Yes
19 January 2005	Yes	Yes
1 February 2005	Yes	No
15 February 2005	Yes	Yes
1 March 2005	Yes	No
15 March 2005	Yes	Yes
29 March 2005	Yes	No
12 April 2005	Yes	Yes

Air testing

MEA plates 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 d.

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

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 d.

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

Microscopy

Upon return to the University of KwaZulu-Natal in Durban, selected seed samples stored in phosphate-buffered 2.5% glutaraldehyde were processed (see Appendices in Chapter 9) for fluorescence and scanning electron microscopy and viewed.

Results

Mapping the population

The population was distributed along a north-east to south-west axis (Figs 5.1 and 5.2). The highest concentration, (and the largest plants) were found in the 'main wash', a declivity running from north to south (Fig. 5.4). Most plants were established in the sandy bottom of the washes although a few occurred on the rocky slopes of the wash (Figs 5.3A-F).

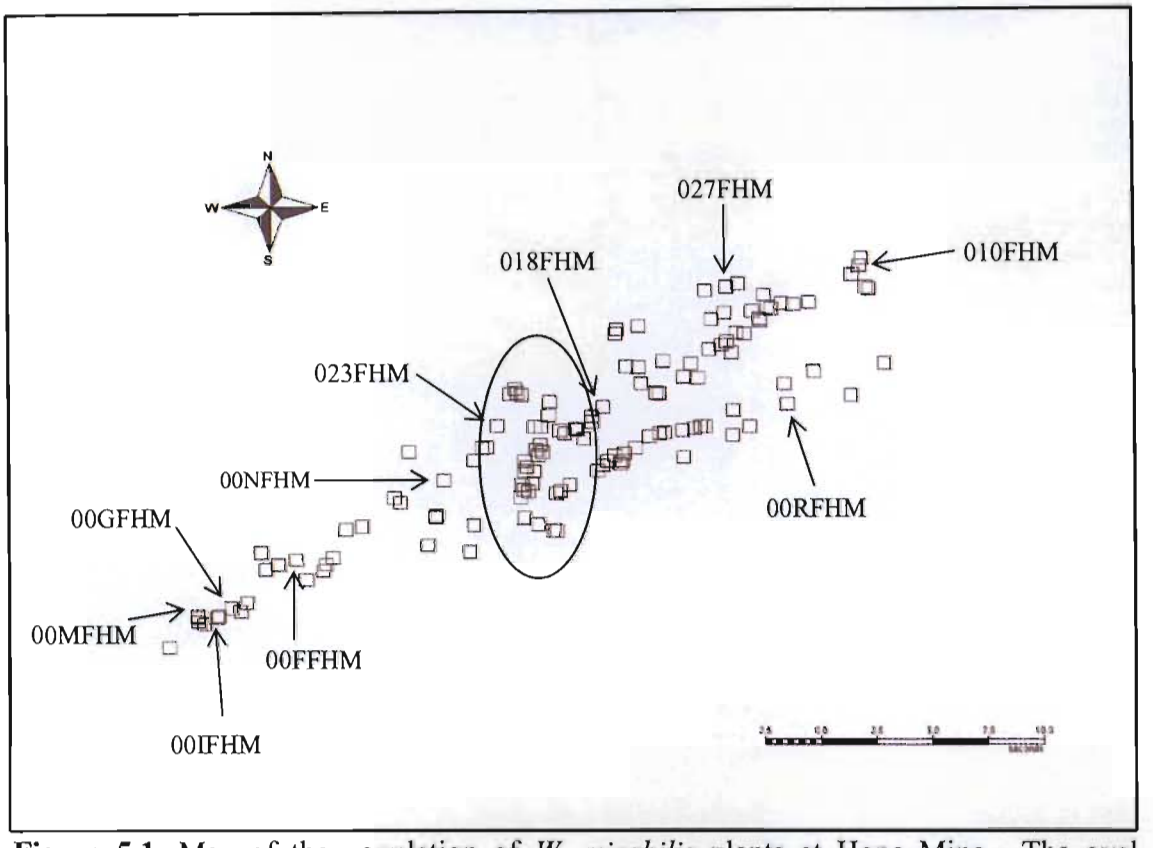

Figure 5.1. Map of the population of *W. mirabilis* plants at Hope Mine. The oval indicates the main wash area. Some female plants are identified by the codes ending 'FHM', and are arrowed on the map.

Several of the plants had previously been allocated numbers, which were inscribed on attached metal tags. Plants that had not previously been allocated numbers were allocated alphabetic designations. After 26 plants had been recorded, the system rolled around to such names as 0AA, 0BB etc. Female plants were identified by the suffix FHM – Female Hope Mine, and male plants by the suffix MHM (Male Hope Mine). The co-ordinates of each plant in the population are lodged at the Gobabeb Training and Research Centre.

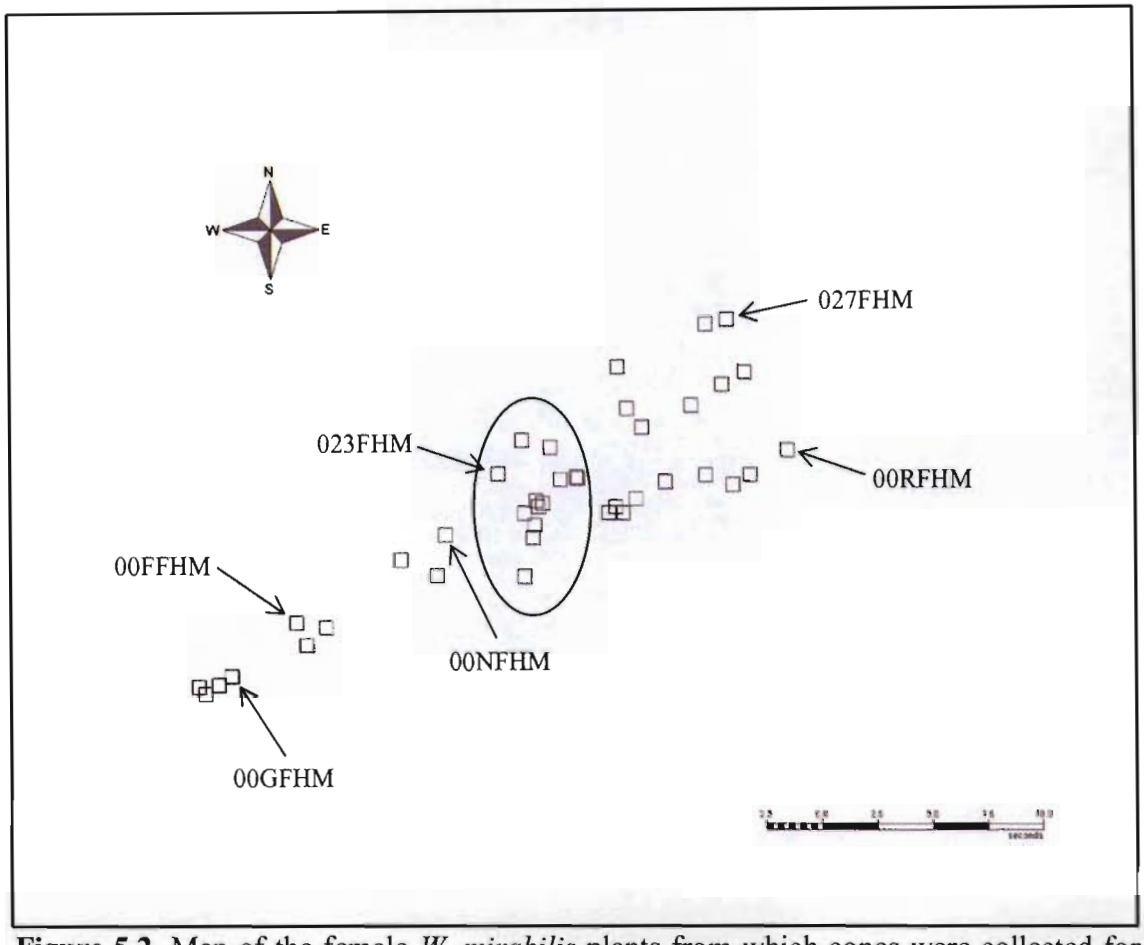

Figure 5.2. Map of the female *W. mirabilis* plants from which cones were collected for culturing and fixation. The oval indicates the main wash area. Some plants are identified and are arrowed on the map.

Figure 5.3. Some individual plants identified in Figs 5.1 and 5.2 are illustrated, showing the surrounding substratum, and some associated plants (E). A: 00IFHM; B: 00GFHM; C: 018FHM; D: 00FFHM; E: 027FHM with *Adenolobus pechuelii*; F: 00RFHM.

Scale (white object) = 300 mm

Figure 5.4. The study site at Hope Mine.

Seed persistence and dispersal in the surrounding environment

A total of 1140 seeds were collected around the plants indicated in Figures 5.5 and 5.6; 188 intact seeds were recovered after removal of the seed coverings. The loss of seeds is attributed to their degraded state which resulted in most crumbling upon removal of the outer coverings. The condition of some cones collected near plant 018FHM is shown in Figure 5.7. The cones were discoloured, dry and brittle. Clouds of black spores were dislodged from the cones when the bracts were separated from the cone axis. All seeds were found to be infected with *A. niger* var. *phoenicis* and only two seeds germinated. In most cases, infection by *A. niger* var. *phoenicis* spores was macroscopically visible. Of the 188 intact seeds recovered, 96% were recovered from the area immediately under the canopy of the adult plants, while the remaining 4% were recovered under the canopy of other plants (Figs 5.8A and B), under rocky overhangs (Fig. 5.9) and from abandoned rodent burrows. Seeds were noted being blown about by the wind.

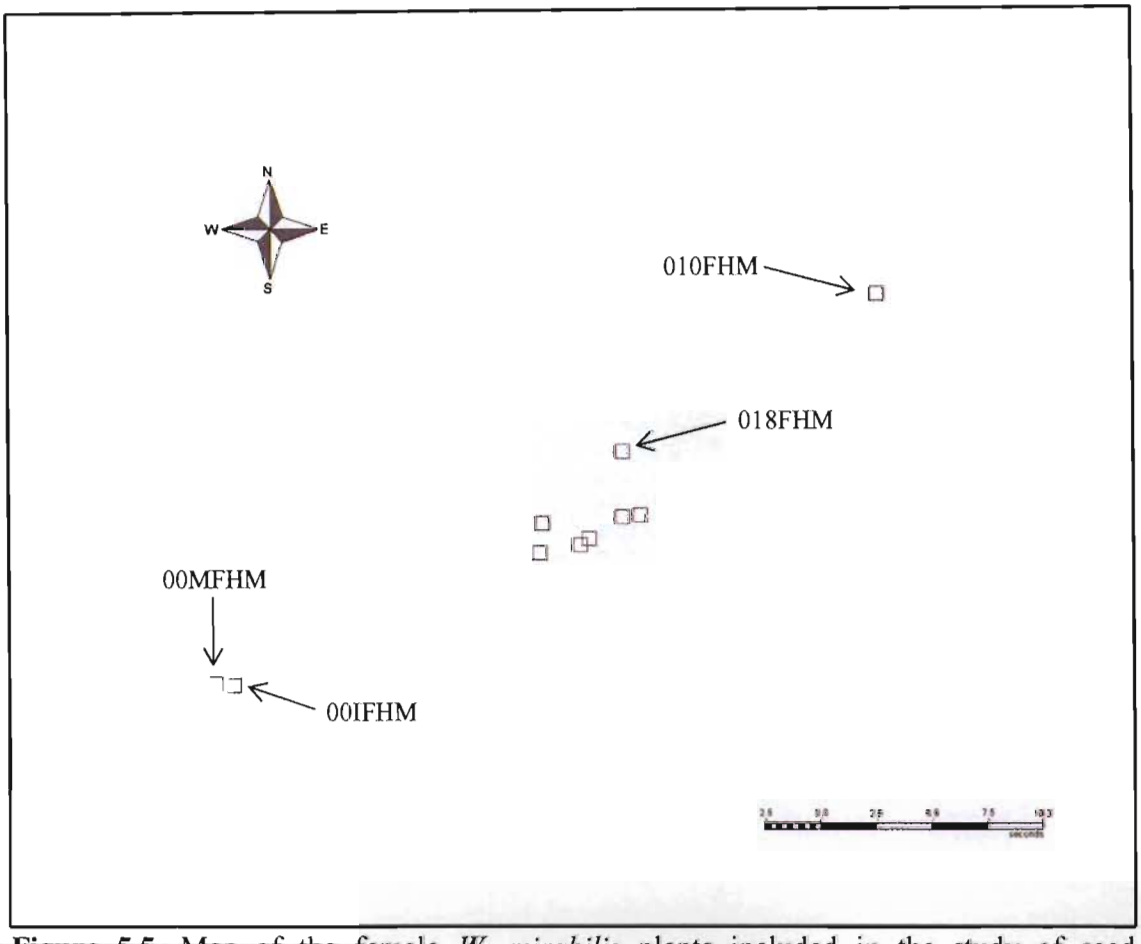

Figure 5.5. Map of the female *W. mirabilis* plants included in the study of seed persistence. Some plants are identified.

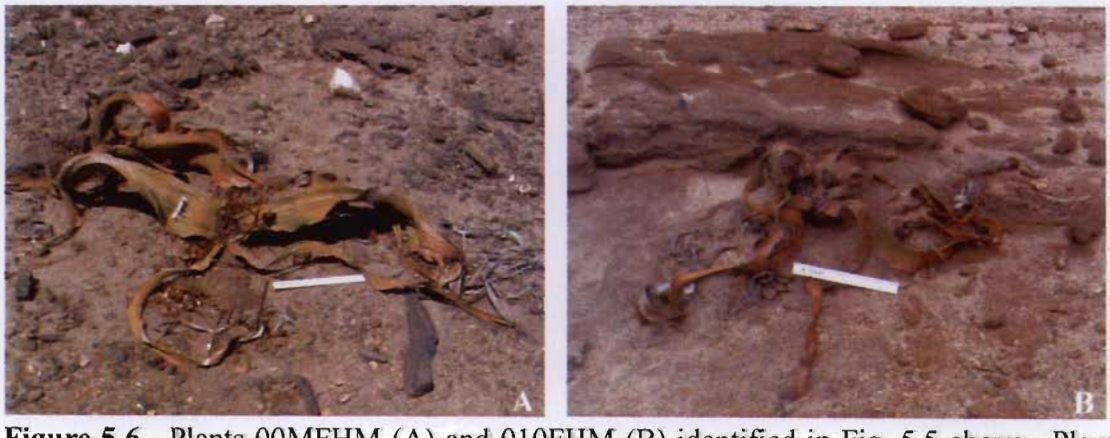

Figure 5.6. Plants 00MFHM (A) and 010FHM (B) identified in Fig. 5.5 above. Plants 018FHM and 00IFHM are shown in Fig. 5.3.

Scale (white object) = 300 mm

Figure 5.7. Cones collected from the soil to the north side of the canopy of plant 018FHM. The condition of the cones is likely to indicate their age, with the past season's cones (May/June 2004) on the extreme left and cones from earlier seasons towards the right.

Scale (white object) = 300 mm

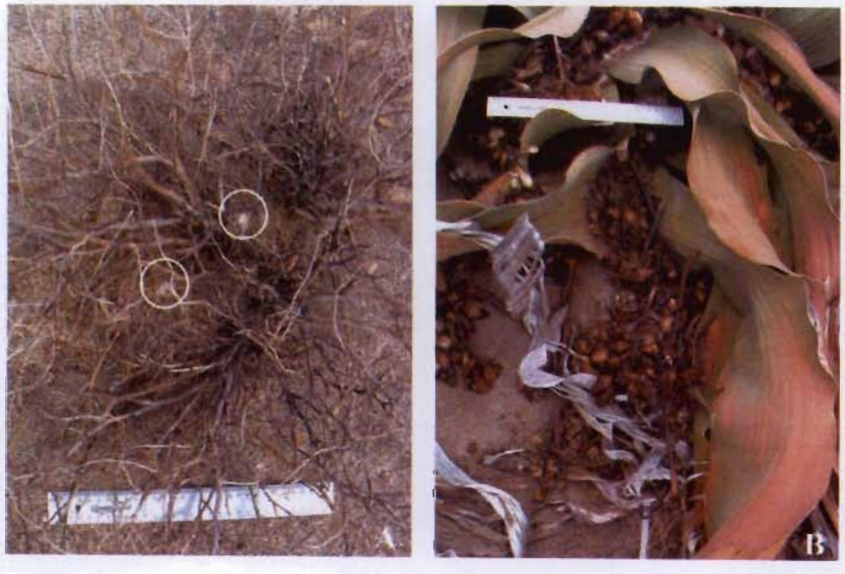

Figure 5.8A and B. A) A plant of *Adenolobus pechuelii* (Kuntze) Torre and Hillcoat with *W. mirabilis* seeds (circled) caught amongst the branches, and B) Cones and seeds below the canopy of a mature female *W. mirabilis* plant (018FHM).

Scale (white object) = 300 mm

Figure 5.9A and B. *W. mirabilis* seeds (circled) caught amongst rocks.
Scale (white object) = 300 mm

Presence and distribution of *Aspergillus niger* var. *phoenicis* colony forming units

An assessment of the *A. niger* var. *phoenicis* colony forming unit (cfu) load of soil surrounding the ten plants of the seed persistence study was completed. The spatial distribution of the spores around the plants was 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^{-1} 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 (Fig. 5.10). This phenomenon is presumed to be related to the presence of greater amounts of decaying plant material (leaves and cones) beneath larger plants which provide the substrate for *A. niger* var. *phoenicis* growth. 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 levels of nutrients were not correlated with plant size in that study. It does not follow that smaller plants were at least risk of infection as all plants were contaminated/infected during the 2004/2005 season. Larger sample sizes could help to resolve this pattern. The *A. niger* var. *phoenicis* colony forming units were distributed throughout the greater local environment which resulted in widespread infection of developing cones (see section "Infection of cones").

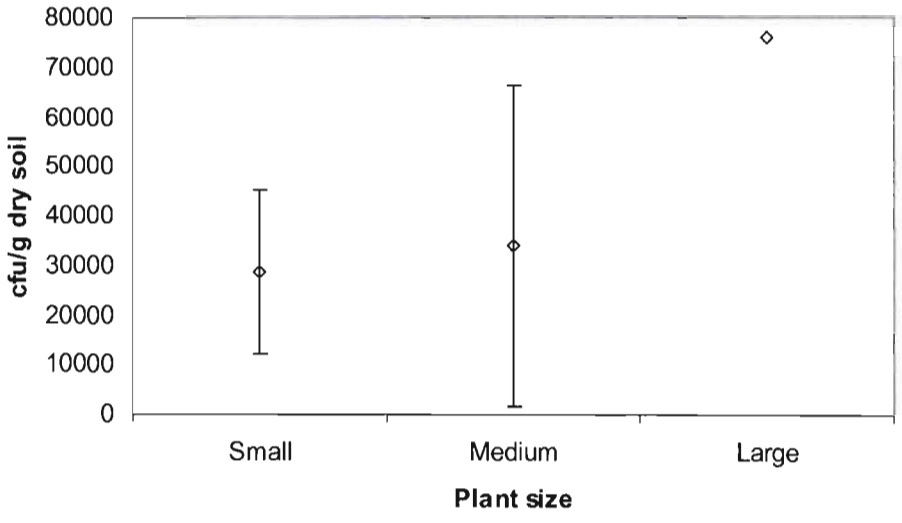

Figure 5.10. The relationship between average number of colony forming units per gram dry soil within a 40 m radius and plant size.

*Sample sizes: Small plants – 6; medium plants – 3; large plants – 1.

*Error bars represent standard deviation.

Insects collected

Probergrothius sexpunctatis, ants, termites, "Australian bug" or fluted scale (tentative identification: *Icerya purchasi* [Family Margarodidae]) (Figs 5.11A and B), spiders (Family Thomisidae) and flies were collected from the *W. mirabilis* plants.

Figure 5.11A and B. A female plant heavily infested with "Australian bug". Both current (CS) and past season (PS) cones are visible (encircled) in the crown.

Insects as vectors

Probergrothius sexpunctatis were observed feeding on both mature seeds from the past season's cones and on the stalks of immature cones (Figs 5.12A and B). Four live *P. sexpunctatis* were placed on separate MEA plates under sterile conditions. The insects were removed after 10 min and the plates sealed and incubated. *Aspergillus niger* var. *phoenicis* colonies were noted after incubation of the plates. Scanning electron microscopy was carried out on two adult and one juvenile *P. sexpunctatis*. Spores, presumably of *A. niger* var. *phoenicis*, were visualised on all three individuals (Figs 5.13 and 5.14). The spores were particularly concentrated in the inverted genitalic capsule found on the lower underside of the abdomen of the adult insects (Figs 5.14A-D).

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 of the cones and seeds.

Figure 5.12A and B. Two *P. sexpunctatis* investigating a seed from the previous season's crop. The insect on the left has inserted its mouthparts into the seed (A). Three *P. sexpunctatis* insects probing a maturing cone (B).

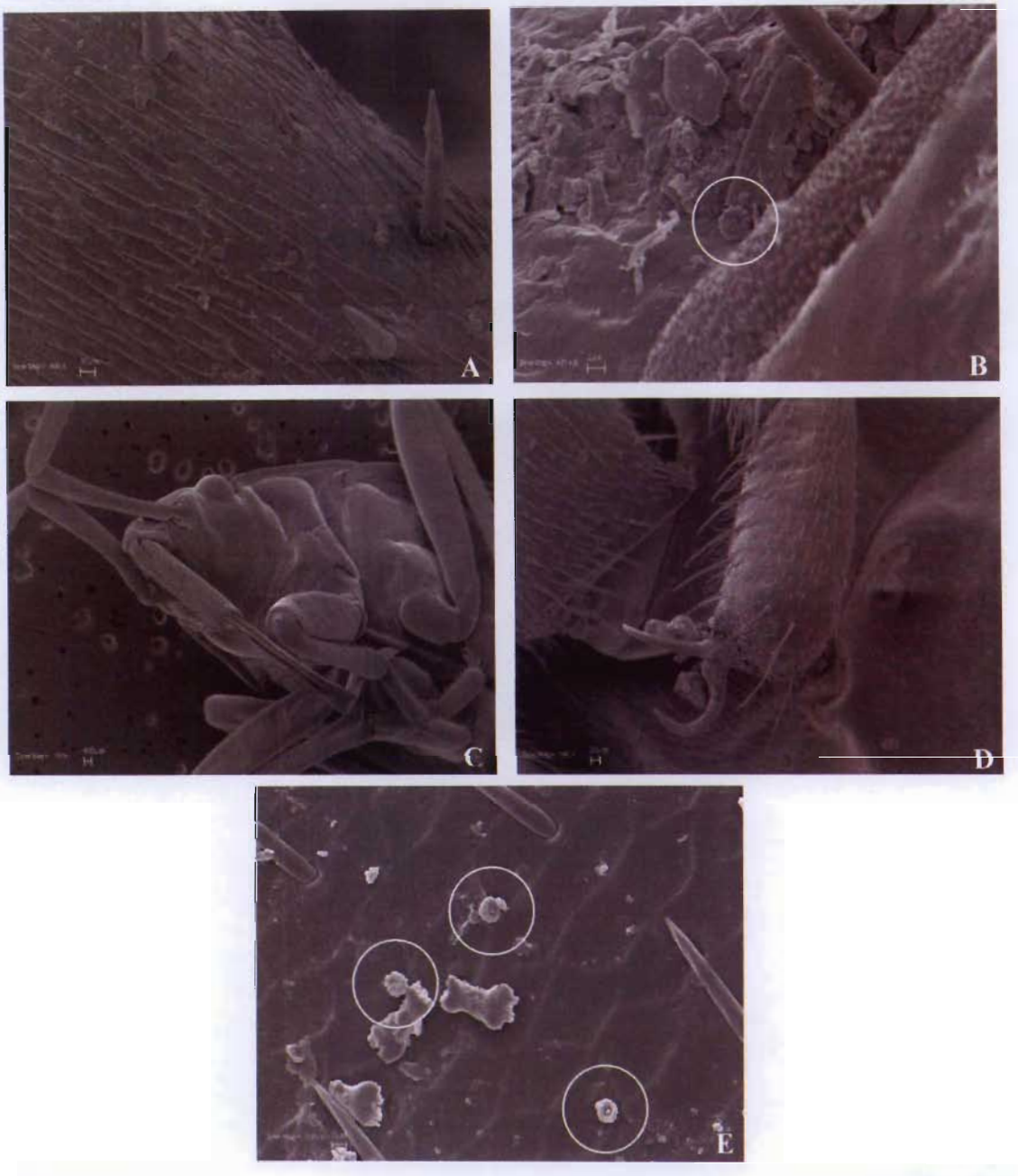

Figure 5.13. A) Surface of an antenna of a juvenile *P. sexpunctatis* with scattered debris (x800). B) Debris, including an *A. niger* var. *phoenicis* spore (circled), in the joint of an antennae of a juvenile *P. sexpunctatis* (x4800). C) The underside of the head of an adult *P. sexpunctatis* (x40). D) The clawed foot of an adult *P. sexpunctatis* with associated debris (x245). E) Spores (circled) on the abdomen of an adult *P. sexpunctatis* (x5000).

Specimen collection dates: A, B, C, D: 9 December 2004, E: 11 November 2004.

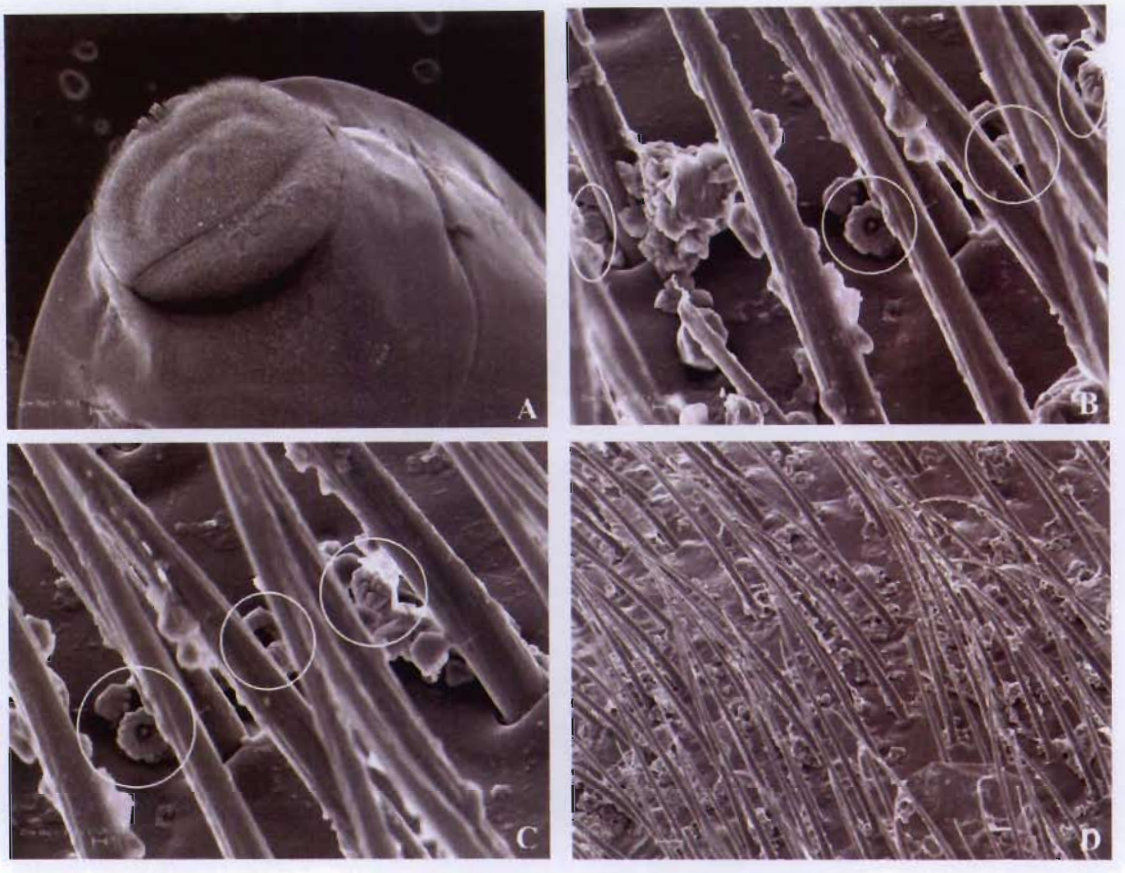

Figure 5.14. A) The inverted genitalic capsule on the ventral side of the abdomen of an adult male *P. sexpunctatis* (x50). B and C) *Aspergillus niger* var. *phoenicis* spores (circled) amongst the hairs of the genitalic capsule of an adult *P. sexpunctatis* (both x6220). D) The inverted genitalic capsule on the abdomen of an adult male *P. sexpunctatis* with debris and spores amongst the hairs (x1340)

Specimen collection dates: All 11 November 2004.

Five ants collected from *W. mirabilis* plants were also placed on MEA. *Aspergillus niger* var. *phoenicis* and another *Aspergillus* species were noted after the plates had been incubated. Scanning electron microscopy of two small (Figs 5.15A-C) and one large ant (Figs 5.15D and E) did not reveal any *A. niger* var. *phoenicis* spores. However, some debris was visualised on the legs and body segments of the ants (Figs 5.15B-E), suggesting that spores could be carried in a similar way by these insects.

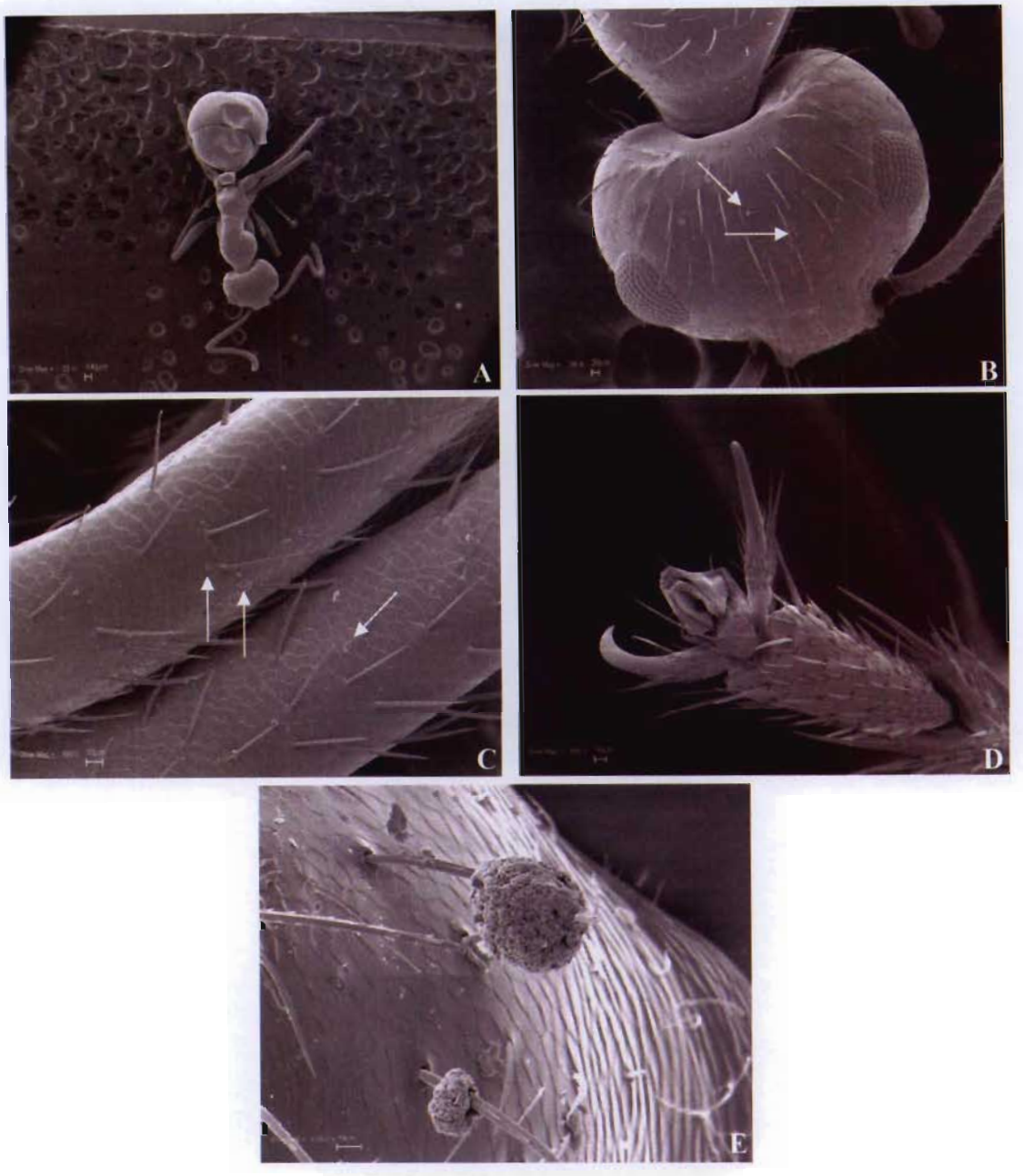

Figure 5.15. A) A view of a whole small ant (x33). B) The head of the ant shown in A; some debris (arrowed) can be seen (x195). C) The legs of a small ant; note some particles of debris (arrowed) (x880). D) A foot of the larger ant (x616). E) An accumulation of debris on the hairs of the thorax (x1320).

Specimen collection dates: A, B, C, D, 5 November 2004; E, 9 December 2004.

Other fauna noted in, or near, the study area

Animals observed included a horned adder (*Bitis caudalis*), springbok, ostriches, several other species of birds (including pied crows and eurasian and barn swallows), a feral horse, rodents (evidenced by burrowing activity, although none were seen) and a scrub hare. The animals observed may possibly act as occasional vectors of *A. niger* var. *phoenicis*; however, this could not be confirmed in the context of this study.

Infection of cones

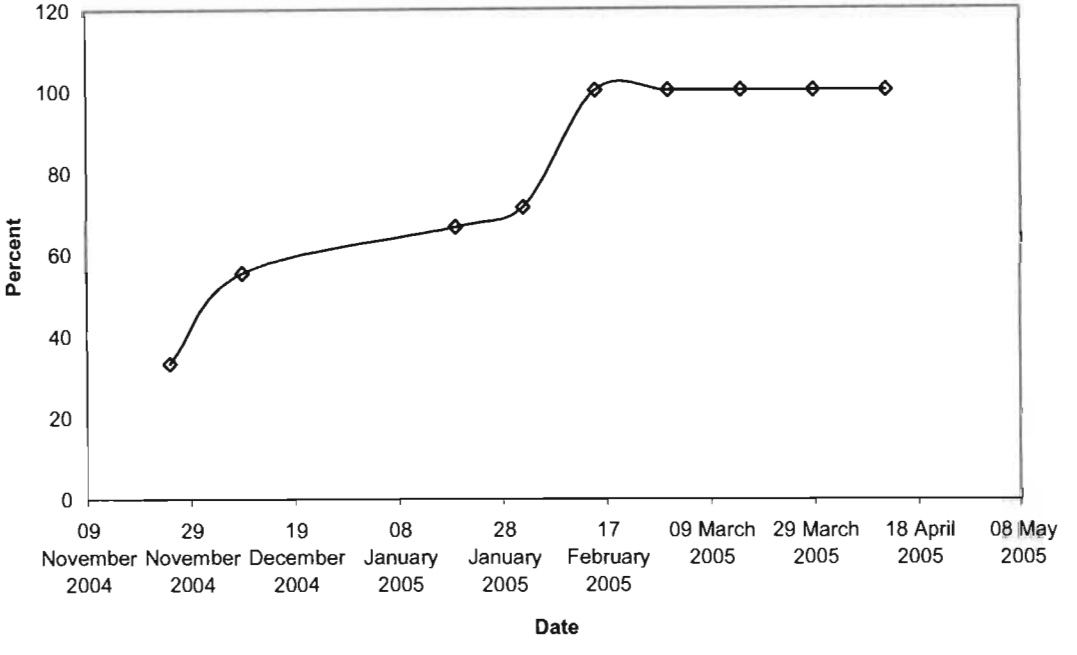

Figure 5.16. Percentage of cones infected by *A. niger* var. *phoenicis* over time.
(sample size varied between datum points because of differences in number of cones produced by each plant)

Figure 5.16 illustrates the progression of infection of cones over time. Infection of *W. mirabilis* cones reached a peak in February, coincident with the appearance of the micropylar drops and with weather conditions conducive to the growth and spread of fungi. A record high temperature of 45.5°C for the Gobabeb Training and Research Centre primary weather station (25 km from Hope Mine) was recorded on 5 February

2005, while heavy falls of rain at Hope Mine occurred on 14 February and at the beginning of April (Fig. 5.17).

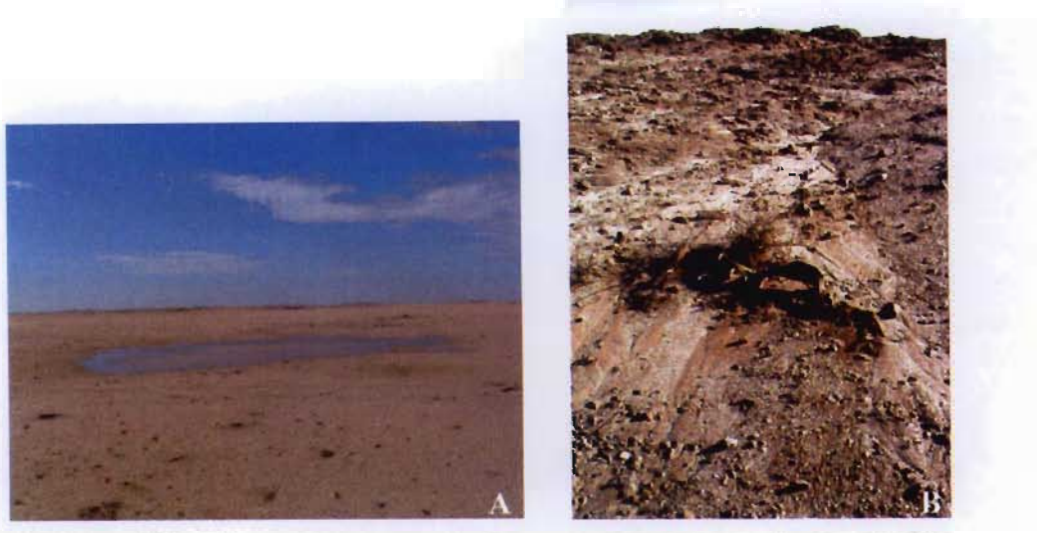

Figure 5.17A and B. Shown above are standing water near Hope Mine on 15th February 2005 (A) and evidence of heavy water flow around a *W. mirabilis* plant in early April 2005 (B).

Air testing

Air testing was performed twice. In the first instance (November 2004) 100% of plates exposed showed 1 or more colonies of *A. niger*. Other fungi and bacteria were also noted. On repeat testing (April 2005), 70% of the plates exposed showed *A. niger* colonies, with the remaining 30% showing other fungal species.

Fungal isolates from surrounding vegetation

Both *Adenolobus pechuelii* and the grass samples (most likely *Stipagrostis* spp.) were found to harbour several readily distinguishable types of fungi including *A. niger*. *Aspergillus niger* has also been noted on the seeds of *Acacia erioloba* (during the course of research undertaken by Petra Moser[2] at Gobabeb Training and Research Centre).

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

Microscopy

Fluorescence microscopy

Infection of the cones by *A. niger* var. *phoenicis* was not microscopically evident (Figs 5.19A-D) until January 2005, after which hyphae and spores became apparent (Figs 5.20A-F). It was found that 66% of cones were contaminated by *A. niger* at the 19 January 2005 sampling (see Fig. 5.16). However, the infection levels surged to 100% of cones sampled on 15 February, some of which were extensively degraded by *A. niger* var. *phoenicis*, with macro- and microscopically visible spores and hyphae (Figs 5.20A-F). Seed quality was extremely poor at this stage; the seed itself was crumbly and degraded (Figs 5.21A and B), and a large space was evident between the seed and the testa (Figs 5.20E and F), indicating that the seed had shrunk in volume over the course of its development. Seeds at this sampling stage (15 February 2005) were noted to be discoloured (Fig. 5.18). Upon microscopical investigation, the discolouration was noted to be most concentrated in an area of the embryo towards the cotyledons (ie. opposite the micropyle. Figs 5.21C and D). This area is associated with the collar cells of the embryo.

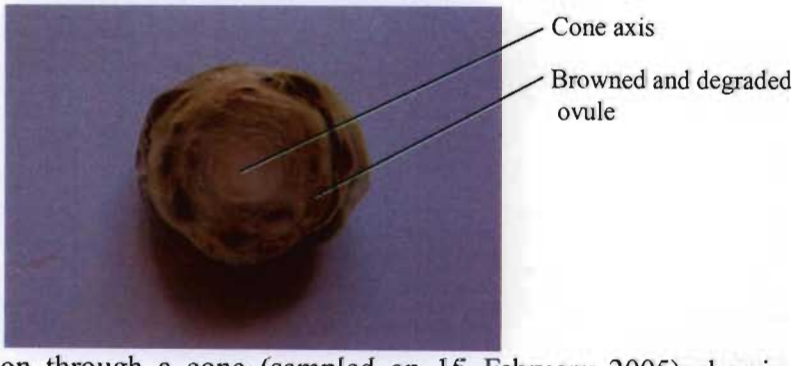

Figure 5.18. Cross section through a cone (sampled on 15 February 2005) showing browned ovules.

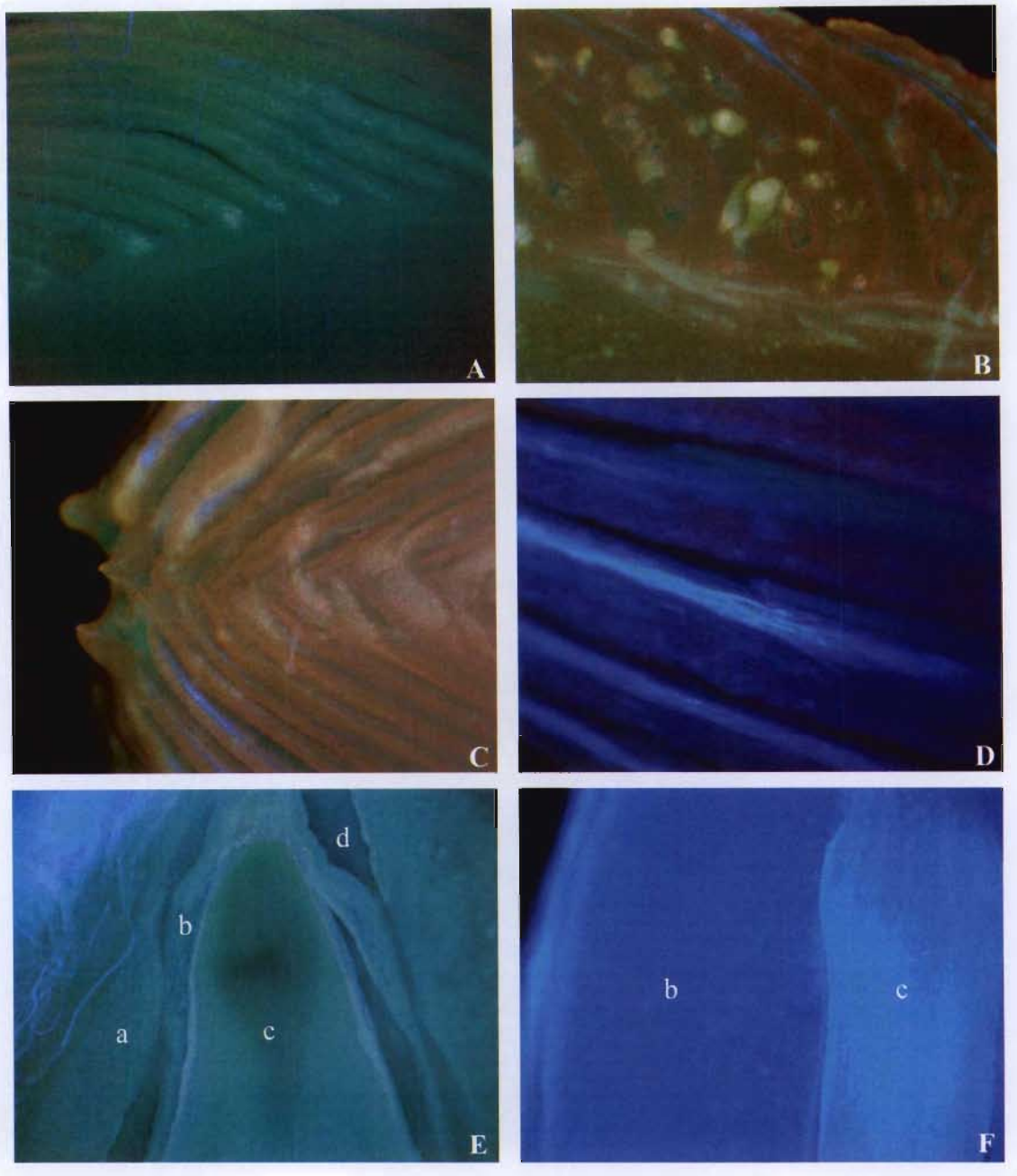

Figure 5.19. Fluorescence micrographs showing aspects of developing cones (A – D) and embryos (E and F). A and B) The point of attachment of bracts to the axis of the cone (x40). C) The tip of a developing cone (x40). D) The micropyle between bracts of the cone (x100). E) The micropylar end of a degraded seed (x40); labels: a) the outer seed covering, b) gametophyte, c) embryo and d) space between gametophyte and outer seed covering. F) An uninfected seed from a different location (x40, accession HK1250; outer seed coverings removed); labels as for E.

Specimen collection dates: A, B, C, 11 November 2004; D, 9 December, 2004; E, 15 March 2005; F, 2002.

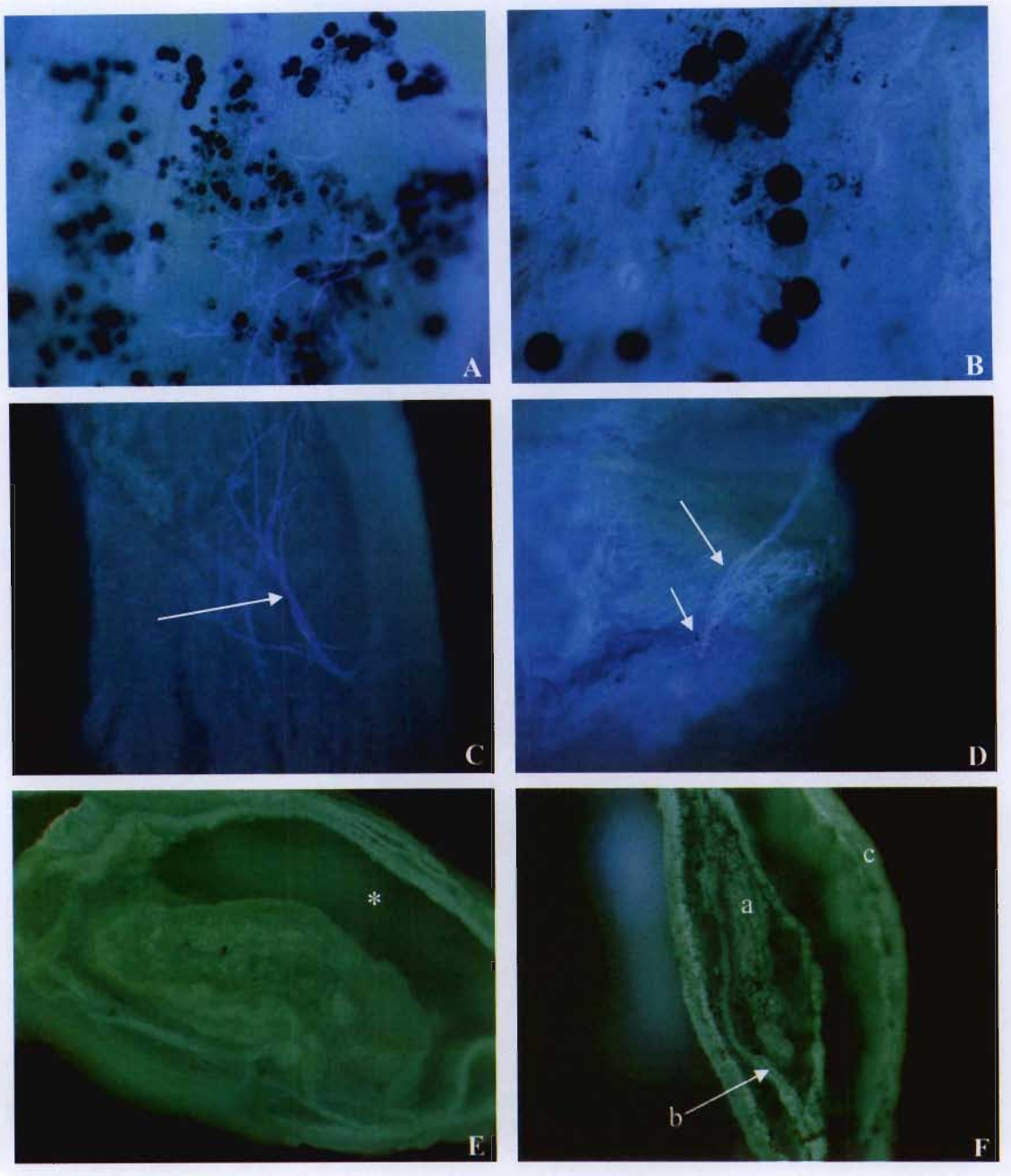

Figure 5.20. A) Hyphae and sporulating conidia on the outer seed coverings (x40). B) Conidia on the wing of a seed (x100). C) Degraded remains of a seed penetrated by hyphae (arrow) (x40). D) A portion of the testa of a seed showing hyphae (long arrow) and spores (short arrow) (x100). E) Cross-section of an infected seed; space between seed and testa indicated with an asterisk (x40). F) Cross-section of a heavily infected seed in which an abundance of spores occurred; labels: a) embryo, b) testa, c) outer seed coverings (x40).

Specimen collection dates: A, B, D, E, F, 15 February 2005; C, 19 January 2005.

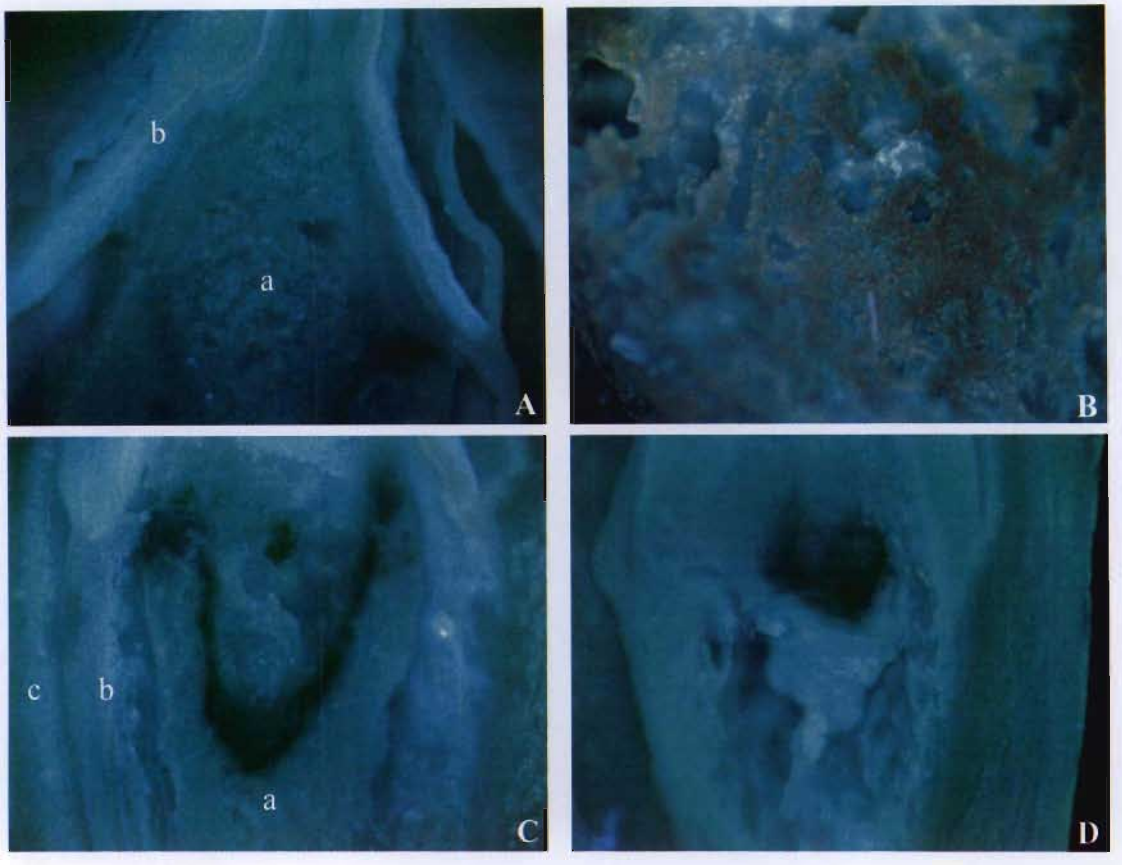

Figure 5.21. Fluorescence micrographs showing aspects of seeds infected and degraded by *A. niger* var. *phoenicis*. A) The micropylar end of an embryo (a) which has shrunken and discoloured; b) outer seed coverings (x40). B) A magnified view of the texture of a degraded embryo (x100). C) A degraded embryo (a) and surrounding seed tissues: b) gametophyte and c) outer seed coverings (x40). D) The cotyledonary end of a degraded embryo (x40).

Specimen collection dates: A, C, D, 15 March 2005; B, 15 February 2005.

Scanning Electron Microscopy (SEM)

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. *phoenicis* spores gained access to the developing ovule via the secretion, since infection increased greatly during this period (see Fig. 5.16).

Figures 5.22A, B and C show a cone in the early stages of development (A and B) and the micropyle of a more mature seed at a later date (C). The apparent lack of any fungal structures at this stage indicated that seed infection was still to occur, as all cones were later revealed to be infected. Figure 5.22D shows the micropyle of a seed sampled in mid-March, a month after the seed shown in 5.22C, when the lumen of the micropyle was almost completely obstructed by dehydrating pollination drop (Fig. 5.22D). Figure 5.22E is a magnified image of part of the micropyle shown in 5.22D, and illustrates what are presumably *A. niger* var. *phoenicis* spores embedded in the dehydrating pollination drop. Spores were found clustered around the entrance to the micropyle (Fig. 5.23A), adhering to the outer surface (Figs 5.23B and C) and in the lumen (Fig. 5.23C: arrow). 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 (Figs 5.23D, E and F; Figs 5.24A, B and C).

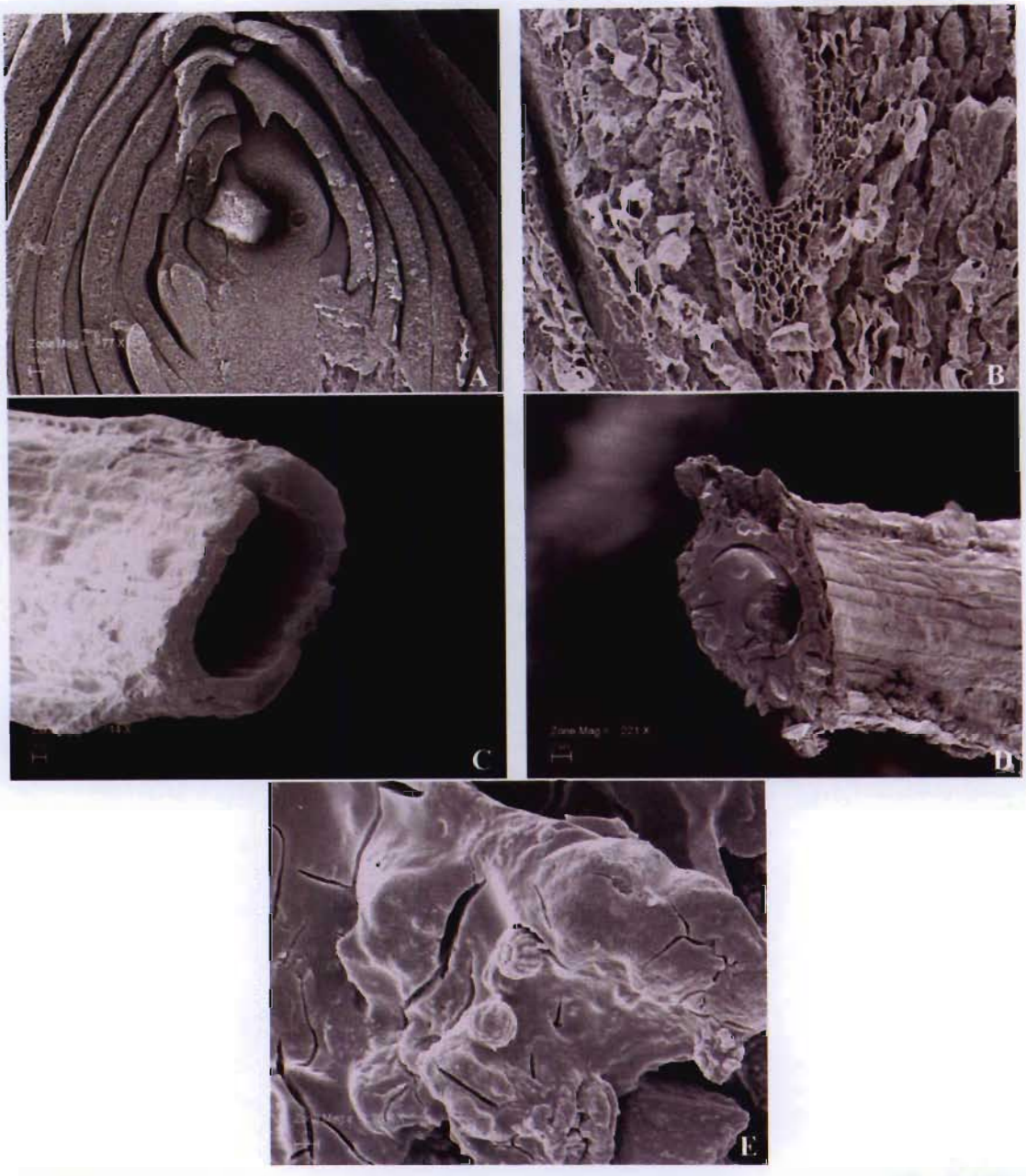

Figure 5.22. A) A longitudinal section of a young cone (x75). B) A longitudinal section through a young cone showing the base of the bracts (x430). C) Micropyle (x715). D) Micropyle showing dehydrating pollination drop with spores embedded in the matrix (x220). E) *A. niger* var. *phoenicis* spores embedded in the dehydrating pollination drop of the seed shown in D (x2700).

Specimen collection dates: A, B, 9 December 2004; C, 15 February 2005; D, E, 15 March 2005.

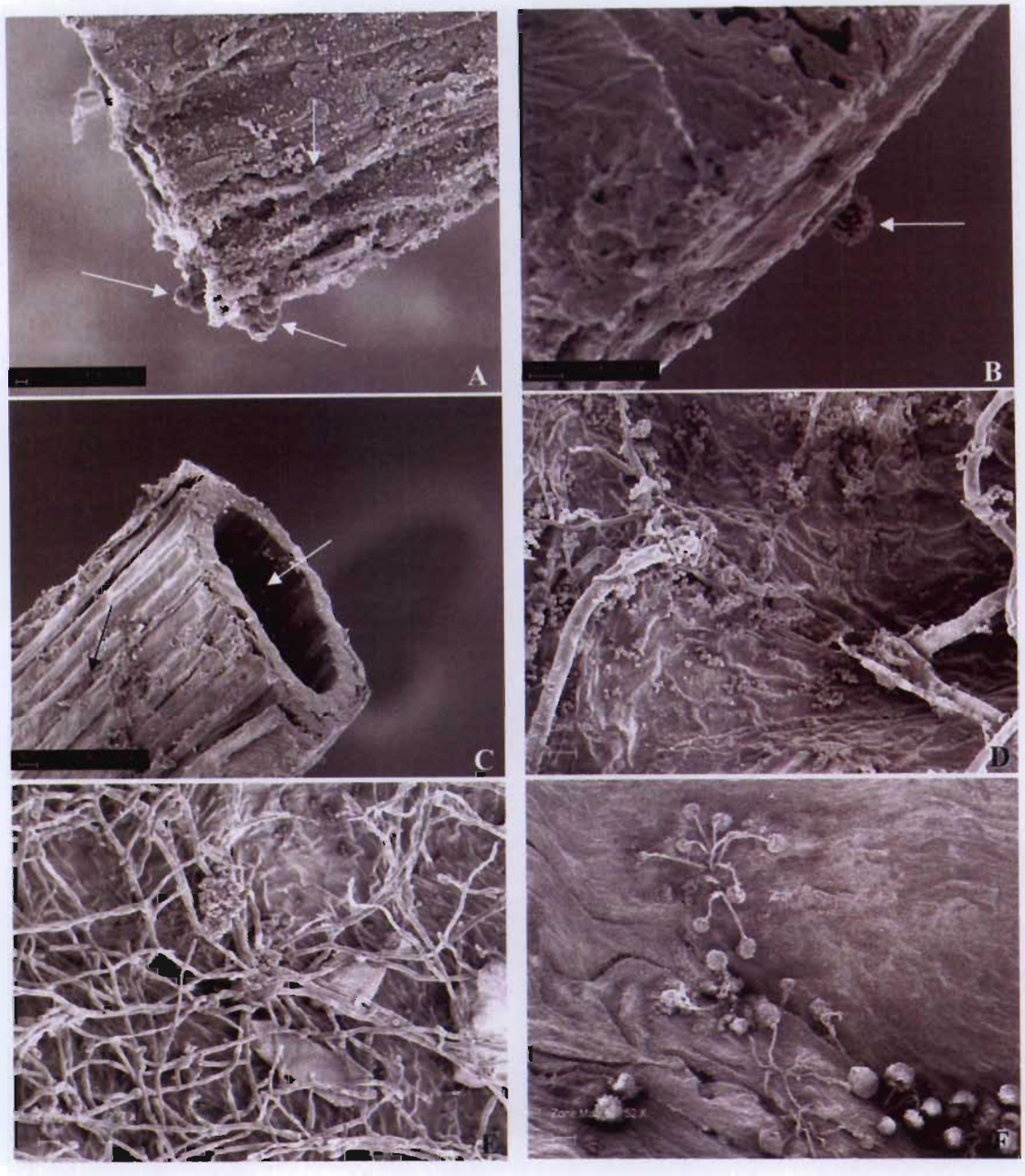

Figure 5.23. A and B) Surfaces of a micropyle showing putative *A. niger* var. *phoenicis* spores (arrows) (x9000 and x6000, respectively). C) The entrance to the micropyle with *A. niger* var. *phoenicis* spores on both the inner and outer surfaces (arrows) (x1000). D and E) *A. niger* var. *phoenicis* spores and hyphae on the testa of a *W. mirabilis* seed (x300, x240 respectively). F) Conidiophores with sporulating heads which appear to have erupted through the testa from the inner seed tissues (x50).

Specimen collection dates: all specimens collected on 15 March 2005.

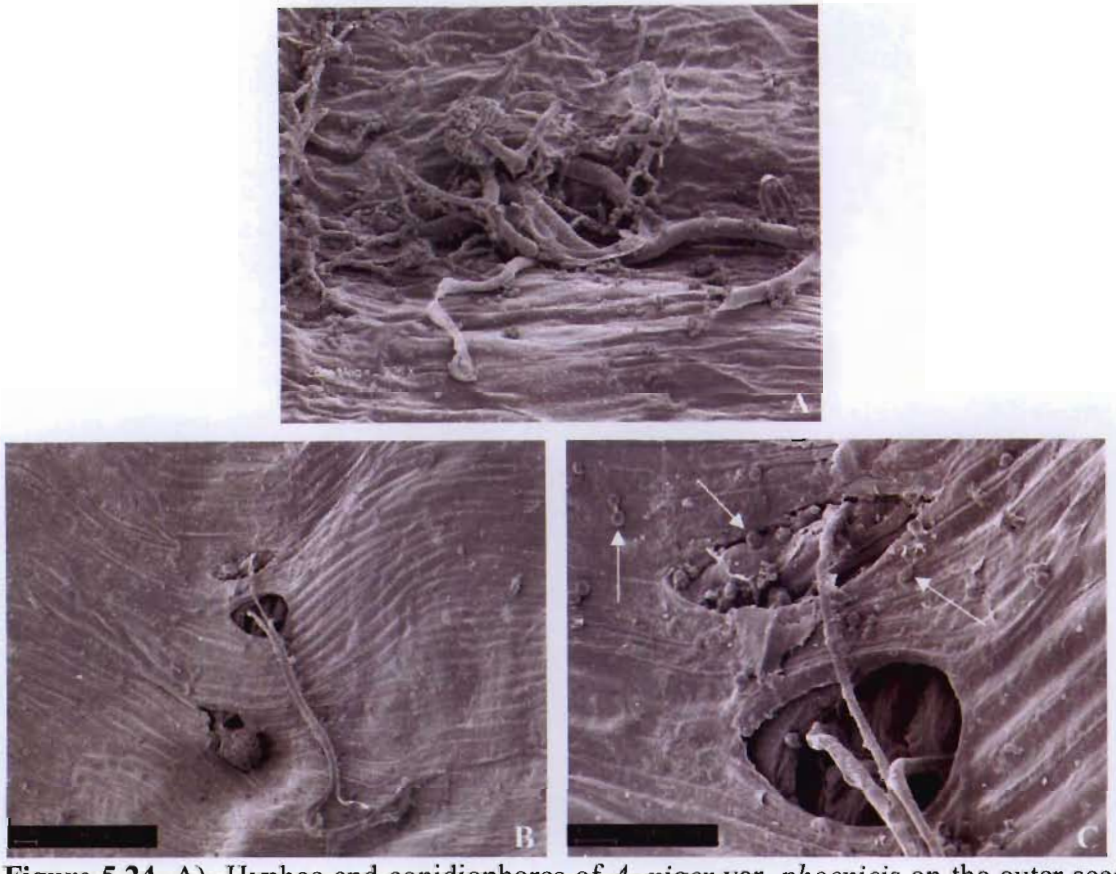

Figure 5.24. A) Hyphae and conidiophores of *A. niger* var. *phoenicis* on the outer seed surface (x330). B and C) Hyphae of *A. niger* var. *phoenicis* interpreted as emerging onto the outer seed coverings from the interior of the seed through an opening possibly created by fungal enzymes. Spores are also visible (arrowed) (x530, x2000 respectively).

Specimen collection dates: All specimens collected on 15 March 2005.

Discussion

There does not appear to be significant seed persistence at Hope Mine, according with previous findings on persistence in desert perennials (Kemp, 1989). Dead seeds were found under the canopy of the *W. mirabilis* plants, under rocky overhangs and in rodent burrows; none were found buried in the soil. The primary dispersal mechanism appeared to be wind (as posited by Bornman [1978]), although water-borne dispersal is also possible (as suggested by Pearson [1906]; see Fig. 5.17B- the seed of the plant visible could have reached that position during a previous rainfall event). The majority of seeds were found under the canopy of the parent plant, probably because the leaves prevented wind dispersal. The adaptive advantage that would be gained by this apparent lack of dispersal is unclear, although it is possible that no advantage would be gained by movement of seeds away from an environment in which parent plants already thrived. Fenner and Thompson (2005) theorise that seed persistence and seed banks are of lesser importance in stable perennial communities when compared with annuals in highly disturbed areas such as arable fields. It might reasonably be suggested that the *W. mirabilis* community habitat could be categorised as stable, given the unchanging nature of the environment. Hypothetically, the risk that all mature *W. mirabilis* plants would be destroyed in any one season is vanishingly small; hence the requirement for a persistent seed bank is low. Individual mature *W. mirabilis* plants may reach ages of 1500 to 2000 years (Bornman *et al.*, 1972), which reduces the necessity for frequent seedling establishment, since a small number of recruitment events within the lifespan of the parent plant will ensure that replacement progeny exist. Theoretical considerations on the nature of seed persistence in perennials (see introduction to this chapter) accord with the observed absence of seed persistence in *W. mirabilis*. Presumably, seed shedding and subsequent germination must occur in the same season, when environmental conditions allow.

Spores of *Aspergillus niger* 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 in infection occurred 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 inoculum introduced by associated insects (Figs 5.12A and B, 5.13A-E and 5.14A-D). Infection via the micropyle is borne out by microscopical evidence (Figs 5.22D and E), which clearly shows *A. niger* var. *phoenicis* spores embedded in the dehydrated pollination drop. 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 occurs on the exterior of the seed surface, from conidiophores that appear to have erupted from the interior (Figs 5.23D-F). A similar disease process characterises *Tilletia caries* – the stinking smut of wheat – which proliferates in the interior of the caryopsis, while leaving the seed wall intact (Ingold, 1971) (consider Figs 5.20E and F). Macroscopically-visible *A. niger* var. *phoenicis* infection was first noted on 1st February 2005, while browning and discolouration of the ovules (Fig. 5.18) was recorded earlier on 19th 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 (Figs 5.19E and 5.20E and F) caused by fungal action. 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; Butler *et al.*, 1979c). 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 these starch reserves. The embryo may be destroyed by fungal action first, following which, the fungus exhausts the gametophyte tissues (which are particularly atrophied in infected seeds – see Figs 5.19E and F for a comparison of the thickness of degraded and healthy gametophyte tissue).

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. Experiments by Hayden and Maude (1992) showed that *Aspergillus 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. It is likely that temperatures up to 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 through this structure 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 (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". *Aspergillus niger* was found to be harboured by *A. pechuelli*, *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), indicating that those of 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 *Aspergillus 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 *Aspergillus niger* enable 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.

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CHAPTER 6

THE POTENTIAL OF VARIOUS TREATMENTS TO ERADICATE *Aspergillus niger* var. *phoenicis* (Corda) Al-musallam FROM SEEDS OF *Welwitschia mirabilis* Hook. fil.

Aspergillus niger

Aspergilli are ubiquitous in nature (Kwon-Chung, 1988; Anon, 1997), and are generally considered to exist in the imperfect (or anamorph) state, although some perfect (teleomorph – sexually reproductive) forms have been found (Anon, 1997). Members of the Aspergilli (including *Aspergillus niger*) are widely distributed across the globe, and have been isolated from soil in tropical, arid savannah and alpine areas (Griffin, 1972). Many species have been implicated as spoilage fungi on seeds (Agarwal and Sinclair, 1987) and other organic material (Hudson, 1986; Anon, 1997; Klich, 2002). The genus *Aspergillus* is composed of asexual saprophytic fungi, producing large black, brown, white or brightly coloured conidia by phialides which are arranged in a globose head radiating from a vesicle or spherical conidiophore (Kwon-Chung, 1988; Anon, 1997). *Aspergillus niger* is among the biseriate Aspergilli, in which conidia-producing phialides arise from metulae, rather than directly from the vesicle, as in the uniseriate species (Fig. 6.1) (Kwon-Chung, 1988; Abarca, 2004).

Classification of the black Aspergilli has been complicated by reliance on morphological characteristics, and the often subtle differences between species, causing confusion around specific names (Abarca, 2004). Frequently, any black *Aspergillus* is identified as *A. niger*, without further investigation of the morphology of the isolate (Abarca, 2004). The black Aspergilli were formerly collectively described as the "*A. niger* group", but are now known as members of "*Aspergillus* section *Nigri*" in the subgenus *Circumdati* (Klich, 2002; Abarca, 2004). While *Aspergillus niger* is a known opportunistic pathogen, varieties are also important in the biotechnological field, being used in the production of fermented food products and industrial production of citric acid (Hudson, 1980; Abarca, 2004). Some species of *Aspergillus* have been implicated as pathogens of man and other animals (frequently birds); *Aspergillus niger* ranks as the third most commonly isolated

pathogenic species in humans, after *A. fumigatus* and *A. flavus* (Pattron, 2006). *Aspergillus* spp. are capable of synthesising a number of toxins including malformin (Curtis *et al.*, 1974), ochratoxin (Varga, 1996) and aflatoxin (Agarwal and Sinclair, 1987).

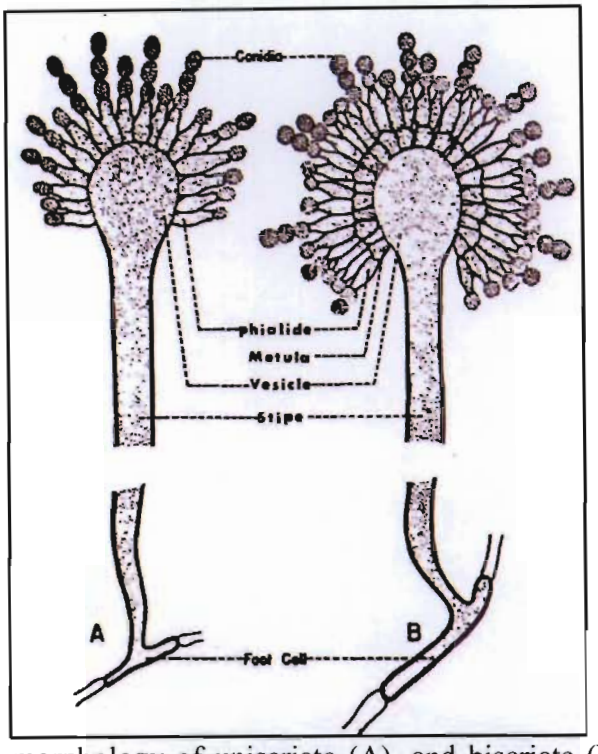

Figure 6.1. The morphology of uniseriate (A), and biseriate (B) Aspergilli. (adapted from Kwon-Chung, 1988)

The presence of a fungus on the seeds of *W. mirabilis* was first recorded nearly 150 years ago in 1863 by Hooker (1863). This fungus was confirmed as *Aspergillus niger* var. *phoenicis* in 1996 by L. Shutte and H. Kolberg (Cooper-Driver *et al.*, 2000). *Aspergillus niger* var. *phoenicis* was known as *A. phoenicis* prior to revision of the genus by Al-Musallam (1980), although use of the original name still persists. *Aspergillus niger* var. *phoenicis*, specifically, is identified as occurring in a range of habitats by Klich (2002), *viz.* forest, wetland, grassland, desert and cultivated land. Abarca *et al.* (2004) provide a comprehensive review of the taxonomy of the black Aspergilli, and comment that the members of the "*A. niger* aggregate" (of which *A. niger* var. *phoenicis* is a member) are extremely difficult to distinguish on the basis of morphology; molecular data indicate that

this aggregate could be divided into two, three or four taxa. Those authors concluded that species identification would therefore be problematic. *Aspergillus niger* varieties are not prominent among the xerotolerant seed (storage) fungi, and are not recorded as major seedling pathogens (Agarwal and Sinclair, 1987). Those authors, however, do mention that *A. niger* penetrates peanut pods and infects the kernels, and that the range 26 - 38°C is optimal for these processes. *Aspergillus niger* has also been found to infect or contaminate onion bulbs and seeds (Curtis *et al.*, 1974; Hayden and Maude, 1992) and infection has been confirmed for peanuts in the Southern African Development Community region (Subrahmanyam *et al.*, 1997). *Aspergillus niger* contamination/infection of seeds has previously been successfully treated using microwave heating (sorghum – More *et al.*, 1992), hot water, and fungicides (onion – Hayden and Maude, 1992).

The presence of the fungus on the seeds of *W. mirabilis* is associated with seed and seedling mortality (Bornman *et al.*, 1972; Bornman, 1978), and the potential threat to the population of plants has recently been highlighted as a cause for concern (Cooper-Driver *et al.*, 2000), since the fungus could pose a serious problem in terms of propagation of the species in its natural habitat, as well as via planting programmes. It is thus crucial that methods be investigated to eradicate *A. niger* var. *phoenicis* from the seeds in order to safeguard these unique plants. The effects of microwave irradiation, dry heat thermotherapy, hot water thermotherapy, chemical surface sterilisation, and fungicidal treatment on both the *W. mirabilis* seeds and *A. niger* var. *phoenicis* were investigated with a view to achieving this aim.

Fungicidal treatments

Inhibitory influences of the environment, or human intervention, can bring the metabolism of fungi to a halt. This is fungistasis, which involves the cessation of growth or reproduction (Müller and Loeffler, [undated]). If the inhibitory influence is not too severe, the fungus may enter a metabolic phase resulting in the formation of resting and/or reproductive structures. Fungi can survive fungistasis: however, if the deleterious influences exceed certain limits, essential molecules or their states of aggregation are

disrupted and, ultimately, the fungi are killed. This is the fungicidal effect (Müller and Loeffler, [undated]), which may be achieved in several ways.

Seed treatments to ameliorate fungi and other contaminants may be categorised as biological, mechanical, chemical or physical processes (Agarwal and Sinclair, 1987). Biological control is commonly achieved through the application of antagonists such as *Bacillus subtilis*, *Chaetomium* spp., *Penicillium oxalicum*, and *Trichoderma* spp. to contaminated or infected seeds, which cause a reduction in the seedborne fungi and an increase in the proportion of healthy, vigorous seedlings produced (Agarwal and Sinclair, 1987). The mechanical control of contaminants is achieved through the processing, screening, and sieving of a seed lot in order to remove associated non-seed material and obviously infected seeds. Chemical control refers to the application of chemicals to the seed in order to eradicate or inhibit pathogens, and is regarded as the safest, cheapest and most effective method available. Physical methods of control refer primarily to types of thermotherapy, such as hot-water, hot-air and radiation (Agarwal and Sinclair, 1987). Hot-water treatments are recommended for seeds with deep-seated infections (Agarwal and Sinclair, 1987), such as is encountered in *W. mirabilis*, but those authors clearly considered such physical methods inferior to chemical treatments.

In the present investigation, chemical and physical methods of fungal control were tested. A degree of mechanical control was also employed, in that seed lots were cleaned of adhering debris and outer seed coats prior to treatment, and, in some cases, obviously degraded seeds were discarded.

Physical methods

Microwave irradiation

Positive fungicidal effects of microwave treatment on the mycoflora of seeds such as Douglas-fir, sorghum and soybean have previously been recorded (Dumroese *et al.*, 1988; More *et al.*, 1992; Reddy *et al.*, 2000), with no attendant deterioration in the quality of the seeds themselves. It is, however, crucial to experiment and control the duration of

radiation and microwave intensity, in relation to seed water content. In this set of experiments, the methods of Reddy *et al.* (2000) were followed.

Thermotherapy

Thermotherapy describes the application of dry or wet heat to seeds and has been developed as a means of eliminating (or at least minimising) the associated mycoflora (e.g. Agarwal and Sinclair, 1987). Generally, however, it is essential that a fine balance be achieved between the temperature to which the seeds are raised and the time of the treatment, which are inversely related (Erdey *et al.*, 1997). For example, those authors reported for maize that thermotherapy (wet heat) at 57 and 60°C was highly effective in eliminating the xerotolerant seed-associated mycoflora, but the treatment could not be applied for longer than 30 and 10 min, respectively, without adverse effects on germination and seedling establishment. Hot water treatments have been found to decrease or eliminate fungi in maize caryopses (Erdey *et al.*, 1997) and acorns of *Quercus robur* (Delatour *et al.*, 1980; Kehr and Schroeder, 1996; Finch-Savage *et al.*, 2003), while *Fusarium graminearum* infection of wheat and barley (Clear *et al.*, 2002) and *Colletotrichum lupini* infection of *Lupinus angustifolius* were successfully treated using dry heat thermotherapy (Thomas and Adcock, 2004).

For orthodox seeds, heating, including microwave treatments, is held to be effective because the fungal inoculum remains hydrated and active, but the seed tissues are quiescent because of their desiccated condition (Christensen and Kaufmann, 1969).

Chemical methods

Chemical treatments

The effects of a range of conventional seed sterilants as well as the effects of three fungicidal compounds were tested. All three of the fungicidal compounds are relatively novel in the context of seed treatment and for fludioxonil the mode of action remains unknown. None of the compounds is described by the manufacturers as a treatment for seed-borne *A. niger* infection, and application concentrations and methods had to be developed here.

Tebuconazole (a triazole fungicide) has been successfully used to treat fungal infections in wheat seeds (pre-harvest *Fusarium* infection – Homdork *et al.*, 2000) and leaves (*Puccinia striiformis* – Han *et al.*, in press). Han *et al.* (in press) also concluded that treatment of wheat with tebuconazole both inhibited the development of the fungus, and enhanced defence reactions of the plant. Triazole fungicides comprise the largest group of SDIs (sterol C^{14}-demethylation inhibiting fungicides), in which the antifungal action is concerned primarily with the inhibition of ergosterol synthesis (Buchenauer, 1987). Ergosterol forms part of the plasmalemma and internal membranes in fungi and is suggested to "order" the phospholipids (Deacon, 1980) probably promoting membrane fluidity under a wide range of conditions (Berjak, pers. comm.[3]). Treatment with SDIs results in changes to membrane fluidity and structure (Buchenauer, 1987). The SDIs produce morphological variation in treated fungi: however, the specific effects on multiplication of sporidia, hyphal growth and spore germination are species-dependent (Buchenauer, 1987). Here, tebuconazole in the form of Orius 200EW (Makhteshim-Agan SA PTY [LTD]), an emulsion oil-in-water systemic fungicide, was applied.

Fludioxonil, a phenylpyrrole fungicide, displays broad-spectrum contact antifungal activity. This class of fungicides is derived from a natural antimycotic isolated from soil microflora, which interferes with the electron transport chain in susceptible fungi such as *Microdochium nivale*, *Fusarium*, *Septoria* (Celest Syngenta, 2006) and *Rhizoctonia* (Brandl and Long, 2003). However, the targets of phenylpyrroles are yet to be identified, although it is suggested that they act upon a component of the osmosensing histidine kinase pathway (Okada *et al.*, 2005). In this study, fludioxonil in the form of Celest 100FS (Syngenta SA, Johannesburg) was applied.

The systemic and translaminar strobilurin fungicides are derived from mushrooms such as *Strobilurus tenacellus* and interfere with the production of ATP by acting upon the electron transport chain, blocking electron transfer at the quinol oxidation site (Vincelli,

[3] Prof. P. Berjak, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, South Africa

2002; Heritage Syngenta, 2006). This highly specific mode of action has resulted in the acquisition of resistance by fungi in certain instances, and has prompted the publication of guidelines for the reduction of such risk (Vincelli, 2002). Azoxystrobin in the form of Heritage (Syngenta SA, Johannesburg) targets and controls a broad range of fungi, including *Rhizoctonia*, *Sclerotium* and *Fusarium* (Heritage Syngenta, 2006). Some strobilurin fungicides display plant growth enhancing effects; in contrast, specific crops such as Macintosh apples and Concord grapes are very sensitive to azoxystrobin and show phytotoxic effects (Vincelli, 2002).

A systemic fungicide is capable of preventing the development of disease on, or in, regions of the plant away from the site of application. The fungicide is applied to the surface of the plant or seed and effective amounts are translocated within the body (Wain and Carter, 1977). The fungicide is taken up by the plant and may be fungitoxic immediately, or may be metabolised to form toxic derivatives within the plant (Dickinson and Lucas, 1977).

When non-systemic fungicidal compounds are applied to a plant, areas missed in the initial application will be unprotected (Wain and Carter, 1977). An additional constraint is that these protectant compounds must be applied before the arrival of the inoculum. The effective use of non-systemic fungicides depends on the prediction of outbreaks of disease, which is not always possible. In addition, these fungicides form surface coatings which are subject to degradation, and, in the field, erosion by light, wind, rain and other environmental factors (Dickinson and Lucas, 1977). It has also 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). Since systemic fungicidal compounds (systemics) should move freely internally from the site of application to distant, untreated tissues, systemic compounds do not have these disadvantages. The main disadvantages of systemics are their narrow spectrum of activity and the rapidity with which fungi develop resistance to them (Wain and Carter, 1977).

Materials and methods

In the experiments described, seeds from two accessions were treated in parallel (unless otherwise stated), in order to determine the effects of the treatment on both *A. niger*-free and -infected populations.

Physical methods

Microwave treatment

The outer coverings of seeds from accessions HK1249 (not infected) and HK1322 (heavily infected) were removed. Sets of 20 seeds from each accession were placed in 90 mm diameter glass Petri dishes and treated separately in a Sharp R-6260 microwave oven (with a rotating turntable) on high power (600 kW at 50Hz). A 500 ml capacity beaker containing 100 ml of tap water was placed in the microwave during treatment to act as a heat sink (Reddy *et al.*, 2000). Seeds were treated for intervals of 15 s up to 60 s and thereafter at intervals of 30 s up to a maximum of 8 min 30 s (510 s). The quantity of water in the heat sink was increased to 150 ml for the longer treatment durations to compensate for loss through evaporation. The seeds were plated onto 1% water agar in 90 mm Petri dishes (five seeds per plate), maintained under ambient light and temperature conditions in the laboratory and monitored periodically for germination and incidence of fungus.

Dry heat thermotherapy

Seeds were subjected to temperatures of 80°C, 90°C, 100°C in a forced air oven for varying periods depending on the temperature.

Sets of 20 or 25 seeds were prepared for treatment by removal of the outer coverings. The seeds were placed in 90 mm diameter glass Petri dishes lined with two sheets of Whatman no. 1 filter paper. Seeds were subjected to temperatures as indicated in Table 6.1. The seeds were then placed on 1% water agar in 90 mm Petri dishes and incubated under ambient light and temperature conditions.

Table 6.1. Temperature and time regime for dry heat thermotherapy of seeds from accessions HK1249 and HK1322.

Treatment (°C)	Time (hours or minutes)
Untreated (Control)	0 h 0 min
85	12 h
	36 h
90	24 h
100	12 h
	3 h
	2 h
	1 h
	10 min
	8 min
	6 min
	4 min
	2 min

Hot water thermotherapy

Seeds were subjected to hot water immersion at temperatures of 65°C, 70°C, 75°C for varying periods depending on the temperature (after Erdey *et al.*, 1997).

The outer coverings of 340 seeds from each of accessions HK1249 (and later HK1250 – neither infected) and HK1322 (heavily infected) to be treated, were removed. Two 500 ml beakers of distilled water were positioned in a large water bath on a raised metal grid. The water bath was covered with heavy duty aluminium foil while being heated with a Haake D1 inflow heater to the required temperature; the seeds were then placed in the beakers of water and 20 were periodically removed at the intervals indicated (Table 6.2) using a small sterile tea strainer. The seeds were then aseptically plated on 1% water agar in 90 mm diameter plastic Petri dishes (five seeds per plate), sealed with Parafilm, incubated under ambient light and temperature conditions in the laboratory, and monitored daily for germination and fungal proliferation.

Table 6.2. Temperature and duration of treatment of seeds for hot water thermotherapy. (n=20).

Temperature (°C)	Time (min)
65±2	10
	20
	30
	40
70±2	10
	12
	14
	16
	18
	20
	30
	40
75±2	2
	4
	6
	8
	10

The most effective antifungal treatment indicated by preliminary trials was repeated six times using contaminated seeds from accession HK1322 to ensure reproducibility of the results. The trials were carried out as described above, with slight modifications as noted in Table 6.3.

Table 6.3. Replication of the hot water thermotherapy experiment. (n=25).

Trial	Conditions
A	Sterile water used in beakers, small water bath
B	As for A
C	Sterile water used in beakers, large water bath
D	Non-sterile water used, large water bath
E	Sterile water used, large water bath, seeds plated individually on 90 mm dishes of 1% water agar
F	As for E
G	Sterile water used, large water bath, five seeds plated per 90 mm dish of 1% water agar

After determination of the most effective temperature and time treatment, seeds from accessions HK1250 and HK1322 were treated, blotted to remove surface water and dried in plastic Ziploc bags containing activated silica gel for 1, 2, 3 and 4 d. Water concentration was assessed gravimetrically (n=10) using a Mettler MT5 six place balance at each time interval, and the time taken to return the seeds to a water concentration approximating that pre-treatment (ie. suitable for storage) was noted. The experiment was repeated, and seeds were set to germinate on 1% water agar after drying to the appropriate level, to determine the effect, if any, of hydration and dehydration on viability.

Seeds from accession HK1250 were used for treatments at 65°C and 75°C, as all seeds from accession HK1249 had been depleted. Untreated seeds from accessions HK1249 and HK1250 showed very minor differences in germinability (see Table 2.2, Chapter 2).

Chemical methods

Surface sterilisation

Except in cases indicated, the coverings were removed from samples of 25 seeds each (accession HK1250), prior to all treatments (Table 6.4), after which the seeds were rinsed three times in sterile distilled water, plated on 1% water agar and assessed for germinability and fungal status. *Aspergillus niger* var. *phoenicis* proliferated freely on both the germinating seeds and the surface of the water agar, thus the fungal status of the seeds could be easily assessed, without the need to subculture onto a nutrient enriched medium. Treatments that were not detrimental to the viability of the seeds were repeated using seeds from accession HK1322 to assess their effectiveness against *A. niger* var. *phoenicis* (Table 6.5). *Aspergillus niger* var. *phoenicis* was not commonly isolated from seeds of either accession HK1249 or HK1250.

Table 6.4. Details of surface-sterilisation treatments applied to *W. mirabilis* seeds from accessions HK1249 and HK1250 after removal of the coverings (*except in the case indicated). (n = 25).

Treatment	Time (min)
Sterile distilled water (control)	10
NaOCl, 2% (v/v), coverings intact*; rinsed (distilled water); coverings then removed	10
2%; rinsed (distilled water)	10
1%; rinsed (distilled water)	5
0.35%; rinsed (distilled water)	5
$Ca(OCl_2)$, 1% (w/v); rinsed (distilled water)	2
Ethanol, 70%	2
$HgCl_2$, 0.02% (w/v); rinsed (distilled water)	0.5
Sporekill[1], 0.02% (v/v); rinsed (distilled water)	5
Sporekill, 0.02% (v/v); rinsed (distilled water)	10

[1]ICA Laboratories, Cape Town

Table 6.5. Details of surface-sterilisation treatments applied to *W. mirabilis* seeds from accession HK1322 after removal of the coverings. (n=25).

Treatment	Time (min)
Sterile distilled water (control)	10
Ethanol, 100%; rinsed (distilled water)	2
Ethanol, 70%; rinsed (distilled water)	2
$Ca(OCl_2)$, 1% (w/v); rinsed (distilled water)	2
$HgCl_2$, 0.02% (w/v); rinsed (distilled water)	0.5
Sporekill, 0.02% (v/v)	10
Sporekill, 0.2% (v/v)	10
Sporekill, 2% (v/v)	10

***Effects of fungicidal compounds tebuconazole (Orius), azoxystrobin (Heritage) and fludioxonil (Celest) on* A. niger *var.* phoenicis in vitro**

Plates were prepared in 90 mm diameter Petri dishes by adding quantities of fungicide to autoclaved malt extract agar to achieve a range of concentrations of the active ingredients (Table 6.6). Ten dishes per concentration per fungicide were then inoculated with *A. niger* var. *phoenicis* under aseptic conditions, stored in the dark at ambient temperatures and monitored daily for 14 d for fungal growth. The diameter of colony growth was measured daily for 14 d using a Vernier calliper, and daily averages of 10 colonies plotted. Fungicides used were Celest (active ingredient fludioxonil; Syngenta SA, Johannesburg); Heritage (active ingredient azoxystrobin; Syngenta SA, Johannesburg) and Orius (active ingredient tebuconazole; Makhteshim-Agan SA, Johannesburg). These particular fungicides were selected on the advice of Mr V. Morton (pers. comm.[4]), who has wide experience in the field.

Data were analysed using the ANOVA function of SPSS 13 for Windows.

[4] V. Morton, MortV@aol.com.

Table 6.6. Fungicides and concentration of active ingredients incorporated in the malt extract agar.

Fungicide	Concentration of active ingredient	Active ingredient
Control: malt extract agar	n/a	n/a
Celest	0.33 $g.L^{-1}$	fludioxonil
Celest	0.44 $g.L^{-1}$	"
Celest	0.66 $g.L^{-1}$	"
Heritage	0.8 $g.L^{-1}$	azoxystrobin
Heritage	1.2 $g.L^{-1}$	"
Heritage	1.6 $g.L^{-1}$	"
Celest+Heritage	0.33+0.8 $g.L^{-1}$	fludioxonil+azoxystrobin
Orius	0.5 $g.L^{-1}$	tebuconazole
Orius	0.1 $g.L^{-1}$	"
Orius	0.01 $g.L^{-1}$	"
Orius	0.005 $g.L^{-1}$	"
Orius	0.0025 $g.L^{-1}$	"
Orius	0.00125 $g.L^{-1}$	"

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

Malt extract agar containing the relevant concentrations of fungicide (Table 6.7) was prepared in 90 mm Petri dishes as previously described.

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 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. Plain malt extract agar acted as the control. Results after 14 d monitoring are presented here.

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

Table 6.7. Concentration of fungicidal active ingredients tested on *W. mirabilis* seeds *in vitro*.

Fungicide (a.i.)	Concentration of active ingredient
Celest (fludioxonil)	0.33 $g.L^{-1}$
Celest (fludioxonil)	0.44 $g.L^{-1}$
Celest (fludioxonil)	0.66 $g.L^{-1}$
Orius (tebuconazole)	0.1 $g.L^{-1}$
Orius (tebuconazole)	0.01 $g.L^{-1}$
Orius (tebuconazole)	0.005 $g.L^{-1}$

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 $g.L^{-1}$ 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 115RPM. 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 on malt extract agar (five seeds per plate) to assess fungal contamination. A control set of 40 seeds 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 d are presented here.

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

Combined soaking and* in vitro *treatment of* W. mirabilis *seeds in a solution and on media containing 0.1 g.L^{-1} tebuconazole

Plates were prepared in 90 mm diameter Petri dishes by adding quantities of fungicide to autoclaved 1% water agar and malt extract agar to achieve a concentration of 0.1 g.L^{-1} 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^{-1} solution of tebuconazole, as described above. All seeds were removed from the solution after 3 h and blotted on sterile laboratory paper under a laminar air flow. Fifty seeds were plated onto 1% water agar plates containing 0.1 g.L^{-1} tebuconazole (two seeds per plate), and the remaining 50 on MEA plates (two seeds per plate), also containing 0.1 g.L^{-1} 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 d are presented here.

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

Results

Physical methods

Microwave treatment

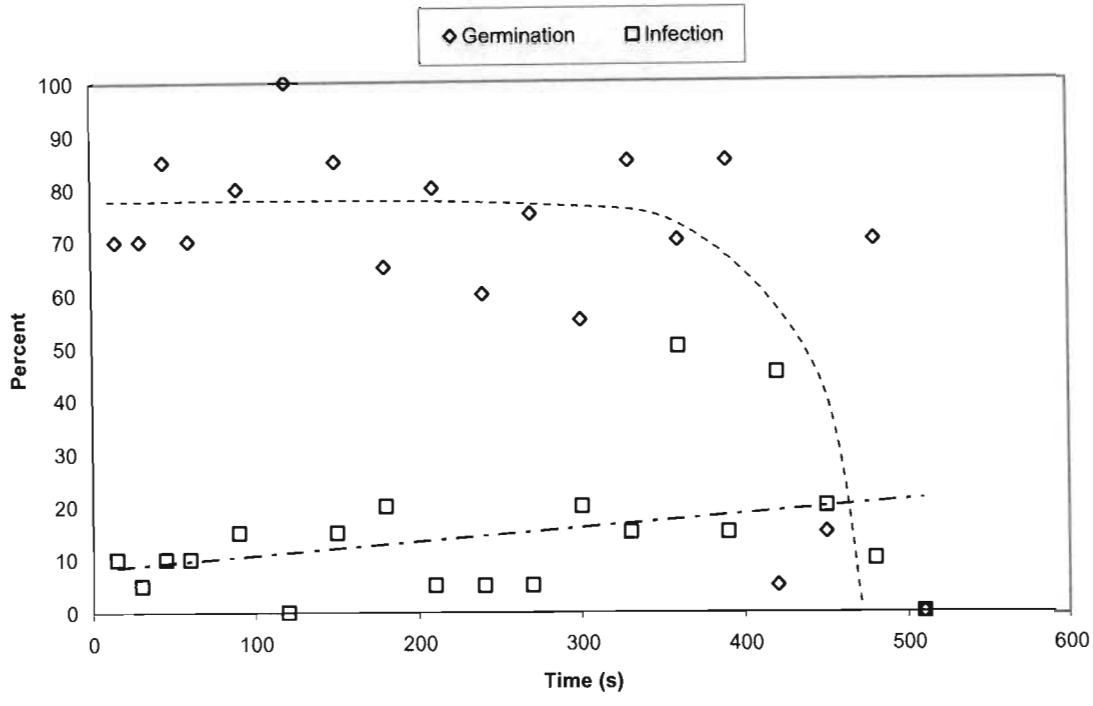

Figure 6.2. Effects of microwave irradiation on germination and fungal infection of seeds from accession HK1249 (n=20).

*Trendlines fitted by Excel.

Seeds from accession HK1249 showed variable (but more than 50%) viability after microwave treatment for up to 6.5 min (390 s), whereafter viability declined sharply (Fig. 6.2). After 8.5 min (510 s) of treatment, none of the seeds had retained viability. The incidence of fungal proliferation of these seeds (by a species of *Penicillium*) increased as treatment time increased, apparently associated with the increasing proportion of dead seeds in any one sample. Seeds from accession HK1322 showed no significant decline in *A. niger* var. *phoenicis* infection throughout the experiment (Fig. 6.3). After 8.5 min (510 s), fungal proliferation remained associated with 95% of seeds from accession 1322.

Microwave treatment was not successful in curtailing the infection of the seeds of *W. mirabilis* by *A. niger* var. *phoenicis*. Significantly, in the dry state, *W. mirabilis* seeds appear somewhat more susceptible to the stress imposed by microwave treatment than the fungus, which is extremely hardy. It is possible that, were the fungal inoculum to be hydrated, it would have become susceptible to damage by the microwave energy. Carefully controlled seed imbibition, for periods that might significantly raise fungal water concentration, but not that of the seed embryo, might theoretically achieve the desired result of elimination of the fungal inoculum. However, in practice, such manipulation of water contents is probably not reliably able to be achieved.

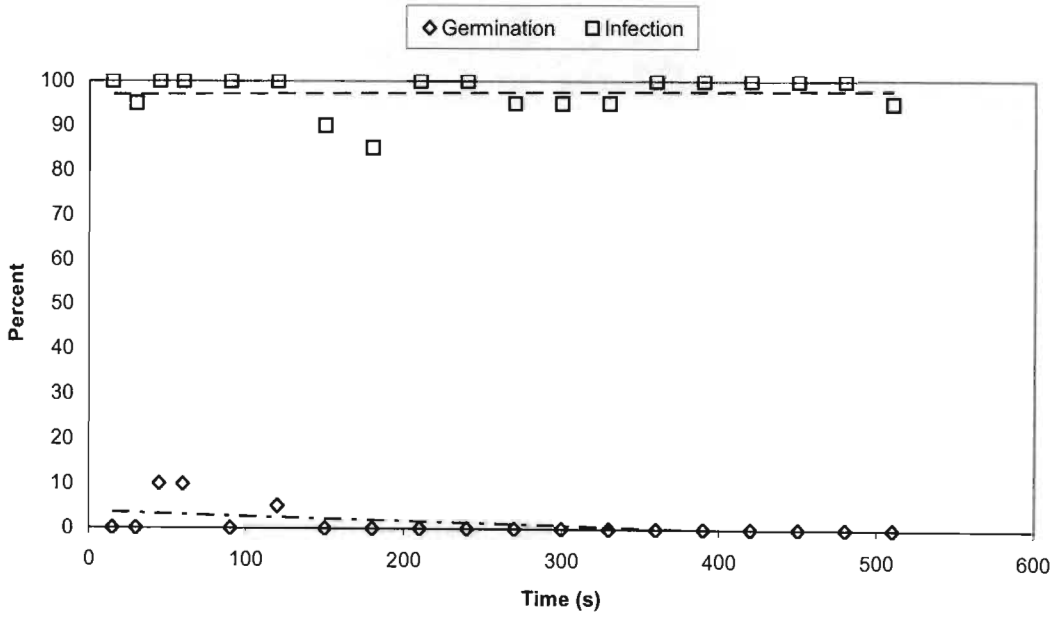

Figure 6.3. Effects of microwave irradiation on germination and infection (by *A. niger* var. *phoenicis*) of seeds from accession HK1322 (n=20).

*Trendlines fitted by Excel.

Dry heat thermotherapy

While treatment at 100°C for 1, 2, 3 and 12 h reduced infection by *A. niger* var. *phoenicis*, the duration of exposure was accompanied by death of the seeds (accession

HK1249) (Table 6.8). Similarly, seed viability was lost after treatment at 90°C for 24 h. Shorter treatment durations at 100°C (4, 6 and 8 min) allowed for higher seed germinability (accession HK1249), but fungal persistence in both accessions was unacceptable. Treatment of seeds for 2 min at 100°C did not appear to affect germination of seeds from accession HK1249 negatively, but fungal persistence was not curtailed for either accession. Germinability of seeds after treatment at 85°C for 12 and 36 h was essentially unaffected (76 and 68% for accession HK1249 respectively); however, infection by *A. niger* var. *phoenicis* was not reduced to acceptable levels (accession HK1322) and an increase in seeds showing proliferation of fungus (accession HK1249) was recorded. This was probably as a result of the debilitation of some lower vigour seeds by the treatment, which allowed the fungus to proliferate more readily.

None of the temperature and time combinations tested was suitable for decontamination of seeds prior, or subsequent to, storage, since acceptable seed germinability was invariably correlated with unacceptably high survival of *A. niger* var. *phoenicis* (see results for 100°C - 2 min and 85°C - 12 and 36 h).

Table 6.8. Germination of, and fungal persistence on/in, *W. mirabilis* seeds after dry heat thermotherapy. Figures recording fungal persistence are presented, with occurrence of *A. niger* var. *phoenicis* specifically, appearing in parentheses. (n=20, unless otherwise stated).

Treatment (°C)	Time (hours or minutes)	Germination (%)		Fungal occurrence (%)	
		HK1249	HK1322	HK1249	HK1322
Control (n=25)		72	0	4 (0)	100 (96)
85	12 h (n=25)	76	0	0	92 (56)
	36 h (n=25)	68	0	24 (0)	88 (56)
90	24 h (n=25)	0	0	0	32 (32)
100	12 h (n=25)	0	0	0	8 (8)
	3 h	0	0	0	0
	2 h	0	0	0	5 (5)
	1 h	0	0	0	5 (5)
	10 min	0	0	5 (0)	100 (100)
	8 min	10	0	25 (0)	100 (100)
	6 min	10	0	15 (0)	100 (100)
	4 min	35	0	5 (0)	100 (65)
	2 min	80	10	20 (10)	100 (90)

Hot water thermotherapy

It was concluded that a suitable balance between eradication of *A. niger* var. *phoenicis* and survival of the *W. mirabilis* seeds might be attained using 10 min of treatment at 70±2°C (Table 6.9). Other treatment temperatures and durations did not produce an acceptable balance between negative effects on germinability, and antifungal activity.

Table 6.9. Trials to ascertain the effects of duration of hot water thermotherapy on germination and fungal persistence of *W. mirabilis* seeds. Figures recording fungal persistence are presented, with occurrence of *A. niger* var. *phoenicis* specifically, appearing in parentheses. (n=20 at each time interval).

Temperature (°C)	Time (min)	Germination (%)		Fungal infection (%)	
		HK1249/50	HK1322	HK1249/50	HK1322
Control (untreated)		100	5	0	100 (100)
65±2	10	45	5	5 (5)	65 (65)
	20	35	0	5 (5)	55 (55)
	30	20	0	10 (10)	15 (15)
	40	0	0	5 (5)	75 (75)
70±2	10	70	0	0	35 (35)
	12	30	0	0	10 (10)
	14	25	0	0	5 (5)
	16	0	0	0	15 (10)
	18	0	0	10 (5)	5 (5)
	20	0	0	0	5 (5)
	30	0	0	0	0
	40	0	0	5 (0)	0
75±2	2	45	0	0	20 (20)
	4	55	0	10 (10)	15 (15)
	6	20	0	15 (15)	10 (10)
	8	10	0	25 (15)	20 (20)
	10	0	0	0	35 (35)

However, repetition of the 70°C/10 min treatment did not show satisfactory reproducibility. In the seven replications of the experiment, 100% contamination (by *A. niger* var. *phoenicis* of seeds from accession 1322) was recorded three times (trials A, B and C), 96% twice (trials D and F), 92% once (trial G), and 12% once (trial E). The results did not appear to be affected by the water used (both sterile [trials A, B, C, E, F and G] and non-sterile [trial D] distilled water was tested), or the number of seeds plated

per Petri dish (the effect on contamination of plating seeds individually [trials E and F] as against five seeds per plate [all other trials] was assessed and found to have no effect). It was concluded that hot water thermotherapy, even without subsequent drying back of the seeds, produced variable results and consequently could not be of practical use for routine disinfection of *W. mirabilis* seeds infected with *A. niger* var. *phoenicis*. However, the experiment was extended to assess the effects of drying back of the seeds after hot water thermotherapy.

Figures 6.4 and 6.5 illustrate the effects of hydration and dehydration on the water concentration of seeds from accessions HK1250 and HK1322 respectively.

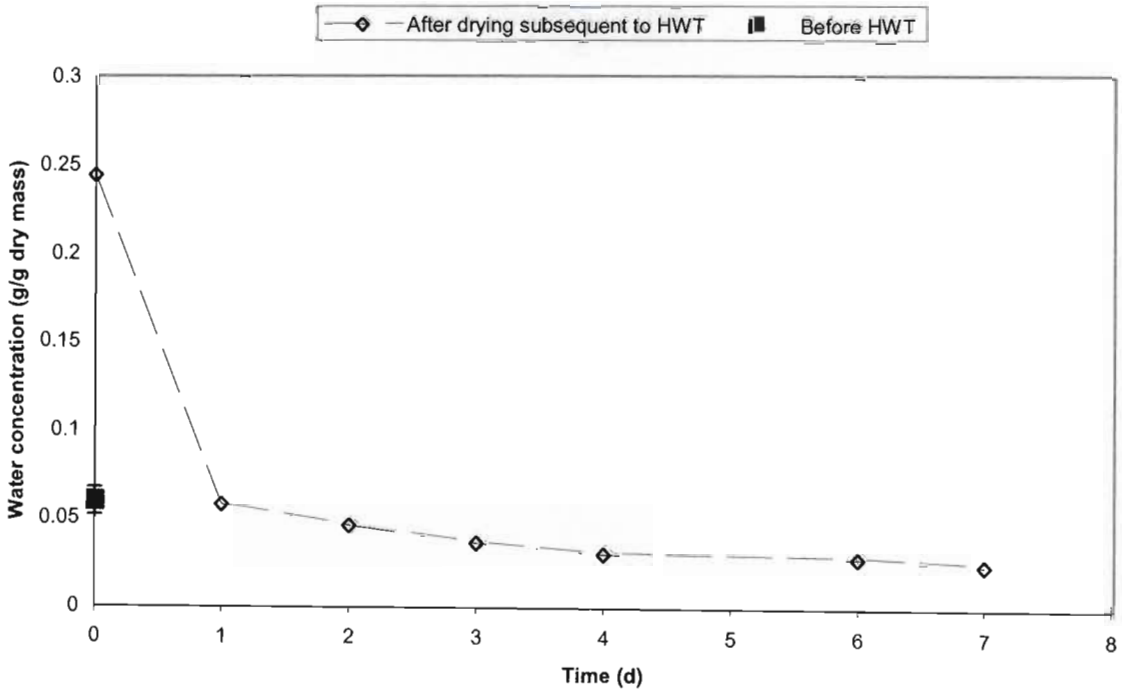

Figure 6.4. Water concentration of seeds from accession HK1250 after hot water thermotherapy (HWT), and subsequent dehydration in silica gel (n=10 at each sampling time).

*Error bars represent standard deviation.

*Error bars for dehydrated seeds were smaller than symbols and are not shown.

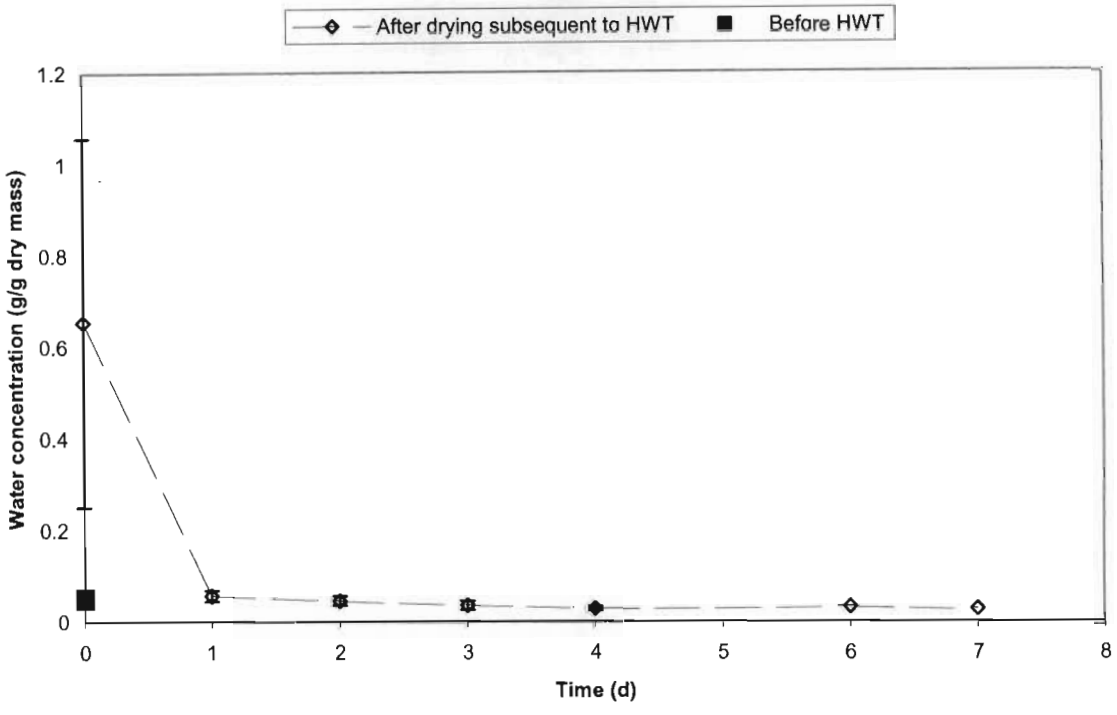

Figure 6.5. Water concentration of seeds from accession HK1322 after hot water thermotherapy (HWT), and subsequent dehydration in silica gel (n=10 at each sampling time).

*Error bars represent standard deviation.

The water concentration of seeds dried for one day subsequent to hot water treatment was not significantly different from that of untreated seeds.

Table 6.10. Effect of hot water thermotherapy (HWT; 70°C, 10 min) followed by dehydration in silica gel (1 day) on germination and fungal infection of seeds from accessions HK1250 and HK1322. Figures recording fungal persistence are presented, with occurrence of *A. niger* var. *phoenicis* specifically, appearing in parentheses. (n=25).

	Germination (%)		**Fungal infection (%)**	
	HK1250	**HK1322**	**HK1250**	**HK1322**
Before HWT	84	8	0	88 (88)
After HWT	32	0	20 (12)	100 (100)
After dehydration (1 d)	36	0	48 (24)	100 (100)

Comparison of the germination of seeds from accession HK1250 shown in Tables 6.9 and 6.10 shows a marked difference after two different hot water thermotherapy trials (70% and 32% respectively). This indicates once again the variable results obtained using this method. Results shown in Table 6.10 indicate that fungal infection was not eradicated in accession HK1322, with isolates from a greater number of seeds from both accessions being obtained in comparison with controls immediately after treatment in this particular trial. Dehydration in silica gel improved germination slightly for seeds from accession HK1250, but not for those from accession HK1322.

Chemical methods

Surface sterilisation of seeds from accession HK1250

Immersion of the intact seed units in 2% NaOCl for 10 minutes did not have adverse effects on germination after removal of the coverings. In contrast, removal of the coverings prior to immersion in NaOCl resulted in the very poor germination totality of 8% (Table 6.11). A reduction in the both the concentration and the time for which the NaOCl was applied to naked seeds resulted in correspondingly less damaging effects in terms of germinability. However, in all cases where NaOCl was used after removal of the seed coverings, germination totality was unsatisfactory compared with the results obtained for seeds decontaminated with this sterilant before removal of the coverings. While NaOCl is the most commonly used surface sterilant for seeds and explants to be cultured *in vitro,* it has been found to be damaging in other instances, for example, in the case of the embryos of another gymnosperm, *Podocarpus henkelii*, [Berjak, pers. comm.[5]], and for seeds of *Warburgia salutaris* (Kioko *et al.*, 2003).

[5] Prof. P. Berjak, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, South Africa

Table 6.11. Effect of various surface-sterilisation treatments of seeds (accessions HK1249 and HK1250) from which the coverings had been removed (*except in the case indicated) on germination and fungal persistence. (n = 25).

Treatment	Germination (%)	Seeds infected (%)	Fungus isolated
Sterile distilled water (control), 10 min	76	8	*Penicillium crustosum*
NaOCl, 2%, 10 min (coverings intact*, but removed after treatment)	72	4	*Aspergillus niger* var. *phoenicis*
2%, 10 min	8	4	*P. crustosum*
1%, 5 min	28	20	*P. crustosum*
		4	unidentified
0.35%, 5 min	52	20	*P. crustosum*
		4	unidentified
$Ca(OCl)_2$ – 1%, 2 min	64	8	unidentified
Ethanol – 70% - 2 min	40	4	unidentified
$HgCl_2$ - 0.02%, 30 sec	68	0	
Sporekill - 0.02%, 5 min	64	0	
10 min	76	0	

Of the range of other surface sterilants used on seeds of *W. mirabilis* after removal of the coverings, 70% ethanol emerged as unsatisfactory (germination totality 40%), but germination totalities in excess of 60% were recorded after application of 1% calcium hypochlorite [$Ca(OCl)_2$] or 0.02% mercuric chloride ($HgCl_2$). However, the best results both in terms of germination totality and apparently complete elimination of *Penicillium crustosum* and another (minor) unidentified fungus, were obtained by the 10 min application of the commercial fungicide, Sporekill (Table 6.11).

Surface sterilisation of seeds from accession HK1322

Almost all seeds from accession HK1322 showed macroscopically visible contamination/infection by *A. niger* var. *phoenicis* and were non-viable. The seeds were treated as outlined in Table 6.12. The NaOCl treatments were excluded, as these had previously been found to be detrimental to seed viability. Three concentrations of

Sporekill were tested against the seeds, and 100% as well as 70% ethanol was used. Treatment with $HgCl_2$ produced a small decrease in the number of seeds infected, but none of the other treatments showed any satisfactory effect (Table 6.12).

Table 6.12. Effect of various surface-sterilisation treatments of seeds (accession HK1322) from which the coverings had been removed on germination and fungal persistence. (n = 25).

Treatment	Germination (%)	Seeds infected (%)	Fungus isolated
Sterile distilled water (control); 10 min	4	92	*A. niger* var. *phoenicis*
$Ca(OCl_2)$ – 1%, 2 min	0	100	*A. niger* var. *phoenicis*
Ethanol – 100% - 2 min	0	100	*A. niger* var. *phoenicis*
Ethanol – 70% - 2 min	0	92	*A. niger* var. *phoenicis*
$HgCl_2$ - 0.02%, 30 sec	0	84	*A. niger* var. *phoenicis*
Sporekill – 0.02%, 10 min	0	100	*A. niger* var. *phoenicis*
Sporekill – 0.2%, 10 min	0	100	*A. niger* var. *phoenicis*
Sporekill – 2%, 10 min	0	100	*A. niger* var. *phoenicis*

***Effects of tebuconazole (Orius), azoxystrobin (Heritage) and fludioxonil (Celest) on* A. niger *var.* phoenicis in vitro**

Tebuconazole and fludioxonil were found to inhibit the growth of *A. niger* var. *phoenicis*, while azoxystrobin, at the concentrations tested, was not found to have a comparable effect (Figs 6.6C, B and A, respectively). Tebuconazole at concentrations of 0.5, 0.1 and 0.01 $g.L^{-1}$ completely inhibited the growth of the fungus and the results are therefore not presented graphically as an individual figure. However, an overview of the effects of all treatments (in cases in which some fungal proliferation occurred) is shown graphically (Fig. 6.6D) from which it may be concluded that 0.005 $g.L^{-1}$ tebuconazole was the most inhibitory fungicidal treatment, compared with the highest concentrations of the other two fungicides.

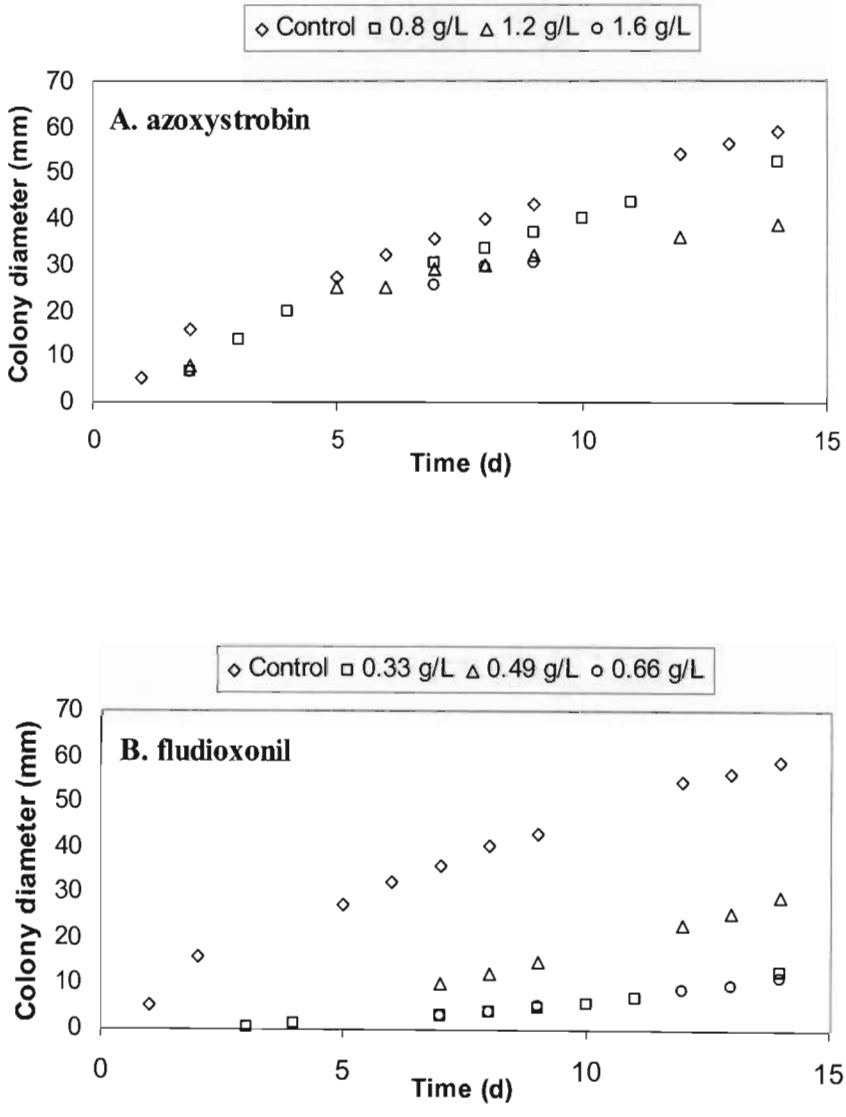

Figure 6.6. A and B show the effects of various concentrations of azoxystrobin and fludioxonil respectively on diameter of *A. niger* var. *phoenicis* colonies (Fig. 6.6 continues overleaf).

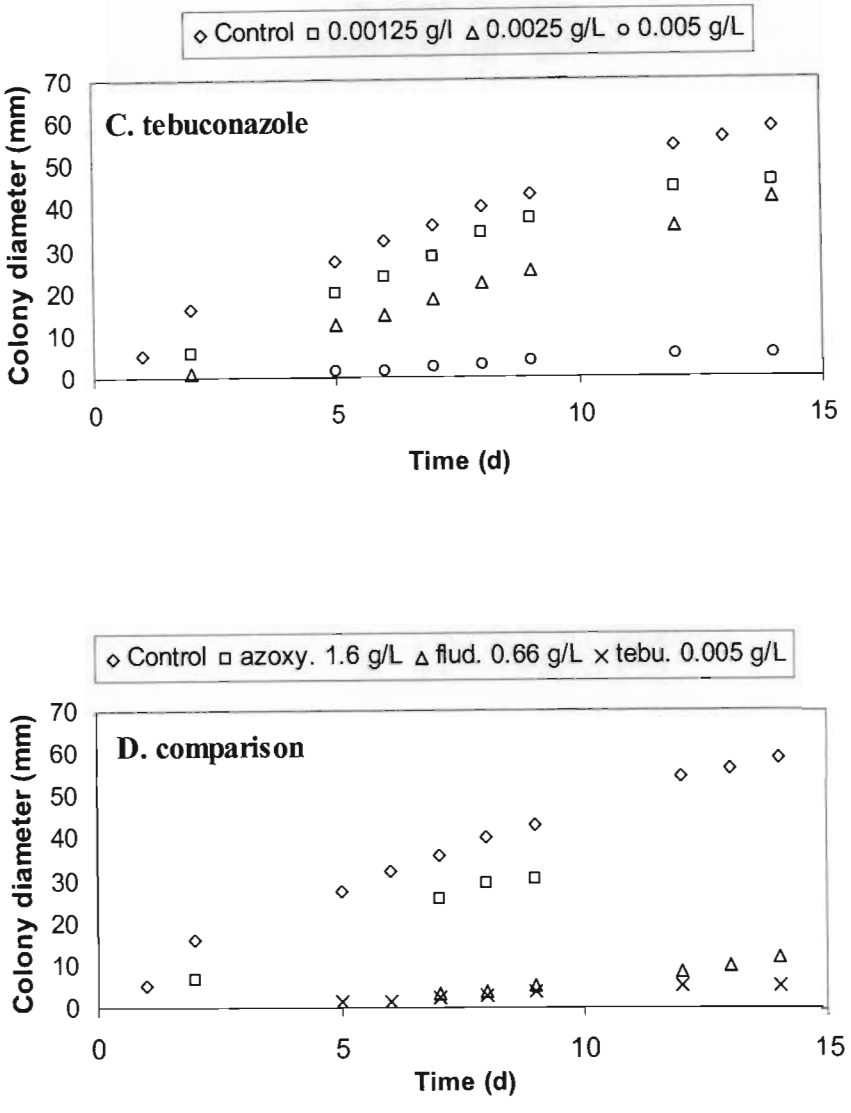

Figure 6.6. C and D show the effects of various concentrations of tebuconazole on diameter of *A. niger* var. *phoenicis* colonies, and a comparison of the diameter of colonies produced by *A. niger* var. *phoenicis* as affected by the highest concentrations of each of the fungicides, respectively.

*Abbreviations: tebu. = tebuconazole; flud. = fludioxonil; azoxy. = azoxystrobin.

The effects of the highest concentrations of each fungicide were statistically analysed, using the ANOVA function of SPSS 13. Analysis was carried out after 9 d since multiple colonies were produced on media containing azoxystrobin after this time, resulting in

interaction effects on colony growth. The results indicated that all fungicidal treatments were significantly different from the control ($p<0.05$). Treatment with 1.6 $g.L^{-1}$ azoxystrobin resulted in colony growth that was significantly different from that produced on media containing 0.66 $g.L^{-1}$ fludioxonil and tebuconazole (all concentrations) ($p<0.05$), but no significant difference existed between the tebuconazole treatments (0.005, 0.01, 0.1 and 0.5 $g.L^{-1}$) and the 0.66 $g.L^{-1}$ fludioxonil treatment ($p>0.05$). It is possible that the latter effect arose from the large standard deviation in colony growth on media containing fludioxonil (SD = 9.863).

Because there was a shortage of viable seeds, only selected concentrations of fungicide could be tested against seeds. Tebuconazole at concentrations of 0.1, 0.01 and 0.005 $g.L^{-1}$ and fludioxonil at concentrations of 0.33, 0.49 and 0.66 $g.L^{-1}$ were selected for *in vitro* tests on viability retention of seeds of *W. mirabilis* from accession HK1603.

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 ($\chi^2 = 0.888$, df = 6, $p>0.05$) (Fig. 6.7).

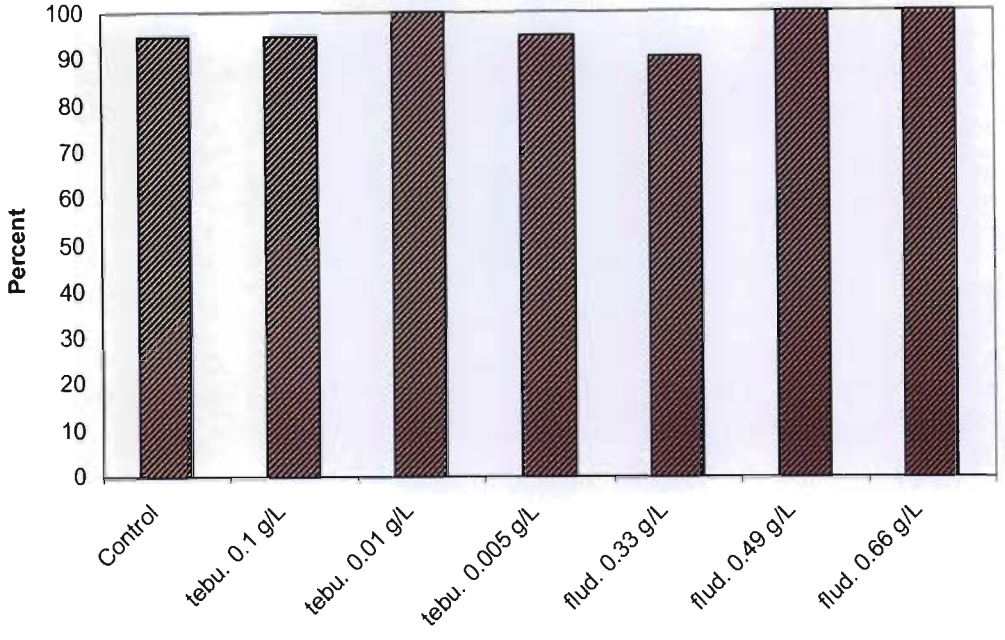

Figure 6.7. Germination of seeds on media (MEA) containing various concentrations of fungicide (n=20).

*Abbreviations: tebu. = tebuconazole; flud. = fludioxonil.

Fungal proliferation was halved from 90% of seeds (control set) to 45% in the 0.1 $g.L^{-1}$ tebuconazole treatment set (Fig. 6.8), and the inhibitory effect of this compound on the fungi declined as the concentration decreased. The inhibitory effect of fludioxonil was constant, regardless of the concentration (65 – 70% of seeds infected; Fig. 6.8). The fungicides exerted a significant effect on infection of seeds ($\chi^2 = 18.602$, df = 6, $p<0.05$). It should be noted that the concentrations of tebuconazole differed by a factor of 10 from one treatment to another, whereas that of fludioxonil increased stepwise by half.

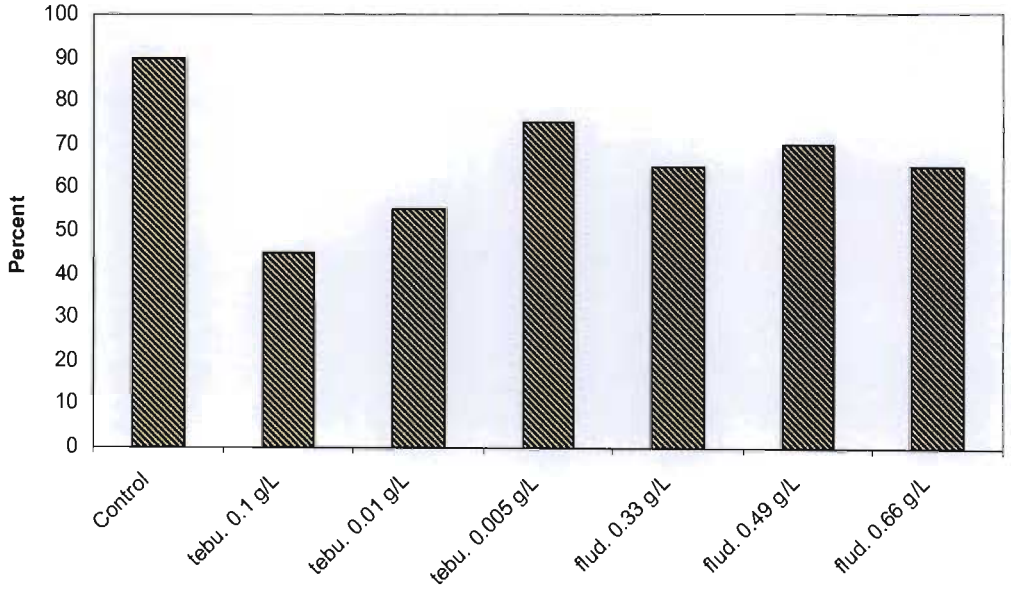

Figure 6.8. Infection of seeds by *A. niger* var. *phoenicis* on media (MEA) containing various concentrations of fungicide (n=20).

*Abbreviations: tebu. = tebuconazole; flud. = fludioxonil.

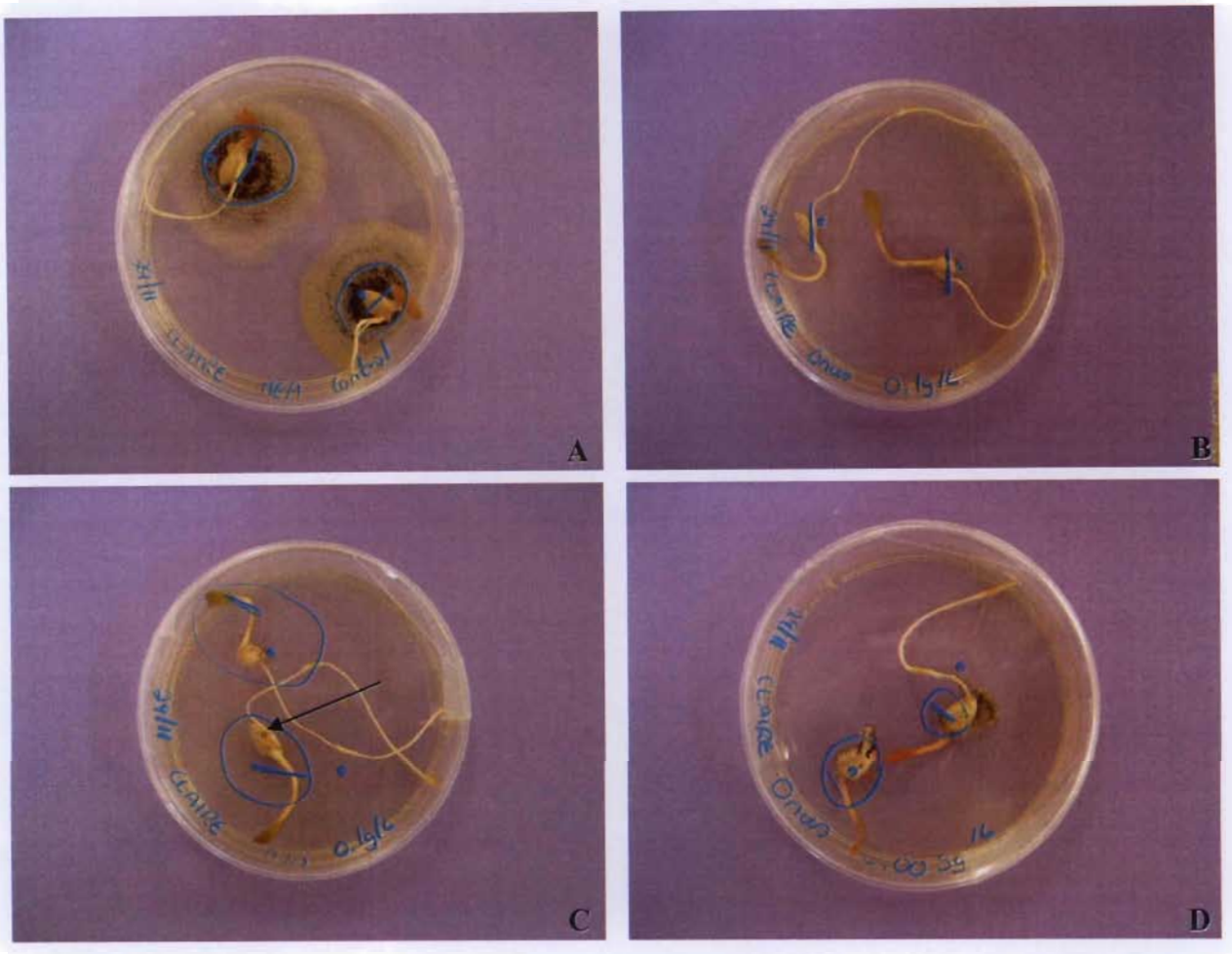

Figure 6.9. The effects of incorporation of tebuconazole in MEA plates, on germination and proliferation of *A. niger* var. *phoenicis*. A) *W. mirabilis* seeds infected with *A. niger* on control medium. B) Seeds on medium containing 0.1g.L^{-1} tebuconazole - no infection is visible. C) Infection limited to points (arrowed) of the seed with the least contact with the medium containing 0.1g.L^{-1} tebuconazole. D) *A. niger* infection was extensive on seeds treated with 0.005g.L^{-1} tebuconazole.

Tebuconazole at 0.1 g.L^{-1} was the most effective treatment as infection was reduced (Fig. 6.8) and the fungicide did not adversely affect germination of the seeds (Fig. 6.7). Fungal proliferation was restricted to points of the seed furthest from contact with the medium (Fig. 6.9C). The potential of tebuconazole to reduce infection further was assessed by soaking the seeds in a solution of the fungicide – effects of this treatment are presented below.

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

Soaking of the seeds from accession HK2031 in a 0.1 $g.L^{-1}$ solution of tebuconazole for 1 h improved viability from 80% to 100% (Fig. 6.10). Concomitantly, infection of the seeds was reduced by nearly half, from 100% of seeds to 55% after 1 h. Germination was reduced essentially to the level of untreated seeds after longer soaking periods (75% after 2 h), while 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) (Fig. 6.10). Germination was not significantly affected by the soaking treatment (χ^2 = 4.402, df = 3, $p>0.05$), while the effects on infection were found to be very significant (χ^2 = 56.086, df = 3, $p<0.05$).

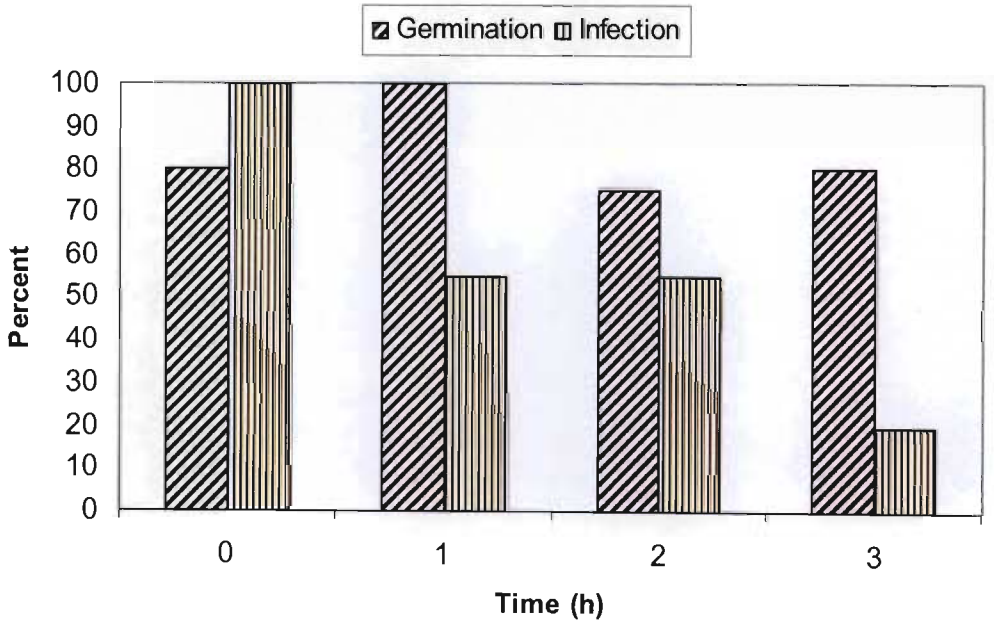

Figure 6.10. Germination and fungal infection of *W. mirabilis* seeds on 1% water agar and MEA respectively, after 14 d incubation following soaking in a 0.1 $g.L^{-1}$ tebuconazole solution for 0, 1, 2 or 3 h (n=20).

Combined soaking and* in vitro *treatment of* W. mirabilis *seeds in a solution and on media containing 0.1 g.L^{-1} tebuconazole

Soaking of seeds from accession HK2031 for 3 h in a 0.1 g.L^{-1} solution of tebuconazole, followed by plating of the seeds on media containing the same concentration of the fungicide, resulted in a decrease in infection by fungi from 100% of seeds in the control group to 8% of seeds. A combined figure is presented for comparison of results obtained for various modes of treatment using 0.1 g.L^{-1} tebuconazole (Fig. 6.11).

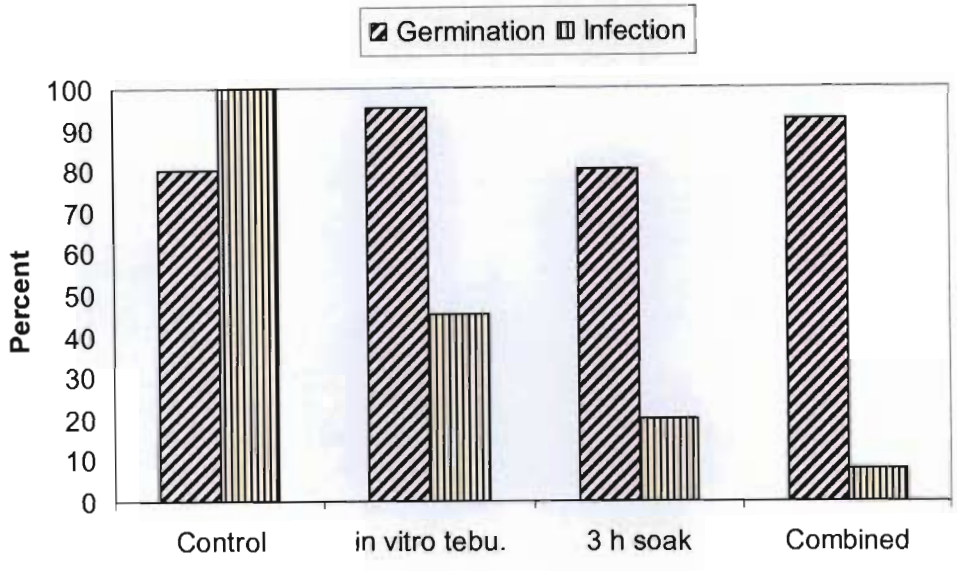

Figure 6.11. Germination and fungal infection of *W. mirabilis* seeds after 14 d incubation following various treatments with 0.1 g.L^{-1} tebuconazole (n=20, except for combined treatment, where n=50).

*Note that seeds utilised in the *in vitro* trial were from accession HK1603, all others from accession HK2031.

It should be noted that infection in all cases other than the combined treatment resulted from exclusively *A. niger* var. *phoenicis* infection. However, infection following the combined treatment was exclusively a species tentatively identified as *A. flavus*, no *A. niger* var. *phoenicis* being noted at all. The appearance and proliferation of *A. flavus* was possibly a consequence of the removal of inter-specific competition between the fungal

species. The effects of treatments on infection were found to be highly significant (χ^2 = 115.763, df = 3, $p<0.05$).

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

Discussion

The spores of *Aspergillus niger* var. *phoenicis* appear to be extremely resistant to microwave irradiation, dry and wet heat thermotherapy and most chemical treatments and fungicides, except tebuconazole.

The survival of seeds of *Welwitschia* under the microwave conditions imposed here is, in itself, remarkable, the seeds showing high viability retention up to treatment times of 6.5 min (Fig. 6.2). Viability of sorghum grains was reduced to zero, and that of soybean seeds much reduced, after only 60 s at the highest power levels tested by More *et al.* (1992) and Reddy *et al.* (2000), respectively. However, the spores of *A. niger* var. *phoenicis* showed no such decline in vigour, and viable inoculum remained associated with 95% of seeds after 8.5 min treatment (Fig. 6.3). It is possible that the internal location of the inoculum within the remains of the seed tissue protected it from damage (Fig. 6.12), although this survival is not without precedent. The spores of Aspergilli have been found to survive the extremes of outer space, under conditions of extreme UV and proton irradiation (Imshenetsky *et al.*, 1979; Koike *et al.*, 1992; Koike and Oshima, 1993). The melanin present in the spore walls of *A. phoenicis* (syn. *A. niger* var. *phoenicis*) is thought to be responsible for their resistance to lysis by other microorganisms, and for their prolonged persistence in harsh natural environments (Bloomfield and Alexander, 1967), as well as for their resistance to ultra-violet (Imshenetsky *et al.*, 1979) and (presumably) other forms of irradiation, as shown here.

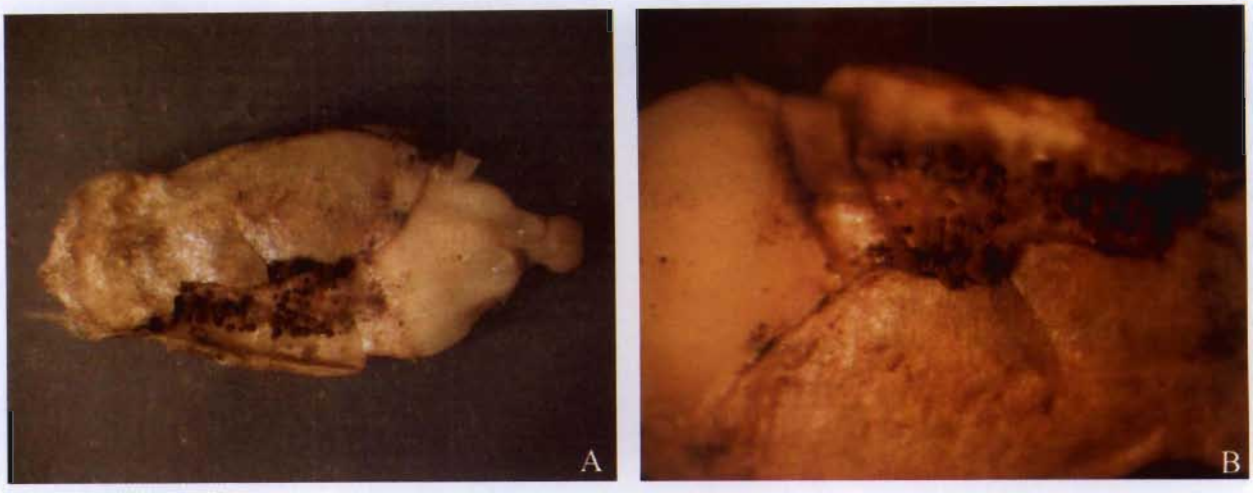

Figure 6.12. A) Conidiophores of *A. niger* var. *phoenicis* emerging from the interior of a *W. mirabilis* seed (HK1322) (x6), and B) A magnified view of the conidiophores (x16).

Dry heat thermotherapy has been successfully employed to eradicate *Fusarium graminearum* from wheat and barley seed (after 5 d at 80°C), while *Cochliobolus sativus*, *Pyrenophora teres* and *Pyrenophora tritici-repentis* could not be eliminated from the seeds at treatment temperatures up to 70°C (Clear *et al.*, 2002). Viability of barley seeds was negatively affected by treatment at 80°C for 5 d, whilst that of wheat was not (Clear *et al.*, 2002). *Colletotrichum lupini* was eliminated from *Lupinus angustifolius* seed after 1 d at 80°C (Thomas and Adcock, 2004), whilst *A. niger* var. *phoenicis* could not be eliminated from *W. mirabilis* seeds after 36 h at 85°C (Table 6.8) illustrating the heat-resistance of the spores and possibly also other fungal structures. *Aspergillus niger* var. *phoenicis* inoculum could be reduced or eradicated from *W. mirabilis* seeds only after treatment at 100°C for between 1 and 12 h, which rendered the seeds non-viable. The extreme resistance of the fungus to dry heat thermotherapy posed a problem in that the seeds were comparatively more sensitive to the treatment (losing viability after only 10 min at 100°C – Table 6.8). A balance between fungal elimination and seed viability could not be found using this approach.

Hot water thermotherapy initially showed promise as a fungicidal treatment (Table 6.9). However, the fungicidal effects of treatment at 70°C for 10 min could not be successfully

replicated, and it was concluded that hot water thermotherapy was not suitable for routine decontamination of *W. mirabilis* seeds harbouring *A. niger* var. *phoenicis*. *Aspergillus niger* infection of onion seeds has previously been controlled by hot water thermotherapy at 60°C for 15 min, with no attendant negative effects on germination (Hayden and Maude, 1992). However, given the results reported in Table 6.9 for effects of treatment at 65°C on fungal infection (65% infection of seeds from accession HK1322 after 10 min), it appears unlikely that a similar regime would have been successful in this case. Treatment of soil isolates of members of the *A. niger* group at 70°C for 10 min on Sabouraud agar resulted in their eradication; however, other members of the Aspergilli were found to tolerate treatment at 90°C for 10 min (Jesenská *et al.*, 1993). In the present case, *A. niger* var. *phoenicis* remained associated with 35% of seeds from accession HK1322 after 10 min treatment at 70°C; complete eradication was achieved only after 30 min treatment at that temperature, at which point the seeds had lost viability (Table 6.9). It is possible that the efficacy of both dry and wet heat thermotherapy is limited by the internal location of the inoculum in *W. mirabilis* seeds, which affords it some protection, as well as the apparently extraordinary hardiness of the particular variety, var. *phoenicis*, infecting those seeds.

Immersion of the intact seed units in 2% sodium hypochlorite (NaOCl) for 10 min did not have adverse effects on germination after removal of the coverings. In contrast, however, if the coverings were removed prior to immersion in NaOCl the seeds responded extremely poorly, with germination totality being reduced to 8%. Reduction in the concentration of NaOCl and the time for which the sterilant was applied to the naked seeds was correspondingly less damaging in terms of germination, but paradoxically, fungal proliferation was associated with 20% of the seeds (Table 6.11), the predominant fungus being *Penicillium crustosum*. These results are difficult to explain, in view of the fact that *P. crustosum* was associated with only 8% of the seeds immersed in distilled water for 10 min (the control), but may be the outcome of the small number of seeds per sample (25) available for these trials.

In all cases where NaOCl was used after removal of the seed coverings, germination totality was unsatisfactory. Of the range of other surface sterilants used on seeds of *W. mirabilis* after removal of the coverings, 70% ethanol emerged as unsatisfactory (germination totality 40%), but germination totalities in excess of 60% were recorded after application of 1% calcium hypochlorite [$Ca(OCl)_2$] or 0.02% mercuric chloride ($HgCl_2$). However, the best results both in terms of germination totality and apparently complete elimination of *P. crustosum* and a minor (unidentified) fungus, were obtained by the 10 min application of the commercial fungicide, Sporekill (Table 6.11).

Seeds of accession HK1322 showed a macroscopically visible aggregation of *A. niger* var. *phoenicis* spores on the surface of the coverings. In this case, Sporekill was equally ineffective, whether applied at concentrations of 0.02, 0.2 and 2.0%, with the fungus surviving on all the seeds which were treated after removal of the coverings (Table 6.12). These results indicated not only that use of this specific fungicide is ineffectual when *A. niger* var. *phoenicis* is the predominant species, but also that the fungus had penetrated (or inoculum was also situated below) the seed coverings at harvest. Despite the fact that Sporekill is claimed to be effective against *Aspergillus* spp. by the manufacturers, this is obviously not so in the case of the *A. niger* var. *phoenicis*.

Hayden and Maude (1992) suggested that surface sterilants are of limited use in cases in which *A. niger* inoculum is internally located, and that chlorine-based sterilants, in particular, were of limited use in cases of *A. niger* contamination. Thus the use of systemic fungicides and/or soaking treatments which facilitate the uptake of the active ingredient are indicated.

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.*, in press), and has been shown here to eliminate a substantial proportion of the infection from *Welwitschia mirabilis* seeds, when applied at a rate of 0.1 $g.L^{-1}$ as a combined treatment. Infection was reduced from 90% to 45% by inclusion of tebuconazole in the medium (Fig. 6.8), without affecting germination (Fig. 6.7). Whilst

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. 6.10). 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 substantial decrease in seed infection (from 100% to 8%) was achieved by the combination of a 3 h soak in 0.1 $g.L^{-1}$ tebuconazole, followed by germination/incubation of seeds on a medium containing the same concentration of fungicide (Fig. 6.11). This treatment eradicated *A. niger* var. *phoenicis* from the seeds, which 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 were un-germinated. 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.* for wheat leaves (in press). 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. The effects of pre-infectional application could not be tested in the context of this 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. As explained in a preceding chapter, *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.

Aspergillus niger is a cosmopolitan species, with global distribution in soil, occurring in humid tropical areas, arid savannah, and cold mountainous zones (Griffin, 1972), to which may be added the hot desert. *Aspergillus niger* is categorised among the mesophilic fungi by Maheshwari *et al.* (2000), who indicated that the temperature optimum for this species is 30°C. Those authors also showed that the oxygen uptake rates of mesophilic fungi (*A. niger* and *A. phoenicis*) were largely unaffected by changes in temperature between 15 and 40°C, and concluded that these fungi would be better adapted to life in the soil (where temperatures vary both spatially and temporally) than thermophilic fungi. One may conclude that the spores and vegetative structures of this fungus possess the ability to resist a wide range of environmental conditions. The mechanisms which enable survival of this fungus are presumably also responsible for its resistance to antifungal treatment. This would appear to apply particularly to the species isolated from *Welwitschia mirabilis*, which could have evolved greater hardiness than species from more mesic environments.

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CHAPTER 7
MISCELLANEOUS OBSERVATIONS AND FINDINGS

During the course of some four years study, several interesting observations have been made on the physiology and fungal contaminants of the seeds of *Welwitschia mirabilis*.

Twin embryos

Twin embryos were thought to be quite rare in *W. mirabilis* (Pearson, 1910). During this study sixteen seeds which produced twin seedlings were observed. Two of the seeds were photographed and dissected (see Fig. 7.1A-D). In one of the seeds, a single radicle from which two plumules emerged was observed (Fig. 7.1A). However, the smaller of the two plumules was shown to have a small radicle which was obscured by the seed tissues (Fig. 7.1B). This arrangement is in accordance with the observations of Pearson (1910), who concluded that a smaller embryo may be produced from a pre-existing one by lateral branching. In the other case, twin radicles could clearly be seen emerging from the seed tissues along with twin sets of cotyledons (Fig. 7.1C). In this seed, a y-shaped suspensor was observed (Fig. 7.1D). Pearson (1910) stated that no instances of twin embryos being produced from branching of the suspensor in *Welwitschia* had been observed at the time of his paper; rather all observed twinning was thought to be the result of bifurcation or lateral branching of the embryo. Additionally, Pearson (1910) noted that "there appears to be no record of the presence of more than one embryo in the mature seed of *Welwitschia* ... all but one sooner or later are crowded out of existence." Thus the current observation is unique. The majority of the twin seedlings observed originated from HK1249 (10 of 16), with the remaining 6 split equally between accessions HK1250 and HK2031. Whilst differences in the size of the samples could potentially bias conclusions (far more seeds were available from accession HK1249 than HK2031), it is possible that a genetic influence is operational, and that twins are more often found in certain localities than in others. Pearson (1906) noted that plants growing in close contact formed "natural grafts". In some cases, both male and female plants were found in a single graft (Pearson, 1906), however, the close association of several plants of the same gender is suggestive of their having developed from twin embryos. Some of the clumps

noted were thought to consist of more than two plants, and twinning in this case could be ruled out.

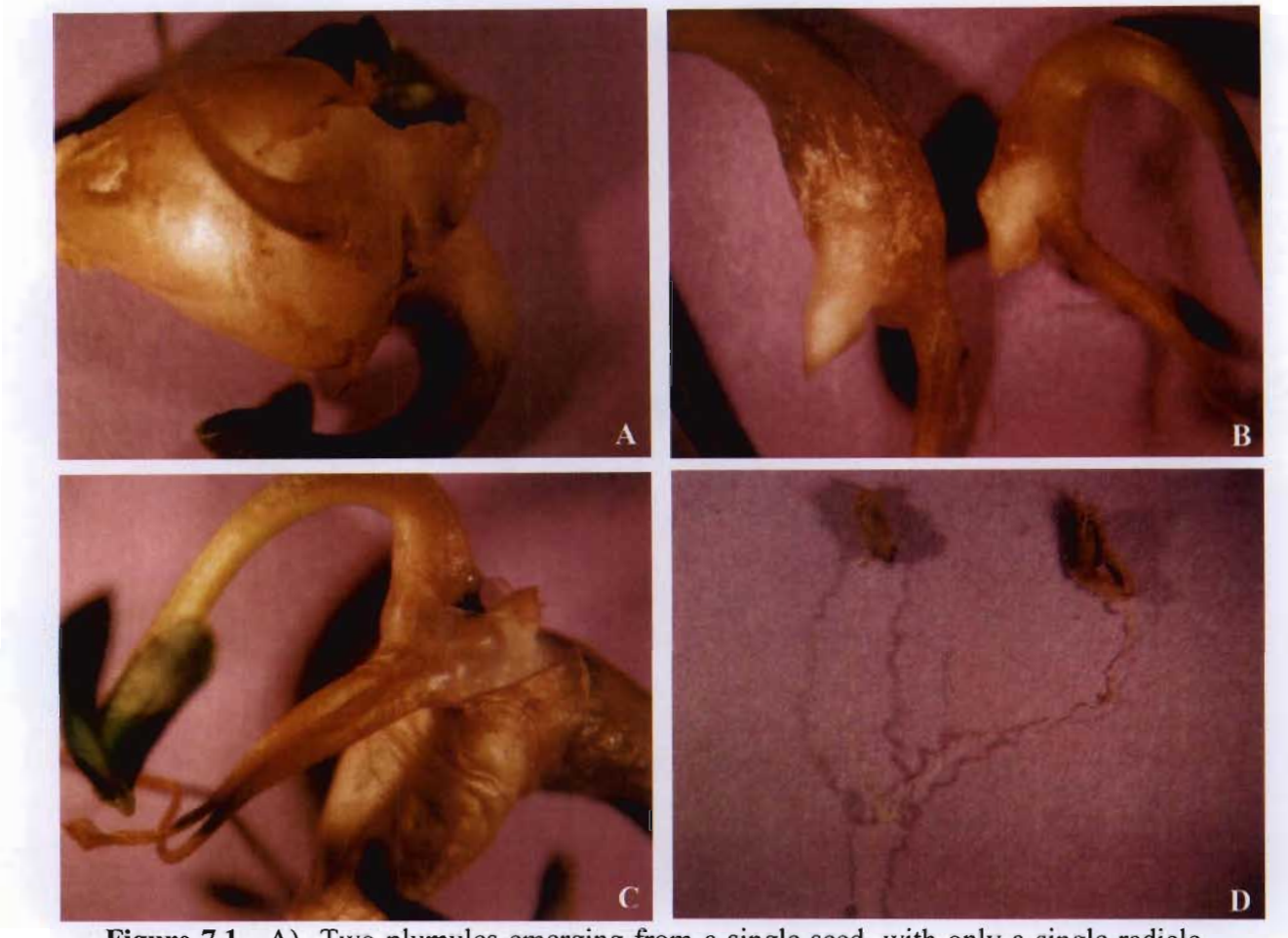

Figure 7.1. A) Two plumules emerging from a single seed, with only a single radicle visible. B) After dissection, the smaller of the two seedlings in A was shown to possess a radicle. C) Twin sets of plumules and radicles emerging from a single seed. D) The Y-shaped suspensor dissected from the seed in C. (All x6).

Achlorophyllous seedlings

Eight instances of seedlings producing achlorophyllous cotyledons (Fig. 7.2) were noted. All 8 originated from accession HK1249.

The cotyledons of *Welwitschia mirabilis* are usually red upon emergence from the gametophyte tissue, and green as germination progresses. In the case of achlorophyllous cotyledons, no pigment was evident upon emergence from the surrounding seed tissues, and cotyledons had not greened upon completion of germination monitoring (after 21 d).

Figure 7.2 . An achlorophyllous seedling and a normal seedling, 21 d after plating on 1% water agar.

The seedlings were not etiolated, and emerged in the same Petri dishes as normal seedlings – an effect of light may be ruled out in this instance. No such abnormal colouration has previously been reported, and it is presumed that these seedlings may represent an achlorophyllous mutant state.

Abnormal foliation

Two distinct types of abnormal foliation patterns have previously been reported from the area south and west of the Brandberg massif (von Willert, 1993). In the first type, the plant produces a second pair of permanent foliage leaves, which emerge from the stem apex. In the second type, a single, narrow leaf emerges between the bases of old

inflorescences, and was suggested to arise from an inflorescence bud which "became transduced to leaf meristem" (von Willert, 1993).

A single seedling (HK1249) showing an abnormal foliation pattern of the cotyledons was observed. The seedling appeared to have three cotyledons (see Fig. 7.3), two of the cotyledons were directly opposed while the third was smaller in size and grew from the outer edge of the seedling stem. In addition, a fourth cotyledon appeared to emerge directly from the gametophyte tissue.

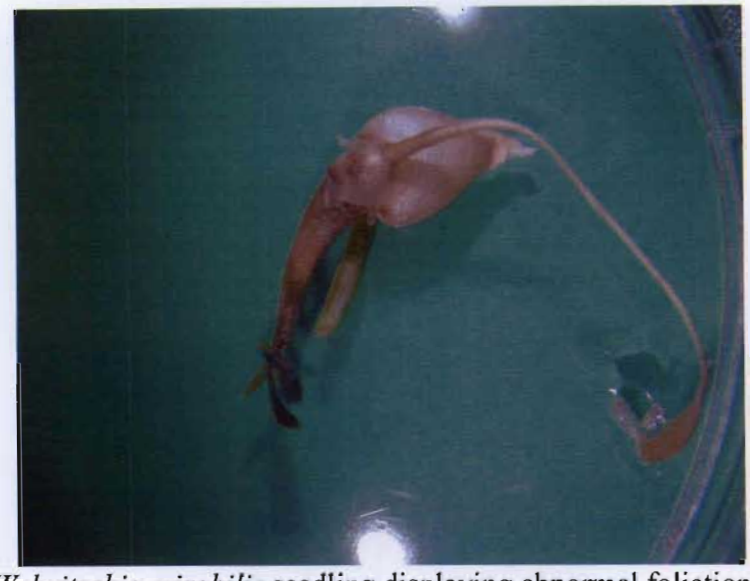

Figure 7.3. *Welwitschia mirabilis* seedling displaying abnormal foliation patterns.

Whilst the foliation pattern does not appear to accord with either of the descriptions of von Willert (1993), the seeds (HK1249) were sourced from a similar locality to that described (south and west of the Brandberg). Von Willert was of the opinion that the concentration of plants presenting abnormal foliation in this area possibly represented a genetic influence. No other similar foliations were noted throughout the present study.

Fungi

The most commonly isolated fungal species were identified by the Plant Protection Research Institute, Pretoria, South Africa. The most common species overall was *Aspergillus niger* var. *phoenicis* (Corda) Al-Musallam, although the prevalence of this species differed from accession to accession. *Aspergillus niger* var. *phoenicis* was not

commonly isolated from accessions HK1249 and HK1250, but was macroscopically visible in association with seeds from HK1322. Minor species isolated from accession HK1249 included *Pithomyces atro-olivaceus* (Cooke and Harkness) M.B. Ellis, *Curvularia ovoidea* (Hiroe and Watan) Muntañola *sensu* M.B. Ellis, and two previously unknown species of non-plurivorous *Cladosporium*. It was suggested by members of the Plant Protection Research Institute that the *Cladosporium* species were probably unique to *W. mirabilis*. *Penicillium* and *Rhizopus* spp. were very occasionally noted.

References

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CHAPTER 8
CONCLUDING COMMENTS

Conservation of the seeds of *Welwitschia mirabilis* may be effectively achieved through their storage at the shedding water content at temperatures of 5°C and below. Conventional recommendations for germplasm conservation suggest that seeds be stored at -18°C or lower (Genebank Standards, 1994), and no obvious injurious effects were observed at -20°C or -196°C. To the contrary, positive effects on germination were recorded for seeds frozen in liquid nitrogen. Storage for up to two years at both 5.5 and -20°C showed no negative effects on either water concentration or germination. Interestingly, nine unused seeds from the batches stored for two years at -20°C were kept in storage at 5.5°C for a further year, and then germinated on soil in a sterile greenhouse. As of writing, five seedlings are four months old and apparently healthy.

The potential of *W. mirabilis* seeds to be used in ultra-dry storage experiments is promising, as harsh drying at high temperatures to very low water concentrations did not adversely affect germination, even after storage for two years. Further research in this area is recommended, as the seeds may provide an ideal model system for further elucidation of the effects and usefulness of ultra-dry storage.

Research in the field has shown that the seeds of *W. mirabilis* were infected by *A. niger* var. *phoenicis* at the earliest stages of development, through the agency of insects and climatic conditions such as wind and rain-splash. Infection peaked in February, coincident with extremely high air temperatures and rainfall, as well as the presence of the pollination drops. These pollination drops evidently trap spores and provide a route to the interior of the developing seed, where extensive proliferation occurs, resulting in the destruction and digestion of the seed tissues. The conidiophores emerge from the seed tissue, through the seed coverings. Since members of the *A. niger* group are considered typical members of the soil microflora (Griffin, 1972), particularly in deserts (Lacey, 1981), the presumptive primary reservoir of the inoculum is the soil surrounding the plants. From this reservoir, inoculum is transferred to nearby plants, including *W.*

mirabilis. The presence of large quantities of decaying leaf and cone material immediately under the canopy of mature plants provides a medium upon which the fungus proliferates in abundance. Results show a trend towards increasing average colony forming unit load within the area immediately surrounding a plant, and the size of the plant – the larger the plant, the greater the average number of cfu per gram dry soil in the surrounding soil. However, this trend requires further investigation, since sample sizes in this study were of necessity small. Also of further interest would be an investigation of the fungal contamination of the male cones, and capture and assessment for contamination of a wider range, and larger number, of insects.

It could be conjectured that the life-history of a *W. mirabilis* plant would begin with the production of seeds from an individual carrying a low *A. niger* var. *phoenicis* burden. The factors governing this infection from year to year cannot yet be predicted. This would coincide with the shedding of the seeds during a year of above average rainfall, or a single extraordinary rainfall event, providing the seeds with sufficient moisture to germinate, and sustaining the seedlings until they have developed a degree of water-stress tolerance. Some degree of abrasion of the outer seed coverings by blowing sand may be necessary in order to break dormancy. Since such rainfall events are isolated and rare, no advantage is to be gained from the development of a persistent seed bank, particularly since surface soil temperatures often reach *c.* 80°C (Henschel, pers comm[5]), a temperature which seeds cannot endure for more than 72 h (data not shown). It is likely that the interval between extraordinary rainfall events would be longer than the life-span of seeds in the environment. Recruitment and establishment of plants is therefore a sporadic and rare event.

The mycelia and spores of *Aspergillus niger* var. *phoenicis* show tolerance for a wide range of anti-fungal measures, including microwave irradiation, dry heat thermotherapy, hot water thermotherapy and chemical surface sterilisation. However, treatment with 0.1 $g.L^{-1}$ tebuconazole as a 3 h seed soak, followed by incubation of seeds on a medium containing the same concentration of tebuconazole resulted in a substantial reduction in

[5] Dr. J. Henschel, Desert Research Foundation of Namibia, Windhoek, Namibia

contamination, from 100% of seeds in the control group to 8% of seeds specifically tested for fungal infection. Infection in the treated seeds arose from a species tentatively identified as *Aspergillus flavus*, and *A. niger* var. *phoenicis* was completely suppressed or eradicated by the treatment. Further refinement of the combined treatment to include planting out and hardening-off of treated seedlings is recommended.

Observation of some anatomical and developmental anomalies is suggestive of a localised genetic effect. The greater number of twin, achlorophyllous and abnormally foliated seedlings noted arose from seeds of accession HK1249. The genetic analysis of this specific population could provide interesting and important data on the developmental biology and control of morphology of *W. mirabilis*.

The primary aims of the present study were to elucidate the seed biology and storage behaviour of the seeds of *Welwitschia mirabilis*, as well as to investigate the relationship between the seeds and *Aspergillus niger* var. *phoenicis*. It is considered that both aims have been achieved, although the complex relationship between *W. mirabilis*, *A. niger* var. *phoenicis* and the environment requires further scrutiny. The potential for fungicidal treatment of *W. mirabilis* plants *in situ* exists, although such intervention in a natural system may not be warranted, and the treatment outlined here may be refined and reserved for cultivated specimens. The highly orthodox nature of the seeds of *W. mirabilis* and their consequent amenability to conventional storage, will ensure the conservation of this unique and wonderful species.

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Lacey, J. (1981). *The Aerobiology of Conidial Fungi*. Pp 373-416 *in* Cole, G. T. and Kendrick, B. (eds), *Biology of Conidial Fungi*, Academic Press, New York.

CHAPTER 9
APPENDICES

Scanning Electron Microscopy

1. Primary fixation: 2-24 h in buffered 2.5% glutaraldehyde
2. Buffer wash: 3x5 min Buffer
3. Postfix: 1x1 h 0.5% Osmium tetroxide
4. Wash: 3x5 min washes with distilled water
5. Dehydrate: 2x5 min 35% Alcohol
   2x5 min 50% Alcohol
   2x5 min 75% Alcohol
   2x5 min 100% Alcohol
6. Critical Point Drying

Specimens were mounted on aluminium stubs using graphite tape and sputter-coated with gold in a Polaron E5300 SEM coating unit. The specimens were viewed with a LEO1450scanning electron microscope and the images captured digitally.

Fluorescence microscopy

The procedure was modified from Rohringer, Kim, Samborski and Howes (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 for 2x 15 min in 50% ethanol
3. washed for 2x 15 min with 0.05M NaOH
4. rinsed 3x with water *
5. soaked in Tris/HCl buffer, pH 5.8 for 30 min
6. stained for 5 min in 0.1% Uvitex in buffer
7. rinsed thoroughly 4x 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)

* The procedure may be interrupted for one or more days without having any negative effect on the stain.

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 380-420nm and barrier filter 450nm) was used to visualise the fungi. Images were digitally captured.

Solutions:

1. Ethanol:dichloromethane; 3:1 v/v + 0.15% trichloroacetic acid
2. Lactophenol; 50 ml lactic acid, 100 ml glycerine, 50 ml distilled water, 10g phenol crystals. Must be stored in a refrigerator
3. 50% ethanol
4. 0.05 M NaOH; 0.05 M = 2 $g.l^{-1}$ NaOH
5. Tris HCl Buffer pH 5.8; 6.05g in 300 ml distilled H_2O. pH adjusted with HCl to 5.8; solution made up to 500 ml and refrigerated
6. 0.1% Uvitex (Ciba) = 0.08 g 130% Uvitex in 100 ml pH 5.8 tris buffer, freshly made each time
7. 25% Aqueous glycerol
8. 50% Glycerol + trace lactophenol

References

Rohringer, R., Kim, W. K., Samborski, D. J. and Howes, N.K. (1977). Calcoflour: An optical brightener for fluorescence microscopy of fungal plant parasites in leaves. *Phytopathology* **67**: 808-810.