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Intraseminal fungal location in maize of selected seed storage fungi in relation to some physiological parameters

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Maize seeds that had been hot-water-treated (30 min at 55°C) to reduce inherent infection, were inoculated with the spores of four storage fungal species of varying xerotolerance. The less xerotolerant species (*Aspergillus oryzae* and *Aspergillus sydowi*) were characterized by vigorous growth on the six single carbon source media tested, and were also associated with rapid and extensive degradation of all the seed tissues. The more xerotolerant species (*Aspergillus chevalieri* and *Penicillium pinophilum*), on the other hand, grew only slowly *in vitro* and were not located in the embryo despite six weeks storage of the artificially infected seeds at 95% relative humidity. Germinability of infected seeds decreased with storage time, as did the dry mass of the resultant seedlings, the extent of the decline increasing with decreasing xerotolerance of the fungal species. The rate of infection of, and ultimate mycelial location in, the seeds are suggested to be related to the extracellular enzyme capabilities of the individual species.

Mieliesaad wat met warm water (30 min teen 55°C) behandel is om inherente infeksie te verminder, is ingeënt met die spore van vier bergingsfungi waarvan die droogtebestandheid varieer. Die spesies wat minder teen droogte bestand is (*Aspergillus oryzae* en *A. sydowi*), is gekenmerkend deur sterk groei op die ses media met 'n enkele koolstofbron elk waarmee getoets is, en het ook vinnige en omvattende afbreking van die saadweefsel tot gevolg gehad. Die fungi wat meer teen droogte bestand is (*A. chevalieri* en *Penicillium pinophilum*), het daarteenoor *in vitro* stadig gegroei en is nie in die embrio opgespoor nie, ondanks 'n bergingsperiode van ses weke van die kunsmatig geïnfekteerde saad teen 95% relatiewe humiditeit. Die kiemkragtigheid van die geïnfekteerde saad het afgeneem met die bergingsduur, soos ook die droë massa van die resulterende saailinge, waar die omvang van die afname toegeneem het met afnemende droogtebestandheid van die fungusspesie. Daar word voorgestel dat daar 'n verband is tussen die ekstrasellulêre ensiemvermoë van die indivuele spesies en die tempo van infeksie van en uiteindelike miseliale ligging in die saad.

Key words: Maize seed, seed deterioration, storage fungi.

Introduction

Seed storage fungi, which appear as a succession, are manifested in a predictable order, from the most to the least xerotolerant. While moisture content is a major factor determining the fungal succession associated with stored seeds (Christensen & Kaufmann 1974), other factors may play an important rôle. In this regard, interspecific fungal interactions and competition have been suggested to be major factors, and these, in turn, may depend on the spectrum of extracellular enzymes elaborated by each species (McLean & Berjak 1987). Furthermore, the precise location of the mycelium of a particular storage fungus within a seed is likely to be dependent on its enzymatic capabilities.

Previous studies have indicated that the less xerotolerant species among the seed storage aspergilli (*i.e.* those that appear later in the succession) are more prolific extracellular enzyme producers than those that initiate the succession (McLean *et al.* 1985). Those authors also showed that the spectrum of enzymes produced changes with increasing xerotolerance of the *Aspergillus* species involved, from those that hydrolyse simple sugars, through those degrading polysaccharides, to lipases and proteases. The composition of a maize grain is not homogeneous, with polysaccharides predominating in the endosperm, while lipids and proteins are concentrated in the embryo. Thus, it might be expected that the more xerotolerant of the seed storage fungi would be primarily located in the endosperm, while the less xerotolerant species, with their broader spectrum of enzymic capabilities, would be capable of becoming established within the scutellum and embryonic axis. It has been shown that the more xerotolerant of the fungal species are associated with incipient deterioration, whereas complete seed spoilage is associated with the activity of the least xerotolerant of the storage fungi (Christensen & Kaufmann 1974; Justice & Bass 1978).

The present contribution details the ultimate location of four seed storage fungi in maize grains in relation to some physiological parameters including extracellular enzyme capabilities. The fungi used were: *Aspergillus chevalieri* (an *A. glaucus* group member); *Aspergillus sydowi* (Bain. and Sart.) (an *A. versicolor* group member); *Aspergillus oryzae* (Ahlb.) Cohn (a member of the *A. flavus* group) and *Penicillium pinophilum*. These species were chosen because they are representative of groups of differing xerotolerance. The aspergilli are listed above in order of decreasing xerotolerance, while *Penicillium pinophilum* might be described as being moderately xerotolerant (Christensen & Kaufmann 1969; 1974).

Materials and Methods

Seeds

Caryopses of *Zea mays* L. (var. Hickory King) were obtained from the Pioneer Seed Company, Greytown, Natal, South Africa and stored at 6 ± 2°C until used. Moisture content

on removal from cold storage was 10.2%.

Hot-water treatment

Following a 4-h presoak in sterile distilled water at ambient temperature, the seeds were hot-water-treated at 55°C for 30 min. After redrying to a moisture content of 11% in a stream of dry air, the seed was surface-sterilized in 2% sodium hypochlorite and 1% sodium dodecyl sulphate for 20 min.

Internal infection

Surface-sterilized seeds were aseptically halved (longitudinally) through the embryo and plated onto potato dextrose agar containing 6% NaCl. The plates were incubated at 25°C, and any fungal growth identified and quantified on a species basis (Mycock *et al.* 1988).

Storage

Experimental seeds were infected by dusting (using a fine paint brush) with the spores of *Aspergillus chevalieri*, *A. sydowi*, *A. oryzae* and *Penicillium pinophilum* prior to storage. Uninoculated seed was used as a control. All the fungal species had been previously isolated from local maize seed and were identified by the Mycological Research Unit of the Department of Plant and Seed Protection, Pretoria. A sufficient quantity of seeds was stored at 95% RH (Thewlis *et al.* 1961) and 25°C to allow fortnightly sampling for six weeks.

Microscopy

Twenty seeds were removed from the storage bins every fortnight, each was halved longitudinally through the embryo and prepared for scanning electron microscopy as follows: fixed overnight in 4% glutaraldehyde and 1% osmium tetroxide made up in 0.1M sodium cacodylate buffer, pH 7.2. Dehydrated through a graded ethanol series (25%, 50%, 75% and 100%) and critically point-dried using liquid CO_2. The dried samples were coated with gold/palladium and viewed with an Hitachi SEM 520 (Mycock & Berjak 1991).

Internal infection

At each sampling 50 seeds from each storage bin were tested for internal infection (see above).

Moisture content

The moisture content of 30 seeds was determined gravimetrically and expressed on a wet-mass basis at each sampling. Each seed was weighed prior to being dried to constant weight in an oven at 80°C. Moisture content was determined using the following equation:

$$\text{Moisture content} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100.$$

Germination and establishment

At each sampling 50 seeds were set to germinate at 25°C on filter paper in Petri dishes containing 8 ml distilled water, all substrates and containers having been sterilized. Percentage germination was scored after 96 h. These seeds, whether germinated or not, were then planted in sterile vermiculite. Seedling dry mass was determined gravimetrically after 14 days by drying in an oven at 80°C.

Extracellular enzymatic capability on solid media

The ability of the four seed storage fungi to utilize protein, starch, lipid, polygalacturonic acid (PGA), cellulose and glucose as sole carbon source was tested using solid-media techniques (McLean *et al.* 1985). All the media contained essential macro- and micronutrients (Hankin & Anagnostakis 1975) and one of the carbon sources, and were solidified with 4% agar. The plates were poured such that each contained 15 ml of medium, and were incubated at 25°C. For each carbon source, colony diameter (mm) (two measurements per colony with 25 replicates) was measured after 10 days of incubation.

Results and Discussion

The growth of a fungus under specific conditions can be measured in various ways such as colony diameter and increase in dry mass. However, the conditions used may not always be optimal in terms of water availability, pH and temperature (Ayerst 1969). Although the temperature (25°C) at which the solid-media tests were conducted was not optimal for growth of each of the four fungal species, it was considered a compromise temperature between the optima for each of the fungal species, and the norm in terms of storage of seed in tropical regions. Additionally, specific enzymes are not identified using media containing single carbon sources; the technique, nevertheless, does demonstrate fungal ability to utilize the particular carbon source, and implies the release of specific extracellular enzymes (Hankin & Anagnostakis 1975).

Of the four species investigated, the least xerotolerant, *A. oryzae*, grew the most rapidly on all the media tested, thereby demonstrating its ability to utilize efficiently carbon sources as diverse as protein, starch, lipid, PGA, cellulose and glucose (Table 1). *A. oryzae*, although capable of sustained growth on PGA, did not utilize this carbon source as efficiently as *A. sydowi*. This latter species, which is representative of aspergilli of intermediate xerotolerance, also grew well on protein-, starch- and glucose-enriched media (Table 1). However, *A. sydowi* grew slowly on the lipid medium. In contrast, *A. chevalieri* and *P. pinophilum* grew slowly on all the media tested.

As has been found previously (McLean *et al.* 1985), the less xerotolerant storage fungal species have greater extracellular enzyme capabilities. *A. oryzae* exhibited a wide range of extracellular enzyme capabilities whereas *A. sydowi* which is more xerotolerant, effiently utilized a narrower range of substrates under the conditions used. *A. chevalieri* had the weakest potential of the aspergilli tested and is also known to be the most xerotolerant (Raper & Fennell 1965, 1974; Christensen & Kaufmann 1974). The penicillia are said to invade seed with moisture contents between 15 and

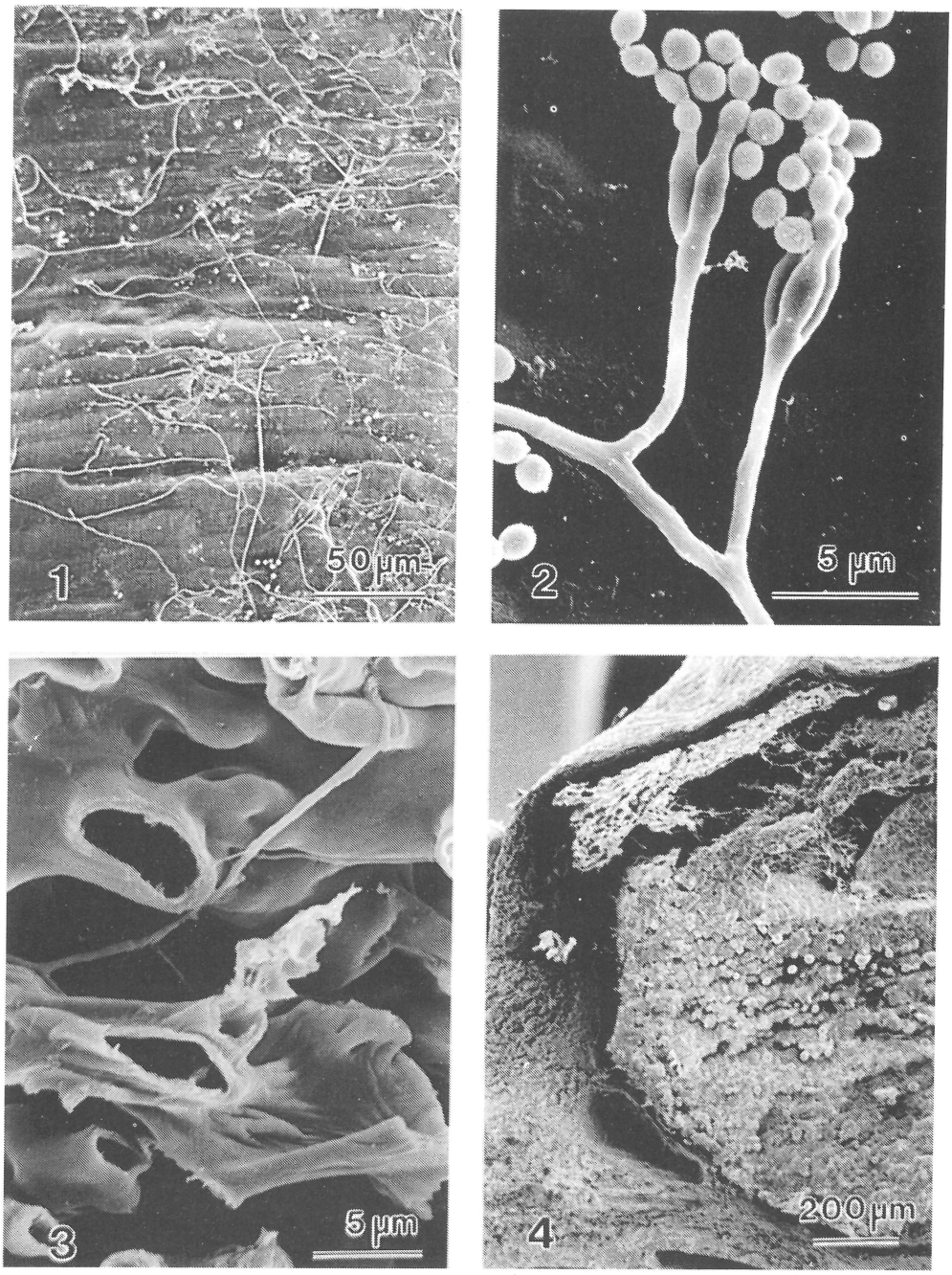
Table 1 Average of 50 diameters (mm) of the four seed storage fungi grown on media containing a single carbon source for 10 days at 25°C

	A. oryzae	*A. sydowi*	*A. chevalieri*	*A. pinophilum*
Protein	57.0 ± 1.3	42.0 ± 0.6	20.0 ± 0.7	19.0 ± 0.1
Starch	88.0 ± 4.4	55.0 ± 1.6	24.0 ± 1.1	20.0 ± 0.5
Lipid	57.0 ± 1.9	23.0 ± 0.8	31.0 ± 0.5	21.0 ± 1.0
PGA	51.0 ± 1.0	60.0 ± 2.1	22.0 ± 1.1	22.0 ± 0.9
Cellulose	58.0 ± 2.3	50.0 ± 1.7	22.0 ± 0.3	22.0 ± 1.0
Glucose	84.0 ± 4.9	78.0 ± 3.2	30.0 ± 1.5	19.0 ± 0.3

18%, and as such are moderately xerotolerant (Christensen & Kaufmann 1969, 1974). It could therefore be expected that *Penicillium* species are capable of utilizing a range of substrates. However, the present results do not support this, and it is possible that *P. pinophilum* is more xerotolerant than expected. Cazalet and Berjak (1983) have found that a number of *Penicillium* species can grow in sugar cane seed at moisture contents below 15%, thereby exhibiting greater xerotolerance.

The untreated seeds showed an internal infection level of 75% comprising *Aspergillus* (61%), *Fusarium* (11%) and *Penicillium* (3%). Hot-water treatment reduced this internal infection level to 25% comprising *Aspergillus* (5%), *Fusarium* (9%) and bacteria (11%). Heat treatment had no effect on germination which was maintained at 98%. Despite the remaining inherent infection, there was no detectable fungal growth on the control seeds at any stage, nor were any of the four fungi used experimentally isolated from this material. However, the four storage fungi under investigation were each isolated at every sampling from the appropriate experimental seed batch.

In all cases, after 14 days of storage some of the spores of each fungal species with which the seeds had been dusted, had germinated and hyphae were seen ramifying over the seed surfaces (Figure 1) and in most cases asexual reproductive structures had developed (Figure 2). The peduncular



Figures 1 – 4 Maize seeds experimentally infected with storage fungi and stored for two weeks under conditions conducive to fungal growth (95% RH and 25°C). **1.** After 14 days in storage some spores of all the species tested had germinated. Hyphae of *Aspergillus sydowi* are shown ramifying over the surface of a seed. **2.** Asexual reproductive structures of *Penicillium pinophilum* produced on the seed surface, are illustrated. **3.** Hyphae of *Aspergillus oryzae* growing on and penetrating the surface of the peduncle. **4.** Hyphae of *Aspergillus oryzae* are shown infecting the peri-embryonic tissues.

tissue was also infected (Figure 3). At this stage, hyphae of *P. pinophilum*, *A. chevalieri* and *A. sydowi* had not penetrated the internal seed tissues, but there was evidence of invasion of the cotyledon and peri-embryonic tissues by *A. oryzae* (Figure 4). The observed damage to the embryonic tissues was corroborated by the increasing decline in germination and the decline in dry mass of plants developed from those seeds (see below).

After one month in storage, the hyphae of *A. sydowi*, *A. chevalieri* and *P. pinophilum* had advanced into the cotyledonary tissue (Figure 5). At that point, however, the more vigorous *A. oryzae*, besides producing sporulating heads on the seed surface, had invaded all the internal seed tissues which were showing considerable deterioration (Figure 6). After an additional two weeks, the mycelium of *A. oryzae* was firmly established and the seeds were extensively degraded (Figure 7). By this final sampling stage, the hyphae of *A. sydowi* were present in the peri-embryonic tissues but not the endosperm. *A. chevalieri*, which generally exhibited a far slower growth on the various carbon sources, had advanced into the seed but had not invaded the embryonic axis or the endosperm (Figure 8). No hyphae of *P. pinophilum* were apparent in either the embryonic axis or the endosperm, with the species appearing to be limited to the scutellum (Figure 9).

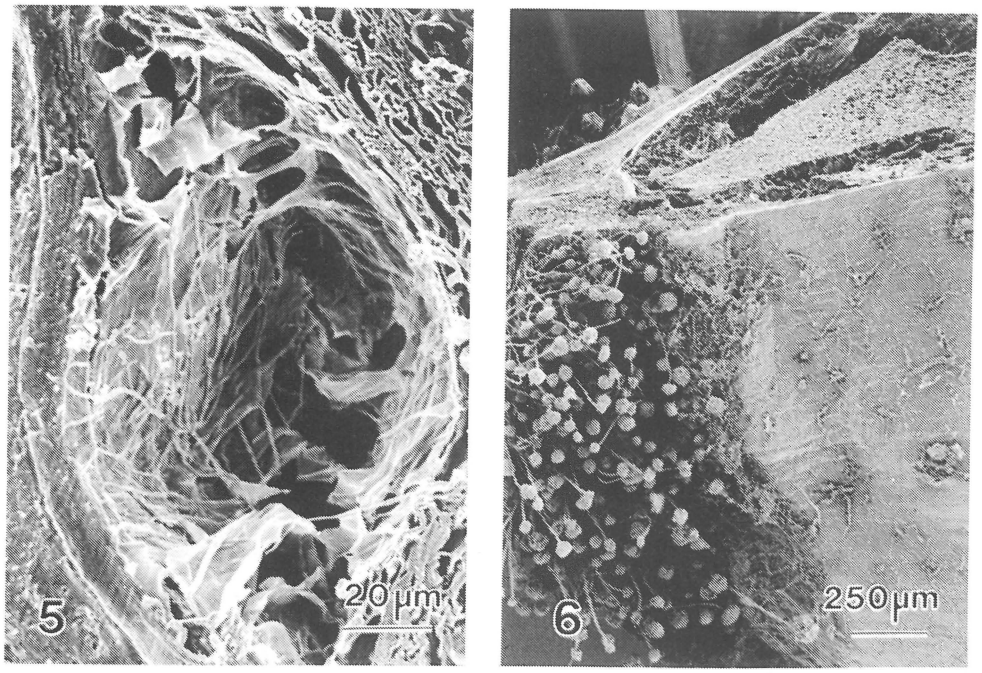
With increasing storage time there was an increase in seed moisture content and in all cases the infected seeds were wetter than those of the controls (Figure 10). Further, the increase in moisture content of the various batches relative to the controls, declined in parallel with the degree of invasion of the seeds by each specific fungus (Figure 10). These data emphasize that the storage fungi not only have an effect on moisture content, but also that the extent to which moisture content is elevated may be related to the metabolic activities of the fungal species involved. This in turn appears to determine the rate of invasion and the location of the mycelium within the seed.

Seed germination declined during the experiment (Figure 11). The drop in germinability after 28 days storage of the control material is suggested to be ascribable to the effects of accelerated ageing. This process is the increase in the rate of natural ageing due to storage under conditions of high temperature and relative humidity (Justice & Bass 1978). Over and above this decline, the loss paralled the extent to which each seed batch was infected by each fungal species (Figure 11). The viability of the sample infected by *A. oryzae* dropped by about 90% to 10%, whereas that infected by *P. pinophilum* dropped by 30%.

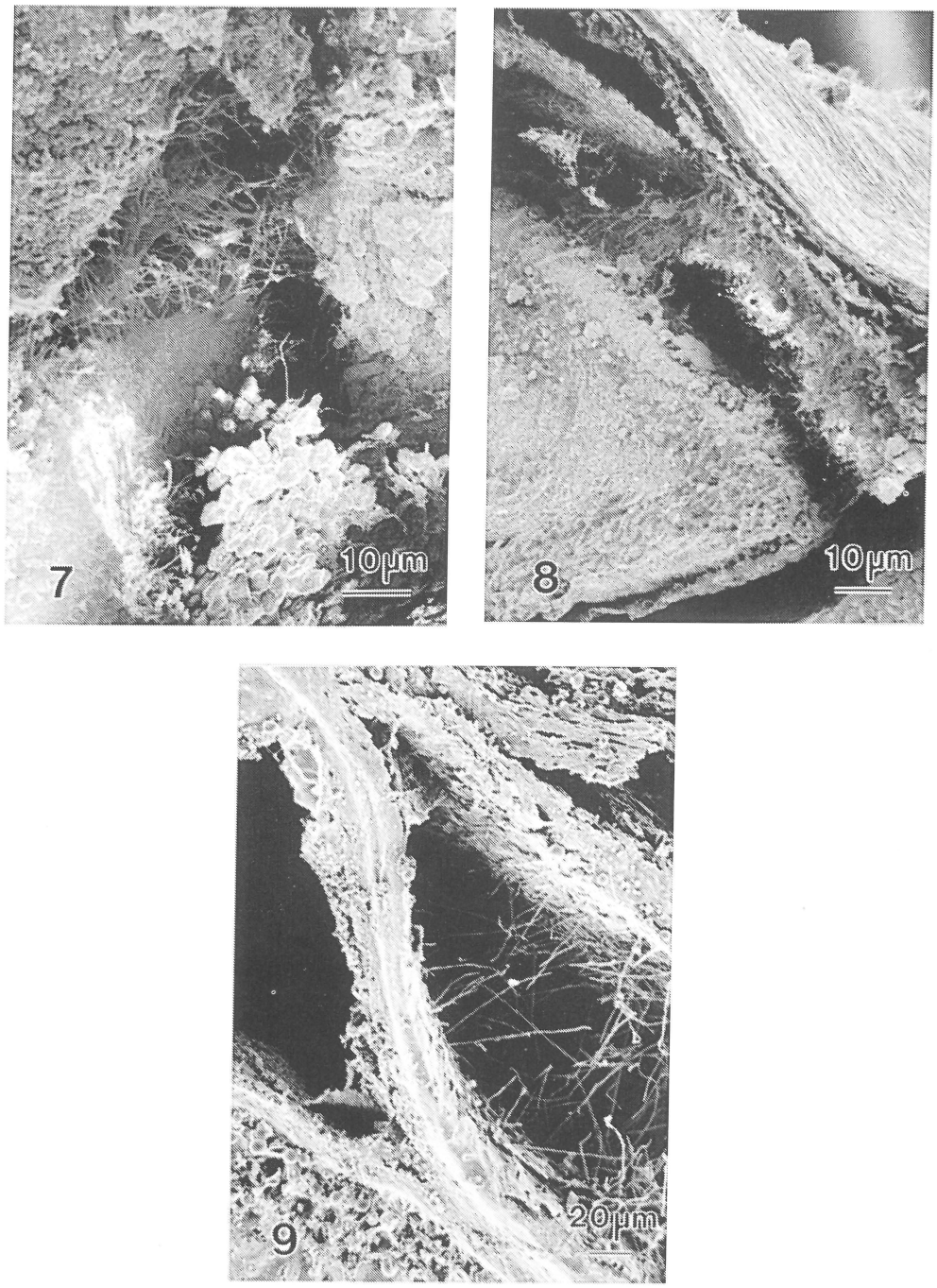
The effect of each storage fungus on the vigour of the surviving seeds was shown by the reduction in dry mass of the seedlings (Figure 12). Once again the seedlings developed from seeds infected with *A. oryzae* showed the greatest reduction compared with the control. Similarly, with the other fungal species the less vigorous the seed infection the less effect on the plants.

All the species tested in this study gained access to the interior of the intact seed via the peduncle and then through the micropyle. This supports the results obtained by Mycock *et al.* (1988) and further indicates that topically located seed storage fungi utilize the path of least resistance in their invasion of the intact seeds. Infection in this manner allows access to the scutellum, embryonic axis and endosperm, in that order. However, the rate of invasion and the location of the mycelium vary with fungal species and this, in turn, may be related to the enzymatic capabilities of the fungi involved.

Despite the residual inherent infection after hot-water treatment, under the experimental conditions used, one fungus dominated each seed batch; consequently, the degree of deterioration could be related to the capabilities of that particular fungus. However, in the natural situation seeds appear to harbour a wide spectrum of fungal propagules



Figures 5 & 6 Experimentally infected maize seeds stored for four weeks at 95% RH and 25°C. **5**. Hyphae of *Aspergillus sydowi* are illustrated extending from the peduncle (p) into the cotyledon (c). **6**. Degradation of the internal tissues by *Aspergillus oryzae*. Note also the abundance of reproductive structures.



Figures 7 – 9 The situation in maize seed stored for six weeks. **7.** Remains of the embryonic axis, *Aspergillus oryzae* infection. **8.** Infection of the scutellum by, and location of, *Aspergillus chevalieri* hyphae in the peri-embryonic space. **9.** Hyphae of *Penicillium pinophilum* penetrating the scutellum.

when they are harvested (McLean & Berjak 1987) and an equally wide spectrum of spores is found in the storage environment (Christensen & Kaufmann 1969; 1974). It is, therefore, highly unlikely that a single species invasion will occur under natural conditions. Furthermore, the succession of fungal species associated with stored grain may well be achieved not only by the increasing seed moisture content and temperature due to fungal metabolism, but also by each participant in the pathway making available substrates for its successor. Extrapolation of the results of the present study indicate that such a succession must inevitably result in extensive seed losses.

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References

AYERST, G. 1969. The effects of moisture and temperature on growth and spore germination in some fungi. *J. Stored Prod. Res.* 5: 127 – 141.

CAZALET, K.R. & BERJAK, P. 1983. Isolation of a seed storage fungus from sugarcane seeds. *Proc. S. Afr. Sugar Technol. Assoc.* pp. 1 – 4.

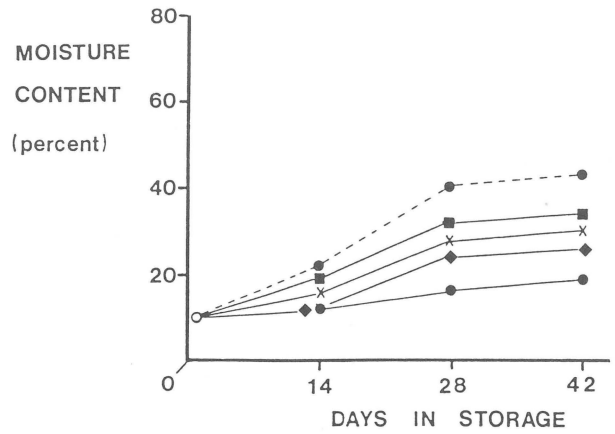

Figure 10 Moisture content of the control and infected seeds over the test period. In no case did standard deviations exceed 0.5%. ○, Initial moisture content; ●—●, Control; ●- - -●, *A. oryzae*-infected; ■—■, *A. sydowi*-infected; ×—×, *A. chevalieri*-infected; ◆—◆, *P. pinophilum*-infected.

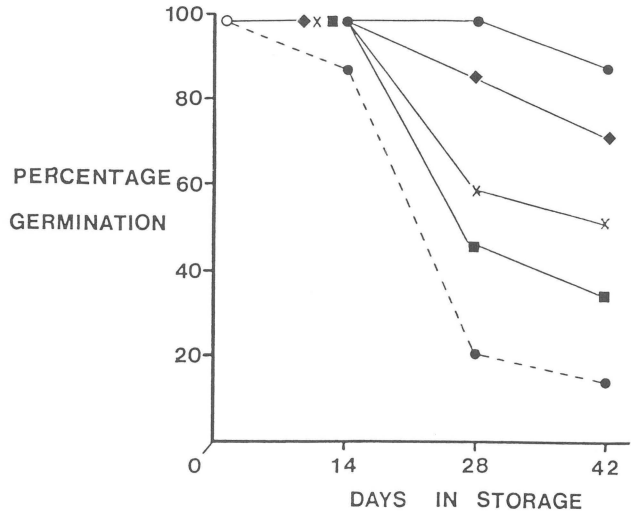

Figure 11 Germination of the stored seeds over the experimental period. ○, Initial germination totality; ●—●, Control; ●- - -●, *A. oryzae*-infected; ■—■, *A. sydowi*-infected; ×—×, *A. chevalieri*-infected; ◆—◆, *P. pinophilum*-infected.

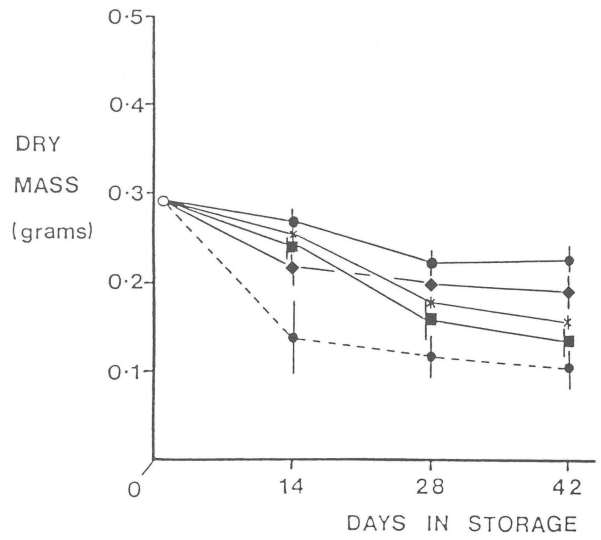

Figure 12 Dry mass of 14-day-old seedlings established from the control and infected seeds. Standard deviations did not exceed 0.04 g. ○, Initial dry mass; ●—●, Control; ●- - -●, *A. oryzae*-infected; ■—■, *A. sydowi*-infected; ×—×, *A. chevalieri*-infected; ◆—◆, *P. pinophilum*-infected.

Chemists, Minnesota.

CHRISTENSEN, C.M. & KAUFMANN, H.H. 1969. Grain storage: The role of fungi in quality loss. University of Minnesota Press, Minneapolis.

CHRISTENSEN, C.M. & KAUFMANN, H.H. 1974. Microflora. In: Storage of cereal grains and their products, ed. C.M. Christensen, pp. 158 – 192. American Association of Cereal Chemists, Minnesota.

HANKIN, H.L. & ANAGNOSTAKIS, S.L. 1975. The use of solid media for the detection of enzyme production by fungi. *Mycologia*, 67: 597 – 607.

JUSTICE, O.L. & BASS, L.N. 1978. Principles and practices of seed storage. Educational Federal Research Agricultural Handbook No. 506.

McLEAN, M., MYCOCK, D.J. & BERJAK, P. 1985. A preliminary investigation of extracellular enzyme production by some species of *Aspergillus*. *S. Afr. J. Bot.* 51: 425 – 431.

McLEAN, M. & BERJAK, P. 1987. Maize grains and their associated microflora – a micro-ecological consideration. *Seed Sci. Technol.* 15: 831 – 850.

MYCOCK, D.J. & BERJAK, P. 1991. In defense of aldehyde-osmium fixation and CPD in seed storage fungal characterisation by scanning electron microscopy. *J. Microsc.* 163: 321 – 331.

MYCOCK, D.J., LLOYD, H.L. & BERJAK, P. 1988. Micropylar infection of post-harvest caryopses of *Zea mays* by *Aspergillus flavus* var. *columnaris* var. nov. *Seed Sci. Technol.* 16: 647 – 653.

RAPER K.B. & FENNELL, D.I. 1965. The genus *Aspergillus*. Williams and Wilkins, Baltimore.

THEWLIS, J., GLASS, R.C., HUGHES, D.J. & MEETHAM, A.R. (eds) 1961. Encyclopaedic Dictionary of Physics, Vol. 3, p. 72. Pergamon Press, Oxford.