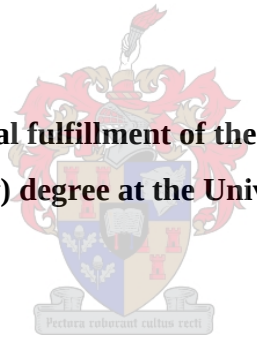
Physical interactions of filamentous fungal spores and unicellular fungi.

By

Rodney S. Hart



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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature :_____ Date :_____

R. S. Hart

SUMMARY

It is known that many hyphomycetous fungi are dispersed by wind, water and insects. However, very little is known about how these fungi may differ from each other regarding their ability to be disseminated by different environmental vectors. Consequently, to obtain an indication of the primary means of spore dispersal employed by representatives of the genera *Acremonium*, *Aspergillus* and *Penicillium*, isolated from soil and indoor environments, we monitored spore liberation of cultures representing these genera in an airflow cell. The experimental data obtained, of plate counts conducted of the air at the outlet of the airflow cell, were subjected to an appropriate analysis of variance (ANOVA), using SAS statistical software. Intraspecific differences occurred regarding aerial spore release. Under humid conditions, however, *Penicillium* species were more successful in releasing their spores than *Aspergillus* and the *Acremonium* strain. Under desiccated conditions the *Aspergillus* took longer to release their spores than representatives of *Acremonium* and *Penicillium*. The taxa that were investigated did not differ from each other regarding the release of spores in physiological salt solution (PSS). Although not proven, indications are that water may act as an important dispersion agent for these fungi, because washing of cultures with PSS resulted in all cases in an immediate massive release of colony forming units.

Subsequently, using standard plate count techniques, conidial adhesion of the fungi mentioned above to synthetic membranes, leaf cuttings and insect exoskeletons differing in hydrophobicity and electrostatic charge were investigated. We found that the different genera showed different adhesion profiles for the series of test surfaces, indicating differences in physico-chemical characteristics of the fungal spore surfaces. In general, the *Penicillium* strains showed a greater ability to adhere to the test surfaces, than the aspergilli, while the representative of *Acremonium* showed the least adherence. No significant difference in the percentage spore adhesion was found between hydrophobic and hydrophilic materials.

Furthermore, evidence was uncovered supporting the contention that, under dry conditions, electrostatic surface charges play a role in the adherence of fungal spores to surfaces, because adherence was positively correlated (Correlation coefficient = 0.70898, p = 0.001) to positive electrostatic charges on the lamellar surfaces. In the next part of the study, standard plate count methods were used to determine the relative adhesion of the above mentioned hyphomycetous fungi, as well as a polyphyletic group of yeasts, to the test surfaces submerged in 10 mM sodium phosphate buffer (pH 7.0).

As was found with the experiments with the dry surfaces, both intraspecific and intergenus differences were uncovered. Overall, the fungi adhered better to hydrophilic surfaces than to hydrophobic surfaces. This indicated that the fungal surfaces were covered with relatively hydrophilic compounds such as carbohydrates. Subsequently, it was demonstrated that all the fungi adhered to plasma membrane glycoprotein coated polystyrene and the presence of fungal carbohydrates on the surfaces of the fungal propagules was confirmed using epi-fluorescence microscopy. Differences in the strategy of the fungal genera to release their airborne spores, as well as differences in their adhesion profiles for the series of test materials, may be indicative of a unique environmental niche for each genus. In future, this phenomenon should be investigated further.

OPSOMMING

Hifomisete fungi is daarvoor bekend om te versprei deur middel van wind, water, en insek vektore. Maar nietemin, daar is bykans geen kennis m.b.t. hoe hierdie fungi van mekaar verskil t.o.v. hul vermoë om versprei te word deur omgewings vektore nie. Gevolglik was spoorvrystelling van kulture, verteenwoordigend van die genera *Acremonium, Aspergillus* en *Penicillium* gemoniteer om ‘n aanduiding te kry van primêre wyse van spoorverspreiding waardeur verteenwoordigers van die onderskeie genera ingespan word. Eksperimentele data ingewin, vanaf plaat tellings wat uitgevoer was op lug afkomstig vanuit die uitlaat-klep van die lugvloei kapsule, was onderwerp aan ‘n toepaslike analise van afwyking (ANOVA), deur gebruik te maak van ‘n SAS statistiese pakket. Intraspesie verskille is waargeneem t.o.v. lug spoorvrystelling. Desnieteenstaande was *Penicillium* meer suksesvol onder vogtige kondisies t.o.v. spoorvrystelling in vergelyking met *Aspergillus* en die *Acremonium* stam. Onder droë kondisies het verteenwoordigers van *Aspergillus* langer geneem om hul spore vry te stel as verteenwoordigers van onderskeidelik, *Penicillium* en *Acremonium*. Geen verskille was waargeneem m.b.t. spoorvrystelling in fisiologiese soutoplossing (FSO) tussen die verskillende filogenetiese stamme nie. Alhoewel dit nie bewys is nie, wil dit voorkom asof water as belangrike verspreidingsagent van die betrokke fungi dien, aangesien die spoel van kulture met FSO tot ‘n oombliklike enorme vrystelling van kolonie-vormende eenhede gelei het.

Gevolglik, deur gebruik te maak van standaard plaattellings tegnieke, was spoor aanhegting van bogenoemde fungi aan sintetiese membrane, blaar snitte en insek eksoskelette wat verskil in terme van hidrofobisiteit en elektriese lading, ondersoek. Daar was gevind dat die aanhegtingsprofiele m.b.t. hierdie reeks toetsoppervlaktes van die verskillende genera verskil, wat op sigself ‘n aanduiding was van verskille in fisies-chemiese eienskappe van die swamspoor oppervlaktes. *Penicillium* stamme het ‘n hoër aanhegtings vermoë aan die toetsoppervlaktes getoon as die aspergilli, terwyl die verteenwoordiger van *Acremonium* die laagste aanhegting getoon het. Geen betekenisvolle verskille i.t.v. persentasie spoor aanhegting was gevind tussen hidrofobiese en hidrofiliese oppervlakte nie.

Daarbenewens was die argument dat spoorvrystelling onder droë kondisies beïnvloed word deur elektrostatiese oppervlak ladings, bevestig deur ons bevindinge, want aanhegting het positief gekoreleer (Korrelasie koëffisient = 0.70898, p = 0.001) met positiewe ladings op die oppervlaktes. ‘n Standaard plaattellingstegniek was aangewend in die volgende fasset van die studie om die relatiewe aanhegting van bogenoemde hifomisete fungi, sowel as ‘n polifilitiese groep giste aan die toetsoppervlaktes, gedompel in 10 mM natrium fosfaat buffer (pH 7.0) vas te stel.

Intraspesie en intragenus verskille was weereens waargeneem, net soos in die geval van die eksperimente met die droë oppervlakte. In die algemeen het die swamme baie beter geheg aan hidrofiliese oppervlaktes in vergelyking met hidrofobiese oppervlakte. Dit was ‘n aanduiding dat die swamspoor oppervlaktes bedek was met relatiewe hidrofiliese verbindings bv. koolhidrate. Verder was daar bewys dat alle swamme ingesluit in hierdie studie die vermoë het om plasmamembraan glikoproteïn bedekte polistireen te bind, en gevolglik was die teenwoordigheid van van koolhidrate op die swamspore bevestig m.b.v epi-fluoresensie mikroskopie. Verskille in die strategie van swamme om spore in die lug vry te stel, sowel as verskille in die aanhegtingsprofiele vir ‘n reeks toetsmateriale, mag net ‘n aanduiding wees van ‘n unieke omgewings nis vir elke genus wat in hierdie studie ondersoek is. Hierdie verskynsel moet dus in die nabye toekoms nagevors word.

Dedicated to my mother

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CHAPTER 1

General introduction and aims of this study

1.1 INTRODUCTION

The roles that fungi play are far more pervasive and diverse than are generally realised, and they interact with all other organisms whether it may be directly or indirectly (Trappe and Luoma, 1992). They serve as food source for many soil organisms, including bacteria, other fungi, nematodes, insects, earthworms, and mammals (Claridge and May 1994). As decomposers, fungi reduce recalcitrant organic substrates to components that other organisms can utilise (Chet and Inbar, 1994). Fungi are among the world's greatest opportunists and do not restrict their feeding to non-living organic material, because some members function as pathogens and parasites (Cromrack and Caldwell, 1992). They are capable of eliciting various disease responses in humans (Rogers, 2003). These omnipresent microbes can potentially have an impact on human health, especially in cases where humans are exposed to airborne fungal propagules.

The incredible success that fungi enjoy can mainly be accredited to two major physical features, of which the first is fungal hyphae (Kendrick, 1992). Hyphae are vegetative, assimilative organs which the fungus deploys to secrete hydrolytic enzymes (including cellulases and xylanases) whilst exploring a newly found substrate (Bakri *et al*., 2003). The strong, waterproof, chitinous hyphae enables it to withstand hydrostatic pressure and are, therefore, perfectly suited to actively penetrate solid substrates in a manner that bacteria cannot match. Secondly, fungi produce spores that permit dispersal of the fungus (Roncal and Ugalde, 2003). The rate of spore production among eumycotan fungi may be very high, therefore, enhancing the chances of survival (Ingold, 1953). Spores can readily be dispersed by wind, water, and animal vectors.

Spores have been shown to be the main mode of removing potential progeny from the direct vicinity of the parent mycelium (Ingold, 1953; Ingold and Hudson, 1993; Moore-Landecker, 1996). Therefore, spores serve to minimise competition amongst siblings as a result of unfavorable nutritional conditions, and thus promote the survival of the organism (Ross, 1979). Moreover, spores have the ability to withstand prolonged periods of unfavorable conditions, such as freezing, starvation and desiccation (Ingold, 1953; Montazeri and Greaves, 2002). Spore dispersal is completed once spores are successfully deposited in a new locale (Ingold, 1953; Moore-Landecker, 1996; Yang *et al.*, 2000). Some spores are inhaled by mammals *e.g.* humans. Once inhaled spores may be deposited into the respiratory tract. Spores are known to adhere to plasma membrane glycoprotein with the aid of glycoprotein on spore surfaces (Coulot *et al*., 1994; Peñalver *et al*., 1996). Inhalation of mold conidia may impact on health since these spores may result in respiratory infection. Attachment of conidia to surfaces has survival value and may be a requirement for colonisation (Kennedy, 1990; Paris *et al.*, 1997). Since spores are the means of vegetative multiplication, dispersal, and survival their physical interactions have great importance in the life-cycle of fungi (Brown and Hovmoller, 2002; Dufrêne *et al.*, 1999; Kendrick, 1992; Sanderson, 2005).

1.2 AIMS OF THIS STUDY

Therefore, the aims of this study was to: 1) isolate, culture and subsequently identify filamentous fungi from soil and indoor environments; 2) conduct a comparative study on hyphomycetous fungal conidia release induced by air currents and aqueous saline solution; 3) conduct comparative studies of the physical interactions of hyphomycetous fungal conidia with various dry test surfaces, as well as the interactions of the hyphomycetous fungi and unicellular fungi with surfaces suspended in an aqueous saline solution; 4) determine the relative adherence of conidia and unicellular fungal cells to plasmamembrane glycoprotein; and 5) deploy fluorescent molecular probes to analise and/or visualise spore surfaces for the presence of adhesives.

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CHAPTER 2

LITERATURE REVIEW - Fungal spore production and dispersal

2.1 Fungal spore production

Fungi belong to three complex biological Kingdoms, one of which is called *Eumycota* (true fungi), which has the same biological rank as plants and animals (Adl *et al.,* 2005; Kendrick, 1985; and Kendrick, 1992). They have long been recognised as important members of terrestrial ecosystems, functioning as decomposers, parasites, pathogens and mycosymbionts (Ale-Agha *et al.,* 2003, Cooke 1977; Cromrack and Caldwell, 1992). The forest floor and belowground is dominated by visible clusters of microscopic filaments (hyphae) that are branched and intertwined (mycelia) in most ecosystems (Harley, 1971; Ingham *et al*., 1989). Fungi are ubiquitous organisms that make up approximately 25 % of the earth's biomass, and can be subdivided by gross morphology into moulds, yeasts, mushrooms, puffballs, truffles and mildews (Marsh, 1968). Their cosmopolitan success can mainly be accredited to their ability to produce large numbers of spores that can be dispersed over long distances (Roncal and Ugalde, 2003). Most fungi do sporulate, though sometimes very special nutritive and environmental conditions are required for the successful formation and dispersion of spores.

2.2 Role of spore formation in the fungal life-cycle

The fungal life-cycle usually involves sexual and asexual stages, and both result in the production of spores (Dyer and Paoletti, 2005; Moore-Landecker, 1982). Sexual and asexual spores (conidia) including their associated reproductive structures are referred to as the teleomorph and anamorph, respectively. Anamorphic and teleomorphic spores have been shown to be morphologically dissimilar.

Conidia are usually produced as an alternative to sexual reproduction, and their formation is brought about as a result of internal controls or by unfavorable nutritional or environmental conditions (Roncal and Ugalde, 2003; Gottlieb, 1978). Sporulation can be functional where spores serve to preserve the organism against unfavorable environmental conditions. Some asexual spores are often thick-walled, as in the case of chlamydospores (Carlile and Watkinson, 1994; Ohara and Tsuge, 2004). Fundamentally spores have been classified into spores that remain dormant at their site of origin and spores that are dispersed in order to secure geographical distrubution (Madelin, 1996; Tsitsigiannis *et al.,* 2004).

Spores, therefore, represent the end of an assimilative growth phase by means of hyphae, which can embark on a speculative investment (Madelin, 1966). They occupy a unique position in the life-cycle as they terminate both reproductive and developmental cycles, and have the inherent potential to develop into a new generation (Moore-Landecker, 1982). Moreover, fungal spores are less susceptible to adverse environmental conditions than the mycelial forms or yeast cells, and germination of spores will not occur until environmental conditions are optimal for their survival. The fungal spore can, therefore, be defined as an entity of a single cell or group of cells of low metabolic activity that are produced by the thallus, has reproductive or survival functions, and can germinate and grow into a thallus under appropriate conditions (Gottlieb, 1978).

It is, therefore, clear that sporulation does not only make provision for the development of new and subsequent generations, but also for the removal of the latter from vicinity of the thallus (Osherov and May, 2001; Ross, 1979). Removing siblings is important since competition among the former and the thallus will not be so intense that starvation occurs among all. Spore dispersal may be grouped into different phases of which the first is the actual dislodging of the spores from the thallus through intervention of physical environmental forces or forcibly discharged. Secondly, once dislodged, spores may be disseminated passively by animals, wind or water to new locales. Lastly, spore dispersal is successfully completed once the spores are deposited on new substrata and germination has taken place under appropriate conditions. However, the laws of probability are against the success of any single spore, and the majority of spores are wasted (Moore-Landecker, 1982).

2.3 Methods of spore release

"Fungi cannot walk or run, but some can swim, most can soar, a few can jump and some must be carried" (Kendrick, 1985). As mentioned previously, fungal spores may either be released from the thallus through external forces in which the sporebearing structures plays a passive role, or the spores may be actively discharged by these structures (Moore-Landecker, 1972 and 1982; Ingold, 1953; Madelin, 1966).

2.3.1 Active mechanisms of spore release

The most profound feature of active spore liberation is the fact that the spores are forcibly shot into the air (Ingold, 1933; 1934; 1953; Trail *et al.,* 2005). The distance (*d*) that the projectile (spore) will travel may be calculated using a simplified mathematical formula: $d = Kr^2$, where *K* is a constant and *r* the radius of the spore (Madelin, 1966). The distance that the spore will be discharged is, therefore, directly proportional to its diameter. Active spore liberation may furthermore be subdivided into three categories, which will be briefly discussed.

2.3.1.1 Explosive mechanism

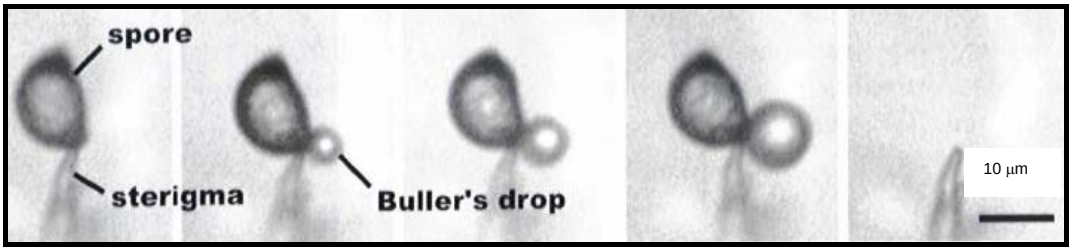
A common means of forcible spore liberation is that in which the subsporangial vesical becomes turgid through increased osmotic concentration and suddenly bursts, carrying the spores away in a jet of water (Fischer *et al.,* 2004; Ross, 1979). The majority of bursting cells are found in the Ascomycetes, and the actual mechanism for all explosive asci is probably through the conversion of insoluble to soluble materials in the epiplasm resulting in an increased tugor pressure. Spores are usually shot 0.5 to 5 cm through the still layer of air directly above the ground into a region of relative turbulent air conducive to effective dissemination.

However, this mechanism is not restricted exclusively to Ascomycetes, as it is also being deployed by coprophilous fungi. A good example is the mucoralean fungus, *Pilobolus,* which posses a highly specialised sporangiophore that functions as a gun capable of projecting the sporangium a considerable distance away (Page, 1964). At maturity the photodirected sporangium which contains from 15 000 to 30 000 spores rests on top of a subsporangial vesicle. Separating the former from the latter is the so called columella, which has a weak zone at its base. At maturity tugor pressure inside the vesicles rapidly increases, until the whole complex is very unstable. A sudden change in light intensity or any other environmental shock exceeds the cohesive strength of the weak zone, which eventually ruptures. The rupture extends all around the vesicle resulting the in an explosive release of pressure in the form of a jet of cell sap up to 2 m in length.

2.3.1.2 Ballistospore discharge

This mechanism of active spore discharge has been observed amongst members of the Hymenomycetes, gelatinous Basidiomycetes and rusts (Haard and Kramer, 1970; Pringle *et al.,* 2005; Webster *et al.,* 1984 and 1989). The basidium consists of four sterigmata which bears basidiospores at a 45^0 angle, and are separated from the basidiospores through a septum and a minute kneelike projection, called the hilar appendix. As basidiospore discharge is being set in motion, a liquid droplet (Buller's drop) starts to accumulate at the base of the hilar appendix. This bubble forms because the outer spore wall weakens prior to its separation from the sterigmata. Once the bubble reach half the size of the basidiospore the latter is suddenly violently projected from the sterigmata, seemingly propelled by the escaping jet of air and/or liquid that results from the sudden release of pressure (see Figure 1 a & b). However, it was recently demonstrated that spore discharge involves the coalescence of Buller's drop and the spore (Pringle *et al.,* 2005). Spores are projected individually from each basidium, and several seconds may elapse between discharges.

a



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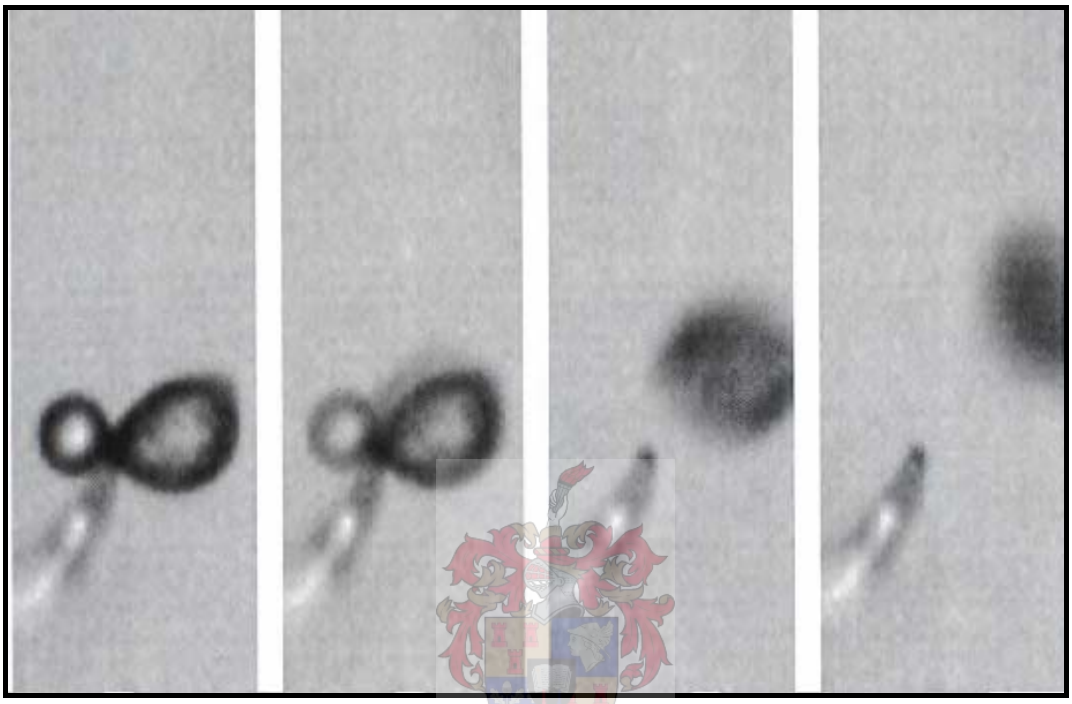


FIG. 1 (a). Photographic illustration of the formation of Buller's drop in *Itersonilia perplexans*, the disappearance thereof, as well as the almost simultaneous discharge of the ballistopsore. (b). The actual discharge of the spore visualised with ultra high speed video (taken from Pringle *et al.,* 2005).

2.3.1.3 Eversion mechanism

This dispersal mechanism is deployed by the basidiomycete, *Sphaerobolus stellatus*, the cannonball fungus (Ingold, 1972). The fungus has a spherical fruiting body, consisting of multiple layers *i.e.* the firm outer cup, the double layered inner membrane, an air layer between the cup and double layered inner membrane, the peridiole (containing the spores) and the fluid in which the peridiole is immersed. At maturity the outer layer covering the sporecontaining periodiole splits radially from the apex of the sphere because the double layered inner membrane expands laterally as it absorbs water.

This creates a toothed cup, which vary between four and eight teeth. Stresses are continually being created within the double layered inner membrane, and eventually there comes a point when the outer cup has opened enough to allow the stresses to be released. Subsequently, the double layered inner cup suddenly turns inside out and in the process the periodiole is flicked forcibly out and upwards.

2.3.2 Passive mechanisms of spore release

The vast majority of spores requires an outside agent to dislodge them from the sporebearing structures *i.e.* wind, water or animals (Fagg, 2004). Passive spore release is often directly related to their method of dissemination, so much that the two processes are almost inseparable. Therefore, the mechanism of actual separation from the thallus can be reflected in the mechanism of dispersal. As rule of thumb, spores that are to be dispersed by air currents are dry at maturity, whereas spores utilising animal vectors tend to be sticky with palatable flavor components (Ingold, 1953). Many conidial fungi that produce conidia in loose heads, tend to secrete drops of slimy exudates around matured conidia that cause the latter to adhere to anything that happens to touch them, including mammals, insects etc. Waterdispersed spores on the other hand tend to be unusually shaped, formed in coherent masses, and some may be dry. Large numbers of conidial fungi produce chains of conidia on top of each other, exposing the higher conidia to air currents (Deacon, http://helios.bto.ed.ac.uk/bto/microbes/biotroph.htm).

The morphology of the fruiting body also plays a role in spore dispersal. Brodie and Gregory (1953) have observed that air currents is much more effective in removing spores from a cup-like fruiting body than from flat surfaces, because an eddy stream forms with the former carrying spores upwards. Some fruiting bodies *e.g.* splashcups of the bird's nest fungus (*Cyathus olla*) are especially adapted to utilise the force of falling raindrops to dislodge spores (Brodie, 1951).

Falling raindrops have been shown to dislodge dry-spore types upon impact on sporebearing structures and even carry some of the spores in their splatter (Dixon, 1961; Geagea *et al.,* 1999; Savary and Janeau, 1986). Some basidiospores are puffed from their fruiting bodies as a result of external pressure being exerted on the latter by foraging animals and/or falling raindrops. Other examples of fungi producing such fruiting bodies include members of the genera *Lycoperdon* (Figure 2a) and *Geastrum* (Figure 2b). However, in order for fungi to become widespread mechanisms must be deployed to ensure that fungi are successfully dispersed over long distances.

2.3.2.1 Spore dispersal by air

Spore dispersal by wind. Fungi have tended largely to be anemophilous, therefore, the most common mechanism, by far, of spore dispersal is by wind (Christensen, 1975; Ingold, 1953; Menezes *et al.,* 2004). Wind-dispersed fungi often produce "dry" spores, which do not readily absorb water or moisture from their direct environment and are said to be hydrophobic. The resistance of these spores to absorb water may enhance their ability to remain afloat in air, since their weight is kept down. When these spores are bombarded by water or falling raindrops (splash-dispersal), the impact dislodges and scatters the spores in all directions and the spores are dispersed by resulting turbulent air (Geagea *et al.,* 1999; Dixon, 1961).

Lamellar layer. In order for spores to be dispersed by air currents, they first need to breach the layer of still air (lamellar layer, Figure 3) directly above the ground or the surface of their growth substrate (Geagea *et al.,* 1999; Moore-Landecker, 1982). Some fungi have evolved in such manner that their spores are elevated on either sporangiophores or conidiophores enabling spores to be released in the turbulent air above the lamellar layer. Some plant associated fungi *e.g.* powdery mildew have evolved in such a manner that the spores are released even higher from the ground since the spores are borne on diseased plants surfaces well above the ground (Huckelhoven, 2005).

a

b

FIG. 2 (a) *Lycoperdon* that allows spore puffing from the ostiole when external pressure is applied on the peridium (www.botany.hawaii.edu/faculty/wong/BOT135) (b) *Geastrum*, the so-called earthstar is another example of a puffball with a pliable peridium from which spores are puffed from the ostiole.

Floatation and sedimentation in still air. Small particles, fungal spores in particular, have the ability to readily stay afloat in relative still air (Gregory, 1973). The air in a closed room is theoretically still, and dust particles can readily be observed staying afloat with ease in the sunlight shining through a window. The convection caused by the light beam perpetuate the floating, and it should not be difficult to imagine that spores, which are far smaller and lighter, would and probably are also present in such a light beam. However, spores will eventually sediment from the air under the influence of gravitation. The sedimentation rate of small spherical objects in relative turbulent air such as fungal spores, can be predicted by using Stoke's Law, which describes the terminal velocity of a smooth spherical object falling in a fluid medium (Gregory, 1973). For microbial aerosols *e.g.* fungal spores, suspended in air, assuming that the density of the medium is negligible compared to the density of the falling spore, Stoke's Law simplifies to: $V_T = 0.0121\ r^2$, where V_T is the terminal velocity (ms^{-1}) and r is the radius (μm) of a spherical aerosol droplet. Previous studies have shown that basidiospores of *Agaricus campestris* fall at a rate of just over 1 mm per second, and at that rate even the slightest air current will disturb the gradual fall under gravity and throw the descending spore into turbulent air. It can, therefore, be envisaged that the rate of sedimentation of a spore, and the path it will travel, is determined by its velocity in response to gravitation and the direction and rate of airflow (Moore-Landecker, 1982). Dispersal of falling basidiospores under the influence of gravitation and a very minute airflow is depicted in Figure 4a & b. The greatest number of spores landed directly below the mushroom, as you might expect, but some spores stayed afloat until reaching the other end of the box.

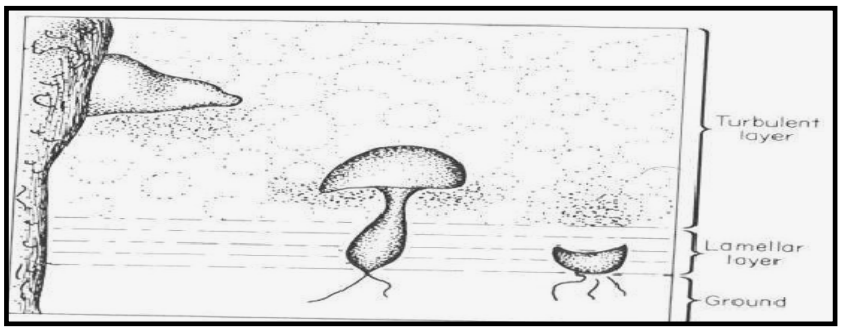
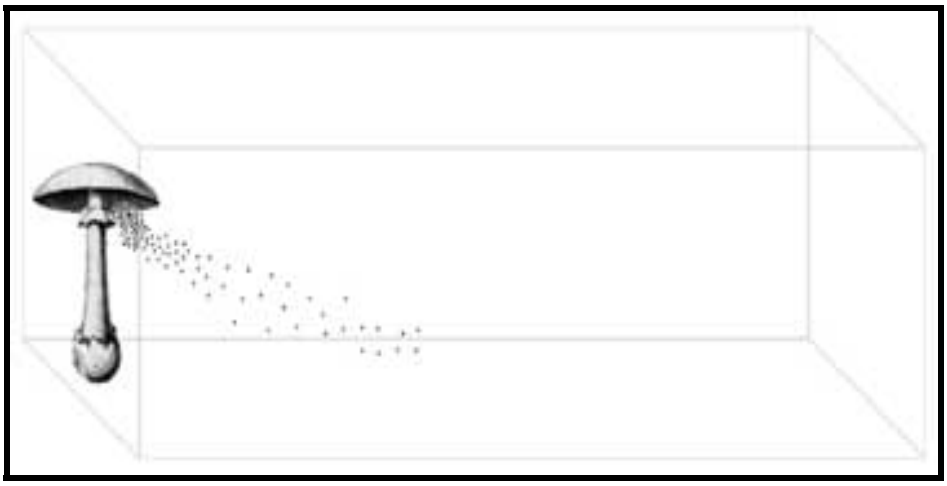


FIG. 3. Schematic illustration of the different layers of air directly above a flat surface in nature (Moore-Landecker, 1982).

a



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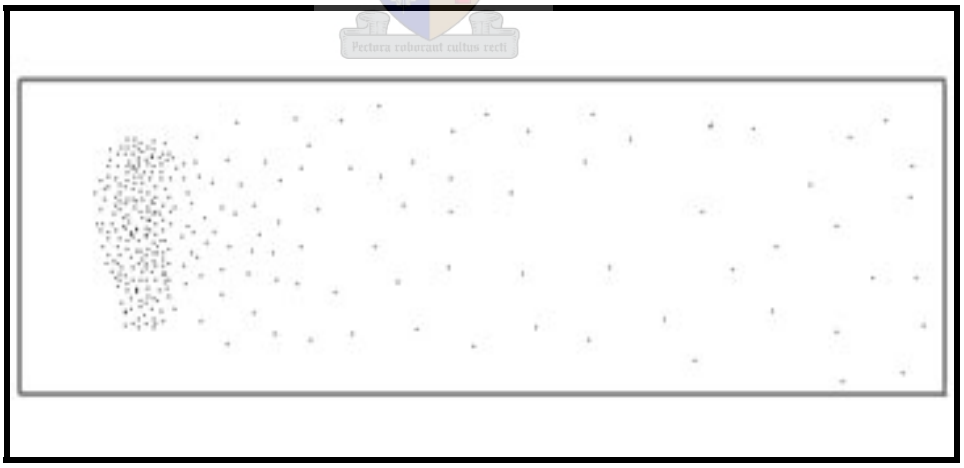


FIG. 4 (a). Mushroom spore dispersal in a covered cardboard box without air circulation (www.botany.hawaii.edu/faculty/wong/BOT135). The spores will land on the cardboard bottom where we can record the number of spores (b). Top view of spores on the bottom of the box.

Distances travelled in the outdoors. Movement of spores in the air from their point of liberation has been compared to the smoke of a chimney, which tends be conical with the apex at the chimney's opening (Ingold, 1953). At the periphery, eddies tend to mix the smoke with air, thereby simultaneously increasing the width of the cloud and dilute it. The horisontal concentration of airborne spores usually approaches zero at 100 to 200 m from the point of liberation, where as the vertical concentration decrease logarithmically with height. Despite most fungal spores being deposited within 200 m from their point of liberation, evidence was uncovered that some spores travel long distances *e.g.* evidence exists that spores of *Puccinia gramminis* may be able to travel from Australia to New Zealand (Viljanen-Rollinson and Cromey, 2002.). Some wind-dispersed mushroom spores are so effective in travelling along air-currents that these fungi have a worldwide distribution (Raper *et al.*, 1958). It is noteworthy that basidiospores, presumably form a significant part of the aerial microflora (Ingold, 1953).

During the early studies of aerobiology (biology of pollen and fungal spores in aerosols) fungal spores were found at altitudes of 3200 meters. Charles Lindbergh, (1930) conducted an experiment over the Arctic Circle and found fungal spores at an altitude of 1000 m (www.botany.hawaii.edu/faculty/wong/BOT135). The altitude is less than the previous experiment, but it gave an indication of how far spores are able to travel since the observation was made above an open ocean. *Cladosporium* and *Penicillium* spores have been observed to be present in eastern winds coming from the United States blowing towards the Atlantic ocean, and the possibility was mentioned that these spores might even be able to cross this ocean on the wind currents (Feinberg, 1946). In 1935 the balloon Explorer II released a spore trapping device at an altitude of 22 000 m that was set to close at 10 000 meters (Rogers and Meier, 1936). Wind velocity measured during this experiment ranged between 65 and 100 km/h, meaning that spores within this air current was able to potentially travel 13 500 km/week. Christensen (1942) calculated that a fungal spore at an altitude of 1600 m carried by winds of approximately 32 km/h will travel a distance of 4 000 km in only nine days. Similar calculations were made by others, but are presumably of little importance since turbulence was not taken into consideration (Gregory, 1945).

Spore deposition. Although wind dissemination is by far the most efficient dispersant in terms of distance that spores travel (Ross, 1979), it was stated that capricious behavior of wind make it a matter of chance that a particular spore will land in a favorable location (Ingold, 1953). Spore dispersal is completed once spores are successfully deposited on a suitable substratum where they can germinate and continue their life cycle. Spore deposition may involve different processes such as impaction on a protruding substratum due to wind-driven spore momentum and sedimentation under the influence of gravitation as previously described (Gregory, 1951). However, only spores with sufficient kinetic energy are able to break free from turbulent air to impact onto the nearest surface (Gover, 1999, http://sydneyfungalstudies.org.au). At low wind speeds only the larger spores impact whilst, smaller spores require greater wind speed. Smaller spores however, were shown to come to rest on leaf surfaces via boundary-layer exchange during which spores pass from turbulent layer to the lamellar layer and sediment out, or they may be deposited with falling raindrops (Fitt and Nijman, 1983; Moore-Landecker; 1982).

2.3.2.2 Water assisted dispersal

Aquatic fungi. In contrast to wind dispersed spores that are known to be hydrophobic, water dispersed spores are usually hydrophilic and able to absorb water (www.botany.hawaii.edu/faculty/wong/BOT135). The latter type of fungus may produce tetraradiate spores, each consisting of four arms diverging from a common point, *e.g.* aquatic hyphomycetous fungi with their hyaline spores. Common aquatic tetraradiate spores isolated from foam in a flowing river are depicted in Figure 5 (Ingold, 1953, Kendrick, 1992). Many aquatic fungi produce their spores within mucilaginous exudates and due to the weight of the latter and the fact that the exudates bind the spores together, the spores are not dispersd by wind. The irregular shape of tetraradiate spores enables them to anchor more easily on a substratum, since it makes contact at three points simultaneously. Furthermore, branched spores tend to be trapped more easily in air bubbles, thus favoring their removal from moving water currents to the foam (Iqbal and Webster, 1973).

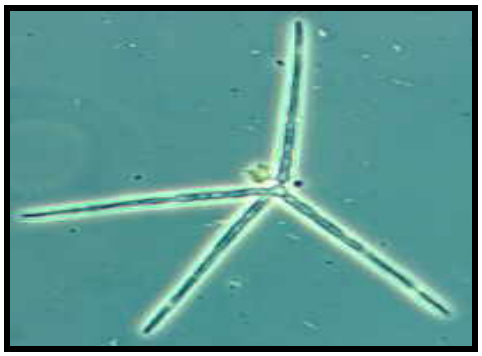


FIG. 5. A tetraradiate conidium of *Lemonniera* spp. isolated from foam in a flowing river (Kendrick, 1992).

Soil fungi. Many terrestrial fungi are disseminated by water, even though their spores seem not primarily adapted for water dispersal (Malloch and Blackwell, 1992). Running water have been shown to carry spores of these fungi from one locale to another *e.g.* the trickling of water down a tree trunk bearing fungi or the seepage of water through soil. Also, spores have been observed to be commonly washed from leaves and branches to the lower parts of the plant (Bertrand and English, 1976; and Spotts, 1980). Furthermore, spores have occasionally been reported to being washed directly from the plant into the surrounding soil (Kuske and Benson, 1983). It has also been reported that spores can readily be transported within the aqueous films covering plant litter, and that many spores may be carried from lower leaves to newly fallen ones (Malloch and Blackwell, 1992). Spore dispersal through relative dense soil occur slowly, however, by having motile zoospores members of the Oomycota are able to disperse in water saturated soil (Benjamin and Newhook, 1982). It is these zoospores that may serve as the primary cause of oomycotan root infections. However, non-motile spores on soil surfaces may be carried with splatters to surrounding host leaves. It can, therefore, be envisaged that once conidia have reached the deeper soil layers, travelling to plant host leaves seems almost impossible, since they cannot be carried by splatters of rain or being propelled as is the case of motile zoospores.

Fungi on the phyloplane. Falling raindrops have been observed to strike spores suspended in water films on leaflets resulting in splash drops that carry many spores in all directions (Savary and Janeau, 1986; Dixon, 1961). Furthermore, the splash droplets tend to be dispersed downwind (Fitt and Nijman, 1983). Many "dry" spores on leave surfaces are regularly dislodged from their colonies by raindrops, and consequently become airborne (Hirst and Stedman, 1963). A positive correlation between the number of airborne conidia and rainfall has been observed (Faulkner and Calhoun, 1976). Splash dispersed spores tend to be dispersed over shorter distances than spores dispersed by dry air currents. However, smaller spore carrying splatters may travel considerable distances in a process called wind-assisted splash dispersal (Fitt and Nijman, 1983).

2.3.2.3 Spore dispersal by Animals

Arthopod dispersal. Smirnoff (1970) have previously reported that arthopods act as vehicles for the fungi on which they feed. Arthopods *e.g.* mites are today best known for the role they play in the dispersal of fungal dissiminules on Petri dishes (Malloch and Blackwell, 1992). Spores of numerous fungal genera were observed to be attached to phoretic gasmid mites *i.e. Arthrobotrys, Coprinus, Dictyostelium, Mucor, Mortierella, Sphaeronaemella,* and *Stylopage.* Fungal spores adhering to the external surface of a mite is a common site when these arthropods are observed with a microscope (Figure 6).

It is known that insects are important vehicles in the dispersal of fungi (Prom and Lopez, 2004). Fungal spores may be carried externally on their body or internally in the gut of the insect (Malloch and Blackwell, 1992). Dispersion via insects was studied in blue-stain fungi, belonging to the family Ophiostomataceae. The interaction of these fungi with bark bettles is probably the best known example of fungi being carried on the exterior of insects. However, bark beetles have also been reported to carry fungal disseminules belonging to the genera *Cryptoporus* and *Fomes* on their exoskeletons (Castello *et al.,* 1976).

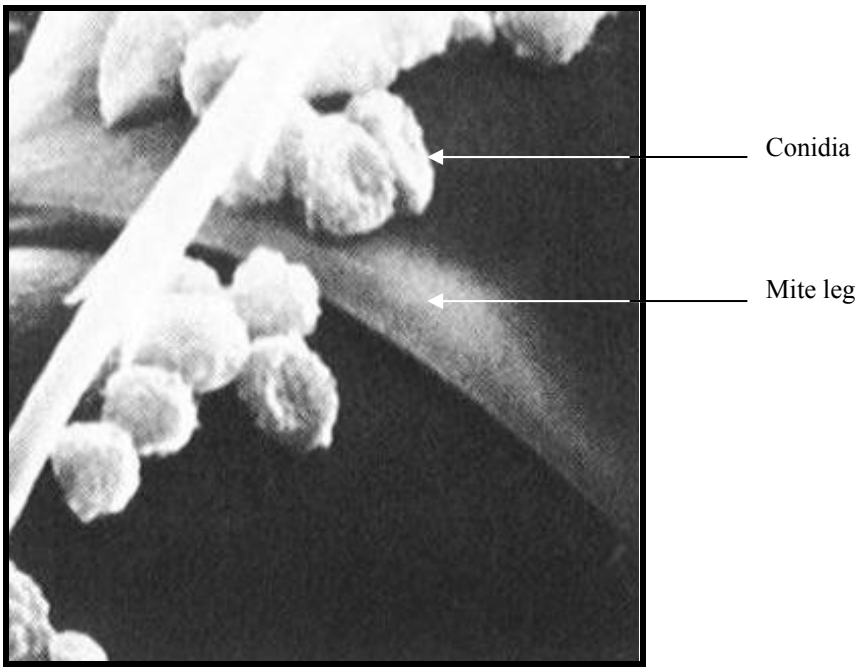


FIG. 6. Conidia on the leg of *Tyrophagus putrescentiae*, a mite (Nilsson, 1983).

A dispersal mechanism deploying insects can also readily be observed in a group of fungi known as stinkhorns (Figure 7a & b) www.botany.hawaii.edu/faculty/wong/BOT135). These foul smelling fungi release their spores into a liver-brown slimy exudate produced on top of a colorful fruiting body. When the spores are matured the odors of the exudates attract insects, usually flies that eat the spore-containing slime. Spores are dispersed by passing undamaged through the digestive system of the insect and being excreted in a new location.

Bird vectors. It has long been suspected that many plant pathogens deploy birds as agents of dispersal (Malloch and Blackwell, 1992; Warner and French, 1970). Migrating birds are known to disperse fungi externally or internally over long distances. Beak, claw and throat swabs of birds plated onto media revealed the presence of Hyphomycetes, which was complemented by a similar study that also revealed the hyphomycetous fungi isolated from claw swabs of mist-netted birds to be the prevalent ones in the atmosphere (Malloch and Blackwell, 1992).

Mammal vectors. Hyphomycetous fungi such as *Penicillium* occur in the fur of small mammals (Beguin *et al.,* 2005; Frisvad *et al.,* 1987). Spores can easily be brushed off on objects in the path of the animal and in the process be dispersed. Similarly, humans in greenhouses have been reported to be the major source of external plant pathogens (Malloch and Blackwell, 1992). Many fungi are also dispersed internally via the gut of herbivores which accidentally consume the fungus whilst foraging. A common example is the dung fungus *Pilobolus kleinii*, depicted in Figure 8. *Pilobolus kleinii* inhabits dung, and the fungus disperse its spores actively far beyond the dung heap. Grazing cattle will eat the spore, and complete the fungal life cycle when the spore passes through the herbivores's digestive system and is being excreted elsewhere. In addition, mammalian guts are wellknown dispersers of large basidiomycetous fungi (Teetor-Barsch and Roberts, 1983).

a

b

FIG. 7 (a). *Phallus rubicundus*, a stinkhorn, has a very strong odor that attracts flies when the spores are mature. The slimy apical portion contains the spores (www.botany.hawaii.edu/faculty/wong/BOT135). (b). *Aseroë rubra*, the red star, produces brownish slime containing spores on top of red pseudo-flowers. This species is a common stinkhorn that also occurs in South Africa (van der Westhuizen and Eicker, 1994).

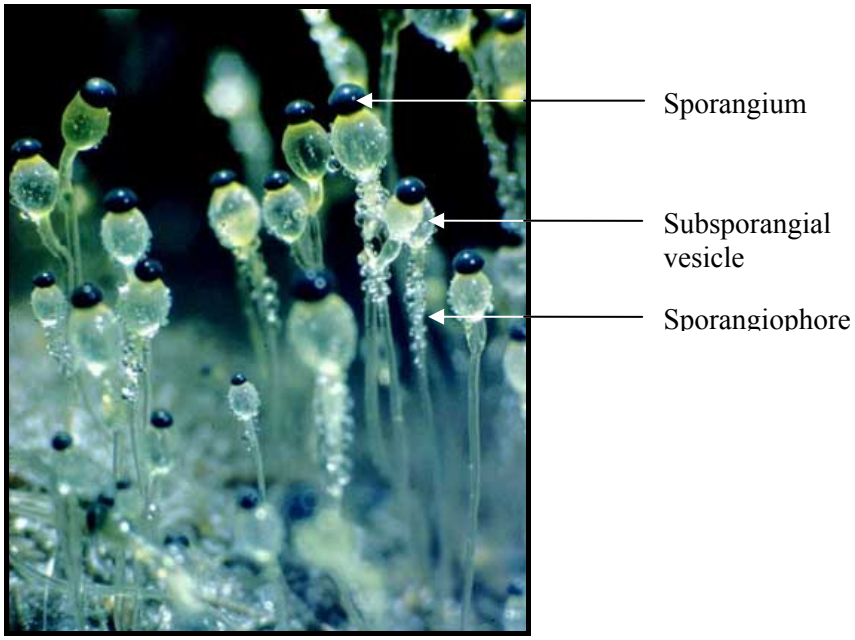


FIG. 8. *Pilobolus kleinii*, a copriphilous fungus, which discharge its spores actively by means of internal energy in its subsporangial vesicle (www.bioimages.org.uk).

2.4 Ability to withstand adverse environmental conditions

Following dispersal and the subsequent deposition from amongst others the air, spores can immediately start to germinate if suitable conditions prevail or they may go into a dormant phase as a result of less favorable conditions (Moore-Landecker, 1982). A critically important feature of many fungal spores is their ability to withstand adverse environmental conditions, by remaining dormant until favorable conditions are encountered. Dormancy is induced by unfavorable conditions such as lack of appropriate nutrition (Feofilova *et al.,* 2004), and/or the presence of growth-inhibiting organic compounds secreted by other micro-organisms within the micro-environment of the spore (Lavermicocca *et al.,* 2000; 2003). Rogers and Meier (1936), observed floating spores at altitudes of least 22 500 m, at temperatures below freezing within thin air. Suprisingly, five viable spores were found among the mass of spores sampled. It can be envisaged that these spores must have also been subjected to elevated levels of UV radiation.

These experiments during the first half of the 20th century already showed that fungal spores are able to survive quite adverse conditions. Some fungi produce survival spores (chlamydospores), encapsulated by a thick spore wall, and contain a fair amount of lipids (Ingold, 1953; Regulez *et al.,* 1980). The morphological adaptations enables the spores to remain dormant for years in extremely hot environments, and can germinate when more favorable conditions prevail, and the fungal life cycle is continued (Ciotola *et al.,* 2000). Furthermore, some spores are able to pass through the gastro-intestinal tract of herbivores, and continue the germination process. One such example is the dung fungus, *Pilobolus kleinii*.

2.5 Germination and dormancy

Germination is the process which enables the fungal spore to develop into vegetative structures (Moore-Landecker, 1972). Transformation of the spores into the thallus is always preceded by the formation of a germ tube (Figure 8a). However, this is only true for mycelial fungi because some unicellular fungi (yeasts) produce a bud that breaks away from the mother-cell once fully grown, creating a bud-scar (Figure 8b). The most significant feature of germination is the metabolic shift from a low rate (the period of dormancy) to a relatively high metabolic rate. Gottlieb (1978) have observed that the latter sometimes surpasses the rate of optimum vegetative growth. Furthermore, the increased rate of anabolism and catabolism are dependant on a variety of factors *i.a.* the presence of innate regulatory mechanisms; the availability of essential nutrients, which can be readily absorbed from the immediate environment; the presence of internal enzymes for respiration and biosynthesis; and proper environmental physical conditions which will allow the metabolic functions to proceed (Gottlieb, 1978; Osherov and May, 2001). Germination can thus be regulated by a number of limiting factors, working in tandem. These include the following (1) Developmental factors such as maturation, sencescene and dormancy; (2) Physico-chemical factors, such as the absence of water, appropriate carbon and nitrogen sources, temperature and osmotic pressure; (4) The presence of toxic compounds (Chitarra, 2003).

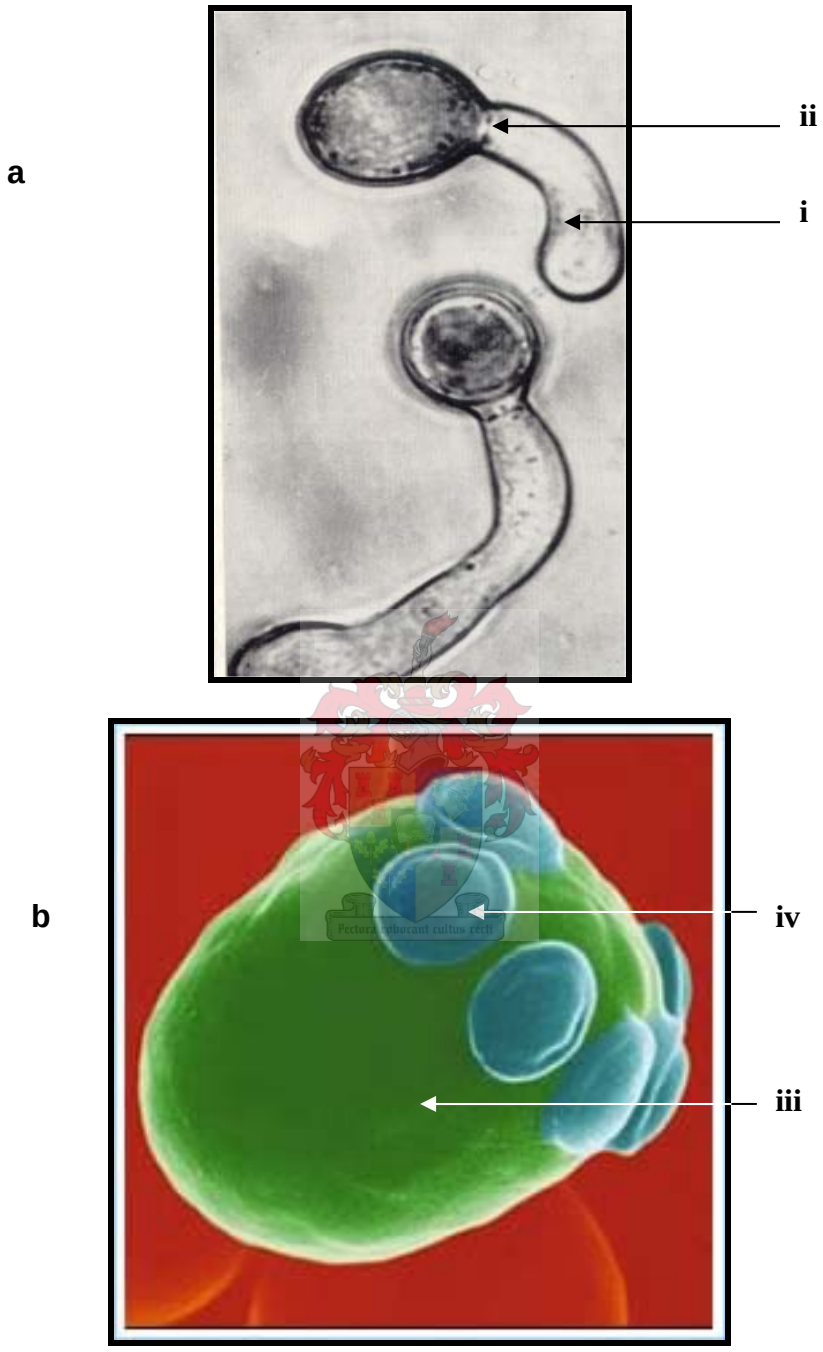


FIG. 8 (a). Germtube (i) formation of *Botrytis* spores, with the cross wall (ii) at the base of the germtube (adapted from Gottlieb, 1978). (b). Schematic depiction of *S. cerevisiae* mothercell (iii) with bud scars (iv)(Harold, 1990).

2.5.1 Developmental factors

Maturation and senescence. Spore liberation occurs usually as result of maturation and spores are expected to germinate unless a dormant state intervenes (Carlile and Watkinson, 1994). The two states differ from each other in the sense that mature spores cannot be activated to germinate, whilst dormant cells can be stimulated to germinate (Jones, 1978; Thanh and Nout, 2004). Senescence is an inherent trait involved in the ability of spores to germinate. Some spores are very short-lived and the percentage germination decreases with increasing time under a given set of environmental conditions (Darby and Mandels, 1955). However, some fungal spores are able to remain viable where moist conditions prevail, as was observed with *Aspergillus* spores (Page *et al*., 1947).

Dormancy. This is the inertia state of spores which doesn't allow spore germination under conditions of limited nutrition after a prolonged period of maturation and following sedimentation on a substrate (Gottlieb, 1978). Sometimes dormancy can be reversed by periods of alternating warm and cold, and/or wet or dry (Necas and Gabriel, 1978). It has been shown that dormancy can artificially be induced in the presence of organic compounds such as aldehydes (Saksena and Tripathi, 1987).

Self-inhibition. Self-inhibition is a phenomenon, whereby micro-organisms belonging to the same species inhibit the germination of others by secreting an inhibiting compound, however, intraspecific stimulation have also been observed (Allen and Dunkle, 1971; Bottone *et al.,* 1998). When spores occur together a in large concentration, it has been observed that individual spores are able to self-inhibit germination by secreting inhibitors. It has been proposed that the latter can be attributed to the fact that spore papillae are less permeable and the inhibitor cannot be leached out (Chung and Wilcoxson, 1969). The self-inhibition factor of *Puccinia graminis* has once been labeled as a volatile compound, which had been isolated and subsequently identified as trimethylethelyne (Forsyth, 1955).

Carlile and Sellin (1963) demonstrated that self-inhibitors are synthesised in the mycelium during the vegetative phase and are incorporated into spores. Self-inhibition can be seen as survival strategy in the sense that the spores do not germinate until they are dispersed away from the parent mycelium to other environments favoring germination. As a result the inhibitor would then diffuse out of the spore to a point lower than its inhibitory concentration and germination will continue normally (Chitarra *et al.,* 2004).

2.5.2 Environmental factors

Time. Different fungal species require different periods before spore germination (Fergus, 1954). An experiment conducted on *Peronospora parasitica* and *Endoconidiophora fagaceatrum* showed that spores from the former had almost completely germinated by the time the latter had even begun. Gottlieb (1978) observed that germination in a population of spores is a function of time. Depending on time, as well as on environmental conditions the individual spores will germinate in a heterogenous manner.

Temperature. It was demonstrated that temperature influences physiological functions and consequently spore germination (Gottlieb, 1978). It was found that high temperatures stimulate biochemical reactions whilst low temperatures inhibit them. Furthermore, the former may inhibit spore germination because it increases reaction rates until it is out of balance, and may also result in conformational changes in proteins, as well as denaturation of these molecules. Optimum spore germination was found to be temperature sensitive (Plaza *et al.,* 2003). While most fungi readily germinate at temperatures ranging from 20 to 30 ^{0}C, some are thermophilic and germinate at higher temperatures (40 to 50 ^{0}C) (Ogundero and Oso, 1980). A number of fungi are known to be cryophilic, having a low optimum growth temperature, whilst others are thermoduric, having a high maximum growth temperature but a lower optimum growth temperature. It was found that spore germination (Figure 9a) is so sensitive to temperature changes, that germination dropped from 100 % to 20 % when incubation temperature was increased by one degree from 32 ^{0}C to 33 ^{0}C.

Oxygen. The effect of oxygen on germination was studied in the zygomycete, *Mucor rouxii* (Wood-Baker, 1955). This fungus, which is known to be fermentative respiring, showed an increase in percentage spore germination with increased atmospheric oxygen concentrations. Likewise, manipulation of the gaseous environment of *Erysiphe graminis* spores in shake flasks showed only 29 % germination, when nitrogen gas was pumped through the spore suspension. However, when oxygen was passed through the suspension spore germination rose to 65 %. In another experiment, conducted with *Fusarium solani f. phaseoli*, percentage germination seemed to reach a plato when the percentage oxygen was increased (Figure 9b) (Cochrane *et al*., 1963). Total anaerobic conditions were found to prevent growth of this species.

Carbon-dioxide. It was found that CO_2 affects spores of different fungi differently, for example germinating spores of *Botrytis cinerea* are able to withstand high concentrations CO_2 (Dock *et al.,* 1998), whilst germination of *Coccomyces hiemalis* spores is inhibited by high levels of this gas (Bourret *et al*., 1978). In contrast, germination of *Fusarium solani* spores is stimulated by CO_2. Studies with ^{14}C-labeled CO_2 indicated that this gas is fixed during germination and is essential for the germination of *Aspergillus niger* spores (Yanagita, 1963). Furthermore, CO_2 fixation has also been claimed to be vital for *Schizophyllum commune* spore germination, whilst *Uromyces phaseoli* fix CO_2 into its purine and pyrimidine bases during germination, and subsequently into its RNA needed for translation (Hafiz and Niederpruem, 1963).

Water relations. Water is an essential component for fungal growth and it was demonstrated that water is crucial for spore germination (Pratt, 1936; Sautour *et al.,* 2001). However, the concentration of water needed for spore germination differs. Armolik and Dickson (1956) measured the relative humidity required for spore germination and found the humidity required for *Aspergillus*, *Penicillium* and *Fusarium* is respectively, 79 %, 81 % and 87.3 %. Moreover, different isolates of *P. verrucosum* were observed to respond significantly different to equilibrium relative humidity, also known as water activity (Pardo *et al.,* 2005).

Yarwood (1950) found that the conidial water content of powdery mildews ranged between 52 and 75 %, whilst that of members belonging to the genera *Uromyces, Peronospora, Penicillium, Aspergillus, Botrytis,* and *Monilinia* ranged between 6 and 25 %. Furthermore, it was observed that powdery mildews conidia require less atmospheric humidities than the other genera in order to germinate. It can, therefore, be envisaged that the relative humidity required for conidial germination is inverse proportional to its water content. The minimum water activity determined on artificial substrates should not be seen as reflecting water requirements for germination in nature. Longree (1983) demonstrated that *Sphaerotheca pannosa* spores require a minimum relative humidity of 96 % on glass slides but only 22 % on leaves, because the relative humidity around the host plant at the time of sporulation may also determine spore water content. Also noteworthy, is the fact that water activity required for germination varies with temperature (Marin *et al.,* 1998; Plaza *et al.,* 2003).

Hydrogen ion concentration. In general hydrogen ion concentration (pH) was found to effect spore germination in the same manner as it effects mycelial growth (Gottlieb, 1978). The optimum usually is in the acidic range and may vary quite wide *e.g.* between pH 3.0 and pH 7.0 (Figure 9c). However, some species have a narrow pH optimum range between pH 3.0 and 4.0. In contrast, a recent study on *Penicillium chrysogenum* demonstrated that pH may not have any significant effect on spore germination under certain experimental conditions *i.e.* temperature (15 or 25 ^{0}C), water activity (0.75 or 0.85) and pH (3.5 or 5.5) (Sautour *et al.,* 2001).

General comments. As soon as appropriate environmental conditions arise, germination takes place, therefore stored, nutrients must be available immediately. Amino acids which are stored within the spore must be readily available to be synthesised into protein (Burleigh and Purdy, 1962). It was demonstrated that protein is a major endogenous reserve in *Mucor racemosus* sporangiospores and that its turnover is a necessary event for glucose-triggered germination (Tripp and Paznokas, 1982). Furthermore RNA and DNA should also be present which will facilitate the immediate synthesis of more proteins and hence growth (Osherov and May, 2000; Sheppard, 1978).

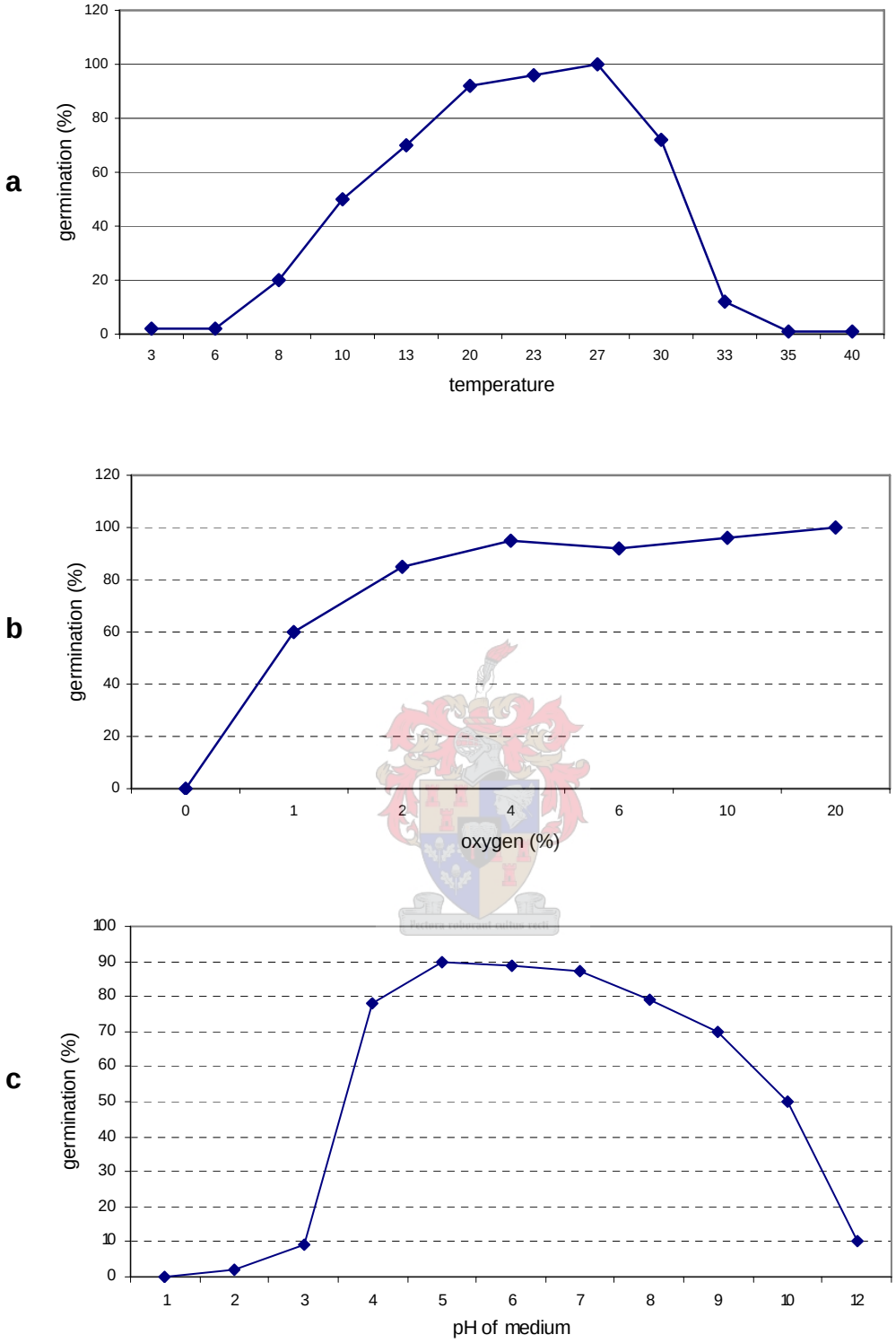


FIG. 9 (a). The effect of temperature on spore germination of *Ceratocystis fagecearum* (adapted from Fergus, 1954). (b). The effect of oxygen on spore germination of *Fusarium solani* (adapted from Cochrane *et al*., 1963). (c). The effect of pH on spore germination of *Penicillium atrovenetum* (adapted from Gottlieb and Tripathi, 1968).

2.6 Toxins produced by airborne fungal spores

Fungal toxins (mycotoxins) are secondary metabolites produced by moulds which result in a deleterious short-term or long-term effect in an animal host if ingested, inhaled or skin contact is made (Bennett and Klich, 2003; Yu and Keller, 2005). These mycotoxins are low-molecular-weight metabolites (less then 1 kDa) and are non-immunologic, because they elicit a toxic reaction that occurs with the first encounter (Woods, 2001). This phenomenon is known as mycotoxicosis and was first discovered when ergotism (ingestion of ergot) was studied (Desjardins and Hohn, 1997; van Dongen and de Groot, 1995). Later, stachybotryotoxicosis was discovered in humans and horses, while aflatoxicosis was discovered in turkeys (Pohland, 1993). Some of these metabolites may be products of enzymatic reactions or may serve as an adaptive strategy to inhibit competing microbes. It may be speculated that in a lot of cases humans are simply caught in the crossfire of "biological warfare" between microbes.

Symptoms associated with mycotoxins from airborne fungal spores include dermatitis, recurring cold/flu, sore throats, headeaches, fatigue, diarrhea, and impaired immune function (Sorenson, 1999). The most worrying factor is that all mentioned symptoms may be caused by many other diseases, therefore, mycotoxicosis can potentially be misdiagnosed, which so often happens. Mycotoxicosis might also be occupational/building-related diseases *e.g.*, Trichothecene intoxication in farmers handling wet hay originates from *Stachybotrys atra* (Bisby, 1943; Nikulin *et al.,* 1996). These fungi enter the interior environment as airborne spores (see fungal spore dispersal), which end up in damp areas and begin to germinate. Toxigenic fungi associated with damp conditions include, amongst others the genera *Aspergillus, Penicillium*, *Acremonium, Alternaria*, and *Trichoderma*. Furthermore, members of the aforementioned genera were shown to thrive on many building materials. Species of *Aspergillus* and *Penicillium* produce toxins such as aflatoxins and ochratoxins, which have been detected in stored grain and peanuts (Bennet and Klich, 2003).

It should be noted that the mycotoxins mentioned above are very potent at low dosage and are known to be cytotoxic, because they disrupt various cellular structures, and interfere with replication, transcription and translation (Woods, 2001). The cytotoxicity that these toxins display may disrupt the physical functioning of the route of entry into the host, which include the respiratory tract, gastro-intestinal route and skin. The combined result from this cytotoxic activity may generally increase the exposed person's susceptibility to infectious disease, and decrease their defense against other contaminants. Health effects associated with these toxins include hepatoxicity, carcinogenesis, heamorrhage, nephrotoxicity.

To conclude, many fungi produce mycotoxins, which can be found in airborne spores, mycelia and in the growth substrates *e.g.* wood or paper in quantities dependent on the specific fungal species (American Conference of Governmental Industrial Hygienists, http://www.mold-survivor.com/). Toxins display three basic mechanisms of action/activity: (1) Direct cytotoxicity, of which the effects can seen within a few hours; (2) Mutagenecity; and (3) Mimicry, where these toxins mimics some mammalian hormones, especially sex hormones. Table 1 list the toxilogical effects of some of the purified mycotoxins, of which more than one may be displayed by the same toxin (Pohland, 1993).

TABLE 1. Toxilogical effects of two common mycotoxins, Pohland (1993).

Mycotoxins	Mutagenicity	Teratogenicity	Carcinogenecity
Aflatoxin	+	+	+
Ochratoxin A	-	+	+

+ Toxin displays activity; - Toxin is unable to display activity

2.7 Allergens on airborne fungal spores

Allergy is another symptom associated with exposure to elevated levels of fungi, and the allergic reaction is generally due to inhalation of conidia (Salvaggio and Aukrust, 1981). Fungal-cell surfaces including conidia contain antigenic proteins that can cause allergic reactions in allergy sensitive individuals (Green *et al.*, 2005).

These reactions include conjunctivitis, rhinitis, bronchitis, asthma and hypersensitivity pneumonitis (Nolard, 2001). Furthermore, mold has been linked to cases of subclinical, acute and chronic respiratory disease. Fungal allergens (myco-allergens) are fungal products that elicits a type I hypersensitivity reaction (allergic sensitisation) in a previously exposed host (Woods, 2001). These allergens are usually proteins or glycoproteins. Exposure to myco-allergens occurs via the same routes as in the case of mycotoxins and hypersensitivity may also be an occupational disease *viz* Cheese washer's lung and Malt washer's lung as a result of *Penicillium* spp. and *Aspergillus* spp., respectively (Harber *et al*., 1996). Furthermore, the hypersensitivity reaction occurs within 30 minutes after exposure, and seldom occurs via the gastrointestinal route.

Aerobiological surveys have shown that aerial fungal spores are a cosmopolitan phenomenon (Woods, 2002). Thus, not surprisingly, along with dustmites fungal allergens are one of the most frequently encountered indoor allergens, while the most frequently encountered outdoor allergens are pollen and fungi. Fungal allergens are a perennial phenomenon, and spore levels and types may vary between seasons. Spore numbers tend to peak during summer and fall in temperate climates, whilst it decrease in areas of snow, at least outdoors. It is not uncommon for spore counts to exceed 4000/m^3. Fungi implicated in Type I hypersensitivity (mycoallergy) include many fungi imperfecti such as *Aspergillus, Penicillium, Cladosporium* and *Alternaria* (Anderson, 1985; Vijay *et al*., 1999).

The route of acquisition is predominantly via the respiratory tract (Salvaggio and Aukrust, 1981). Evidence that indoor dampness and mold growth are associated with respiratory health has been accumulating, but few studies have been able to examine health risks in relation to measured levels of indoor mold exposure (Jacob *et al.,* 2002). Many fungal genera have been implicated in allergy of the respiratory tract (Gravesen, 1979). Table 2 lists some fungi which have been implicated in allergenicity. Exposure via the respiratory tract displays two clinical manifestations (Horner *et a*l., 1995). The first health effect manifests itself in the upper respiratory tract (rhinitis), which is characterised by exudation of fluid and swelling of surrounding tissue.

TABLE 2. Taxonomic distribution of some allergenic fungi (Horner *et al.*, 1995).

TRUE FUNGI	
Phylum: Zygomycota	
Class: Zygomycetes	
Order: Mucorales	*Mucor, Rhizopus*
Phylum: Dikaryomycota	
Subphylum: Ascomycotina	
Class: Ascomycetes (including fungi imperfecti)	
Order: Dothidiales	*Alternaria, Cladosporium, Epicoccum, Drechslera, Semphylium, Wallemia*
Order: Eurotiales	*Aspergillus, Penicillium*
Order: Helotiales	*Botrytis*
Order: Hypocreales	*Fusarium*
Order: Onygenales	*Trichophyton*
Class: Saccharomycetes	*Saccharomyces, Candida*
Subphylum: Basidiomycotina	
Class: Holobasidiomycetes	
Order: Agaricales	*Coprinus, Lentinus, Pleurotus, Psilocybe*
Order: Aphyllophorales	*Ganoderma, Merulius*
Order: Lycoperdales	*Calvatia, Geaster*
Class: Phragmobasidiomycetes	*Dacrymyces*
Class: Teliomycetes	
Order: Uredinales	Rusts
Order: Ustilaginales	Smuts, red yeast (*Sporobolomyces*)
PROTISTAN FUNGI	
Phylum: Oomycota	
Class: Oomycetes	
Order: Peronosporales	*Phytophthora, Plasmopara* (plant downy, or false mildews)

The second health effect is associated with the lower respiratory tract (asthma), which is characterised by bronchial smooth muscle contraction, mucus plugging of the bronchioles, which in turn may be delibitating and life threatening. As illustrated in Figure 10, asthma is generally caused by the smaller fungal spores (< 10 μm), whilst the rhinitis is caused by larger spores (>10 μm).

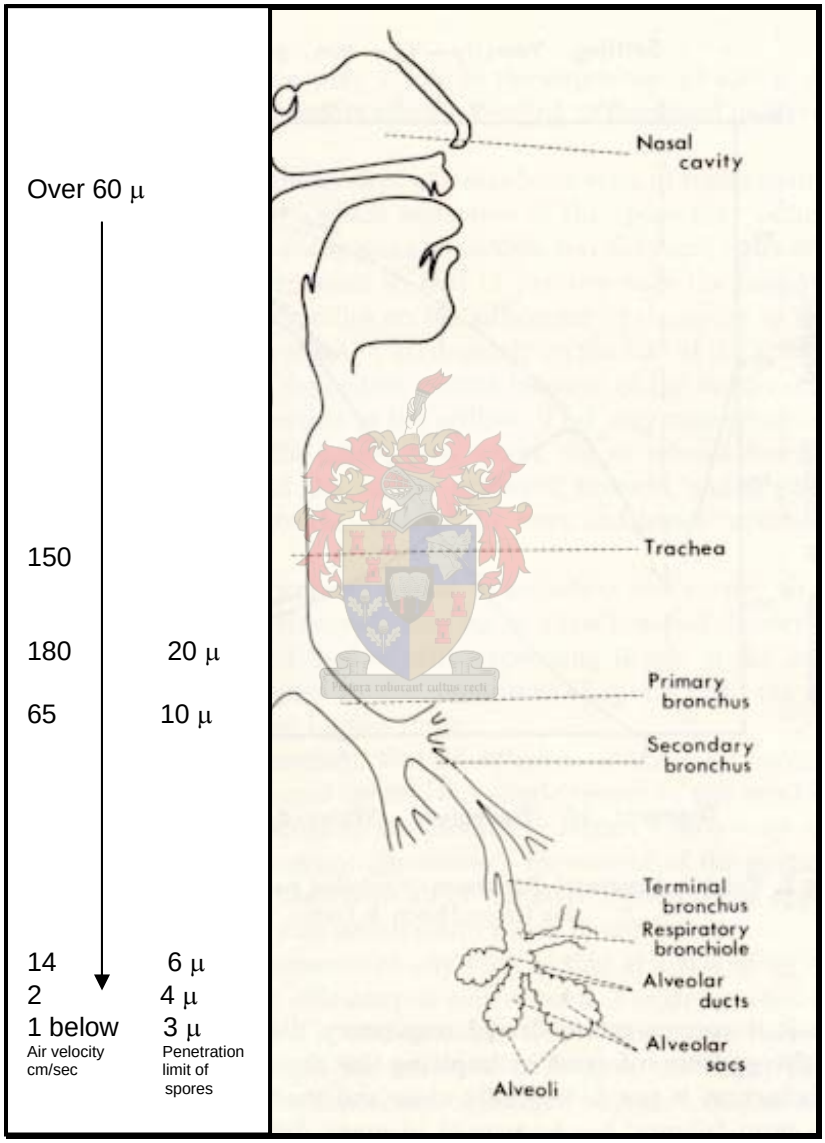
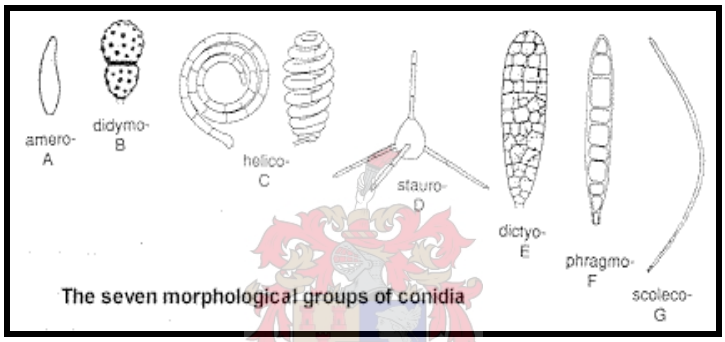


FIG. 10. Schematic illustraton of locations within the respiratory tract where spores of different diameters tend to settle (adapted from Madelin, 1966).

2.8 Spore morphology

Airborne fungal spores: The shape of fungal spores includes a variety of geometric forms, where spherical to ellipsoidal forms are more commonly occurring among single-celled spores (Gottlieb, 1978). Generally fungal spores may be classified into seven categories according to their shape and septation which are illustrated in Figure 11a (www.botany.hawaii.edu/faculty/wong/BOT135). It has been observed that individual spores that are elongated, tend to clump together when in groups. Figure 11b gives a schematic representation of conidia from a variety of fungal species known to cause mycoses and isolated from human lungs tissue (Madelin, 1966).

a



b

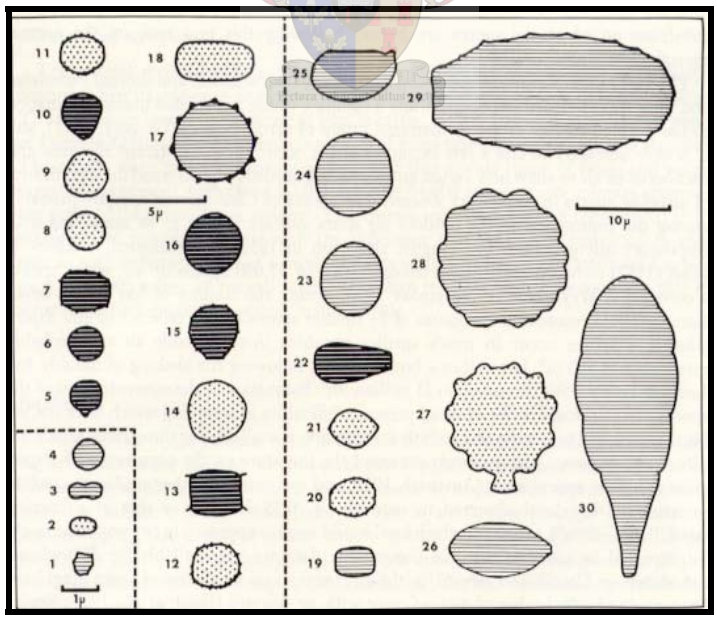


FIG. 11 (a). Schematic illustration of the seven morphological groups of fungal conidia, where tetraradiate spores, and banana-shaped spores are marked D and G respectively (www.botany.hawaii.edu/faculty/wong/BOT135). (b). Schematic representation of fungal spores with their corresponding diameters isolated from lung tissue (Madelin, 1966).

Dark shading = pathogenic species; light shading = allergenic species; and dotted = isolated from mammal lungs

2.9 Mycotic infection by airborne fungal spores

Mycotic infection can be defined as the entry of the fungus into the host body tissue, followed by multiplication (Cooke, 1977). Usually the infection is clinically unapparent, but may result in disease due to cellular injury as a result of the competitive fungal metabolism, production of toxins or the response of the host immune system (Vennewald and Wollina, 2005). Fungal infections are classified according to the site of infection, route of acquisition (endogenous or exogenous), and type of virulence (primary or opportunistic). The primary mode of infection have been shown to be via the respiratory tract, and it has, therefore, proved useful to create a unit for particle size as deposited in the respiratory tract, namely the aerodynamic diameter (Morrisa *et al*., 2000). It was reported that the aerodynamic diameter (dae) of *Penicillium* conidia, measured at different humidities, ranges between 1.9 and 3.9 (m. However, other experiments conducted with this genus revealed a dae value of less than 2.2 (m. The implications of these observations, are that airborne conidia from the same genus may manifest in different tissues of the respiratory tract if inhaled. The relative distance that fungal spores, differing in diameter, travel into the respiratory tract has been illustrated in Figure 10.

Humans first exposure to endogenous fungi occurs during birth whilst passing during the vaginal canal (Chapman, 2003; Walsh and Dixon, 1996). The fungus usually is *Candida albicans*, a known opportunistic pathogen, and is only implicated in mycoses in patients with a coexisting immune, endocrine, or delibitating disorder. Exogenous fungi on the other hand is divided in a number of subgroups: (1) Superficial fungal infection localised on skin, hair, and nails caused by fungi such as *Trichophyton* (Borgers *et al.,* 2005); (2) Cutaneous fungal infection caused by *Aspergillus* is usually confined to the outer layers of the skin, nail or hair, which rarely invades the deeper tissue (Stiller *et al.,* 1994); (3) Subcutaneous fungal infection caused by *Sporothrix schenckii* is confined to the subcutaneous tissue and rarely spread systemically (Alves *et al.,* 2004).

It should be noted though that fungi associated with this type of infection are soil saprophytes which are introduced through trauma to feet and legs and not airborne fungal spores; (4) Systemic fungal infection caused by *Candida* is associated with the deep organs and occurs as a result of inhaled fungal propagules (Zupanic-Krmek and Nemet, 2004). These propagules are lodged on the mucous membrane of the respiratory tract where they are phagocytosed. The infection usually involves the lungs, bones, central nerve system and can become widely disseminated. The most common portals of entry are the respiratory tract, gastrointestinal tract, and blood vessels; (5) Opportunistic infections occur in immunocompromised hosts (Zupanic-Krmek and Nemet, 2004). The fungi implicated in these infections include *Cryptococcus*, *Candida*, *Aspergillus* and *Mucor*.

2.10 MOTIVATION

From the preceding literature review it can be concluded that fungal spores interact with their environment in various ways. After their production these structures, adapted for dispersal and survival, are dislodged from their parent mycelium. Depending on the fungal species, the latter process may be brought about by endogenous changes within the thallus, or by exogenous forces such as moving air, water or animals including insects. Once dislodged, the spores may travel via various vectors to end up on an appropriate substrate containing sufficient nutrients in an environment which favor growth. During this dispersal process, the spore will inevitably encounter different substrates with which it interacts. These substrates may range from synthetic polymers, to plant materials, insects and even fibrinogen from mammalian cells. With the above as background the first goal of this study was to study spore liberation from colonies of hyphomycetous fungi representing the genera *Acremonium*, *Aspergillus* and *Pencillium*, into the air under dry and humid conditions (Chapter 3). The aim was to determine whether the taxa differed in their ability to release their spores under different conditions and to determine differences in spore release in an aerial and aqueous environment.

The fungal strains were then studied for their different abilities to adhere, in dry conditions, to various test materials differing in hydrophobicity and electrostatic surface charges (Chapter 4). The test materials included six lamellar materials *i.e.* nitrocellulose filter membrane, nylon, Parafilm®, and polyethersulfone, citrus leaves, and cricket exoskeleton. The adherence of the filamentous fungal propagules, as well as the adherence of yeasts, onto these test materials was also studied within an aqueous solution (Chapter 5). In addition, the adherence of the fungi to plasma membrane glycoprotein (fibrinogen) coated polystyrene was studied. Also, using epi-fluorescence microscopy, the fungal propagules were investigated for the presence of carbohydrates on their surfaces.

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CHAPTER 3

Hyphomycetous spore release induced by air currents and aqueous saline solution.

3.1 INTRODUCTION

It has long been recognised that many fungi liberate their spores in a passive manner (Ingold, 1953). During this process millions of spores are released through the energy of wind, rain and animals. It was suggested that spores that are released in this manner can be classified in two groups; the first group contains those spores that are imbedded in slimy exudates and are mostly dispersed by water or insects; the second contains "dry" spores that are essentially free of slimy exudates which may readily be dispersed by wind (Webster, 1980). Many hyphomycetes are known to produce conidia that belong to this second group of spores.

Currently we know that hyphomycetes may be considered as common airborne fungi occurring in both indoor and outdoor environments (Shelton *et al*., 2002). Sometimes the occurrence of these fungi in air may have health implications, since a number of them may cause mycotic illnesses (Chiewchanvit *et al.,* 1991; Pitt, 1974; Supparatpinyo *et al*., 1994). It was, therefore, essential to obtain knowledge of the environmental conditions that promote hyphomycetous spore release. Hyphomycetous fungi have been known to liberate their hydrophobic conidia under desiccated environmental conditions, and with the aid of rain drops by means of splash dispersal (Fitt and Nijman, 1983). *Penicillium* and *Aspergillus* have been shown to occur commonly in "dry" air samples (Fogelmark *et al*., 1994; McNeel and Kreutzer, 1996).

It was suggested that the most effective means of spore dissemination among soil hyphomycetes is through the movement of rain water (Horn *et al*., 2000; Sutton *et al*., 1976). We were, therefore, interested to obtain an indication of the primary means of spore dispersal employed by representatives of the genera *Acremonium*, *Aspergillus* and *Penicillium*, isolated from soil and indoor environments in the Western Cape, South Africa. Also, since all of these fungi contain different sporogenous structures, we were interested whether the quantity of colony forming units released into the air and into an aqueous solution containing a low concentration of salt differed among the isolates.

3.2 MATERIALS AND METHODS

Fungal strains used. Hyphomycetous fungi originating from soil were obtained from the fungal culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa (Table 1). In addition, fungi were isolated from the interior of wine cellars in the Western Cape, South Africa. Fungal growth was scraped from the walls using a sterile spatula and the scrapings were subsequently used to inoculate 2 % malt extract agar (MEA) plates. The plates were incubated at 22 ^{0}C and the resulting colonies were purified by consecutive transfer and incubation on MEA plates at 22 ^{0}C.

Identification of isolates using morphological criteria. Single-spore cultures were prepared from the fungal isolates. Each single spore culture was inoculated on differential media and subsequently incubated for seven days as required for the identification of these fungi (Pitt, 1974). Following incubation, colonies were visually examined for characteristic features as described in literature (Thom, 1930; Pitt, 1979). In addition, microscopic characters, conidial colour, presence of exudates, mycelial growth and coloration were used to identify the isolates according to the descriptions and keys in literature.

Identification of isolates using molecular criteria. Each isolate was grown at 22 ^{0}C in 100 ml liquid malt extract until the pellicle formed by the mycelia covered the surface of the medium. The mycelia were harvested through a sterile mira-cloth, and subjected to the DNA extraction method of Raedar and Broda (1985). Taxon-specific sequences of the ribosomal DNA (rDNA) were amplified with universal primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') in conjunction with ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), respectively (Skouboe *et al*., 1999; White *et al.,* 1990).

The reaction conditions used were 1.5 U Expand *Taq* polymerase, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 0.2 mM of each dNTP. The DNA templates were amplified for 25 consecutive cycles, which included, denaturing for 45 sec at 94 ^{0}C, annealing for 30 sec at 55 ^{0}C and elongation for 2 min at 72 ^{0}C. In addition, amplification of the larger subunit of the rDNA region (D1/D2), also proved useful in identifying some of the isolates with primer pair, LR3 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and F63 (5'-GGT CCT TGT TTC AAG G-3') (Fell *et al*., 2000). The reaction conditions were the same as above, except for an annealing temperature of 58 ^{0}C. The sequences of ITS1-ITS4 and LR3-F63 products were determined by purification from agarose gels using the Nucleospin® Extract columns (Machery-Nagel). The purified products were sequenced, by using an ABI Prism 310 automated sequencer and an ABI Prism Dye terminator cycle sequencing ready reaction kit (Applied BioSciences). Sequence editing and analysis were performed using the PC-based software program DNAMAN, version 4.13. (http://www.lynnon.com/pc/framepc.html; Lynnon Biosoft Corp., USA). Homologues to the sequences were identified using the software program BLASTN (nucleotide-nucleotide BLAST), version 2.2.9. [freeware: http://www.ncbi.nlm.nih.gov/BLAST; National Center for Biotechnology Information (NCBI), USA].

Measurement of spore liberation into air. A comparative analysis of spore liberation of all strains, was conducted in an airflow cell (Figure 1). This airflow cell consisted of a horizontal tubular growth chamber (450 mm in length, 70 mm in diameter) containing 100 ml MEA (surface area of 165 cm^2). A spore suspension (10 ml; containing ca. 5×10^6 spores/ml as enumerated using a heamocytmeter) of a week old culture was used to inoculate each chamber. The sporecount was determined microscopically, by using a haemocytometer. The chamber was incubated at 22 ^{0}C for 48 hours, where after it was connected at the one end to an air pump and incubated further. Sterile air, with a relative humidity of 36, was subsequently pumped through the chamber at a rate of 0.6 liter.min^{-1} (laminar flow rate of 16.3 cm.min^{-1}) via a 0.45 μm Midistart® 2000 PTFE filter (Sartonet Sa. Cc), a Gabler airflow regulator, and sterile water in a conical flask.

After two weeks, the water-containing flask was replaced with an anhydrous calcium chloride ($CaCl_2$) moisture trap in order to subject the fungal culture in the airflow cell to desiccated air for a further two week period. Spore liberation of the fungal culture in the airflow cells was monitored by exposing a set of nine MEA containing Petri dishes consecutively to air at the flow cell's outlet for 15 minutes respectively. The MEA plates were subsequently incubated for one week at 22 ^{0}C and the number of colony forming units was enumerated. Monitoring of spore release was conducted on five days of every week over the four week incubation period.

Measurement of spore liberation into aqueous saline. After the four-week incubation period, the ability of the cultures to release spores into an aqueous solution was determined. Fifteen (15) ml of physiological salt solution (PSS) (0.85 % NaCl) was gently transferred into each airflow cell . The number of spores in the resulting suspension was microscopically enumerated using of a haemocytometer.

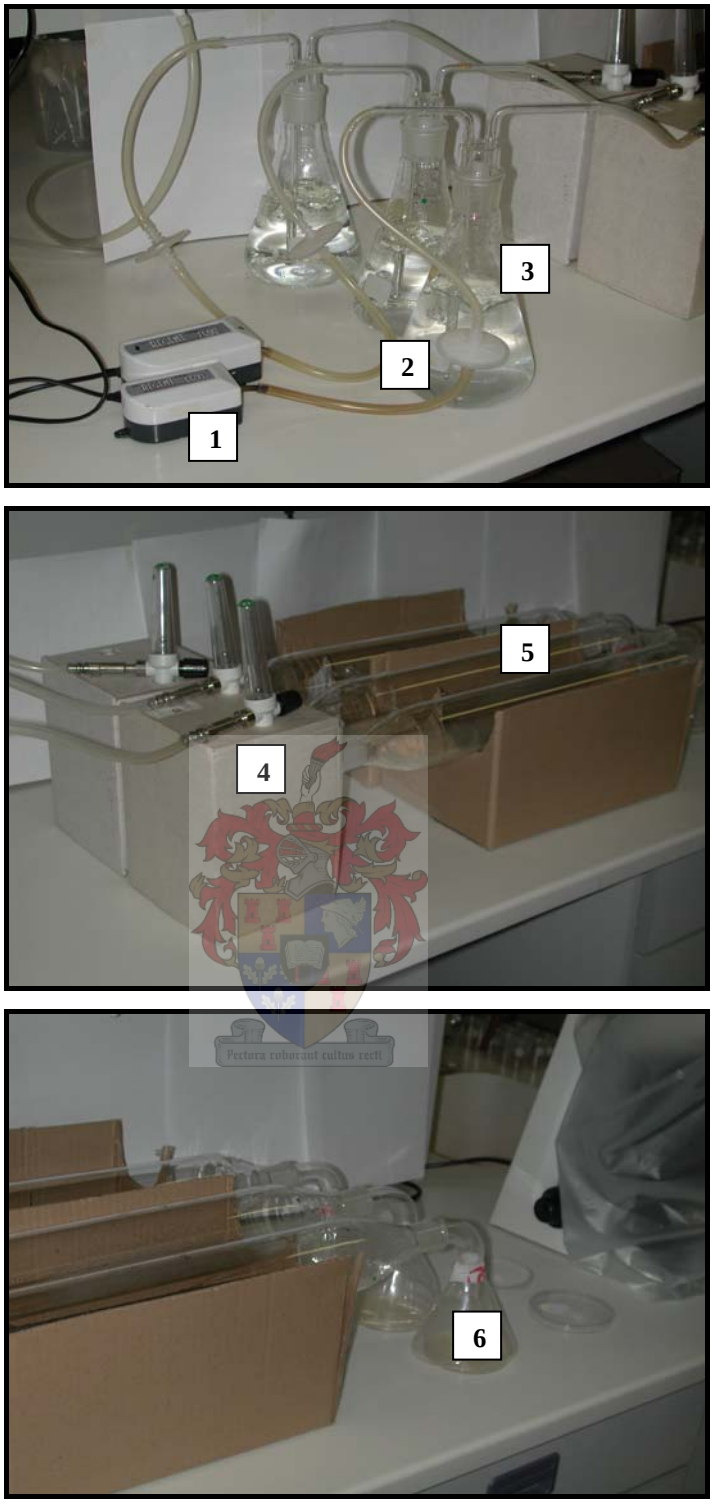


FIG. 1. The components of the airflow cell used in the experimentation (a). Air pump, sterile 0.24 µm Millipore filter and the sterile distilled H_2O containing flasks. (b). Air flow regulators and the incubation chamber containing the fungal growth medium and inoculum (test organism). (c). Air flow cell outlet, allowing liberated spores to be trapped on MEA-containing Petri dishes.

1 = pump; 2 = sterile filter; 3 = water containing flask; 4 = airflow regulator; 5 = growth chamber; and 6 = air flowcell outlet

Statistical analyses. To test spore liberation on environmental conditions, strains and time, a completely randomised experiment was conducted with the treatments in a 2 × 17 × 2 factorial with three random replications (Snedcor and Cochrane, 1967). The factors were two environmental conditions (humid and dry aeration); 17 strains/isolates which were grouped into three genera namely, *Acremonium* (ACR), *Aspergillus* (AA, AC, AT, AN, and AF), and *Penicillium* (F2, R4, FG16, R1, R2, AB0268, ABO272, ABO275, AB0486, ABO487, PC), and two time periods (week 1 and 2). Total spore liberation was recorded and transformed by a Log_{10} (x + 1) transformation before subjected to an appropriate factorial analysis of variance (ANOVA), using SAS statistical software (SAS Institute Inc., 1999).

The above experimental units for the 17 strains and the 3 replications thereof were flushed with saline solution, and the spores liberated. The data was subjected to the appropriate ANOVA. Shapiro-Wilk test was performed on the residuals to test for non-normality (Shapiro and Wilk, 1965). In order to compare the means of significant effects, the Student's t-LSD (least significant difference) was calculated at a 5 % significance level. The means of the statistical analysis are presented in tables and figures.

3.3 RESULTS AND DISCUSSION

Identification of fungal isolates. Morphological characteristics revealed that the isolates were hyphomycetes that belonged to the genera *Acremonium, Aspergillus* and *Penicillium* respectively (Pitt, 1979; Klich, 2002). Molecular analyses were used to support the findings of the morphological analyses and the identities of the isolates, together with the other strains obtained from the culture collection, are listed in Table 1.

TABLE 1. Fungal isolates and strains used in the experimentation

Isolate/Strain Code	Species	Origin
ACR	*Acremonium alternatum*	Fynbos soil
AA	*Aspergillus aculeatus*	DSM 2344
AC	*A. carneus*	Fish River plains
AF	*A. fumigatus*	Soil from Zimbabwe
AN	*A. niger*	ATCC10864
AT	*A. terreus*	Soil Northern Province, South Africa
ABO486	*Penicillium camemberti*	Walls of cheese factory
PC	*P. candidum*	Commercial strain for cheese making
AB0272	*P. citrinum*	Wine Cellar 1
R1	*P. citrinum*	Wine Cellar 1
R2	*P. citrinum*	Wine Cellar 1
F2	*P. commune*	Fynbos soil
FG16	*P. commune*	Fynbos soil
R 4	*P. glabrum*	Fynbos soil
ABO268	*P. spinulosum*	Wine Cellar 1
ABO275	*P. sumatrense*	Wine Cellar 1
ABO487	*P. westlingii*	Wine Cellar 2

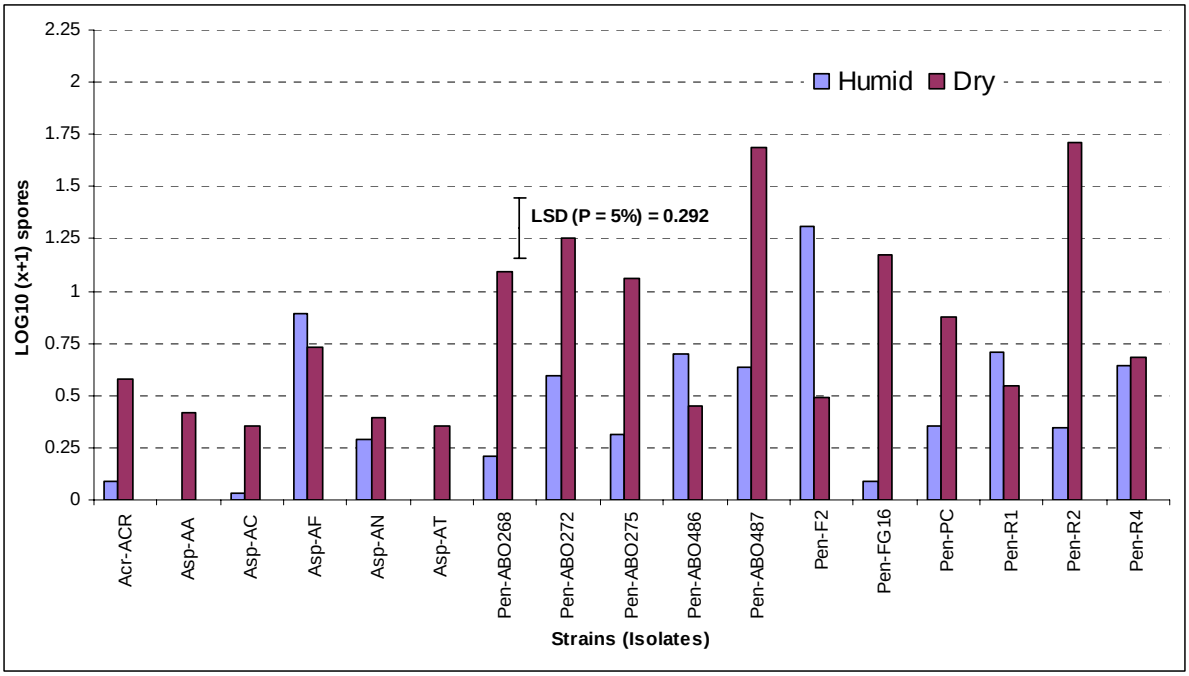
Spore liberation. The Shapiro-Wilk test on spore liberation data revealed deviation from normality, subsequently outliers were removed until the residuals had a normal distribution or were symmetric (Glass *et al.,* 1972). Significant three-factor interaction was found (Appendix I, Table 1). A comparative analysis of three factor interaction (time × strain × environmental condition) between time period 1 (week 1) and time period 2 (week 2) during humid and desiccated aeration, respectively, showed significant differences between the time periods. Thus, spore liberation during the first week of incubation under humid conditions differered significantly ($p < 0.0001$) from spore liberation during week 2 (Figure 2a). Furthermore, intraspecific differences were observed, *e.g. P. commune* F2 liberated more spores during period 1 than period 2, whilst *P. commune* FG16 showed a inverse spore liberation pattern compared to the former. *P. citrinum* R1 and *P. citrinum* R2 liberated significantly more spores during period 2, whilst *P. citrinum* ABO272 did not differ significantly between period 1 and period 2 regarding spore liberation.

It can tentatively be speculated that spore liberation of strains R1 and R2 is dependant on the age of culture, whilst spore liberation of strain ABO272 is not influenced by the age of the culture. Furthermore, a species known to thrive in humid conditions, *Aspergillus fumigatus* (Wasylnka and Moore, 2000), released significantly more spores during time period 1 than time period 2, whilst *Aspergillus aculeatus* AA, a fungus occurring in habitats such as plant debris and indoor environments, released no spores in the presence of humid air.

As was found for environmental condition 1, the comparative analysis for environmental condition 2 between time period 1 (week 1) and time period 2 (week 2) also showed a significant ($p < 0.0001$) difference between time periods (Figure 2b). Similar to the findings obtained for humid aeration, intraspecific differences were observed as demonstrated by the results obtained for *P. citrinum* where one strain liberated more spores during time period 1, whilst the others were consistent with regard to period 1 and period 2.

Figure 3 provides a summary of the mean total number of spores released for both environmental conditions. Interestingly, except for *Aspergillus fumigatus* AF, *A. aculeatus* AA released the most spores in the air, despite no spores being released during humid aeration (environmental condition 1). As a result of the intraspecific diversity observed, any conclusions on the possible correlation between species or morphology with the numbers of spores released should be made with caution. Overall, members belonging to the genus *Penicillium*, released more spores than *Aspergillus* spp., which in turn released more spores the member representing *Acremonium.*

a



b

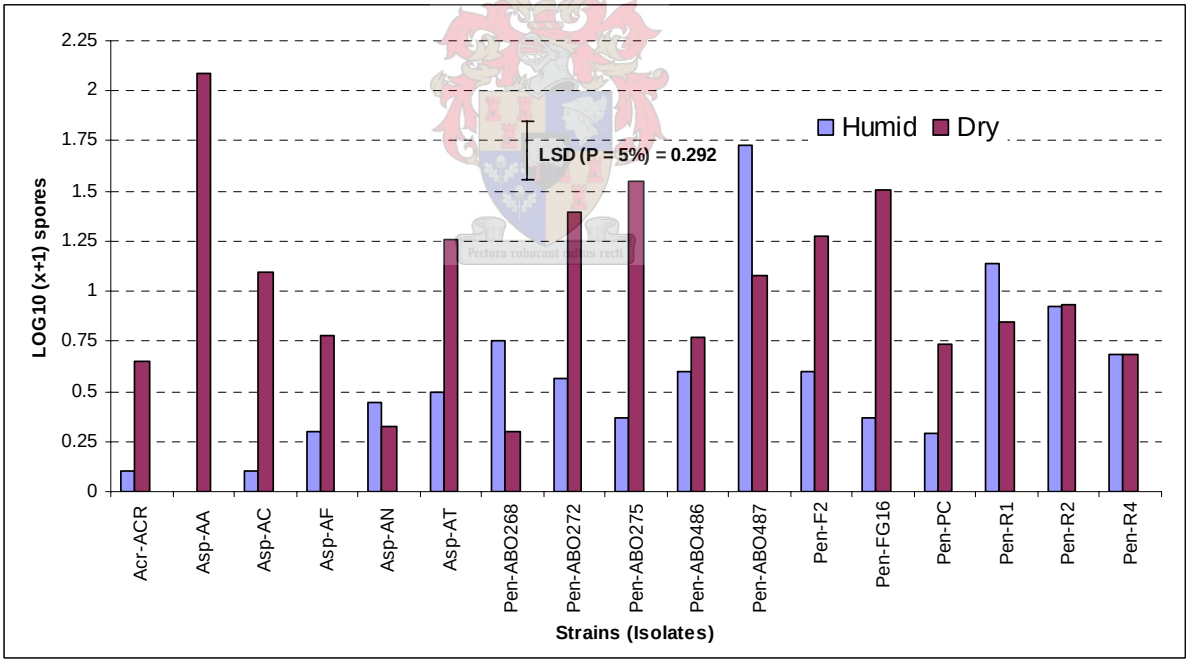


FIG. 2a & b. Three factor interaction (time × strain × environmental condition) means of total spores liberated during period 1 and period 2 with (a) humid and; (b) dry aeration. The pre-fixes "Acr, Asp and Pen-" indicates the genera, *Acremonium*, *Aspergillus* and *Penicillium*, respectively.

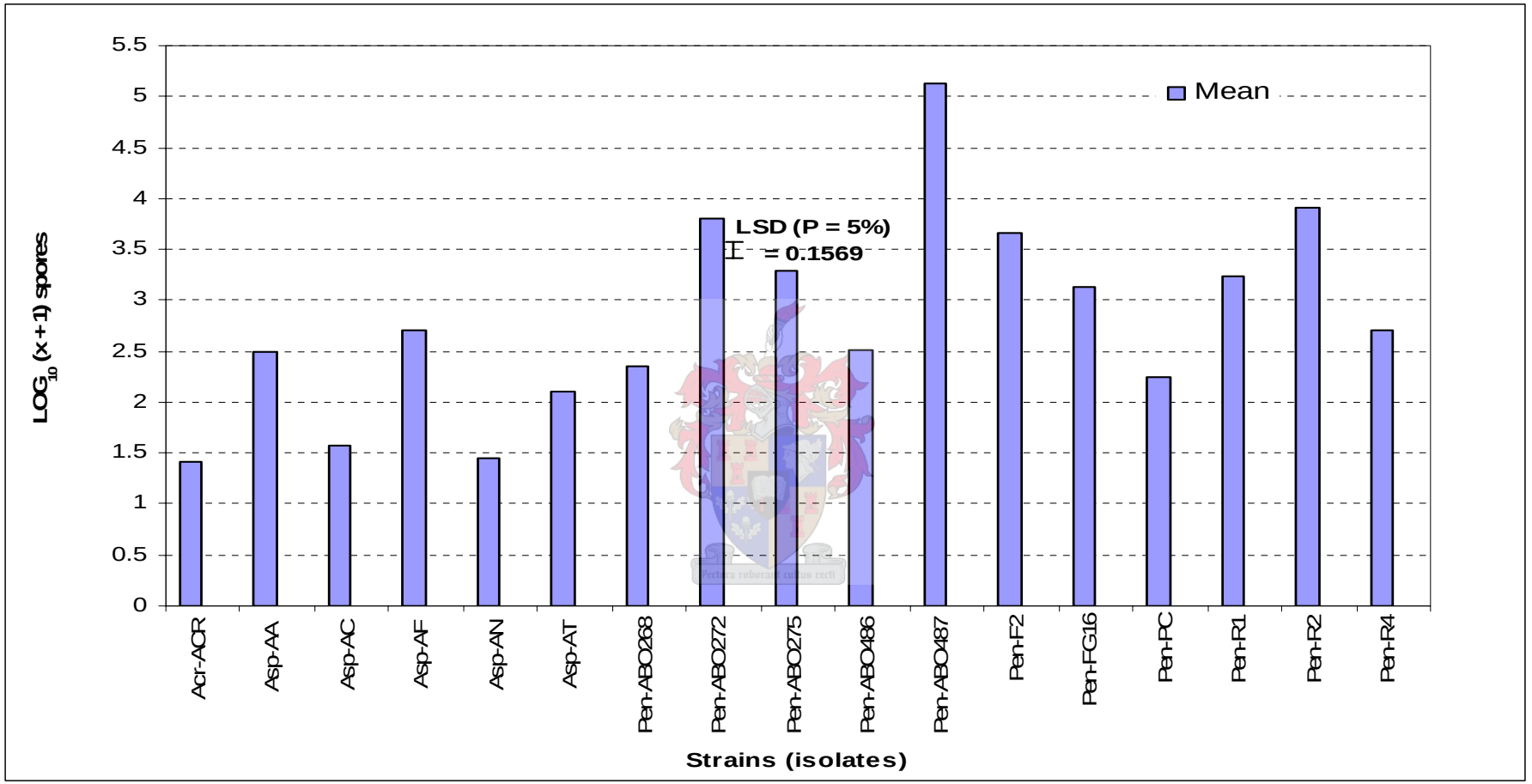
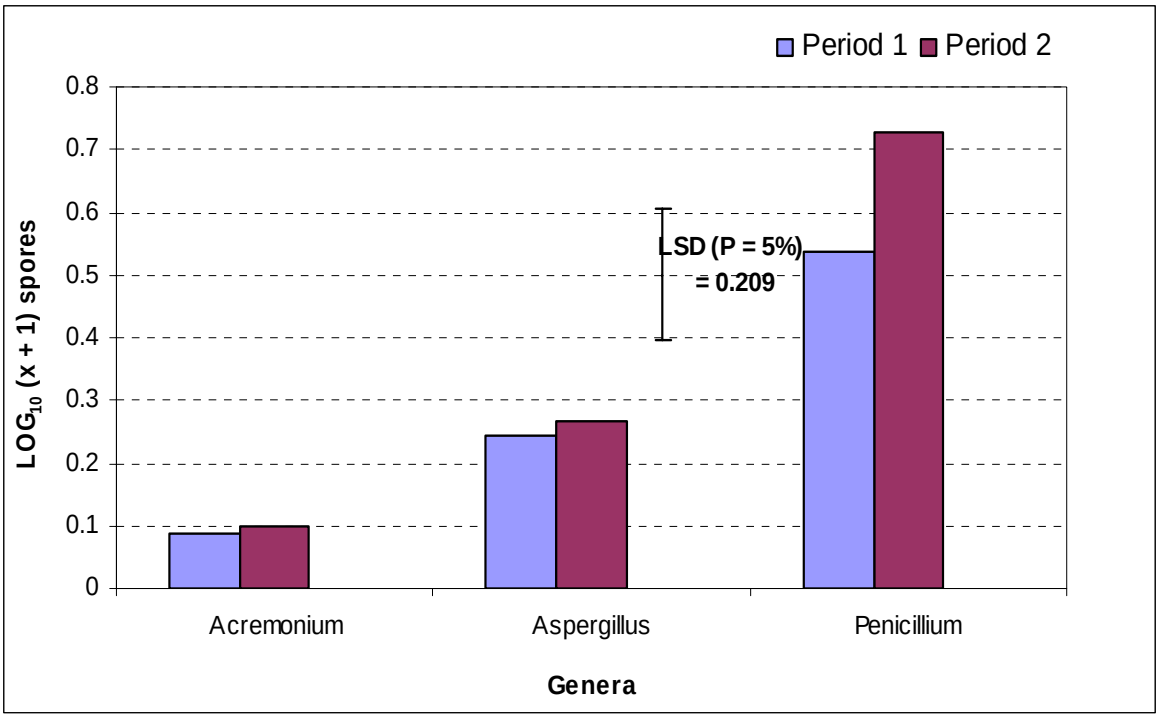


FIG. 3. Means of total spores liberated during humid and dry aeration. The pre-fixes "Acr, Asp and Pen-" represents the genera, *Acremonium, Aspergillus* and *Penicillium*, respectively.

Therefore, to investigate a possible correlation between fungal taxa and spore release, the strains were grouped into genera and spore release under humid and desiccated conditions were analysed. A comparative analysis of three factor interaction (time × genus × environmental condition) between time period 1 (week 1) and time period 2 (week 2) for environmental conditions 1 and 2 (humid and desiccated aeration), respectively, showed a significant difference between time periods ($p < 0.012$). In contrast to representatives of the genus *Penicillium*, members belonging to the genera *Acremonium* and *Aspergillus* did not differ between the two time periods (week 1 and week 2) during humid aeration (Figure 4a). Under humid conditions, the *Acremonium* strain and members representing the genus *Aspergillus* did not differ significantly, but the *Penicillium* species were significantly more successful in dispersing their spores. All genera tended to release more spores during time period 2 than time period 1, though not significantly more. These results complement the observation of continuous spore release of mature fruiting bodies belonging to the basidiomycetous macrofungi (Ingold, 1953).

However, monitoring of spore liberation under desiccated aeration, representatives of the genus *Aspergillus*, released more spores during time period 2 than time period 1, whilst the other two genera *i.e. Acremonium* and *Penicillium*, did not differ (Figure 4b). The *Aspergillus*, released significantly more spores during time period 2 of desiccated aeration. This indicates that the sporogenous structures of this fungus is adapted for passive spore release upon changes in humidity and age of the culture. Furthermore, members belonging to the genus *Penicillium* differed significantly from the *Acremonium* strain, but not from *Aspergillus* spp, with regard to the number of spores released. This difference in spore liberation may be ascribed to differences in the numbers of spores produced and to major differences in spore morphology. The *Aspergillus* and *Penicillium* strains used all produce globose to spheroidal conidia with a diameter of 2 to 5 μm, while the *Acremonium* strain formed fusiform conidia normally 3 μm wide and 8 μm in length.

a



b

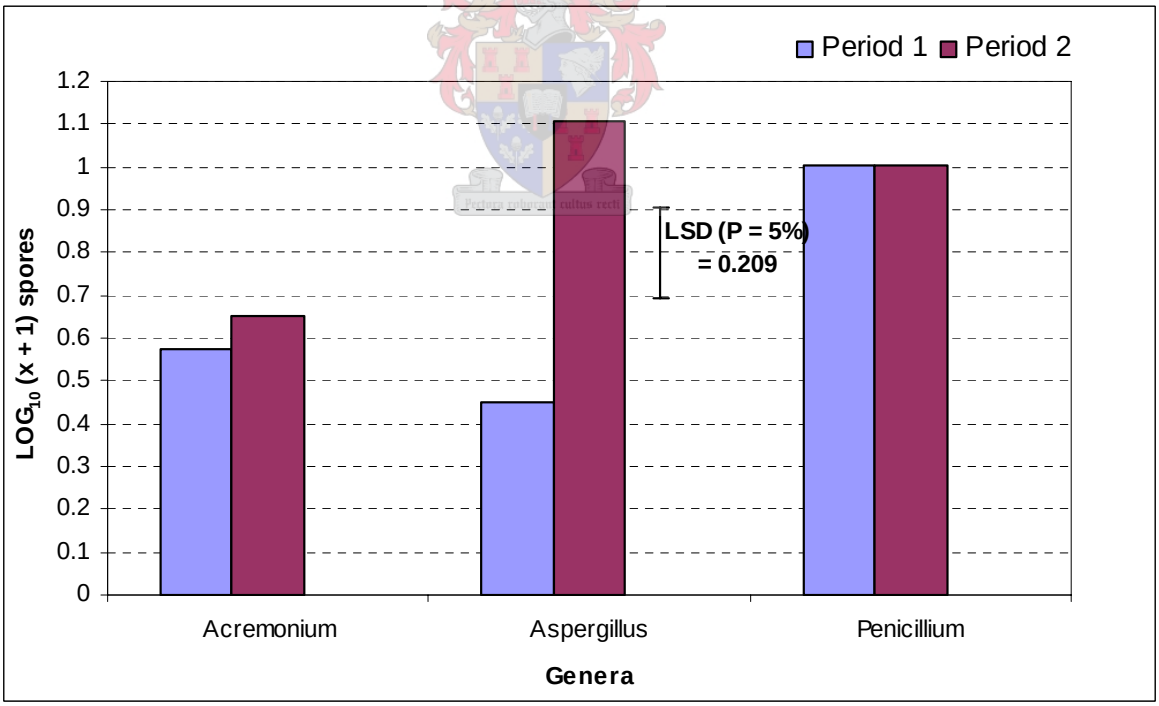


FIG. 4a & b. Three factor interaction (time × genus × environmental condition) means of total spores liberated.

a = humid aeration

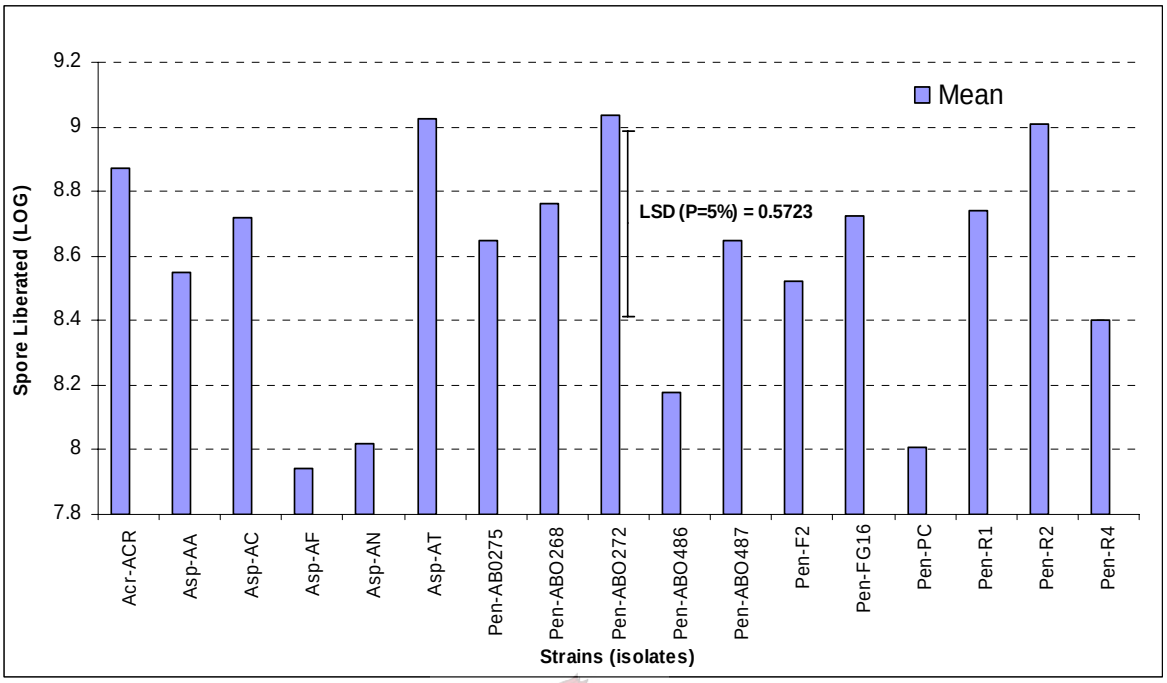
b = dry aeration

Furthermore, it is tempting to speculate that this difference may be ascribed to a longer time period needed for the sporogenous structures of *Aspergillus* to reach the desiccated condition needed for increased spore release, than the time period needed for the sporogenous structures of *Penicillium* and the *Acremonium* strain. In contrast to *Aspergillus*, the sporogenous structures of neither *Acremonium* nor *Penicillium* is characterised by a pronounced swollen vesicle at the tip of the conidiophore (Klich and Pitt, 1988; Larone, 1995), which will take longer to reach a desiccated condition than filamentous structures. It must however, be borne in mind that only one representative of the genus *Acremonium* was tested and that the results may differ if more representatives of this genus are included in the analysis. In support of the theory that splashing rain may disperse microfungal spores (Fitt and Nijman, 1983), washing the culture with PSS resulted in all cases in an immediate massive release of colony forming units from the cultures (Figure 5a & b). However, no significant difference could be observed between cultures regarding the release of colony forming units into the aqueous solution of salts (Appendix, Table 2). The $\log_{10}$ (x + 1) of average number of *Acremonium, Aspergillus* and *Pencillium* spores released per cm^2 during the two weekly periods of airflow was 0.004, 0.006, and 0.009 respectively, while this number was notably more 0.054, 0.052, and 0.051 respectively, when the cultures were washed with PSS.

3.4 CONCLUSION

Although intraspecies differences were uncovered regarding aerial spore release among the hyphomycetous taxa investigated, we were able to demonstrate for the genera investigated that more spores are usually released as the culture matures. Furthermore, it was observed that in most cases older cultures release more spores under dessicated conditions. These findings support the results of others on the common occurrence of *Aspergillus* and *Penicillium* in dry air samples (Fogelmark *et al*., 1994; McNeel and Kreutzer, 1996). Interestingly, we also found that the genera responded differently to differences in humidity regarding their aerial spore release.

a



b

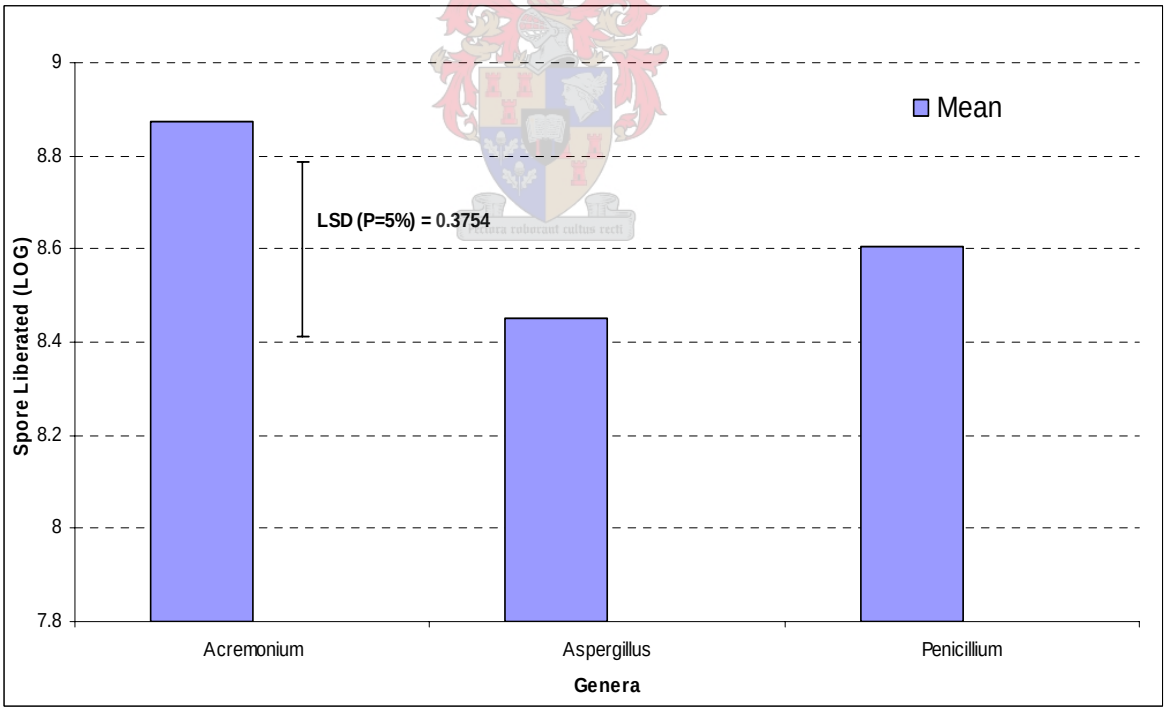


FIG. 5a & b. Means of total spores liberated when colonies were flushed with a saline solution. The pre-fixes "Acr, Asp and Pen-"represents the genera, *Acremonium, Aspergillus* and *Penicillium*, respectively.

a = level of strains (isolates)

b = level of genera

Under humid conditions *Penicillium* species were more successful in releasing their spores than *Aspergillus* and the *Acremonium* strain. Under desiccated conditions the *Aspergillus* took longer to release their spores than representatives of *Acremonium* and *Penicillium.* This may be as a result of the distinct morphology of the sporogenous structures of *Aspergillus*. The taxa that were investigated did not differ from each other regarding the release of spores in PSS and it seems that water may act as an important dispersion agent for the strains representing the genera *Acremonium, Aspergillus* and *Pencillium.* These findings support the views of others recorded in literature (Horn *et al*., 2000; and Sutton *et al*., 1976). The differences in airborne spore release, that were observed between the hyphomycetous genera investigated in this study, may be a result of different strategies to disperse their spores in nature and should be investigated further to elucidate the environmental niche of each genus. Therefore, since it is known that insects and other animal vectors, as well as moving plant material, may disperse fungal spores (Abbott, 2002) we were interested whether the hyphomycetous fungi investigated in this study would differ regarding the ability of various materials to physically dislodge spores from sporogenous structures. This aspect is the subject of the next chapter in this thesis.

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CHAPTER 4

Adherence of filamentous fungal spores to insect cuticle and other test surfaces.

4.1 INTRODUCTION

Compact disseminules *i.e.* fungal spores are the main mode of removing progeny from the direct vicinity of parent mycelium, called the thallus (Ingold, 1953; 1971; Moore-Landecker, 1972). This serves as to minimise competition amongst siblings as a result of unfavorable nutritional conditions, and thus promoting the survival of the organism (Ellis, 1971; Gottlieb, 1978; Ross, 1979). However, spores need to be physically removed from the thallus, whereafter it can be dispersed by some means to a near or far distance. A vast majority of spores are released from parent structures in some way that requires physical intervention from the environment, during which the thallus plays a passive role (Ingold, 1953). Most "dry" conidia are dislodged into and disseminated by turbulent air, water and animals. In some instances conidia are so firmly attached to conidiophores that even relative strong winds fail to dislodge them, especially on flat surfaces (Ingold, 1953).

Previous studies have shown that many terrestrial fungi use insects to varying extent to disseminate their spores (Ingold, 1971; MacNamara, 1924). It can be envisaged that the external force exerted by insects is sufficient enough to dislodge conidia from their conidiophores. Insects, therefore, act as important dispersal agents by brushing against spore-bearing structures whilst foraging, and thereby picking up spores on their integument (Jacot, 1930; Malloch and Blackwell, 1992; Moore-Landecker, 1972; Shaw, 1992). It was demonstrated that the physico-chemical characteristics of fungal spore surfaces impact on the physical interactions of these structures (Dufrêne *et al*., 1999).

Fungal spores have the ability to adhere to a number of different surfaces, including teflon, polystyrene, glass, cellophane, nutrient agar, water agar, and plant leaves (Braun and Howard, 1994; KerChung and Hoch, 1996). We are interested in the relative adherence of different filamentous fungal spores to insect cuticles and other test surfaces differing in hydrophobicity and electrostatic charges. Knowledge of these aspects would provide insight into the mode of spore dispersion, as well as into natural habitats of these fungi. Subsequently, the adherence of filamentous-fungal spores (representing the genera *Acremonium*, *Aspergillus* and *Penicillium*) to membranes, leaf cuttings and insect exoskeletons differing in hydrophobicity and electrostatic charge were investigated in this study.

4.2 MATERIALS AND METHODS

Fungi used. The same hyphomycetous fungal strains belonging to the genera *Acremonium*, *Aspergillus* and *Penicillium* and originating from soil and wine cellars, which were reported on in the previous chapter, were used in this study. Cultures were maintained on 2 % malt extract agar (MEA) plates at 22 ^{0}C.

Determination of surface hydrophobicity. Repulsion of water was determined by means of the water droplet method comprising measurement of the contact angle of the water to a test surface. One square centimeter of test surface was cut from each of the following six lamellar materials; nitrocellulose filter membrane (Micron Sep, product no. AP10047S0, pore size 0.45 μm), nylon (Magna product no. N00HY320FX), Parafilm® (Pechiney Plastic Packaging, Menasha,WI, USA) and polyethersulphone (Osmonics^{TM} Inc. product no. SO4SP04700, pore size 0.45 μm), citrus leaves (*Citrus sinensis*) and two-spotted cricket exoskeleton (forewings of *Gryllus bimaculatus*).

The materials were fixed on a microscope slide with double-sided adhesive tape, and the assembly was mounted on a platform that comprise of aluminium. In each case 1 μl of distilled water was placed on the test surface and a photo was taken using a COHU High Performance CCD camera connected to a personal computer. The contact angle of the water droplet with the surface of the substrate was thus measured and reported as angle theta (θ), with the computer software, Scion Image Beta 4.02 Win (Scion Image Inc., Frederick, Maryland, USA)(Figure 1). Test surface hydrophobicty was measured by subtracting the outside angle theta (θ) 180^0. All experiments were replicated three times.

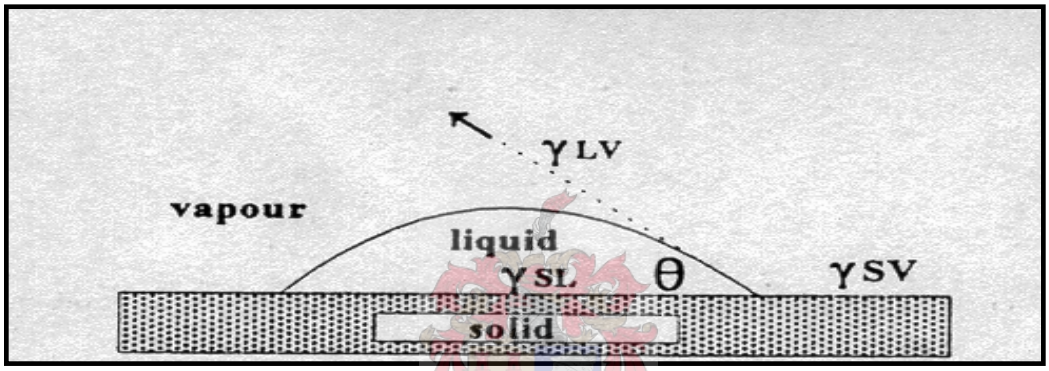


FIG. 1 Hydrophobicity calculation of water droplet on a test surface (adapted from Exl and Kindersberger, 2005).

Estimation of relative electrostatic charges on surfaces. The relative electrostatic charges on the test surfaces were determine by cutting 1 cm^2 of each, using the same scissors, and putting it in a glass Petri dish. Using a metal pincet each material was dipped into photocopier toner (MT Toner 1003A; Konica Minolta Business technologies Inc., Tokyo, Japan) also contained in a glass Petri dish. Photocopier toner is known to have slight electrostatic charge and is repelled from areas of negative charge and attracted to positive charge (McGonigle *et al.,* 2002). The test materials containing variable quantities of photocopier toner were then each submerged in 5 ml 95% ethanol contained in a cuvett. The cuvett was then agitated on a Vortex Genie® 2 agitator (Scientific Industries, Inc.) for 10 seconds at setting 3, and the absorbance of the suspension was determined at 540 nm using a spectrophotometer (Genesys 20, Thermo Spectronic). All determinations were repeated thrice.

Adherence assays on test surfaces. Briefly, 1 cm^2 of each of the above mentioned six lamellar test materials was gently placed on the surface of sporulating cultures of each of the fungal strains used in this study. The cultures had been growing for two weeks on MEA plates incubated at 22 ^{0}C. The test materials were subsequently transferred to 2 ml microcentrifuge tubes (Quality Scientific Plastics) containing 1 ml of phosphate buffer at pH 7.2 (Buck and Andrews, 1999). The tubes were then agitated on a Vortex Genie® 2 agitator (Scientific Industries, Inc.) for 10 seconds at setting 3, after which, the concentration of spores suspended in the buffer was microscopically determined by means of a haemocytometer. Positive and negative controls were included in the experiments which respectively comprised one square centimeter of MEA containing the two week old sporulating culture, and one square centimeter test surface that was not placed on surfaces with sporulating cultures. All experiments were repeated thrice.

Statistical analyses. To test the level of adherence on strains and test surface, a complete randomised experiment with three replicates was conducted with the method in 17 × 8 factorial (Snedcor and Cochrane, 1967). The factors were; 8 test surfaces (positive control, negative control, citrus leaf, exoskeleton, parafilm, nitrocellulose, nylon and polyethylsulphone), and the 17 hyphomycetous fungal strains which have been grouped into three genera namely; *Acremonium* (ACR), *Aspergillus* (AA, AC, AT, AN, and AF), and *Penicillium* (F2, R4, FG16, R1, R2, AB0268, ABO272, ABO275, AB0486, ABO487, PC). Total spore adherence was recorded and transformed by a ln [(% + 0.5) / (100 + 0.5)] transformation before subjected to a appropriate factorial analysis of variance (ANOVA), using SAS statistical software (SAS Institute Inc., 1999). A Shapiro-Wilk test was performed on the residuals to test for non-normality (Shapiro and Wilk, 1965). In order to compare the means of significant effects, the Student's t-LSD (least significant difference) was calculated at a 5 % significance level. The means of the statistical analysis are presented in tables and figures.

4.3 RESULTS AND DISCUSSION

Surface hydrophobicity of test materials. The relative hydrophobicity, determined by measuring the contact angle of a water drop, of the six different test surfaces is depicted in Figure 2. The most hydrophobic were the insect exoskeleton and the Parafilm, while the citrus leaf was less hydrophobic, but cellulose, nylon and polyethersulfone were hydrophilic.

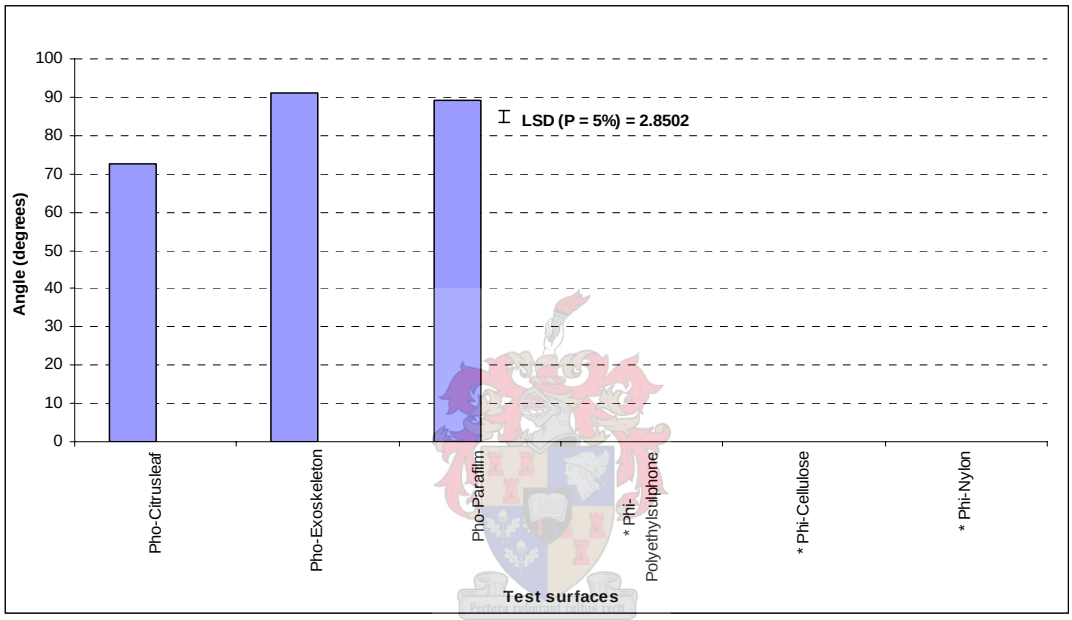


FIG. 2. The relative hydrophobicity determined by measuring the contact angle of a water drop on six different test surfaces is. The pre-fix "pho-" indicates a hydrophobic surface, while the pre-fix "phi-" indicates a hydrophilic surface.

*The contact angle of the water droplet on these hydrophilic surfaces was to small to be measured, thus the ~0 value.

Relative electrostatic charges on surfaces of test materials. The relative electrostatic charge as determined using adherence of photocopier toner to each test surface and the subsequent spectrophotometric determination of the relative quantities of toner attached to each surface is depicted in Table 1. The most positively charged surfaces were that of the insect exoskeleton and the citrus leaves while the least positively charge surfaces were that of nylon and nitrocellulose.

TABLE 1. Relative electrostatic charge of test surfaces as determined using adherence of photocopier toner and subsequent spectrophotometric determination of the relative quantities of toner attached to each surface.

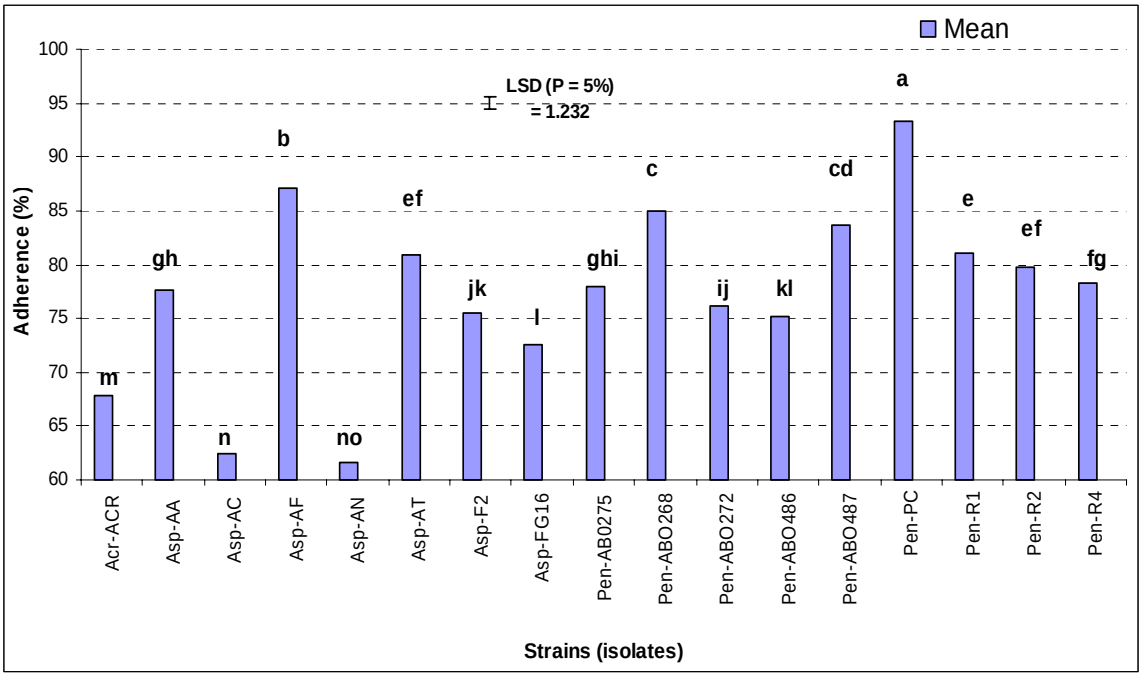
Test Materials	*Absorbance at 540 nm	Relative positive charge
Exoskeleton	0.391 (± 0.053)	+++++
Citrus leaves	0.350 (± 0.090)	++++
Parafilm	0.350 (± 0.018)	++++
Polyethersulfone	0.266 (± 0.033)	+++
Nylon	0.204 (± 0.029)	++
Nitrocellulose	0.169 (± 0.054)	+

* Each value represents the mean of three replications. Standard deviations are indicated between brackets.

Adherence assays on test surfaces. The Shapiro-Wilk test on spore adherence data revealed no deviation from normality (Glass *et al.,* 1972; Shapiro and Wilk, 1965). Significant two factor (genus × test surface and strain × test surface) interaction was found (Appendix I, Table 3). Discernible differences in the overall adherence of some strains to test surfaces were observed (Figure 3a), indicating differences in physico-chemical characteristics of the fungal spore surfaces. It was observed that the commercial fungal strain, *Penicillium candidum* PC showed the highest percentage (%) overall adherence to test surfaces, followed by the toxigenic fungus, *Aspergillus fumigatus* AF.

Interestingly, intraspecific differences regarding overall adherence to the test surfaces were observed for *Penicillium commune* and *Penicillium citrinum.* In general, the *Penicillium* strains showed a greater ability to adhere to the test surfaces, than the *Aspergillus* spp., while the representative of *Acremonium* showed the least adherence to the test surfaces (Figure 3b). When the test surfaces were compared regarding the adherence (%) of fungal spores (Figure 4a), significant differences were observed. No significant difference in the adherence (%) was found between hydrophobic and hydrophilic materials. In general, isolates adhered slighty better to insect exoskeleton than to parafilm and polyethylsulphone, with the least adherence to nitrocellulose. This highlights the role of insects as vectors for these fungi, as suggested by others (Ingold, 1971; MacNamara, 1924).

a



b

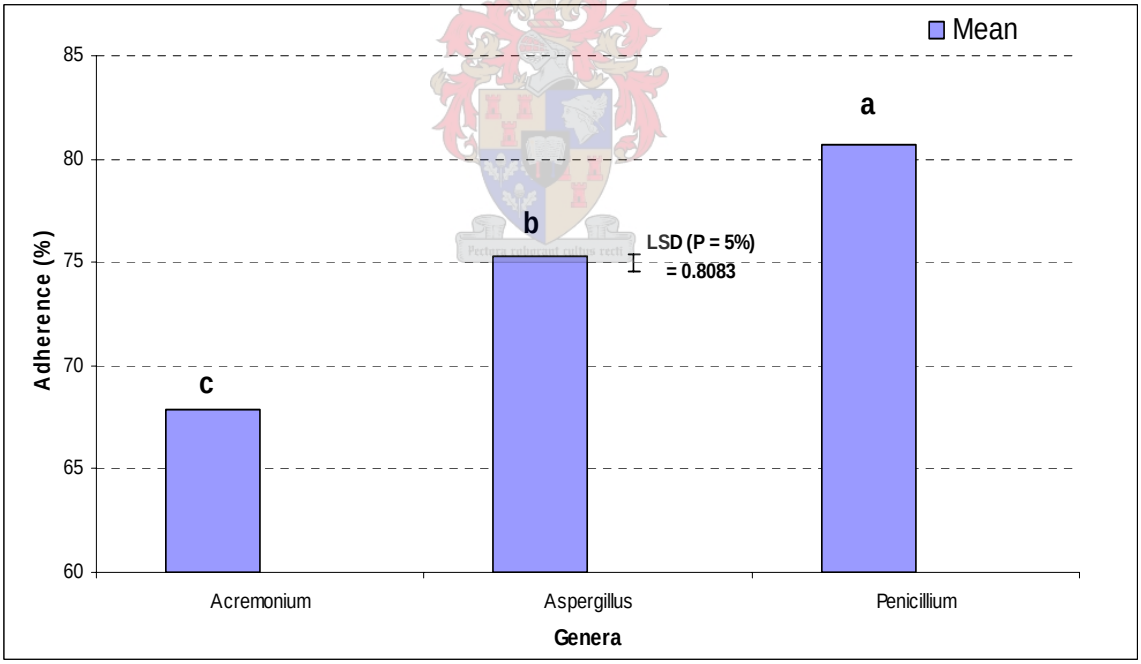
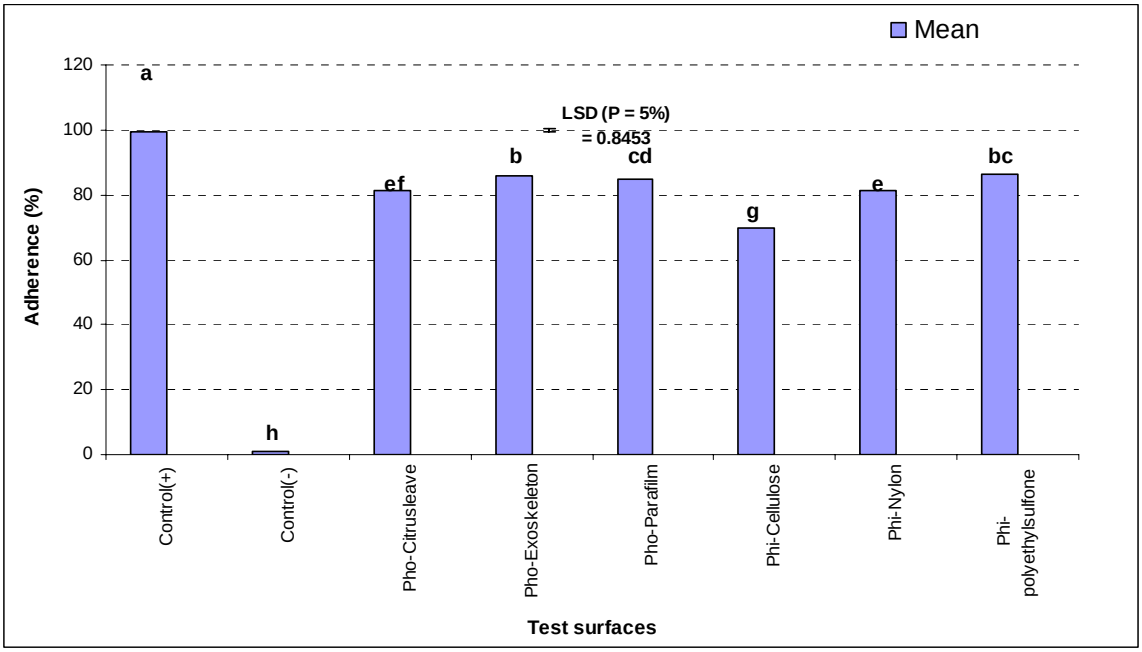


FIG. 3(a). Means of total adherence (%) of various strains included in this experiment. The pre-fixes "Acr-", "Asp-", and "Pen-", represent the genera *Acremonium, Aspergillus* and *Penicllium*, respectively. (b). Means of total adherence (%) of genera (means were back transformed by a ln [(% + 0.5) / (100 + 0.5)]).

*Means with the same letter are not significantly different at a 5% significance level.

a



b

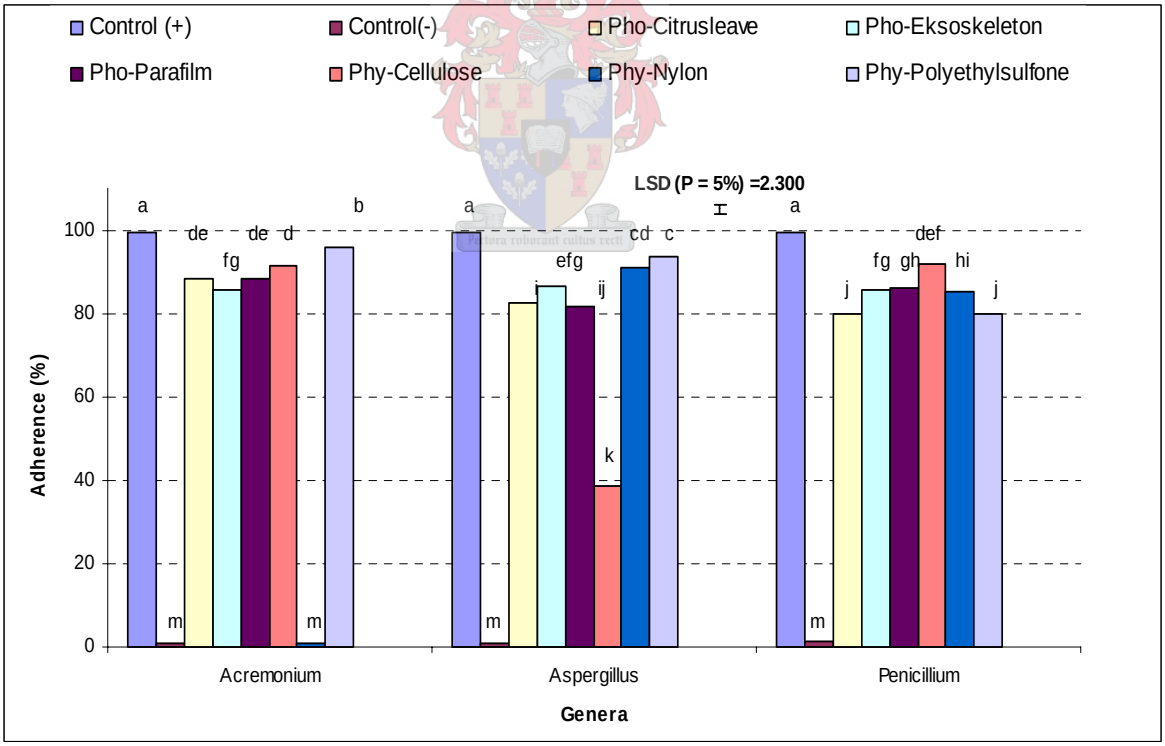


FIG. 4(a). Means of total adherence (%) to the various test surfaces. The pre-fix "pho-" indicates a hydrophobic surface, while the pre-fixes "phi-" indicates a hydrophilic surface. (b). Means of total adherence (%) of genera to the various test surfaces (means were back transformed by a ln [(% + 0.5)/(100 + 0.5)]).

*Means with the same letter are not significantly different at a 5% significance level.

The different genera showed different adherence profiles for the series of test surfaces investigated (Figure 4b), again indicating differences in physico-chemical characteristics of the fungal spore surfaces. A factor that also may have played a role in the adherence of these spores to the test surfaces is the electric charge of the test materials and their relative position in a triboelectric series. Thus, a Pearson correlation analysis of the percentage adherence and the relative electrostatic charges on the test surfaces was conducted which revealed a positive correlation between the two variables (R^2 = 0.5027, p = 0.001). A plot of the correlation is depicted in Figure 5. The number of data points for this analysis is small. The corresponding data does, however, interpolate well from the resultant regression line. Indications are that adherence (%) of the fungal spores is directly proportional to the positive electrostatic charge, represented by the absorbance of the photocopier toner at 540 nm. However, despite rigorous attempts to handle all test materials in an identical manner, conclusions about the electric charge on the surface of the test materials should be made with caution, since these charges are influenced by relatively small changes in temperature and relative humidity (McGonigle and Jackson, 2002).

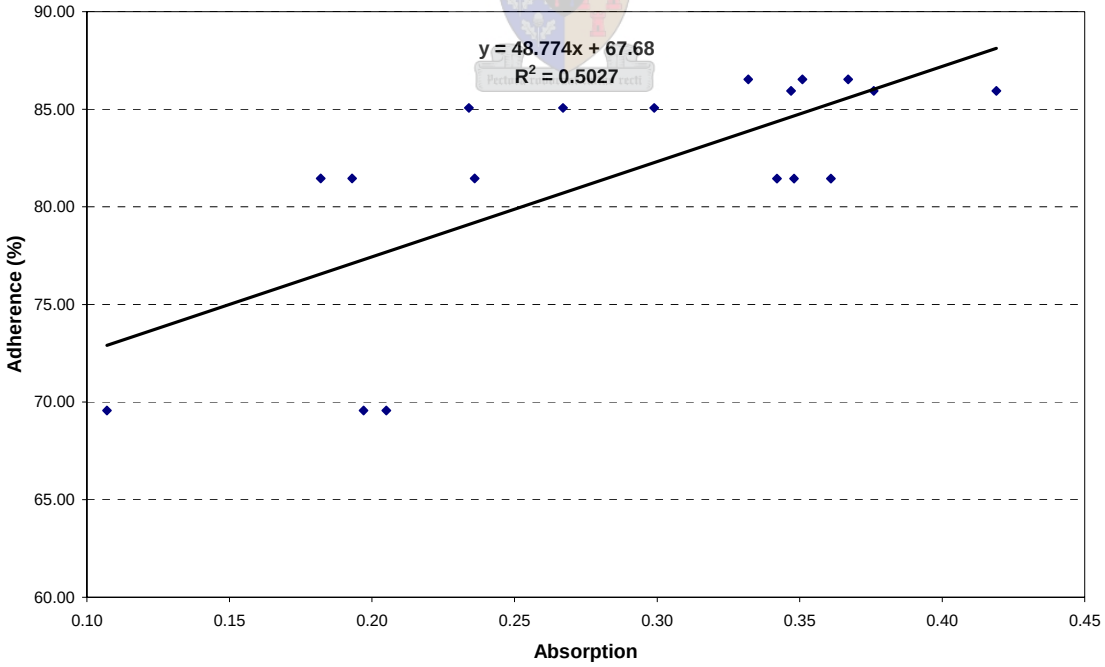
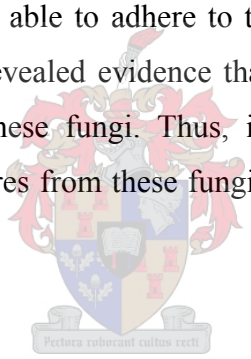


FIG. 5. Pearson's correlation plot of adherence (%) as a function of relative electrostatic charges on the test surfaces, represented by the absorbance of the photocopier toner at 540 nm. The correlation coefficient is 0.70898 (n = 18, p = 0.001).

4.4 CONCLUSION

In this study we demonstrated that fungal spores representing the genera *Acremonium*, *Aspergillus* and *Penicillium* differ in their relative adherence to different test surfaces under dry conditions. Although no significant difference in the percentage spore adherence was found between hydrophobic and hydrophilic materials, evidence was uncovered supporting the contention that electrostatic surface charges plays a role in the adherence of fungal spores to surfaces. Fungal spores are known to have a negative charge and ionic attraction has been implicated in the adherence of these spores to positively charged substrates (KerChung and Hoch, 1996; Smith *et al.,* 1998; Wasylnka *et al.,* 2001). Our results also support the findings of others that insects act as vectors for these fungi, as the fungal spores were able to adhere to the cuticles of these animals. However, in chapter three we revealed evidence that water may be the primary means of spore dispersal for these fungi. Thus, in the next chapter we will investigate the ability of the spores from these fungi to adhere to surfaces within watery environments.



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CHAPTER 5

Adherence of Fungi Suspended in an Aqueous Saline Solution to Various Surfaces.

5.1 INTRODUCTION

Since the middle of the twentieth century, the "dry" spores of hyphomycetous fungi, such as certain representatives of *Acremonium*, *Aspergillus* and *Penicillium,* were thought to be readily dispersed by air currents (Ingold, 1953). However, in Chapter 3 we demonstrated that the majority of spores may remain firmly attached to their conidiophores even after two weeks of constant air flow in airflow chambers. Supporting the contention of others that spore liberation and dissemination is apparently brought about by the presence of water (Frey and Keitt, 1925; and Tenzer and Gessler, 1999), we found that the addition of physiological salt solution immediately releases massive amounts of spores from the thalli of *Acremonium*, *Aspergillus* and *Penicillium* (Chapter 3). In addition, Gregory (1945) has demonstrated that heavy rain is more effective in expelling "dry" spores from the fruiting body of puffballs than wind alone.

Spore dispersal is completed once the spores are successfully deposited (Ingold, 1953; Moore-Landecker, 1972). The latter thus implies that the fungal propagules, dispersed by water, are able to adhere to a synthetic or natural substrate within an aqueous environment. Although the attachment and adherence of yeasts to surfaces has been studied in watery environments (Buck and Andrews, 1999), very little is known about the relative adherence of *Acremonium*, *Aspergillus* and *Penicillium* to different surfaces under aqueous conditions. However, in (Chapter 4) we demonstrated that, under dry conditions, fungal spores representing these genera do differ in their relative adherence to test surfaces differing in hydrophobicity and electrostatic charge.

Airborne fungal spores may also be deposited on a substrate in an aqueous environment. An example is when fungal conidia are inhaled by mammals (*i.e.* humans) and deposited in the respiratory tract where they adhere to the epithelial tissue via host surface glycoprotein (Bromley and Donaldson, 1996; Coulot *et al*., 1994; Peñalver *et al*., 1996). Experiments conducted by others, revealed that exhaled air is substantially cleared of particles in its passage through the respiratory tract, where most inhaled material, including conidia, are deposited (Kendrick, 1992). Inhalation of mold conidia holds serious health implications, since they can elicit respiratory infections. Larger spores are usually deposited in the upper respiratory tract (nose and trachea), whilst the smallest are deposited in the lower bronchioles and alveoli (Madelin, 1966).

However, spores are hygroscopic and have been shown to increase in aerodynamic diameter as the air carrying them into the lungs becomes saturated with moisture (Reponen *et al.,* 1996). Implications of these observations are that airborne conidia from the same genus can manifest in different tissue of the respiratory tract if inhaled. Gil *et al.* (1996) demonstrated that the mechanism of adherence of these spores is dependant on glycoprotein-glycoprotein interaction. Kennedy (1990) concluded that attachment of the conidia to surfaces is an essential event in the establishment of infection in a host and is a prerequisite for colonisation and fungal survival.

With above as background, the aim of this study was to determine the relative adherence of phylogenetic diverse filamentous fungi and yeasts in the presence of water to test surfaces differing in hydrophobicity and electrostatic charge. The ability of propagules representing these fungi to adhere to plasma membrane glycoprotein (fibrinogen) coated polystyrene was also studied and the presence of fungal carbohydrates on the surfaces of these propagules was confirmed using epi-fluorescence microscopy.

5.2 MATERIAL AND METHODS

Fungi used. The same hyphomycetous fungal strains belonging to the genera *Acremonium*, *Aspergillus* and *Penicillium* and originating from soil and wine cellars, which were reported on in Chapter 3, were used in this study. In addition, a polyphyletic group of unicellular fungi (yeasts) was included in the experimentation. The latter (Table 1) was obtained from the culture collection at the Department of Microbiology, University of Stellenbosch, and the Medical Research Council, Tygerberg. Cultures were maintained on 2 % malt extract agar (MEA) plates at 22 ^{0}C.

TABLE 1. Yeast isolates and strains included in the experiment.

Isolate/Strain Code	Species	Origin
CA	*Candida albicans*	Department of Viticulture and Oenology, University of Stellenbosch
CL	*Cryptococcus laurentii*	Fynbos soil
CN	*Cryptococcus neoformans*	Type strain of species (CBS 132T) originating from fruit juice.
CNC1	*Cryptococcus neoformans*	Clinical sample from patient with meningitis.
CNC2	*Cryptococcus neoformans*	Clinical sample from patient with meningitis.
CNE1	*Cryptococcus neoformans*	Vegetative debris
CNE2	*Cryptococcus neoformans*	Vegetative debris
CP	*Cryptococcus podzolicus*	Fynbos soil
RG	*Rhodotorula glutinis*	Fynbos soil
SC	*Saccharomyces cerevisiae*	Department of Viticulture and Oenology, University of Stellenbosch
YL	*Yarrowia lipolytica*	Department of Viticulture and Oenology, University of Stellenbosch

Adherence to test surfaces differing in physical properties. The adherence assay that was performed is the same as was previously described (Buck and Andrews, 1999; Kwon and Epstein, 1993). Conidial suspensions of filamentous fungi and unicellular fungi (yeasts) were prepared in the following manner. Cultures were grown on MEA plates for 5 days until there was sufficient conidial formation and/or growth. Conidia or yeast cells were suspended in 10 mM sodium phosphate (NaH_2PO_4) buffer (pH 7.0) at a concentration of 1×10^6 conidia or cells/ml. Spore counts were enumerated, microscopically by using a haemocytometer. These suspensions were applied in 50 µl aliquots to 1 cm^2 of each of six lamellar test materials *i.e.* nitrocellulose filter membrane (MicronSep, product no. AP10047S0, pore size 0.45 µm), nylon (Magna product no. N00HY320FX), Parafilm® (Pechiney Plastic Packaging, Menasha,WI, USA) and polyethersulphone (Osmonics™ Inc. product no. SO4SP04700, pore size 0.45 µm), citrus leaves (*Citrus sinensis*) and two-spotted cricket exoskeleton (forewings of *Gryllus bimaculatus*). Thereafter, the drops and test surfaces were incubated for 90 minutes in a moist chamber, and subsequently placed into 2-ml microcentrifuge tubes (Quality Scientific Plastics) along with 950 µl of the sodium phosphate buffer. As positive control, 50 µl of the original conidial/cellular suspension was transferred directly into a 2-ml microcentrifuge tube containing 950 µl buffer.

Microcentrifuge tubes were agitated with a Vortex Genie® 2 agitator (Scientific Industries, Inc.) for 10 seconds at setting 3. The test surfaces were removed, and conidia/yeast cells in the remaining suspension were enumerated by using a haemocytometer (counting chamber). The adherence (%) to each test surface was expressed in terms of the number of conidia removed from test surfaces by agitation compared to the original inoculum that was applied. The experiment was completely randomised with the test surfaces in a 28 × 8 factorial with three replications (Snedcor and Cochrane, 1967).

The factors were; 8 test surfaces (positive control, negative control, and six lamellar test materials); and 28 strains which have been grouped into three genera namely, *Acremonium* (ACR), *Aspergillus* (AA, AC, AT, AN, and AF), *Candida* (CA), *Cryptococcus* (CNC1, CNC2, CNE1, CNE2, CL, CN, CP), *Penicillium* (F2, R4, FG16, R1, R2, AB0268, ABO272, ABO275, AB0486, ABO487, PC), *Rhodotorula* (RG), *Saccharomyces* (SC), and *Yarrowia* (YL). Total conidial/cells adherence was recorded and transformed by a ln [(% + 0.5) / (100 + 0.5)] transformation, and the data subjected to a appropriate factorial analysis of variance (ANOVA), using SAS statistical software (SAS Institute Inc., 1999). Shapiro-Wilk test was performed on the residuals to test for non-normality (Shapiro and Wilk, 1965).

Adherence to fibrinogen coated polystyrene. Adherence assays were performed as previously described using fibrinogen coated polystyrene (Coulot *et al*., 1994; Hamilton *et al*., 1998; and Hamilton *et al*., 1999). The latter was prepared by filling each well of a ninety-six-well plate (Titertek®, Flow Laboratories) with a solution of fibrinogen (Sigma-Aldrich, Co.) at a concentration of 200 µg/ml in phosphate buffered saline (PBS), and incubating the plate at 37 ^{0}C for 1 hour. Thereafter, the plates were washed for 5 minutes in PBS. This step was repeated thrice. Unoccupied sites on the polystyrene surface were subsequently blocked by filling the wells with a solution of 0.1 % bovine serum albumin (BSA) in PBS and incubating it for 1 h at 37 ^{0}C and then for 14 h at 4 ^{0}C. Following 3 washes with PBS, each well received 200 µl of a 3 day old conidial/cellular suspension (1×10^6 conidia or cells/ml PBS) of a different fungal strain. Furthermore, to test the effect of soluble fibrinogen on spore adherence, conidia/cells were suspended in a solution of fibrinogen (200 µg/ml). For this purpose, each well received 200 µl of a 3 day old conidial/cellular suspension (1×10^6 conidia or cells/ml fibrinogen dissolved in PBS). Plates were then incubated for 30 minutes at 37 ^{0}C, after which non-adherent conidia were removed by washing the well for 5 minutes with 200 µl PBS containing 0.05 % (w/w) Tween 80.

Subsequently, adherent conidia or cells were fixed with 2.5 % glutaraldehyde in PBS for 15 minutes and counted by phase contrast microscopy at a 200 × magnification using a Nikon Eclipse E400 microscope. The latter was connected to a Samsung Sync master 700p PLUS monitor via a COHU High Performance CCD camera. Images were visualised by means of the computer software, Scion Image Beta 4.02 Win (Scion Image Inc., Frederick, Maryland, USA). Three wells were allocated to each test organism, of which three randomly selected fields (150 μm^2 each) were counted for each well. Moreover, for each test organisms one control well, uncoated with fibrinogen but saturated with BSA, was overlaid with the conidial/cellular suspension for the same incubation period as the experiment. The experiment was completely randomised with the method in a 28 × 2 factorial with three replications (Snedcor and Cochrane, 1967). The factors were; 2 treatments (positive control and fibrinogen treatment); and 28 strains/isolates. Spore adherence to fibrinogen was expressed as relative percentages.

Determination of cell surface polysaccharides. The presence of specific cell surface polysaccharides was determined by studying the adherence of Fluoresecein isothiocyanite (FITC)–labelled lectin (Concanavalin A) to the fungal cells (Buck and Andrews, 1999). For this purpose conidia or yeast cells were suspended in 1 ml ammonium bicarbonate (NH_4HCO_3)–buffer and agitated in a Braun homogeniser at setting three (Horner *et al*., 1993). Thereafter, cells were pelleted by centrifugation (10 000 g, 10 min) and washed in 1 ml lectin buffer (500 mM Tris-HCl; 10mM $CaCl_2$; in pyrogen free 3% NaCl solution). The conidial pellet was suspended and incubated with 200 µg/ml FITC-labelled lectin overnight at 22 ^{0}C and subsequently subjected to epi-fluorescence microscopy to assess fluorescence. A Nikon Eclipse E400 epi-fluorescence microscope, capable of three-color analyses (Dapi, Texas Red, FITC) was used. A Nikon digital camera was attached to the microscope and images were captured at 600 × magnification and visualised with Adobe Photoshop (Adobe Systems) imaging software run on a designated computer.

5.3 RESULTS AND DISCUSSION

In Chapter 4 it was revealed that a positive correlation exists between the adherence of filamentous fungal spores to the dry test surfaces and the relative positive charges on these surfaces. In contrast, a Pearson correlation analysis of the adherence (%) in an aqueous solution and the relative electrostatic charges on the test surfaces (as reported in Chapter 4) revealed a negative correlation (R^2 = 0.4502, p = 0.0023) between the two variables (Figure 1). The presence of the aqueous medium, therefore, seemed to diminish the electrostatic forces of attraction between the fungal propagules and the test surfaces.

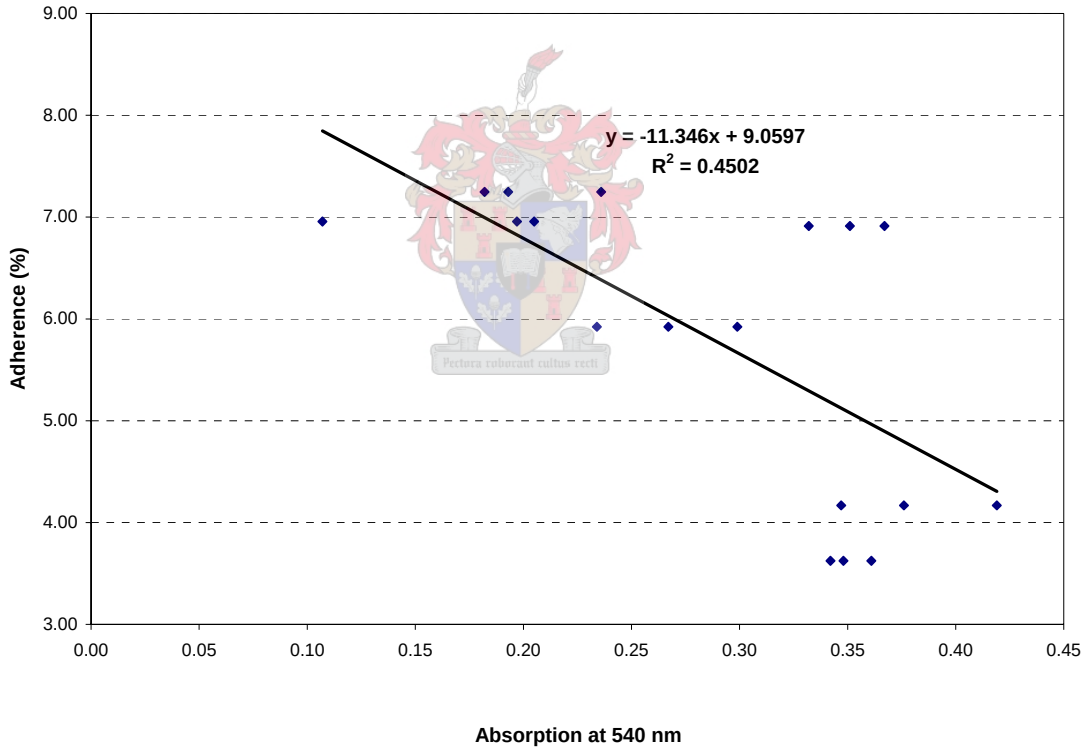


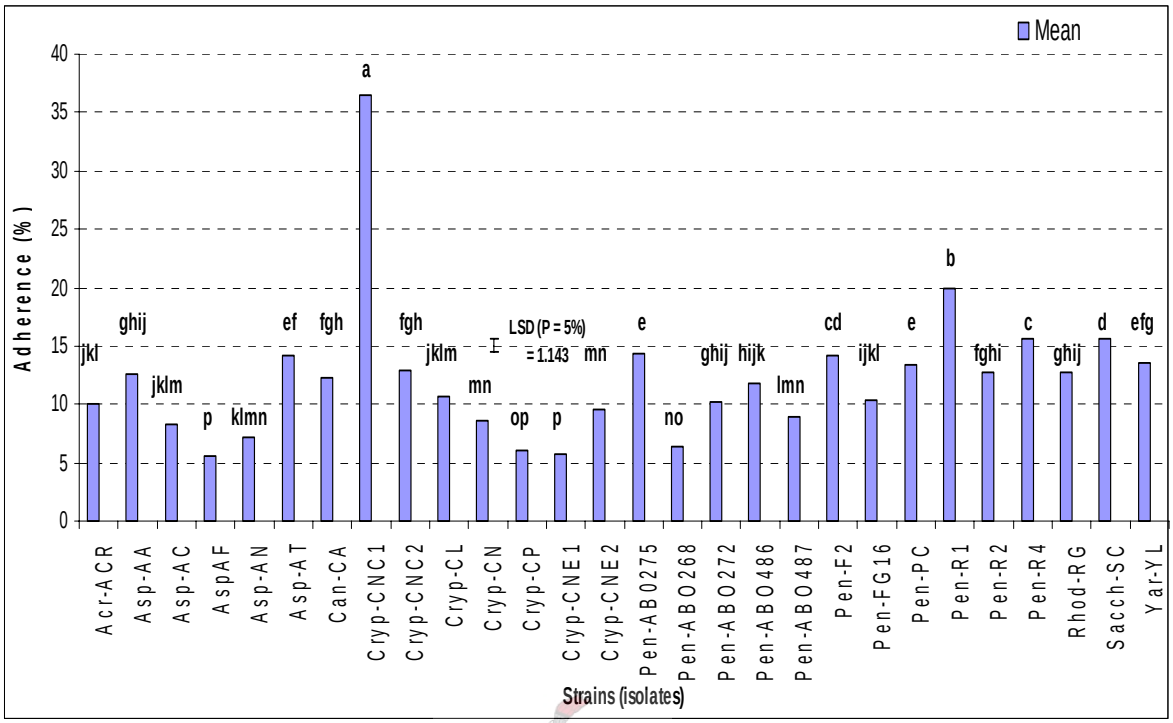
FIG. 1. Pearson's correlation plot of adherence (%) as a function of relative electrostatic charges on the test surfaces, represented by the absorbance of the photocopier toner at 540 nm. The correlation coefficient is -0.6709 (n = 18, p = 0.0023).

Adherence to test surfaces differing in physical properties. The relative adherence of the fungi, under aqueous conditions, to test surfaces differing in hydrophobicity and electrostatic charge (see Chapter 4) are depicted in Figures 2 & 3, Table 2, and Appendix I, Table. 4. The Shapiro-Wilk test on the adherence data revealed no deviation from normality (Glass *et al.,* 1972; Shapiro and Wilk, 1965) and significant intraspecific and intergenus differences were observed (Appendix I, Table. 4). Similar to the results reported in Chapter 4, the filamentous fungi showed intraspecific differences with regard to conidial adherence to the test surfaces *i.e. Penicillium commune* F2 and *P. commune* FG16; and *Penicillium citrinum* R1 and *P. citrinum* R2 (Figure 2a). Intraspecific differences of adherence to the tests surfaces were also observed among representatives of *Cryptococcus neoformans.* This yeast is an opportunistic human pathogen (Kwon-Chung, 1998).

The natural habitat of *C. neoformans* seems to be trees (Lazera *et al.,* 1996; Trilles *et al.*, 2003), although representatives of this species have been isolated from a wide diversity of environmental samples ranging from bird droppings to fruit related habitats (Kwon-Chung, 1998). Interestingly, the two strains representing this species that originated from clinical samples (*C. neoformans* CNC1 and *C. neoformans* CNC2), showed better adherence to the test surfaces than the two isolates originating from the vegetative debris (*C. neoformans* CNE1 and *C. neoformans* CNE2) and the type strain from fruit juice (*C. neoformans* CN). This phenomenon may be the result of adaptations that occurred in the capsules of these yeasts, making the clinical isolates more virulent than the environmental isolates.

Studies of others indicated that biochemical changes in the capsule, the main virulence factor, may have a considerable effect on the virulence of the yeast (Janbon *et al.,* 2001). Despite the high adherence (%) observed for *C. neoformans* CNC1 mentioned above, the cryptococci in general, as well as the representatives of *Acremonium* and *Aspergillus,* showed the least adherence to the test surfaces (Figure 2b). *Saccharomyces* was significantly more adherent than any of the other genera investigated.

a



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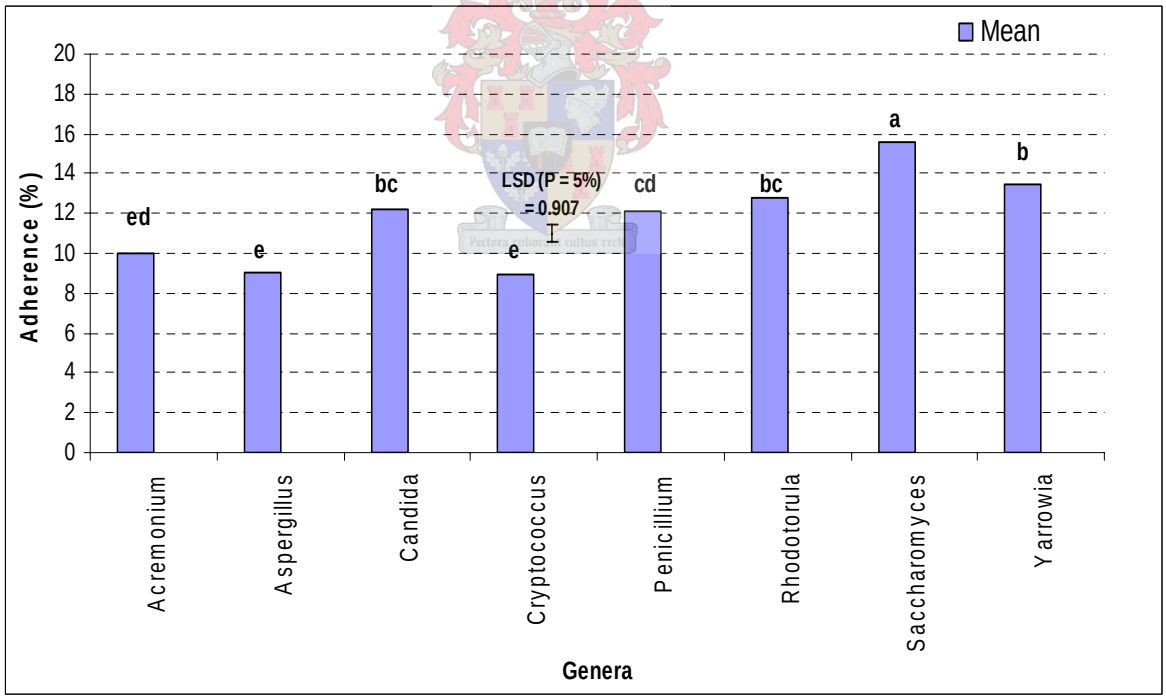


FIG. 2(a). Means of total adherence (%) of various strains included in this experiment. The prefixes "Acr-", "Asp-", "Can-", "Cryp-", "Pen-", "Rhod-", "Sacch-" and "Yl-", represents the genera *Acremonium, Aspergillus, Candida, Cryptococcus, Penicllium*, *Rhodotorula, Saccharomyces,* and *Yarrowia,* respectively. (b). Means of total adherence (%) of genera (means were back transformed by a ln [(% + 0.5) / (100 + 0.5)]).

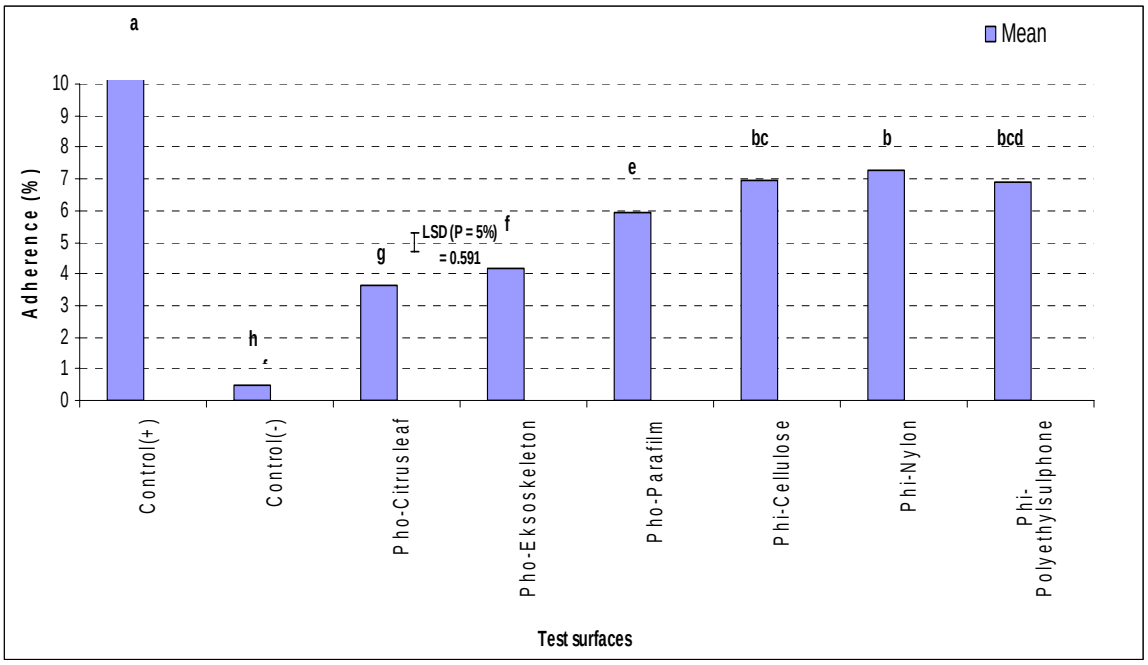
*Means with the same letter are not significantly different at a 5% significance level.

However, more representatives of this genus need to be investigated before any definite conclusions can be made on the relative adherence of this genus compared to the other fungi investigated. When the test surfaces were compared on the basis of the fungal adherence, it was obvious that the fungi adhered significantly more to hydrophilic surfaces than to hydrophobic surfaces (Figure 3a). These findings are in contrast to the results obtained in Chapter 4. In the latter case no significant difference was observed between hydrophobic and hydrophilic surfaces regarding the adherence of fungal conidia under dry conditions. The fact that the fungal propagules suspended in a watery environment showed a greater affinity for hydrophilic surfaces than for hydrophobic surfaces, indicated that the fungi were covered with relatively hydrophilic substances. It is commonly known that both conidia and yeasts are covered with polysaccharide complexes (Buck and Andrews, 1999; Van der Aa *et al*., 2002), however, the presence of these substances on the surfaces of all the fungal propagules examined in this study has not been previously confirmed.

In contrast to the findings reported in Chapter 4, the greatest fungal adherence was obtained for cellulose (Figure 3a). It is tempting to speculate that this phenomenon, where fungal propagules showed the most adherence to cellulose in the presence of water, may be a survival strategy to ensure successful colonisation of plant residues in the presence of available water. Similarly, since it is known that insects may act as vectors for fungal dispersal (Ingold, 1953; Prom and Lopez, 2004) the presence of water may facilitate enhanced deposition of fungal propagules from insects. One of the least adhesive surface in the presence of water was insect exoskeleton (Figure 3a).

The adherence profiles of the genera differed from each other (Figure 3b). In the presence of water, representatives of *Acremonium, Penicillium, Candida* and *Yarrowia* showed the most adherence to cellulose. Accept for the representative of *Candida, i.e*. *Candida albicans*, these fungi are all known to utilise cellulose as substrate and carbon source. (Domsch *et al.,* 1980; Kurtzman and Fell, 1998). It has been suggested that the natural habitat of *Candida albicans* may not be the mammalian intestine as is commonly assumed, but a habitat associated with natural vegetation (Blignaut, 2005).

a



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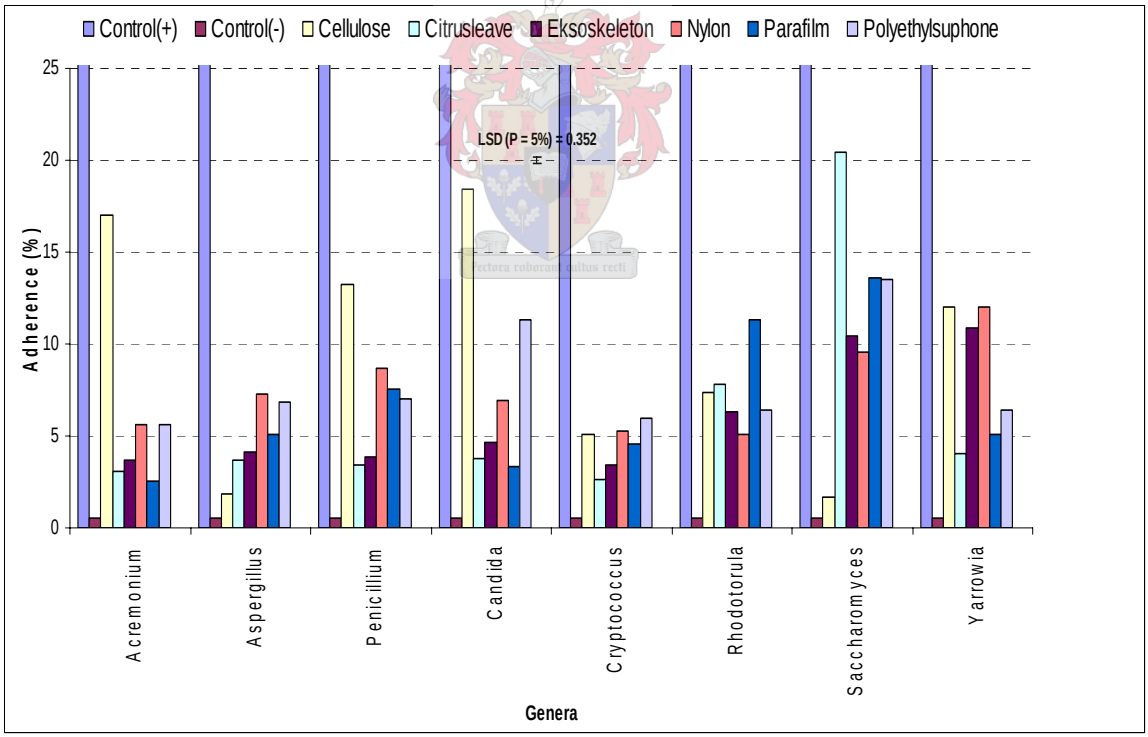

FIG. 3(a). Mean of total adherence (%) to the various test surfaces. The positive control is 100% although not shown on the graph, and the pre-fix "pho-" indicates a hydrophobic surface, while the pre-fix "phi-" indicates a hydrophilic surface. (b). Mean of total adherence (%) of genera to test the various test surfaces (means were back transformed by a ln [(% + 0.5)/(100 + 0.5)]).

*Means with the same letter are not significantly different at a 5% significance level.

Since vegetation is the source of one of the most abundant substrates in nature, *e.g.* cellulose (Crawford, 1981) it is understandable that fungi were able to adhere to this polymere. Among the yeasts, strains representing the genus, *Candida* adhered best to cellulose (Table 2) which has a hydrophilic surface, indicating that hydrophilic substances occur on the surface of this yeast. However, *Candida* is known to possess hydrophobic components on its cell surface, and conversion from surface hydrophilicity to hydrophobicity may be acquired by alterations in the hydrophilic fibrillar protein (Hazen and Hazen, 1993). The filamentous fungi, *i.e. Acremonium* and *Penicillium* also showed good adherence to cellulose, and the former is well-known for its association with plants (Latch, 1994).

TABLE 2. Means of total adherence (%) of genera to cellulose (Means with the same letter are not significantly different at a 5% significance level).

Genus	Mean	t-Grouping
Acremonium	16.8	cd
Aspergillus	5.1	nopqrst
Candida	18.1	bc
Cryptococcus	5.2	nopqrst
Penicillium	15.1	de
Rhodotorula	7	klmno
Saccharomyces	1.2	vw
Yarrowia	11.6	fg

Compared to all the fungi tested, *Saccharomyces* adhered the best to citrus leaves, which has a relative hydrophobic surface, indicating that hydrophobic substances occur on the surface of this yeast (Table 3). Another fungus that showed a relatively high adherence (%) to citrus leaves was the representative of the basidiomycetous yeast, *Rhodotorula.* This is not surprising since *Rhodotorula* strains are known to colonise plant surfaces (Biswas *et al*., 2001). Furthermore, leaf surfaces are naturally inhabited by a large number of saprophytic yeasts (Buck and Andrews, 1999; de Azeredo *et al.,* 1998). These unicellular fungi must have evolved mechanisms that gave them an advantage with regard to adherence to plant surfaces. Compared to the representatives of *Rhodotorula* and *Saccharomyces*, the filamentous fungi showed less adherence to citrus leaves. This is a suprising observation, since hyphomycetous fungi are known pathogens of citrus plants (Wuryatmo *et al.,* 2003).

TABLE 3. Means of total adherence (%) of genera to citrus leaf (Means with the same letter are not significantly different at a 5% significance level).

Genus	Mean	t-Grouping
Acremonium	2.7	tuv
Aspergillus	3.5	qrstuv
Candida	3.3	rstuv
Cryptococcus	3.3	rstuv
Penicillium	4	pqrstu
Rhodotorula	7.6	jklmn
Saccharomyces	20.2	b
Yarrowia	3.7	qrstuv

From the fungi tested, representatives of *Saccharomyces* and *Yarrowia* showed the highest adherence (%) to insect exoskeleton, a hydrophobic substrate (Table 4), emphasising the role of insects as vectors for these fungi (Lachance *et al.,* 2003). Ascomycetous and basidiomycetous yeasts have been shown to co-exist with insects and utilise them as vectors. Compared to *Saccharomyces*, *Yarrowia* did not show good adherence to the hydrophobic citrus leaf, and this phenomenon may be ascribed to differences in the physico-chemical properties between the cell surfaces of these yeasts.

TABLE 4. Means of total adherence (%) of genera to exoskeleton (Means with the same letter are not significantly different at a 5% significance level).

Genus	Mean	t-Grouping
Acremonium	3.3	rsuv
Aspergillus	3.9	qrstu
Candida	4.2	opqrstu
Cryptococcus	3.5	qrstuv
Penicillium	4.1	opqrstu
Rhodotorula	5.9	mnopq
Saccharomyces	10.1	ghij
Yarrowia	10.7	fghi

The fungi showing the most adherence to nylon a hydrophilic substrate, were representatives of the hyphomycetous fungus *Penicillium* and the ascomycetous yeast *Yarrowia* (Table 5). *Yarrowia* is known for its hydrophilic cell surface, and this study complements the findings of others (Aguedo *et al.,* 2005). However, *Yarrowia* also showed good adherence to the hydrophobic insect exoskeleton, and this observation may have been as a result of alterations to the hydrophilic fibrillar protein on the cell surfaces (Hazen and Hazen, 1993).

TABLE 5. Means of total adherence (%) of genera to nylon (Means with the same letter are not significantly different at a 5% significance level).

Genus	Mean	t-Grouping
Acremonium	5.5	nopqr
Aspergillus	8.4	ijklm
Candida	6.5	lmnop
Cryptococcus	5.1	nopqrst
Penicillium	10.3	ghi
Rhodotorula	4.8	opqrst
Saccharomyces	9.2	ghijkl
Yarrowia	11.6	fg

The representative of *Saccharomyces* showed the most adherence to the hydrophobic parafilm (Table 6). These findings support the results obtained for the adherence tests to the other hydrophobic surfaces, *i.e.* citrus leaves (Table 3) and insect exoskeleton (Table 4), where the representative of *Saccharomyces* also showed a relatively high adherence (%). Interestingly, although it has been known for quite some time that polysaccharide complexes occur on the surfaces of these yeasts (Buck and Andrews, 1999; Palecek *et al.,* 2002), it has recently been demonstrated that lipids acting as adhesives, that aid in flocculation, also occur on the surfaces of *Saccharomyces cerevisiae* (Kock *et al*., 2000).

TABLE 6. Means of total adherence (%) of genera to parafilm (Means with the same letter are not significantly different at a 5% significance level).

Genus	Mean	t-Grouping
Acremonium	2.1	uvw
Aspergillus	5.1	nopqrst
Candida	2.9	stuv
Cryptococcus	4.9	opqrst
Penicillium	9	hijkl
Rhodotorula	11.1	fgh
Saccharomyces	13.2	ef
Yarrowia	4.9	opqrst

Cell surface hydrophobicity of microbes has previously been positively correlated with increased adherence (Doss *et al.*, 1993). However, the adherence of filamentous fungi to hydrophobic surfaces was not positively correlated to substratum hydrophobicity (Braun and Howard, 1994). With the exception of *Penicillium* the results of our study thus generally support the findings of others. The adherence (%) to the hydrophobic test surfaces were generally lower for the filamentous fungi than for the yeasts (Tables 3, 4, and 6).

TABLE 7. Means of total adherence (%) of genera to polyethylsulphone (Means with the same letter are not significantly different at a 5% significance level).

Genus	Mean	t-Grouping
Acremonium	5.3	nopqrs
Aspergillus	8.6	hijkl
Candida	11	fgh
Cryptococcus	6.6	lmno
Penicillium	9.9	ghij
Rhodotorula	6	mnopq
Saccharomyces	13.2	ef
Yarrowia	6	mnopq

Adherence to fibrinogen coated polystyrene. Fungal conidia have been shown to be the causative agent of invasive lung disease in humans, because of the ability of the fungi to adhere to extracellular matrix (ECM) protein (Wasylnka and Moore, 2000). Fungi are also known to bind to purified extracellular matrix (ECM) protein *i.e.* fibrinogen (González *et al.*, 2005). This assay revealed that conidia and yeast cells could bind immobilised fibrinogen, confirming the presence of "fibrinogen-receptors" on cell surfaces. In contrast to the results obtained with the other tests surfaces examined in this study, no significant difference could be detected between the fungal strains and genera regarding the ability to adhere to fibrinogen coated polystyrene (Figure 4a & b; Appendix I, Table. 5). Indications are that all isolates included in this study have the potential to adhere to ECM protein, if inhaled.

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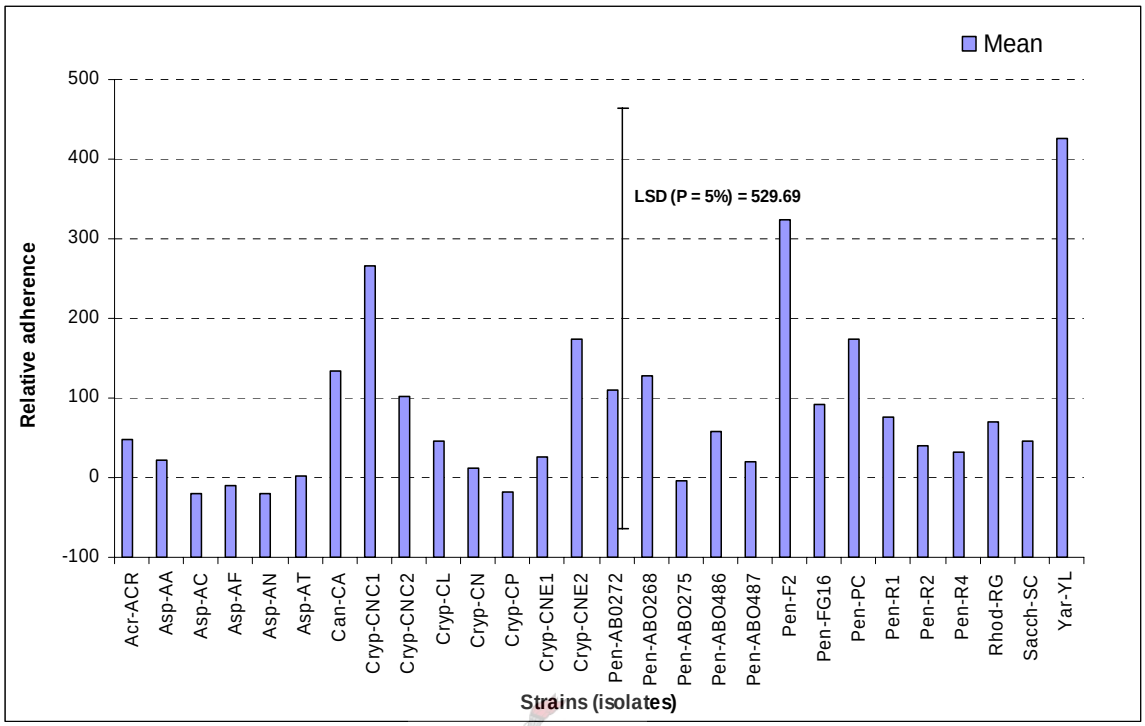

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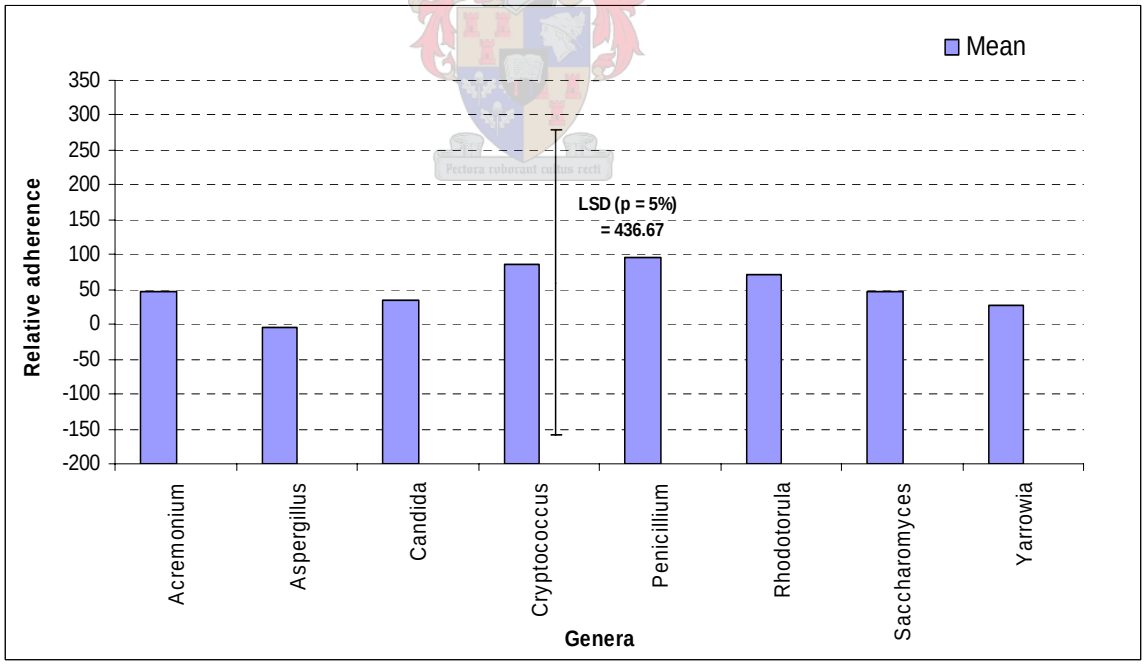


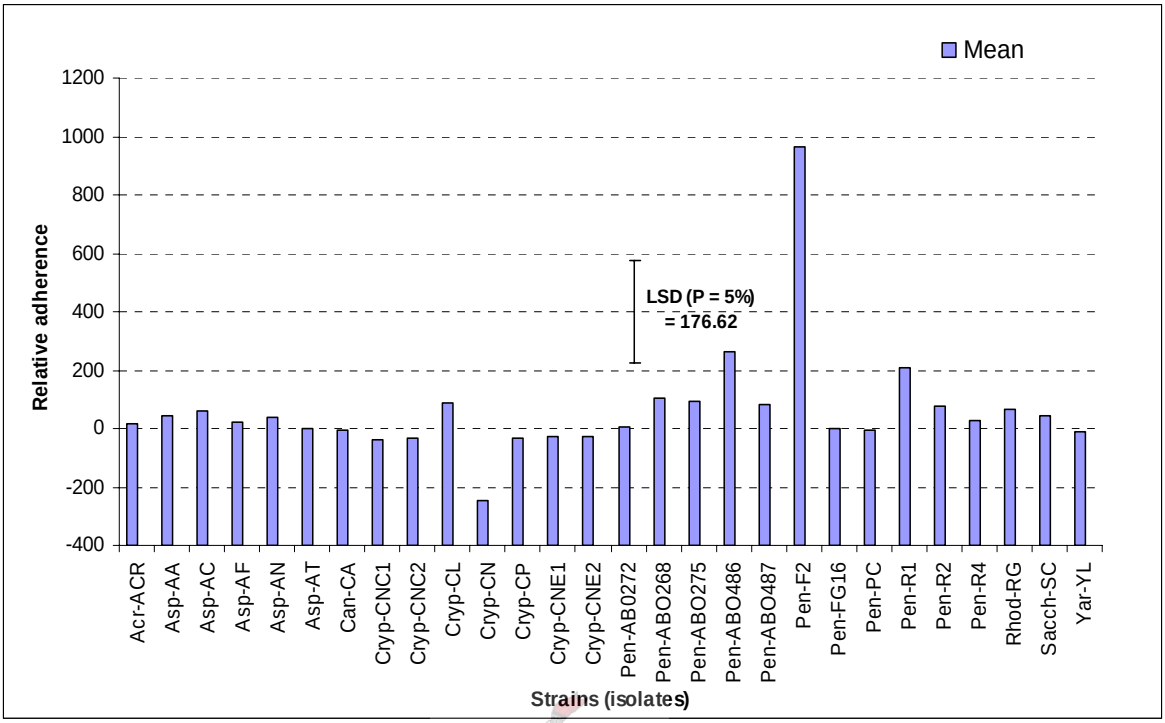
FIG. 4(a). Relative adherence of conidia/ yeast cells of different strains (isolates) to fibrinogen. The pre-fixes "Acr-", "Asp-", "Can-", "Cryp-", "Pen-", "Rhod-", "Sacch-" and "Yar-", represents the genera *Acremonium, Aspergillus, Candida, Cryptococcus, Penicllium*, *Rhodotorula, Saccharomyces*, and *Yarrowia,* respectively. (b). Relative adherence of genera to fibrinogen.

The effect of soluble fibrinogen on spore adherence to immobilised fibrinogen mostly revealed no significant differences between the fungal strains and genera (Appendix I, Table 6). In some cases relative adherence decreased, which indicated that the most of "fibrinogen-receptors" on the fungal surfaces were occupied by specific adherence (Coulot *et al.,* 1994) of the soluble fibrinogen (Figures 5a & b). Lima *et al.* (2001) suggested that that conidial surface glycoconjugates participated in the adherence of conidia to other carbohydrate moieties. González *et al.,* (2005) demonstrated that polypeptides on spore surfaces facilitated the adherence to ECM protein *i.e.* fibrinogen. The ECM has been shown to have a high glycoprotein content, which play a role in the adherence process (Tronchin *et al.,* 2002). It can, therefore, be postulated that adherence is brought about by glycoprotein-glycoprotein interaction.

The results of the study, therefore, indicated that the fungal strains included in this experiment are capable of binding fibrinogen. Wasylnka *et al*. (2000) demonstrated that conidial adherence to purified ECM protein paralleled adherence to intact ECM protein. It would seem that fibrinogen can successfully be applied as a model ECM protein to study conidial and yeast cell adherence. However, the results obtained do not imply that carbohydrate interactions are alone responsible for adherence. Nevertheless, carbohydrate may occur in substantial quantities on the cell surfaces of the fungi investigated in this study.

Determination of cell surface polysaccharides. To confirm the presence of polysaccharides on the surfaces of the fungi investigated in this study a fluorescent labeled lectin (Concanavalin-A) was deployed, because this lectin is known to bind carbohydrates (Buck and Andrews, 1999). Dûfrene *et al*., (1999) suggested that the presence of strong adherence forces on spore surfaces can be attributed to the presence of polysaccharides. In our study the surfaces of both the fungal conidia and yeasts emitted fluorescence when visualised under a epi-fluorescence microscope with the FITC-color analysis. These results confirmed the presence of polysaccharides on all the fungal propagules investigated in this study (Table 2). Images of fluorescence emitted by Concanavalin-A stained spores are included as a compact disc compilation (Appendix II).

a



b

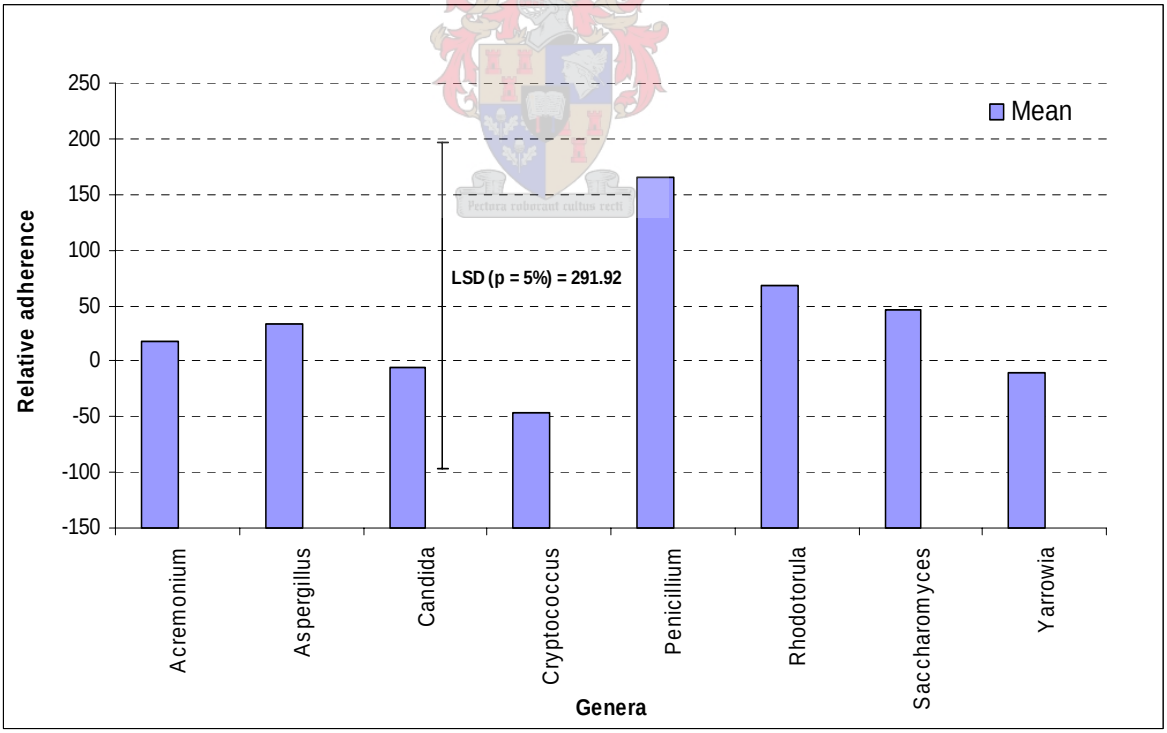


FIG. 5(a). Effect of soluble fibrinogen on the relative adherence of strains to immobilised fibrinogen. The pre-fixes "Acr-", "Asp-", "Can-", "Cryp-", "Pen-", "Rhod-", "Sacch-" and "Yar-", represents the genera *Acremonium, Aspergillus, Candida, Cryptococcus, Penicllium*, *Rhodotorula, Saccharomyces,* and *Yarrowia,* respectively. (b). Effect of soluble fibrinogen on the relative adherence of genera to immobilised fibrinogen.

TABLE 2. The occurrence of cell surface polysaccharides on conidia and yeast cells as determined by epi-fluorescence microscopy using FITC-labelled Concanavalin-A

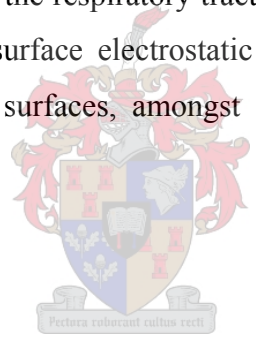
Strains/ Isolates	Species	Fluorescence	
		Epi-fluorescence	Light microscopy
AC	*Aspergillus carneus*	+	-
AF	*A. fumigatus*	+	-
AN	*A. niger*	+	-
AT	*A. terreus*	+	-
ACR	*Acremonium alternatum*	+	-
AA	*A. aculeatus*	+	-
CNE1	*Cryptococcus neoformans*	+	-
CNE2	*C. neoformans*	+	-
CNC1	*C. neoformans*	+	-
CNC2	*C. neoformans*	+	-
CP	*C. podzolicus*	+	-
CA	*Candida albicans*	+	-
CL	*C. laurenti*	+	-
CN	*C. neoformans*	+	-
ABO486	*Penicillium camemberti*	+	-
PC	*P. candidum*	+	-
R1	*P. citrinum*	+	-
R2	*P. citrinum*	+	-
ABO272	*P. citrinum*	+	-
FG16	*P. commune*	+	-
R4	*P. glabrum*	+	-
ABO268	*P. spinulosum*	+	-
ABO275	*P. sumatrense*	+	-
ABO487	*P. westlingii*	+	-
F2	*P. commune*	+	-
RG	*Rhodotorula glutinis*	+	-
SC	*Saccharomyces cerevisiae*	+	-
YL	*Yarrowia lipolytica*	+	-
Negative control	Parafilm	-	-

+ fluorescence emitted; - no fluorescence emitted

Yeast cells appeared to emit more fluorescence, than the conidia, which can cautiously be attributed to their glycoprotein rich cell walls. *Saccharomyces* for instance is known to engage in cell-cell adherence during conditions of nitrogen deficiency, which entails the attachment to carbohydrates in the cell walls of neighboring yeast (Palecek *et al.,* 2002). Strains suspended in the buffer were incubated for 90 minutes on test surfaces, and it can tentatively be said that this would be ample time to put a yeast cell in a state of nitrogen deficiency. In addition, the cryptococci are known for the production of extracellular carbohydrates that have been used in the taxonomy of these yeasts (Kurtzman and Fell, 1998).

5.4 CONCLUSION

Both intraspecific and intergenus differences were observed when the relative adherence, to test surfaces, of phylogenetic diverse filamentous fungi and yeasts were studied in the presence of buffered water. Overall, the fungi adhered better to hydrophilic surfaces than to hydrophobic surfaces. This indicated that the fungal surfaces were covered with relatively hydrophilic compounds such as carbohydrates. Subsequently, it was demonstrated that all the fungi adhered to plasma membrane glycoprotein (fibrinogen) coated polystyrene and the presence of fungal carbohydrates on the surfaces of the fungal propagules was confirmed using epi-fluorescence microscopy. This observation may have medical implicaton for humans, since inhalation of these fungal propagules may result in adherence to the basal lamina of the respiratory tract. Furthermore, evidence was uncovered that suggested that surface electrostatic charges plays a role in the dislodging of spores from test surfaces, amongst others insect exoskeleton in watery environments.



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CHAPTER 6

6.1 CONCLUDING REMARKS

It is known that representatives of hyphomycetous taxa such as *Acremonium*, *Aspergillus* and *Pencillium*, usually release more colony forming units into the air under dry conditions than under humid conditions (Fogelmark *et al*., 1994; McNeel and Kreutzer, 1996). We demonstrated that representatives of these genera released more spores after two weeks of incubation compared to after one week (Chapter 3). During humidified aeration *Penicillium* strains were more successful in releasing their spores than the strains representing *Aspergillus* and *Acremonium*, while during desiccated aeration, the *Aspergillus* took longer to release their spores than representatives of *Acremonium* and *Penicillium.* This phenomenon may be as a result of differences in the morphology of the sporogenous structures of these fungi (Klich and Pitt, 1988; Larone, 1995). *Aspergillus,* for example is characterised by a swollen vesicle from which the metulae and conidiogenous cells arise. It is tempting to speculate that these vesicles may take longer to dry and release conidia into the air than the more filamentous sporogenous structures of *Acremonium* and *Penicillium.*

Once released, conidia from representatives of these genera also differed in their ability to adhere to various test materials differing in hydrophobicity and electrostatic surface charges (Chapter 4). In general, the *Penicillium* strains showed a greater ability to adhere to the test surfaces, than the aspergilli, while the representative of *Acremonium* showed the least adherence to these surfaces. Interestingly, insect exoskeleton was one of the test materials onto which the fungal conidia showed the best adherence, highlighting the role of insects as vectors for these fungi. This phenomenon is currently being studied by others to understand the spreading of fungal diseases via insect vectors among agricultural crops (Engelbrecht *et al.,* 2004).

Although we could find no correlation between hydrophobicity and the level of conidial attachment to the test surfaces, we did find a positive correlation between the percentage spore adherence and the relative positive charges on the surfaces of the test materials (Chapter 4). It must be noted that it is known that fungal spores may have a negative charge, and that ionic attraction has been implicated in the adherence of these spores to positively charged substrates (KerChung and Hoch, 1996; Smith *et al.,* 1998; and Wasylnka *et al.,* 2001). Although not proven, our results also support the contention that an important dispersion agent for these filamentous fungi may be water (Fitt and Nijman, 1983), since the addition of physiological salt solution resulted in an immediate and massive release of spores from the colonies of strains representing *Acremonium, Aspergillus* and *Pencillium* (Chapter 3).

However, in order for spores to be dispersed successfully, they have to be deposited from their dispersion agents, *i.e.* water, and must be able to attach to the new substrate. Consequently, we studied the adherence of the filamentous fungal propagules, as well as the adherence of yeasts, onto the test materials within an aqueous solution (Chapter 5). Yeasts are a polyphyletic group of unicellular fungi adapted for growth in watery environments (Lachance and Starmer, 1998). We found similar levels of adherence to the tests surfaces among the filamentous and unicellular fungi. In general, the fungi adhered better to hydrophilic surfaces than to hydrophobic surfaces (Chapter 5). This indicated that the fungal surfaces were covered with relatively hydrophilic compounds such as carbohydrates. Subsequently, we demonstrated that all the fungi adhered to plasma membrane glycoprotein (fibrinogen) coated polystyrene and the presence of fungal carbohydrates on the surfaces of the fungal propagules was confirmed using epi-fluorescence microscopy. Furthermore, evidence was uncovered that suggested that surface electrostatic charges play a role in dislodging the fungal propagules from the test surfaces, amongst others insect exoskeleton in watery environments. In general it can be concluded that the filamentous fungal genera differed in their strategy to release airborne spores, and that the adherence profiles obtained for each genus, indicated that insects may be used as vectors.

Water however, serves as the primary means of spore dispersal and in an aqueous environment the adherence of the fungal propagules to insect cuticles was significantly less than to the other test materials. The fact that the representatives of the different fungal genera differed in their strategy to release airborne spores, as well as in their adherence profiles for the different test materials, indicate that each of the genera occupy a different environmental niche. This phenomenon should be investigated further in future and the challenge now is to find correlations between the conidiophore morphology of each fungus and characteristics of its niche. A point of departure may be to study differences in spore dispersal within artificial ecosystems in which the abiotic and biotic components are manipulated. Microbiological culture techniques (Pitt, 1979), electron microscopy (Martinez *et al.,* 1982), serological methods in combination with epi-fluorescense microscopy (Prigione *et al.,* 2004), as well as atomic force microscopy (Razatos *et al.,* 1998) may then be used to monitor changes in dispersion, and subsequent adherence of the fungal propagules.

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APPENDIX I

Analysis of variance (ANOVA)

TABLE. 1.ANOVA of total spores liberated during dry and humid aeration.

Source					d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* Exp Error)	Significance level(P)
Species					16	33.123	2.070	18.050	<0.0000
	Genera				2	15.597	7.798	13.640	<0.0001
	Remainder				14	18.684	1.335	2.330	<0.0064
		Penicillium			10	15.987	1.599	16.290	<0.0001
		Aspergillus			4	2.696	0.674	6.210	<0.0001
		Acremonium			0	0.000	0.000	0.0000	<0.000
Method					1	27.834	27.834	48.68	<0.0001
Species*Environmental condition					16	27.947	1.747	3.060	<0.0002
	Genera* Environmental condition				2	0.853	0.427	0.750	<0.4760
	Remainder				14	18.684	1.335	2.330	<0.0064
Time					1	4.110	4.110	7.190	<0.0082
Time*Species					16	12.358	0.772	1.350	<0.1759
	Time*Genera				2	1.938	0.969	1.700	<0.1874
	Remainder				14	18.684	1.335	2.330	<0.0064
Time* Environmental condition					1	0.178	0.178	0.310	<0.5776
Time* Environmental condition *Species					16	29.107	1.819	3.180	<0.0001
	Time* Environmental condition *Genera				2	5.226	2.613	4.570	<0.0120
	Remainder				14	18.684	1.335	2.330	<0.0064
Experimental Error (Tube)					136	77.7535	0.572	5.770	<0.001
Sample Error (Triplicate)					405	40.1187	0.099		
Total Corrected					608	253.706			

* Dependant Variable: L_SporeLiberation = (LOG_{10} (x + 1)

TABLE 2. ANOVA of total spores liberated in aqueous saline solution.

Source	d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* Exp Error)	Significance level (P)
Species (Isolates)	16	6.105	0.382	3.210	<0.0021
Genera	2	0.535	0.268	2.250	<0.1210
Remainder	14	5.569	0.398	3.350	<0.0020
Penicillium	10	2.997	0.299	2.070	<0.0740
Aspergillus	4	2.572	0.643	8.170	<0.0034
Acremonium	0	0.000	0.000	0.000	<0.0000
Experimental Error(Tube)	50	4.043	0.118		
Corrected Total	10	10.149			

*Dependant Variable = [Log (X + 1)]

TABLE 3. ANOVA of the adherence (%) of spores to test surfaces.

Source				d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* ExpError)	Significance level (P)
Species				16	102.7649	6.4228	48.6500	<0.0001
	Genera			2	15.7742	7.8871	59.7400	<0.0001
	Remainder			14	86.9906	6.2136	47.0700	<0.0001
		Penicillium		10	49.9566	4.9956	37.8300	<0.0000
		Aspergillus		4	37.0340	9.2585	70.1300	<0.0000
		Acremonium		0	0.0000	0.0000	0.0000	<0.0000
Test surface				7	2636.5267	376.6466	2852.9200	<0.0001
Species* Test surface				112	814.0518	7.2683	55.0500	<0.0001
	Genera*Test surface			14	255.6515	18.2608	138.3200	<0.0001
	Remainder			98	558.4002	5.6979	43.1600	<0.0001
Experimental Error (Test surface)				272	35.9098	0.1320		
Total Corrected				407	3589.2533			

Dependant Variable: L_% Adherence = ln[(% + 0.5)/(100 - % + 0.5)]

TABLE 4. ANOVA of adherence (%) of spores suspended in an aqueous solution to test surfaces.

Source	d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* Exp Error)	Significance level (P)
Species (Isolates)	28	139.7240	4.9901	70.06	<0.0001
Genera	7	23.4750	0.0010	47.09	<0.0001
Remainder	21	116.2480	0.0001	77.72	<0.0001
Penicillium	10	28.805	2.8805	40.44	<0.0000
Aspergillus	4	17.3790	4.3449	61.01	<0.0000
Acremonium	0	0.0000	0.0000	0.0000	<0.0000
Cryptococci	7	70.0630	10.0090	140.53	<0.0000
Yarrowia	0	0.0000	0.0000	0.0000	<0.0000
Rhodotorula	0	0.0000	0.0000	0.0000	<0.0000
Candida	0	0.0000	0.0000	0.0000	<0.0000
Saccharomyces	0	0.0000	0.0000	0.0000	<0.0000
Test surface	7	5714.9230	816.4176	11462.8000	<0.0001
Species* Test surface	188	259.0600	1.3779	19.3500	<0.0001
Genera*Test surface	49	81.7470	1.6683	23.4200	<0.0001
Remainder	139	177.3130	1.2756	17.9100	<0.0001
Experimental Error(Test surface)	448	31.9080	0.0712		
Total Corrected	671	6145.6160			

* Dependant Variable: L_Sporecount = ln[(% + 0.5)/(100 - % + 0.5)]

TABLE 5. ANOVA of relative adherence of spores to immobilised fibrinogen.

Source	d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* Exp Error)	Significance level (P)
Species (Isolates)	27	931794.035	34510.890	0.333	<0.999
Genera	7	490991.680	70141.668	0.668	<0.697
Remainder	20	440802.355	22040.117	0.210	<0.999
Penicillium	10	251478.545	25147.854	0.239	<0.991
Aspergillus	4	3845.333	961.333	0.009	<0.999
Acremonium	0	0.000	0.000	0.000	<0.000
Cryptococci	6	185478.476	30913.079	0.294	<0.937
Yarrowia	0	0.000	0.000	0.000	<0.000
Rhodotorula	0	0.000	0.000	0.000	<0.000
Candida	0	0.000	0.000	0.000	<0.000
Saccharomyces	0	0.000	0.000	0.000	<0.000
Experimental Error(Well)	56	5872958.000	104874.250		
Corrected Total	83	6804752.036			

* Dependant Variable: RelativePercentage

TABLE 6. ANOVA of the effect of soluble fibrinogen on relative adherence of spores to immobilised fibrinogen.

Source		*df* (n-1)	Sum Square	Mean Square	F-ratio (*df*/ *df* Exp Error)	Significance level (P)
Species (Isolates)	27		3164945.476	117220.203	2.505	<0.0019
Genera	7		640377.764	91482.538	1.954	<0.0779
Remainder	20		2524567.713	126228.386	2.697	<0.0018
Penicillium		10	2336140.970	233614.097	4.991	<0.0126
Aspergillus		4	6283.600	1570.900	0.033	<0.9595
Acremonium		0	0.000	0.000	0.000	<0.0000
Cryptococci		6	182143.143	30357.191	0.648	<0.7620
Yarrowia		0	0.000	0.000	0.000	<0.0000
Rhodotorula		0	0.000	0.000	0.000	<0.0000
Candida		0	0.000	0.000	0.000	<0.0000
Saccharomyces		0	0.000	0.000	0.000	<0.0000
Experimental Error (Well)	56		2620754.667	46799.190		
Corrected Total	83		5785700.143			

*Dependant Variable: RelativePercentage

TABLE 7. ANOVA of test surface water affinity.

Source	d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* Exp Error)	Significance level (P)
Substrates (membranes)	5	32596.862	6519.372	2539.860	<.00010
Water affinity	1	31980.891	31980.891	12459.300	<.00010
Remainder	4	615.971	153.993	59.990	<.00010
Hydrophobic	2	615.971	307.986	59.990	<0.0001
Hydrophyllic	2	0.000	0.000	0.000	<0.0000
Experimental Error(Tube)	12	30.802	2.567		
Corrected Total	17	32627.664			

*Dependent Variable: Angle

APPENDIX II

Compact disc compilation of Epi-fluorescence microscopy images

APPENDIX I

Analysis of variance (ANOVA)

TABLE 1. ANOVA of total spores liberated during humid and dry aeration.

Source	d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* Exp Error)	Significance level(P)
Species	16	33.123	2.070	18.050	<0.0000
Genera	2	15.597	7.798	13.640	<0.0001
Remainder	14	18.684	1.335	2.330	<0.0064
Penicillium	10	15.987	1.599	16.290	<0.0001
Aspergillus	4	2.696	0.674	6.210	<0.0001
Acremonium	0	0.000	0.000	0.0000	<0.000
Method	1	27.834	27.834	48.68	<0.0001
Species*Environmental condition	16	27.947	1.747	3.060	<0.0002
Genera* Environmental condition	2	0.853	0.427	0.750	<0.4760
Remainder	14	18.684	1.335	2.330	<0.0064
Time	1	4.110	4.110	7.190	<0.0082
Time*Species	16	12.358	0.772	1.350	<0.1759
Time*Genera	2	1.938	0.969	1.700	<0.1874
Remainder	14	18.684	1.335	2.330	<0.0064
Time* Environmental condition	1	0.178	0.178	0.310	<0.5776
Time* Environmental condition *Species	16	29.107	1.819	3.180	<0.0001
Time* Environmental condition *Genera	2	5.226	2.613	4.570	<0.0120
Remainder	14	18.684	1.335	2.330	<0.0064
Experimental Error (Tube)	136	77.7535	0.572	5.770	<0.001
Sample Error (Triplicate)	405	40.1187	0.099		
Total Corrected	608	253.706			

* Dependant Variable: L_Sporeliberation = (LOG_{10} (x + 1)

TABLE 2. ANOVA of total spores liberated in aqueous saline solution.

Source	d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* Exp Error)	Significance level (P)
Species (Isolates)	16	6.105	0.382	3.210	<0.0021
Genera	2	0.535	0.268	2.250	<0.1210
Remainder	14	5.569	0.398	3.350	<0.0020
Penicillium	10	2.997	0.299	2.070	<0.0740
Aspergillus	4	2.572	0.643	8.170	<0.0034
Acremonium	0	0.000	0.000	0.000	<0.0000
Experimental Error(Tube)	50	4.043	0.118		
Corrected Total	10	10.149			

*Dependant Variable = [Log (X + 1)]

TABLE 3. ANOVA of the adherence (%) of spores to test surfaces.

Source				d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* ExpError)	Significance level (P)
Species				16	102.7649	6.4228	48.6500	<0.0001
	Genera			2	15.7742	7.8871	59.7400	<0.0001
	Remainder			14	86.9906	6.2136	47.0700	<0.0001
		Penicillium		10	49.9566	4.9956	37.8300	<0.0000
		Aspergillus		4	37.0340	9.2585	70.1300	<0.0000
		Acremonium		0	0.0000	0.0000	0.0000	<0.0000
Test surface				7	2636.5267	376.6466	2852.9200	<0.0001
Species* Test surface				112	814.0518	7.2683	55.0500	<0.0001
	Genera*Test surface			14	255.6515	18.2608	138.3200	<0.0001
	Remainder			98	558.4002	5.6979	43.1600	<0.0001
Experimental Error (Test surface)				272	35.9098	0.1320		
Total Corrected				407	3589.2533			

Dependant Variable: L_% Adherence = ln[(% + 0.5)/(100 - % + 0.5)]

TABLE 4. ANOVA of adherence (%) of spores suspended in an aqueous solution to test surfaces.

Source				d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* Exp Error)	Significance level (P)
Species (Isolates)				28	139.7240	4.9901	70.06	<0.0001
	Genera			7	23.4750	0.0010	47.09	<0.0001
	Remainder			21	116.2480	0.0001	77.72	<0.0001
		Penicillium		10	28.805	2.8805	40.44	<0.0000
		Aspergillus		4	17.3790	4.3449	61.01	<0.0000
		Acremonium		0	0.0000	0.0000	0.0000	<0.0000
		Cryptococci		7	70.0630	10.0090	140.53	<0.0000
		Yarrowia		0	0.0000	0.0000	0.0000	<0.0000
		Rhodotorula		0	0.0000	0.0000	0.0000	<0.0000
		Candida		0	0.0000	0.0000	0.0000	<0.0000
		Saccharomyces		0	0.0000	0.0000	0.0000	<0.0000
Test surface				7	5714.9230	816.4176	11462.8000	<0.0001
Species* Test surface				188	259.0600	1.3779	19.3500	<0.0001
	Genera*Test surface			49	81.7470	1.6683	23.4200	<0.0001
	Remainder			139	177.3130	1.2756	17.9100	<0.0001
Experimental Error(Test surface)				448	31.9080	0.0712		
Total Corrected				671	6145.6160			

* Dependant Variable: L_Sporecount = ln[(% + 0.5)/(100 - % + 0.5)]

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Source	d*f* (n-1)	Sum Square	Mean Square	F-ratio (d*f*/ d*f* Exp Error)	Significance level (P)
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*Dependent Variable: Angle

APPENDIX II

Compact disc compilation of Epi-fluorescence microscopy images

APPENDIX II

COMPACT DISC COMPILATION

Filamentous fungi

Isolate code	Species	Origin
ACR	*Acremonium alternatum*	Fynbos soil

Filamentous fungi

Isolate code	Species	Origin
AA	*Aspergillus aculeatus*	DSM 2344

Filamentous fungi

Isolate code	Species	Origin
AC	*Aspergillus carneus*	Fish river plains

Filamentous fungi

Isolate code	Species	Origin
AF	*A. fumigatus*	Soil from Zimbabwe

Filamentous fungi

Isolate code	Species	Origin
AN	*A. niger*	ATCC10864

Filamentous fungi

Isolate code	Species	Origin
AT	*A. terreus*	Soil from the Northern Province, South Africa

Filamentous fungi

Isolate code	Species	Origin
ABO486	*Penicillium camembertii*	Walls of cheese factory

Filamentous fungi

Isolate code	Species	Origin
PC	*P. candidum*	Commercial strain for cheese making

Filamentous fungi

Isolate code	Species	Origin
ABO272	*P. citrinum*	Wine cellar 1

Filamentous fungi

Isolate code	Species	Origin
R1	*P. citrinum*	Wine cellar 1

Filamentous fungi

Isolate code	Species	Origin
R2	*P. citrinum*	Wine cellar 1

Filamentous fungi

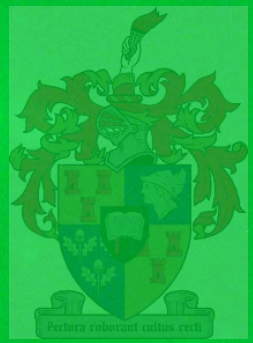
Isolate code	Species	Origin
F2	*P. commune*	Fynbos soil

Filamentous fungi

Isolate code	Species	Origin
FG16	*P. commune*	Fynbos soil

Filamentous fungi

Isolate code	Species	Origin
R4	*P. glabrum*	Fynbos soil



Filamentous fungi

Isolate code	Species	Origin
ABO268	*P. spinulosum*	Wine cellar 1

Filamentous fungi

Isolate code	Species	Origin
ABO275	*P. sumatrense*	Wine cellar 1

Filamentous fungi

Isolate code	Species	Origin
ABO487	*P. westlingii*	Wine cellar2

Unicellular fungi (yeast)

Isolate code	Species	Origin
CA	*Candida albicans*	Department of Viticulture and Oenology, US

Unicellular fungi (yeast)

Isolate code	Species	Origin
CL	*Cryptococcus laurentii*	Fynbos soil

Unicellular fungi (yeast)

Isolate code	Species	Origin
CN	*Cryptococcus neoformans*	Type strain of species (CBS 132T)

Unicellular fungi (yeast)

Isolate code	Species	Origin
CNC1	*Cryptococcus neoformans*	Clinical sample from meningitis patient

Unicellular fungi (yeast)

Isolate code	Species	Origin
CNC2	*Cryptococcus neoformans*	Clinical sample from meningitis patient

Unicellular fungi (yeast)

Isolate code	Species	Origin
CNE1	*Cryptococcus neoformans*	Vegetative debris

Unicellular fungi (yeast)

Isolate code	Species	Origin
CNE2	*Cryptococcus neoformans*	Vegetative debris

Unicellular fungi (yeast)

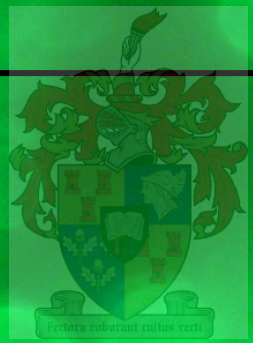
Isolate code	Species	Origin
CP	*Cryptococcus podzolicus*	Fynbos soil

Unicellular fungi (yeast)

Isolate code	Species	Origin
RG	*Rhodotorula glutinis*	Fynbos soil

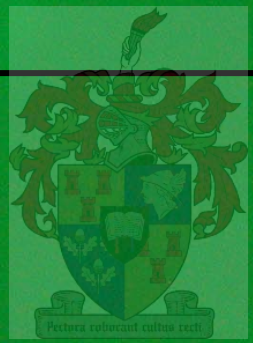
Unicellular fungi (yeast)

Isolate code	Species	Origin
SC	*Saccharomyces cerevisiae*	Department of Viticulture and Oenology, US



Unicellular fungi (yeast)

Isolate code	Species	Origin
YL	*Yarrowia lipolytica*	Department of Viticulture and Oenology, US



Negative control

Substrate	Origin/supplier
Parafilm®	(Pechiney Plastic Packaging, Menasha,WI, USA)