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Hyphomycetous fungi spore release induced by air currents and aqueous solution

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Hyphomycetous fungi originating from South Africa were morphologically characterised and ascribed to the genera *Acremonium*, *Aspergillus* and *Penicillium*, respectively. The primary means of spore dispersal employed by these isolates was investigated by quantifying colony forming units released into the air and into an aqueous solution. Measurement of spore liberation during humid aeration, revealed significant (P < 0.0001) differences among the hyphomycetous taxa investigated. Isolates of the genus *Penicillium* were more successful in releasing their spores than the Aspergilli and the *Acremonium* isolate. Spore liberation during desiccated aeration also showed a significant (P < 0.0001) difference between the respective isolates. Overall, isolates belonging to the genus *Penicillium* released more viable spores than *Aspergillus* spp., which in turn released more spores than *Acremonium*. In support of the theory that splashing rain may dislodge and disperse microfungal propagules, washing respective cultures with physiological salt solution resulted in an immediate massive spore release. However, the taxa investigated did not differ. These differences in airborne spore release that were observed between the hyphomycetous genera may be a result of different strategies to disperse their spores in nature. 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 their niche.

Key words: *Acremonium*, *Aspergillus*, Hyphomycetes, *Penicillium*, spore dissemination.

INTRODUCTION

Conidia are the means of asexual multiplication, dispersal, survival and their physical interactions have great importance in the life-cycle of fungi (Brown and Hovmoller, 2002; Sanderson, 2005; Elbert et al., 2007). Currently, we know that hyphomycetes may be considered as common airborne fungi occurring in both indoor and outdoor environments (Shelton et al., 2002; de Ana et al., 2006). *Aspergillus* and *Penicillium* spores have been shown to occur commonly in “dry” air samples (Fogelmark et al., 1994; Shen et al., 2007). Some spores are inhaled by mammals; for example, humans and may be deposited into the respiratory tract. Inhaled spores are known to adhere to host plasma membrane once deposited, and attachment of conidia to host matrix

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Table 1. Fungal isolates used in the experiment.

Isolate code	Species	Source
ABOACR	*Acremonium alternatum*	Culture collection
ABOAA	*Aspergillus aculeatus*	Culture collection
ABOAC	*A. carneus*	Culture collection
ABOAF	*A. fumigatus*	Culture collection
ABOAN	*A. niger*	Culture collection
ABOAT	*A. terreus*	Culture collection
ABO486	*Penicillium camemberti*	Culture collection
ABOPC	*P. candidum*	Culture collection
AB0272	*P. citrinum*	Culture collection
ABOR1	*P. citrinum*	Culture collection
ABOR2	*P. citrinum*	Culture collection
ABOF2	*P. commune*	Culture collection
ABOFG16	*P. commune*	Culture collection
ABOR4	*P. glabrum*	Culture collection
ABO268	*P. spinulosum*	Culture collection
ABO275	*P. sumatrense*	Culture collection
ABO487	*P. westlingii*	Culture collection

*Culture collection = Fungal Culture Collection of the Department of Microbiology, the University of Stellenbosch, South Africa.

surfaces has survival value and may be a requirement for colonisation (Peñalver et al., 1996; Kukreja et al., 2007; Tronchin et al., 2008). This deposition of spores may impact on health since these spores may result in respiratory tract infection (Supparatpinyo et al., 1994; Denning et al., 2006; Bellanger et al., 2009).

Hyphomycetous fungi have been known to liberate their hydrophobic conidia under desiccated environmental conditions, and also with the aid of rain drops by means of splash dispersal (Fitt and Nijman, 1983; Tadych et al., 2007). It was also suggested that the most effective means of spore dissemination among soil hyphomycetes is through the movement of rain water (Sutton et al., 1976; Horn et al., 2001). It was, therefore, essential to obtain knowledge of the environmental conditions that promote hyphomycetous spore release.

This study was undertaken to investigate the primary means of spore dispersal employed by representatives of the genera *Acremonium*, *Aspergillus* and *Penicillium*, isolated from Fynbos soil and indoor environments in the Western Cape, South Africa. Also, since all of these fungi contain different sporogenous structures, another objective of the study was to investigate whether the quantity of colony forming units released into the air and into an aqueous physiological saline solution differed among the isolates.

MATERIALS AND METHODS

Fungal isolates used

Hyphomycetous fungi were obtained from the fungal culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa (Table 1). The cultures in the culture collection originated from various sources in the Western Cape, South Africa, amongst others, Fynbos soil and interior of cellars. These cultures were subsequently used to inoculate 2% (w/v) malt extract agar (MEA) Petri-dish. The plates were incubated at 22°C and the resulting colonies were purified by consecutive transfer and incubation on MEA plates at 22°C.

Identification of isolates using morphological criteria

Single-spore cultures were prepared from the fungal cultures (Pitt, 1974). Each single spore culture was inoculated on differential media and subsequently incubated for seven days as required for the identification of these fungi. Following incubation, colonies were microscopically examined for characteristic features as described in the literature (Thom, 1930; Raper and Thom, 1949; Pitt, 1979; Domsch et al., 1993). In addition, microscopic and macroscopic characteristics such as conidial colour, presence of exudates, mycelial growth and coloration were used to identify the isolates according to the descriptions and keys in literature.

Measurement of spore liberation triggered by air

A comparative analysis of spore liberation of all isolates was conducted in an airflow cell as schematically illustrated (Figure 1). This airflow cell consisted of a horizontal, tubular growth chamber (450 mm in length, 70 mm in diameter). The growth chamber was aseptically filled with 100 ml MEA resulting in a surface area of 165 cm^2 after the MEA was allowed to solidify, whilst the growth chamber was positioned horizontally. A 10 ml conidial suspension (containing ca. 5 x 10^6 conidia/ml) of a week old culture was used to inoculate each chamber aseptically. Spore counts for the inoculum were determined microscopically using a haemocytometer. The chamber was incubated at 22°C for 48 h to allow conidial germination (colony establishment) and formation of fungal mycelia, whereafter it was connected at the one end to an air pump to

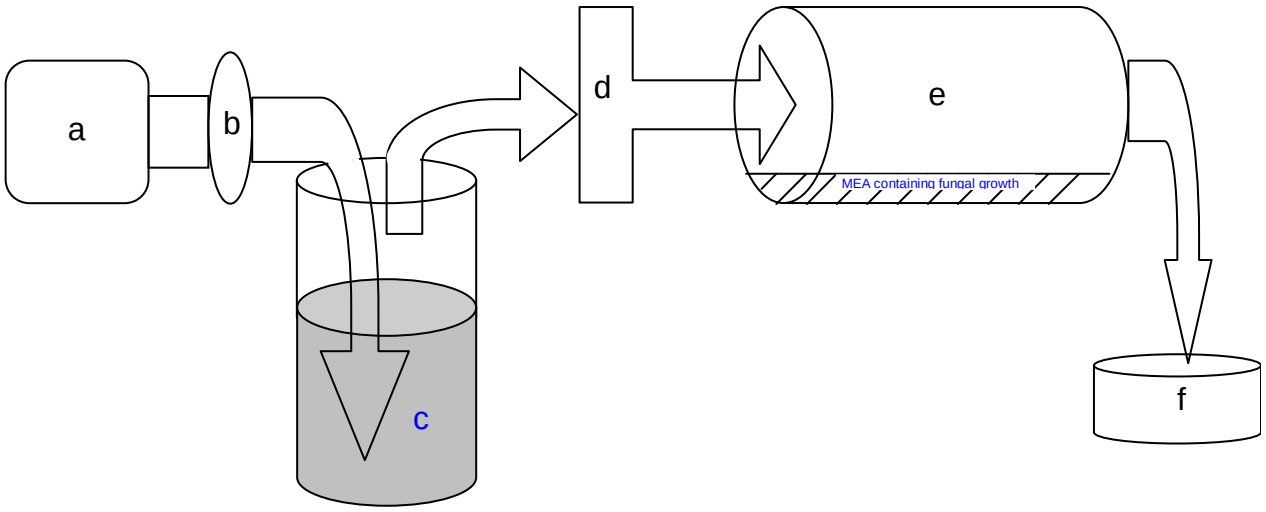

Figure 1. Schematical illustration of the airflow cell and accompanying componets. (a) fish-tank pump; (b) polytetrafluoroethylene (PTFE) 0.45 μm filter; (c) sterile water (or anhydrous $CaCl_2$) in flask; (d) airflow regulator; (e) airflow cell with fungal culture on MEA (surface area of 165 cm^2); and (f) outlet onto malt-extract agar (MEA) containing Petri dishes.

initiate the respective trials. Sterile air with a relative humidity of 36% was subsequently pumped through the chamber at a rate of ± 0.6 l/min (laminar flow rate of 16.3 cm/min) via a 0.45 μm Midistart® 2000 PTFE filter (Sartonet Sa. Cc), sterile water in a conical flask and a Gabler airflow regulator.

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 was monitored by exposing a set of nine MEA containing Petri dishes consecutively to air at the flow cell's outlet for 15 min, respectively. The MEA plates were subsequently incubated for one week at 22°C and the number of colony forming units was counted. Spore release was monitored for five consecutive days per week over a 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 physiological saline (isotonic salt) solution was determined. Pre-liminary investigations showed that 15 ml aqueous physiological salt solution (PSS) (0.85% (w/v) NaCl) was sufficient for this purpose. Therefore, 15 ml of PSS was gently transferred into each airflow cell. The number of spores in the resulting suspension was microscopically counted using a haemocytometer.

Statistical analysis

To test the effect of environmental conditions on spore liberation of isolates over time, a completely randomised experiment was conducted with the treatments in a 2 x 17 x 2 factorial with three random replications (Snedcor and Cochrane, 1967). The factors were two environmental conditions (humid and dry aeration); 17 isolates/strains which were grouped into three genera namely, *Acremonium* (ABOACR), *Aspergillus* (ABOAA, ABOAC, ABOAT, ABOAN, and ABOAF) and *Penicillium* (ABOF2, ABOR4, ABOFG16, ABOR1, ABOR2, ABO268, ABO272, ABO275, AB0486, ABO487 and ABOPC), and two time phases (weeks 1 and 2).

Total spore liberation was recorded and transformed by a Log_{10} (x + 1) transformation before being subjected to an 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). In order to compare the means of significant effects, the Student's t-least significant difference (LSD) was calculated at a 5% significance level. The means of the statistical analysis are presented in figures.

RESULTS

Identification of fungal isolates

Morphological characteristics revealed that the isolates were hyphomycetes that belonged to the genera *Acremonium*, *Aspergillus* and *Penicillium*, respectively (Pitt, 1979; Domsch et al., 1993; Klich, 2002) (Table 1).

Spore liberation into air

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.

A comparative analysis of three factor interaction (time x isolate x environmental condition) showed significant ($P < 0.0001$) differences in spore liberation between the weeks, during humid and desiccated aeration, respectively. Thus, spore liberation during week 1 of incubation under humid conditions differed significantly from spore liberation during week 2 (Figure 2). Furthermore, intraspecific differences were observed, for example

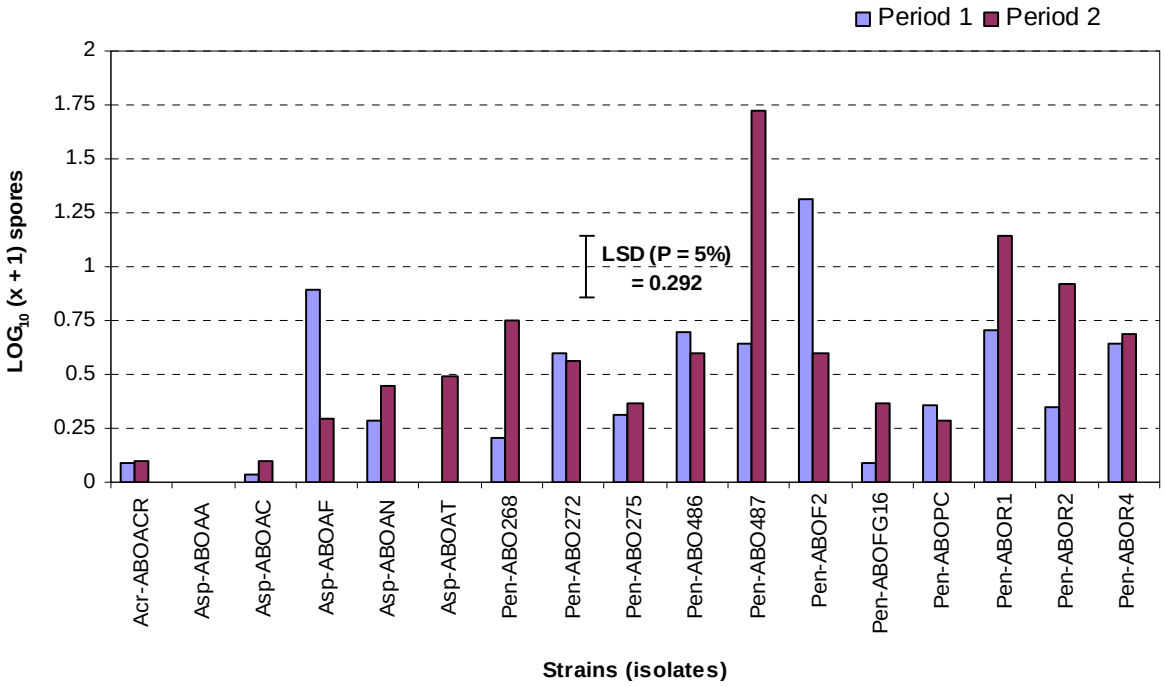

Figure 2. Three factor interaction (time x isolate x environmental condition) means of total spores liberated after weeks (period) 1 and 2 during humid aeration. The pre-fixes "Acr, Asp and Pen-" indicates the genera, *Acremonium*, *Aspergillus* and *Penicillium*, respectively.

Polytrichum commune ABOF2 liberated more spores during week 1 than week 2, whilst for ABOFG16 the spore liberation was reversed. *Penicillium citrinum* ABOR1 and ABOR2 liberated significantly more spores during week 2, whilst for ABO272 no significant differences was found. *Aspergillus aculeatus* ABOAA was the only *Aspergillus* isolate that released no spores in the presence of humid air, whilst *A. terreus* ABOAT only released spores during the second week. As was found for environmental condition 1, the comparative analysis for environmental condition 2 between week 1 and week 2 also showed a significant ($P < 0.0001$) difference between weeks (Figure 3).

Intraspecific similarities were observed; for example, *P. commune* ABOF2 and ABOFG16 liberated more spores during week 2 than week 1. However, intraspecific differences were also observed, for example, *Penicillium citrinum* ABOR1 and ABO272 liberated more spores during week 1, whilst ABOR2 showed a reversed spore liberation pattern compared to the former isolates. All aspergilli with the exception of *Aspergillus niger* ABOAN released significantly more spores during desiccated aeration as compared to humid aeration. Interestingly, *A. aculeatus* ABOAA released the most spores in the "dry" air, despite no spores being released during humid aeration.

Therefore, to investigate a possible correlation between fungal taxa and spore release, the isolates were grouped into genera and spore release under humid and desiccated conditions analysed. A comparative analysis of three factor interaction (time x genus x environmental condition) between weeks 1 and 2 for humid - and desiccated aeration, respectively, showed a significant ($P < 0.012$) difference in spore liberation between weeks.

In contrast to isolates of the genus *Penicillium*, members belonging to the genera *Acremonium* and *Aspergillus* did not differ between the two weeks (weeks 1 and 2) during humid aeration. Furthermore, spore liberation of the *Acremonium* isolate and organisms representing the genus *Aspergillus* did not differ significantly, but the *Penicillium* species were significantly more successful in dispersing their spores. *Aspergillus* isolates released significantly more spores under desiccated aeration during week 2 than week 1, whilst isolates of the other two genera, that is, *Acremonium* and *Penicillium*, did not differ. Also noteworthy is that *Penicillium* isolates differed significantly from the *Acremonium* isolate, but not from that of *Aspergillus*, with regard to the number of spores released.

Spore liberation into aqueous saline

Gently washing the culture with an isotonic salt solution in all cases resulted in an immediate massive release of colony forming units (Figure 4). However, no significant difference could be observed between cultures regarding the release of colony forming units into the aqueous

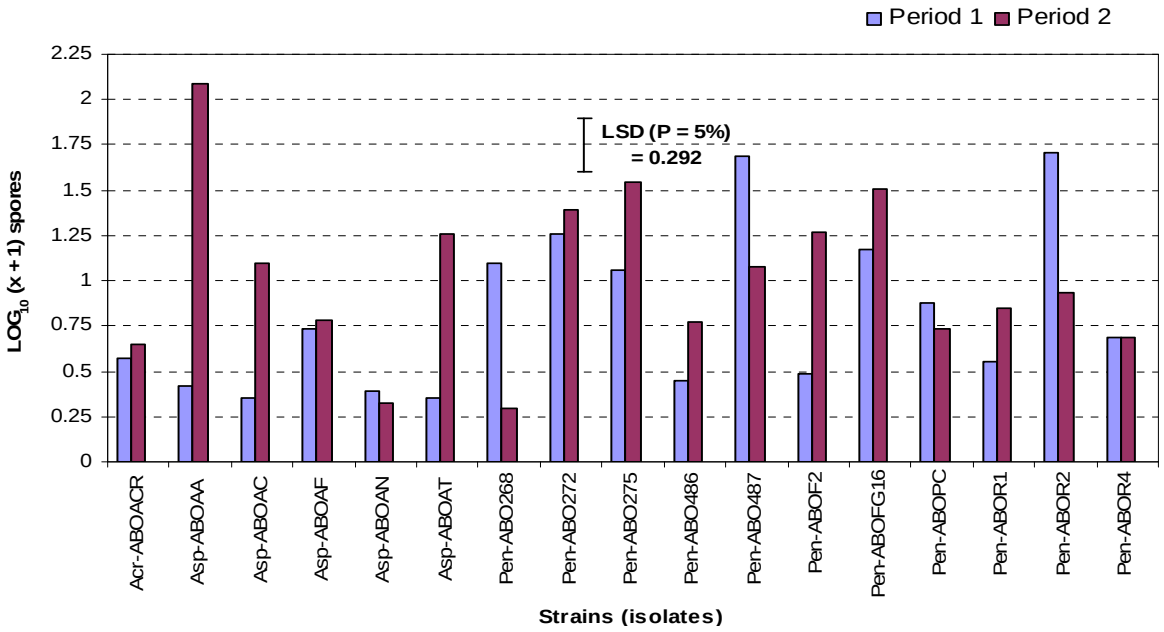

Figure 3. Three factor interaction (time x isolate x environmental condition) means of total spores liberated after weeks (period) 1 and 2 during desiccated aeration. The pre-fixes "Acr, Asp and Pen-" indicates the genera, *Acremonium*, *Aspergillus* and *Penicillium*, respectively.

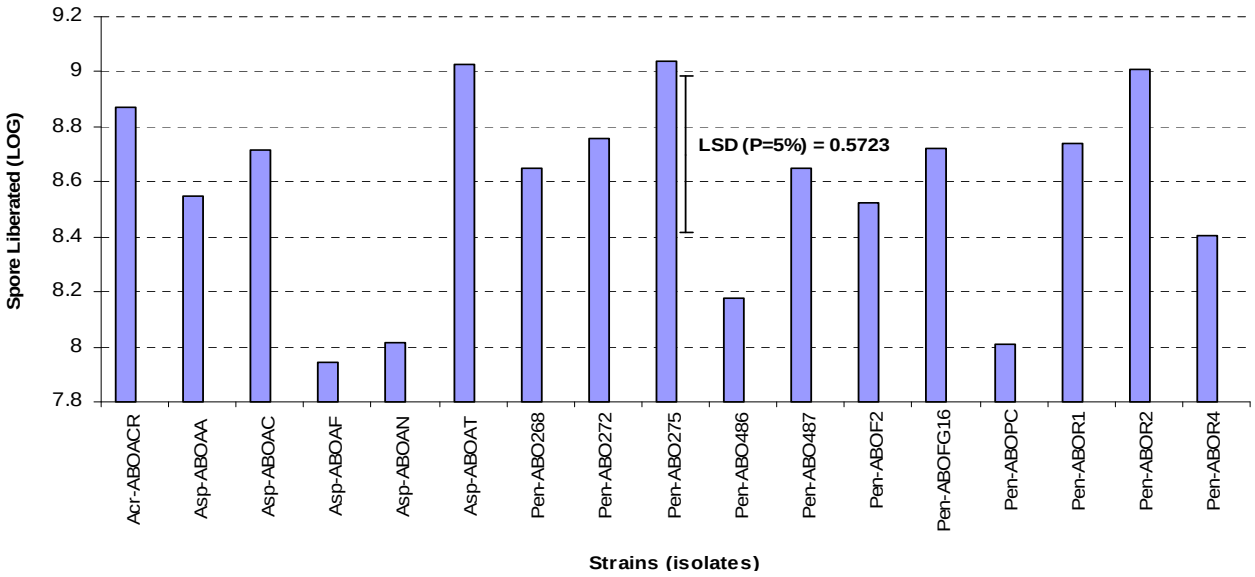

Figure 4. Means of total spores liberated when colonies were flushed with a saline solution. The pre-fixes "Acr, Asp and Pen-"represents the genera, *Acremonium*, *Aspergillus* and *Penicillium*, respectively.

saline solution (data not shown). The $\log_{10}$ (x + 1) of average number of *Acremonium*, *Aspergillus* and *Penicillium* spores released per cm^2 during the two fortnightly phases of airflow was 0.004, 0.006, and 0.009, respectively. Notably more spores were released when the cultures were washed with PSS, that is, 0.054, 0.052 and 0.051, respectively.

DISCUSSION

Fungal spores are the main mode of removing potential progeny from the direct vicinity of the parent mycelium (Ingold, 1953; Moore-Landecker, 1996; McGinnis, 2007). Spore disposal serve to minimise competition amongst siblings as a result of unfavourable nutritional conditions,

and thus promote the survival of the organism and increasing the habital range (Glenn et al., 2004; Gover, 2013). It has long been recognised that many hyphomycetous fungi liberate their spores in a passive manner (Ingold, 1971; Magyar, 2002; Tadych et al., 2007). During this process, millions of spores are released through the physical action of wind, rain and animals. This study was undertaken to obtain an indication of the primary means of spore dispersal employed by isolates of the genera *Acremonium*, *Aspergillus* and *Penicillium*, originating from the fungal culture collection of the Department of Microbiology at the University of Stellenbosch, South Africa. Also, since all these fungi contain different sporogenous structures, we were interested on whether the quantity of colony forming units, released into the air and into an aqueous isotonic salt solution, differed among the isolates.

Measurement of spore liberation during humid aeration, revealed significant ($P < 0.0001$) differences among the hyphomycetous taxa investigated regarding aerial spore release (Figure 2). Intraspecific differences were also observed as demonstrated by the results obtained for *P. citrinum* where two isolates, that is, ABOR1 and ABOR2 liberated more spores during week 2, whilst the other isolate, that is, ABO272 was consistent with regard to weeks 1 and 2. It can, therefore, tentatively be said that liberation of spores into humid air by some *Penicillium* isolates is dependant on the age of culture, whilst in spore liberation of other isolates, amongst others, ABO272 is not influenced by time and/or the age of the culture.

Intraspecific differences with regard to aerial conidial dispersal of *Penicillium* isolates have not been reported previously. Furthermore, a species known to thrive in humid conditions, *A. fumigatus* ABOAF (Wasylnka and Moore, 2000; Gamboa et al., 2005; Stark et al., 2006), released significantly more spores during week 1 than in week 2, whilst *A. aculeatus* ABOAA, a fungal species of the section *Nigri* that commonly occurs on vine fruit (Serra et al., 2006; Bufflier et al., 2007), did not release spores.

As was found for humid aeration, the comparative analysis for desiccated aeration between weeks 1 and 2 also showed a significant ($P < 0.0001$) difference in spore liberation by the respective isolates (Figure 3). Intraspecific differences were also observed as demonstrated by the results obtained for *P. citrinum* where one isolate liberated more spores during weeks 1 than 2, whilst the remainder did not differ between weeks. *A. aculeatus* ABOAA released most spores into the air amongst the aspergilli, despite no spores being released during humid aeration.

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. Although intraspecies differences were uncovered, this study demonstrated for the genera investigated, that more spores are usually released as the culture matures, and in most cases older cultures released more spores under dessicated conditions. It was also found that the genera responded differently to differences in humidity regarding their aerial spore release. Under humid conditions, *Penicillium* isolates were more successful in releasing their spores than *Aspergillus* and *Acremonium* isolates.

Under desiccated conditions, the isolates representing *Aspergillus* took longer time to release their spores than the *Penicillium* isolates and the *Acremonium*. This may be as a result of the distinct morphology of the sporogenous structures of *Aspergillus*. In contrast to *Aspergillus*, the sporogenous structures of neither *Acremonium* nor *Penicillium* are characterised by a pronounced swollen vesicle at the tip of the conidiophore (Klich and Pitt, 1988; Larone, 1995). It can be speculated that this difference may be ascribed to longer time needed for the sporogenous structures of *Aspergillus* to reach the desiccated condition needed for increased spore release, than the time needed for the sporogenous structures of *Penicillium* and *Acremonium*.

Indications, therefore, are that the sporogenous structures of aspergilli are adapted for passive spore release upon changes in humidity and age of the culture. As only one isolate of the genus *Acremonium* was tested, results may have differed if more representatives of this genus were available for the investigation. Differences in spore liberation may also be tentatively ascribed to differences in the numbers of spores produced and to major differences in spore morphology. Also noteworthy is that *Aspergillus* and *Penicillium* spp. produce dry conidia that can be easily dispersed as opposed to that of *Acremonium* produced within a mucus (Davies et al., 2003; Summerbell et al., 2011).

The *Aspergillus* and *Penicillium* isolates used all produce countless globose to spheroidal conidia with a diameter of 2 to 5 µm (Eltem et al., 2004), while the *Acremonium* isolate formed a lesser number of ellipsoidal to fusiform conidia normally 3 µm wide and 8 µm in length (Schroers et al., 2005). Overall, *Penicillium* isolates, released more spores than *Aspergillus* isolates which in turn released more spores than the *Acremonium.* This observation can be ascribed to differences in sporogenous structure (condidiophore) morphology, as the genera *Aspergillus* and *Penicillium* have polyphialidic conidiophores bearing significantly more conidia than the monophialidic condiophore of *Acremonium* (Domsch, 2007; Sigler et al., 2010).

These findings support the results of others on the common occurrence of *Aspergillus* and *Penicillium* conidia in air samples (Shen et al., 2007; Spicer and Gangloff, 2008). It was also observed for some isolates that the airflow did not cause sufficient disturbance to dislodge and/or liberate a large proportion of the conidia (Tucker et al., 2007). Therefore, in support of the theory that splashing rain may dislodge and disperse microfungal

spores (Ntahimpera et al., 1998; Travadon et al., 2007), washing the culture with PSS resulted in all cases in an immediate massive release of colony forming units from the cultures (Figure 4). However, the taxa 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 isolates representing the genera *Acremonium*, *Aspergillus* and *Pencillium.* These findings support the views of others recorded in literature (Sutton et al., 1976; Horn et al., 2001).

Conclusion

It is known that species 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. This study demonstrated that isolates of these genera released more spores after two weeks of incubation as compared to after one week. Also, noteworthy is that conidia are phialidically produced, and that appically positioned conidia are easiliy liberated by the slighest of air turbulence (air movement) after undergoing a maturation phase (Davies et al., 2003). It can be envisaged that younger conidia near the base of the phialide will remain attached until matured, but a stronger air flow will induce their forceful release. 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 time to release their spores than representatives of *Acremonium* and *Penicillium.* Also, noteworthy is that *Aspergillus* spore release is influenced by conidial maturation. However, this phenomenon may be as a result of differences in the morphology of the sporogenous structures of these fungi. *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 time to dry and release conidia into the air than the more filamentous sporogenous structures of *Acremonium* and *Penicillium.*

Although not proven, the results also support the contention that an important dispersion agent for these filamentous fungi may be water, 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*. In general, it can be concluded that the filamentous fungal genera differed in their strategy to release airborne spores. Water however, may serve as the primary means of spore dispersal. The fact that the representatives of the different fungal genera differed in their strategy to release airborne spores, 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, electron microscopy, serological methods in combination with epi-fluorescense microscopy, as well as atomic force microscopy may then be used to monitor changes in dispersion, and subsequent adherence of the fungal propagules.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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