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The influence of water activity and temperature on germination, growth and sporulation of *Stachybotrys chartarum* strains

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Abstract

The objectives were to determine the influence of water activity (a_w, 0.997-0.92) and temperature (10-37°C), and their interactions on conidial germination, mycelial growth and sporulation of two strains of *Stachybotrys chartarum in vitro* on a potato dextrose medium. Studies were carried out by modifying the medium with glycerol and either spread plating with conidia to evaluate germination and germ tube extension or centrally inoculating treatment media for measuring mycelial growth rates; and harvesting whole colonies for determining sporulation. Overall, germination of conidia was significantly influenced by a_w and temperature and was fastest at 0.997-0.98 a_w between 15 and 30°C with complete germination within 24 hours. Germ tube extension was found to be most rapid at similar a_w levels and 25-30°C. Mycelial growth rates of both strains were optimal at 0.997 a_w between 25-30°C, with very little growth at 37°C. Sporulation was optimum at 30°C at 0.997 a_w. However, under drier conditions this was optimum at 25°C. This shows that there are differences in the ranges of a_w x temperature for germination and growth and for sporulation. This may help in understanding the role of this fungal species in damp buildings and conditions under which immune-compromised patients may be at risk when exposed to such contaminants in the indoor air environment.

Introduction

Fungal colonisation of many household materials is a common problem in the UK and worldwide and has been found to cause aesthetic problems, including the discolouration and deterioration of materials that have been contaminated. This has raised public health concerns regarding the quality of indoor air of those exposed to spores in their homes [1, 2, 3]. Fungi are ubiquitous in the outdoor environment and act as natural decomposers of organic matter. They enter homes through open windows, doors, and on clothes. Once in the home these organisms utilise many of the household materials as a food source and break them down into basic compounds for growth [4]. Many moulds including *Penicillium, Cladosporium, Alternaria* and *Aspergillus* species are commonly detected in homes with condensation and ventilation problems [5, 6]. New build energy efficient homes can also potentially be affected by mould growth and subsequently result in poor indoor air quality due to lower rates of natural ventilation from the outside [7].

Damp buildings due to flash flooding have become more common in recent years and this has resulted in contamination of surfaces by a range of fungi including *Stachybotrys chartarum.* This fungus produces cytotoxic compounds, the macrocyclic trichothecenes, which have the potential to cause serious health problems to those exposed including a variety of debilitating respiratory and non-respiratory symptoms [8, 9]. *Stachybotrys* has also been associated with cases of pulmonary haemorrhage in infants in Cleveland, Ohio, USA [10]. Two different chemotypes of *S. chartarum* exist: Chemotype S which primarily produces the macrocyclic trichothecenes (satratoxins and roridins), and Chemotype A which produces the non-trichothecene toxins (atranone and dolabellanes). Certain strains of both chemotypes produce simple non-macrocyclic trichothecenes such as trichodermal [11].

In the indoor environment fungal development on surfaces is likely to be initially influenced by key abiotic factors of which water activity (a_w) and temperature are the most important. There are numerous reports on the effect of moisture and temperature on the growth of contaminant fungi, especially xerotolerant *Penicillium*, *Aspergillus* and *Eurotium* species [12, 13]. Some of these have been implicated in human health problems in the indoor environment, especially asthma [14, 15, 16]. For many fungi which may cause problems under wet conditions it is important to understand the marginal conditions under which establishment may occur and also the effect of drying environments on sporulation. This is especially important to help determine the risk from mould species such as *S. chartarum* under condensation conditions, excessive humidity, water leaks, or flooding [17, 18]. There is surprisingly little knowledge of the effect of interacting conditions of a_w x temperature on the life cycle of *Stachybotrys* species.

The objectives of this study were to examine the effect of interacting conditions of a_w (0.997-0.92) and temperature (10-37^{o}C) on (a) conidial germination, (b) germ tube extension, (c) mycelial growth and (d) sporulation of two strains of *S. chartarum* (chemotypes S and A) *in vitro* on a potato dextrose agar medium.

Materials and methods

Fungal isolates

Initially, four strains, two from a culture collection, and two isolated from damp buildings in the UK, were examined. Because they grew in a similar manner, the two typical strains of *S. chartarum* were used. *S. chartarum* IBT 7711 were chosen as good representative macrocyclic trichothecene producer (Chemotype S) and *S. chartarum* IBT 14915 was chosen as a good representative non-macrocyclic producing isolate which, however, can produce trichodermol and atranones (Chemotype A). These isolates were kindly provided by The Mycology Group, Systems Biology Department, Technical University of Denmark.

Media and inoculum preparation

The medium used in this study was potato dextrose agar (PDA). This was made by boiling 39g L^{-1} of PDA in dH_20 with sterilisation by autoclaving at 121^oC for 15 minutes.

The PDA was modified with the addition of the non-ionic solute glycerol to obtain a_w levels of 0.997, 0.98, 0.95 and 0.92 a_w. The a_w of all media were determined with a Thermconstanter (NovaSina Sprint). Autoclaved media were poured into 9 cm diameter sterile Petri plates (15 ml/plate)

Fungi were grown on PDA for 7-10 days at 25˚C to obtain heavily sporulating cultures. Spores were then suspended in sterile distilled water containing one drop of a wetting agent (Tween 80). Stock spore suspensions (2ml) were added to 4 ml sterile water previously modified with glycerol to the required water availability level. The final water activities of the treatments were 0.997, 0.98, 0.95 and 0.92 a_w and the final concentration of the spores was in the range of 1-5 x 10^6 spores ml^{-1}.

Germination studies

A 0.1 mL aliquot of the spore suspension was pipetted onto PDA plates of the same a_w and spread on the surface of the agar medium with a sterile bent glass rod [19]. Petri dishes of the same a_w treatments were enclosed in polyethylene bags and incubated at 15, 20, 25, 30 and 37˚C. Experiments were carried out with three sub-samples and three replicates per treatment.

On a 12 hourly basis three agar discs were aseptically removed from each replicate plate using a sterile cork borer (1 cm diameter), placed on a glass slide, stained with lactophenol/methylene blue and examined microscopically. Fifty single spores per disc (=150/replicate) were examined. Spores were considered to have germinated when the germ tube was equal to or greater than the diameter of the spore [19]. Mean germ tube lengths were also measured every 12 hours for all treatments for a maximum of 72 hrs.

Growth

A 3µl aliquot of the spore suspension (1-5 x 10^6 spores ml^{-1}) was point inoculated at the centre of three replicate PDA plates for all treatments. The final a_w of the treatments were

0.997, 0.98, 0.95 and 0.92 a_w. Inoculated plates of the same a_w treatment were sealed in polyethylene bags and incubated at 10,15, 20, 25, 30 and 37˚C for 14 days. The replicates were examined every two days, and two diameters at right angles were measured for each colony. The temporal mycelial extension data were used to determine the rates of growth (mm day^{-1}) by linear regression.

Sporulation assessment

A 3µl aliquot of the spore suspension (1-5 x 10^6 spores ml^{-1}) was point inoculated at the centre of three replicate PDA plates for all treatments. The final a_w of the treatments were 0.997, 0.98, 0.95 and 0.92 a_w. Plates of the same a_w were sealed in polyethylene bags and incubated at 15, 20, 25, 30 and 37˚C for 14 days. Replicate plates were each flooded with 10 ml sterile water and spores were agitated into solution using a sterile inoculating loop. The spore suspension was then filtered into sterile Universal bottles through a sterilised funnel with glass wool to remove all mycelia/hyphal fragments. Using this technique >95% of spores are recovered based on subsequent washing of glass wool filters. The total number of spores was determined by using a coulter counter. To determine spores per mm^{-2} the total number of spores was divided by the area of the colony [20].

Statistical treatment of results

The effects of a_w and temperature on germination, mycelia growth and sporulation was analysed for each strain. ANOVA was used to evaluate whether a_w, temperature, strain, and two and three way interactions were significant (P=0.001).

Results

Effects of a_w x temperature effects on germination and germ tube extension

Overall, when examined at a variety of interacting a_w x temperature conditions, conidia of chemotype S was found to germinate over a wider range of conditions than the chemotype A strain (Table 1).

At 20^{o}C both strains germinated rapidly at 0.997 and 0.98 a_w levels with all conidia having germinated within 24 hrs (Figure 1). The effect of different temperatures (15-37^{o}C) showed that rapid germination occurred within 72 hours over the range tested with freely available water (0.997 a_w; Figure 2). Optimal conidial germination occurred at between 25 and 30^{o}C for both strains. No conidia germinated at 0.92 a_w at any of the temperatures tested. Statistical analyses showed that a_w, temperature, strain and two and three way interactions were all statistically significant factors (data not shown).

Germ tube extension was rapid between 0.997 and 0.98 a_w for both strains at 20^{o}C (Figure 3). However, this was significantly reduced at 0.95 a_w. The chemotype A strain had shorter germ tubes, and none were produced at 0.95 a_w and 20^{o}C, reflecting the germination results.

Germ tube extension was rapid at 20-37oC at a steady state a_w of 0.997 (Figure 4). The conidial germ tube extension rate was slightly slower at 15^{o}C. The chemotype A produced most rapid germ tube extension at 30^{o}C, with slower extension observed at 20, 25 and 37^{o}C.

Statistical analyses of the effect of a_w, temperature, strain and two and three way interactions were found to all be statistically significant (P=0.01).

Effect of a_w x temperature on mycelial growth

The effect of a_w x temperature parameters on mycelial growth rates of both strains are shown in Figure 5. Overall, optimum growth was at 0.997 a_w and 25^{o}C. However, under slight water stress (0.98 a_w) optimum temperature for growth was at 30^{o}C for both strains. Slow growth occurred at 0.95 a_w and some growth at 20oC for both strains at 0.92 a_w.

Analysis of variance of the effect of a_w, temperature, strains and their two and three way interactions showed that all single, two- and three-way interactions were statistically significant (P=0.01).

Sporulation

Colonies grown at 0.995, 0.98 a_w had covered the agar plates by the end of the 14 day experimental period at 25-30^{o}C. With freely available water were optimum conditions for maximum spore production at 30^{o}C (Figure 6). Interestingly, at 0.98 and 0.95 a_w this was at 25^{o}C. Maximum sporulation at 0.95 a_w occurred at lower temperatures (20, 25^{o}C) for chemotype S than for chemotype A (25, 30^{o}C).

Statistical analyses (ANOVA) of the effect of a_w, temperature, strains and their two- and three-way interactions showed that all these factors were statistically significant (P=0.01).

Discussion

This is the first study to examine in detail the effect of interacting factors of a_w x temperature on the life cycle of strains of *S. chartarum*. This has shown that to a large extent the type S and A strains behave in a relatively similar manner. They were both influenced by a_w x temperature interactions and marginal conditions of a_w for germination and growth (>0.92 a_w) and for sporulation (>0.95 a_w) were identified. It was particularly interesting to find that while growth was optimum when water was freely available at 25^{o}C, when slight water stress was imposed (0.98 a_w) this changed to 30^{o}C. At 0.95 a_w growth was inhibited by more than 80% at optimum temperatures, regardless of strain. Some germination and growth was observed to occur at 37^{o}C in contrast to the findings by Grant et al. [21].

In terms of minimum conditions for growth, both strains could actively grow at 0.95 a_w. Previously Ayerst [12] reported the minimal a_w for growth at 0.94 and an optimal temperature of 23^{o}C for a *Stachybotrys atra* strain isolated in the U.S.A. In the present study we observe only very slow growth at 0.92 a_w over the experimental period used. It may be that this is influenced both by nutritional conditions and by length of incubation.

The detailed analysis of conidial production was particularly interesting and important in understanding the role of this species in colonisation and contamination of damp buildings. The strains grow optimally at 30^{o}C with freely available water. This

allows both colonisation and spore production. As the a_w decreases, growth can be reduced by almost 70-80% at 0.95 a_w. However, sporulation is still at levels which occur with freely available water. This suggests that during the drying out of flooded or damp buildings, spore release into the atmosphere may become a serious problem in an indoor environment. However, even in cooler conditions sporulation can occur at 15-20oC and 0.98-0.95 aw. This certainly has implications for remediation of damp buildings and surfaces and suggests that aerosols of such spores could represent a health hazard under a relatively wide range of damp aw x temperature conditions.

Previous studies by Nielsen et al. [22] investigated the effect of temperature (5-25^{o}C) and low a_w levels (0.65 – 0.95a_w) on colonisation of 21 building materials. They found that only *Penicillium*, *Aspergillus* and *Eurotium* species grew at <0.95 a_w. *Stachybotrys* growth was only detected on gypsum board at >0.95 a_w. This again suggests that nutritional conditions may also influence the conditions over which growth might occur. The present study and those of Nielsen et al. [22] support previous studies that relatively damp conditions are required for *S. chartarum* to grow [23, 24].

In this study it appeared that *S. chartarum* chemotype S and A strains may behave slightly differently to changes in a_w x temperature conditions. This has not been demonstrated or studied previously. This could be important in developing a better understanding of the importance and role of *S. chartarum* in damp buildings [23, 24]. It may well be the case that in damp buildings freely available water allows very good colonisation over a wide range of conditions and as conditions dry out sporulation occurs in the ranges identified. Disturbance would allow bioaerosols of these spores to be produced. Perhaps, fluctuating conditions of temperature and a_w need to be now examined for a better understanding of the role of these conidia in causing ill health in immune-compromised people exposed to damp environments. Indeed, surveys of damp buildings in the UK have identified *S. chartarum* as an important component (Frazer and Aldred, unpublished data).

The data obtained in this study provide a good framework for the conditions which are conducive and non-conducive to germination, growth and sporulation of strains of *S. chartarum*. This can be used to develop models of risk of exposure related to environmental conditions in different good and poorly managed home environments. Data is now required on the conditions of a_w x temperature over which the trichothecenes, especially satratoxins, may be produced by strains of this species.

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Table 1. Minimum water activity at different temperatures for the germination of two *S. chartarum* strains after 72 hrs incubation.

		Temperature (°C)				
Species	**Strain**	**15**	**20**	**25**	**30**	**37**
S. chartarum	7711	0.98	0.95	0.95	0.95	0.95
S. chartarum	14915	0.98	0.98	0.95	0.95	0.95

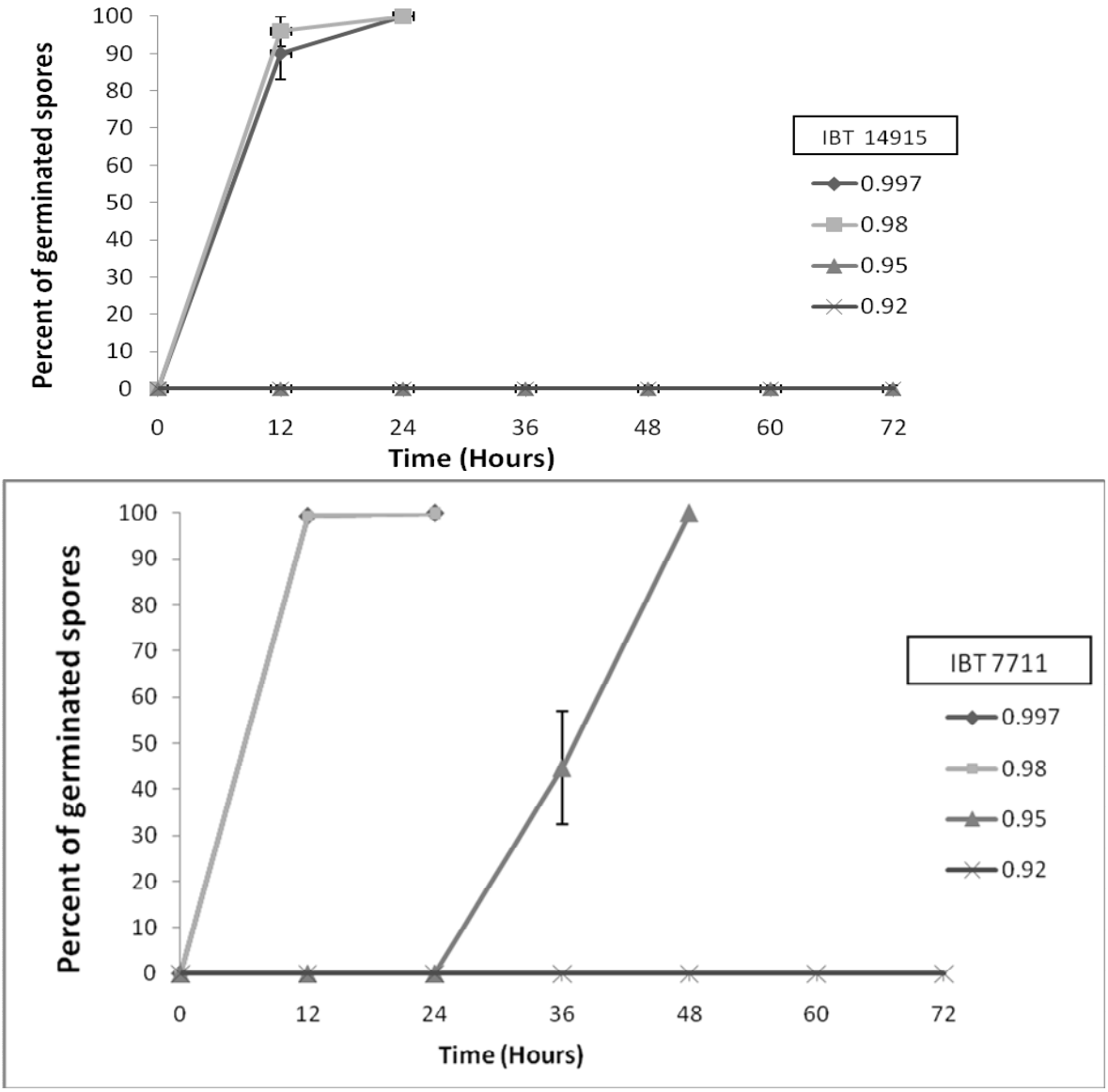


Figure 1. Mean percentage germinated conidia of (a) *S. chartarum* IBT 7711and (b) *S. chartarum* IBT 14915 at various water activity levels over a 72 hr period at 20^{o}C on PDA medium. The data analyses were carried out actual data. The percentages are plotted for presentation purposes. Bars indicate standard error of the mean.

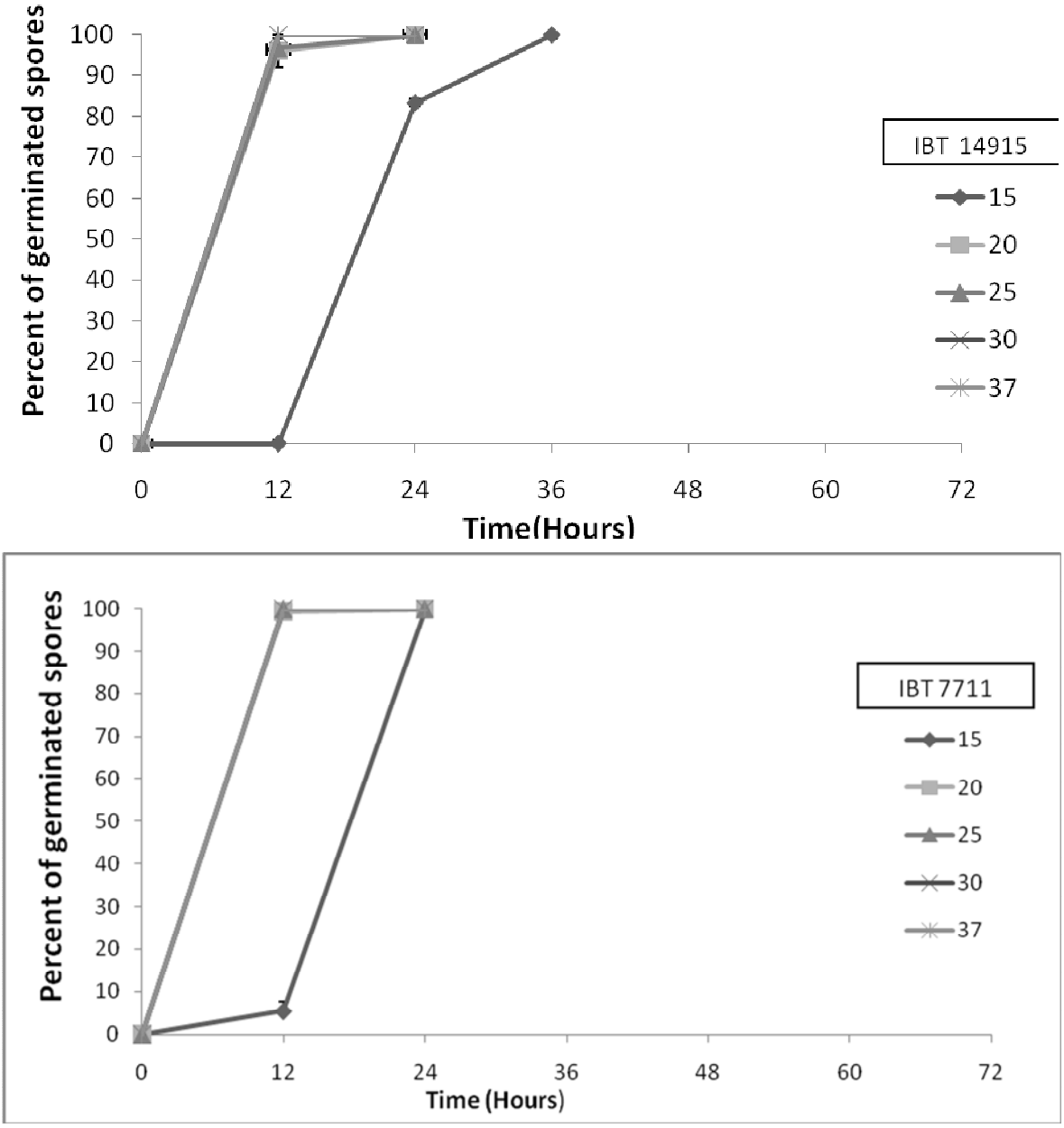


Figure 2. Effect of temperature on the temporal conidial germination of (a) *S. chartarum* IBT 7711 and (b) *S. chartarum* IBT 14915 at 0.98 water activity over a 72 hour period. Bars indicate standard error of the mean.

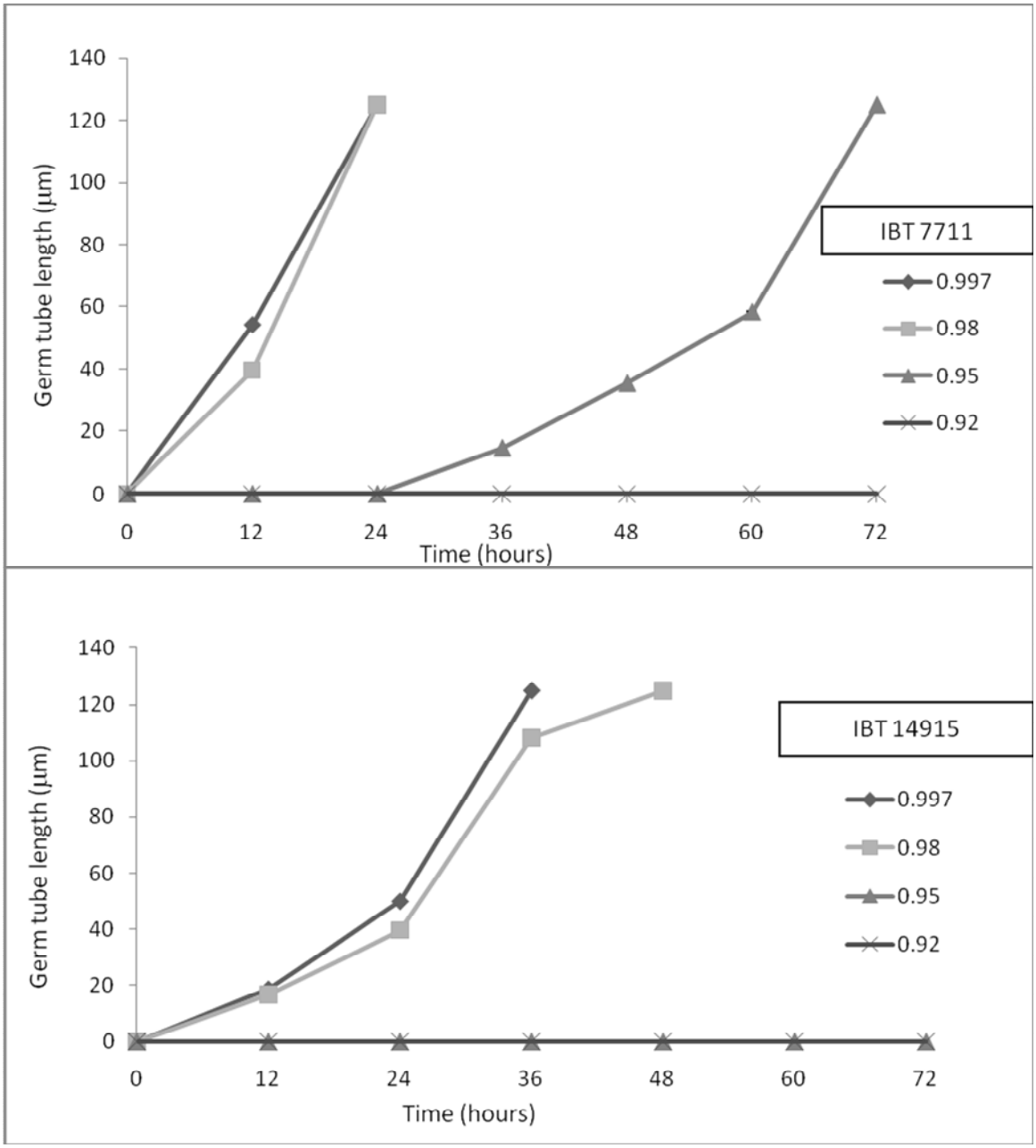


Figure 3. Effect of water activity on germ tube extension (µm) of (a) *S. chartarum* (IBT 7711) and (b) *S. chartarum* (IBT 14915) over a 72 hour period at 20^{o}C.

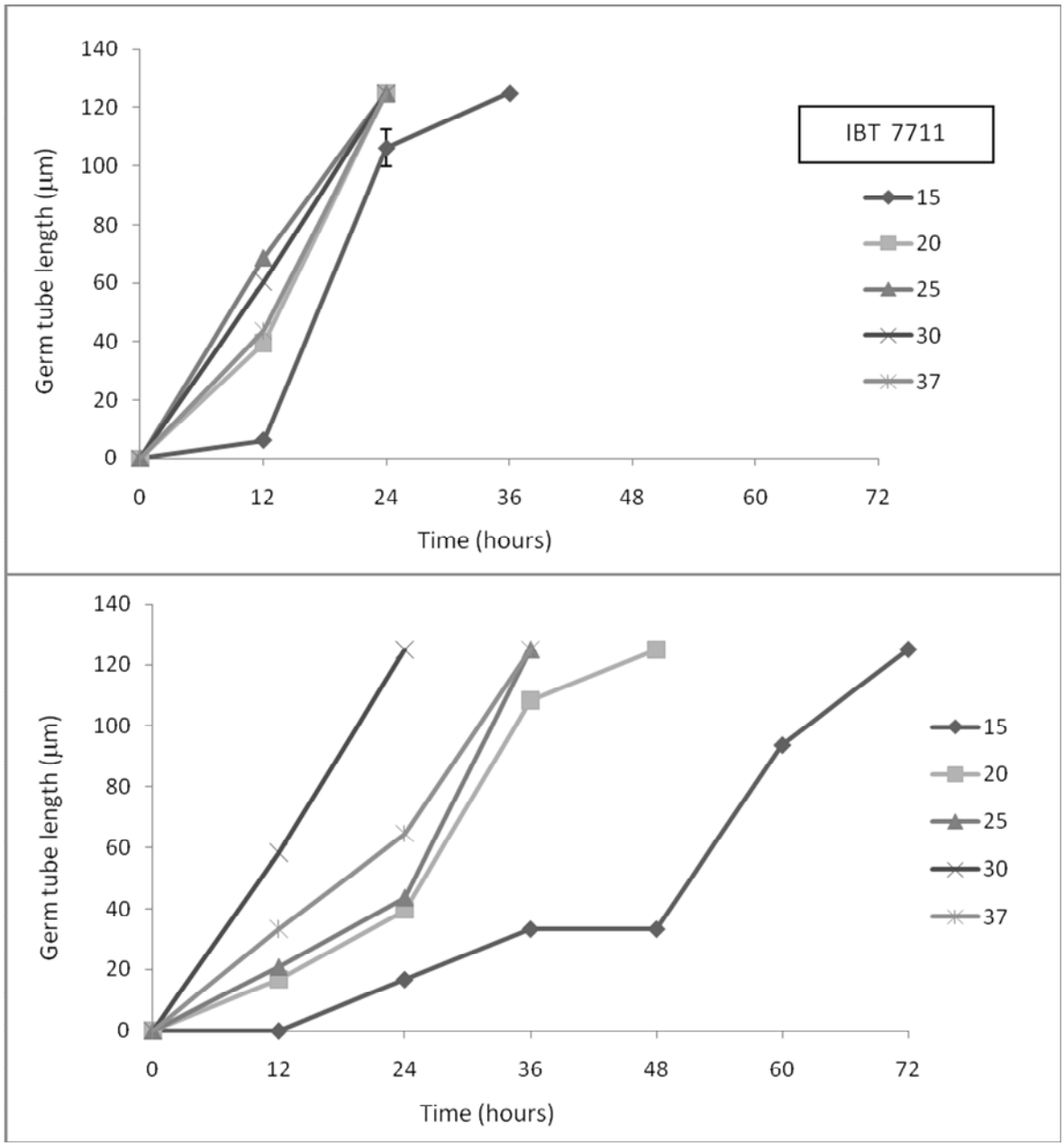
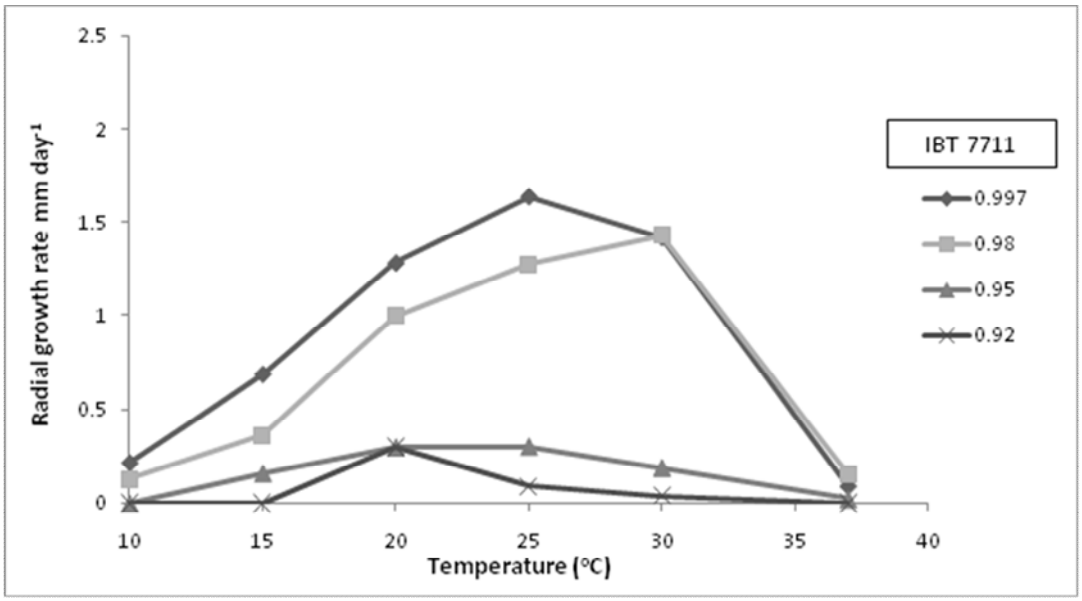


Figure 4. Effect of temperature on germ tube extension (µm) of (a) *S. chartarum* (IBT 7711) and (b) *S. chartarum* (IBT 14915) at 0.98 a_w over a 72 hour period.



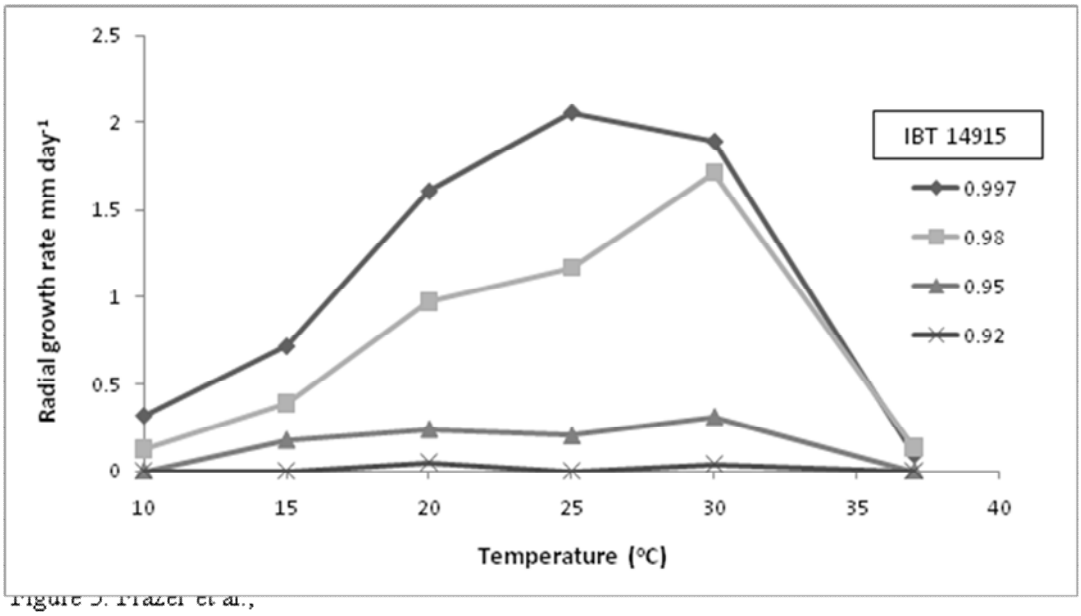


Figure 5. Frazel et al.,

Figure 5. Comparison of water activity x temperature interactions on growth rate (mm day^{-1}) of (a) *S. chartarum* (IBT 7711) and (b) *S. chartarum* (IBT 14915) on a PDA medium.

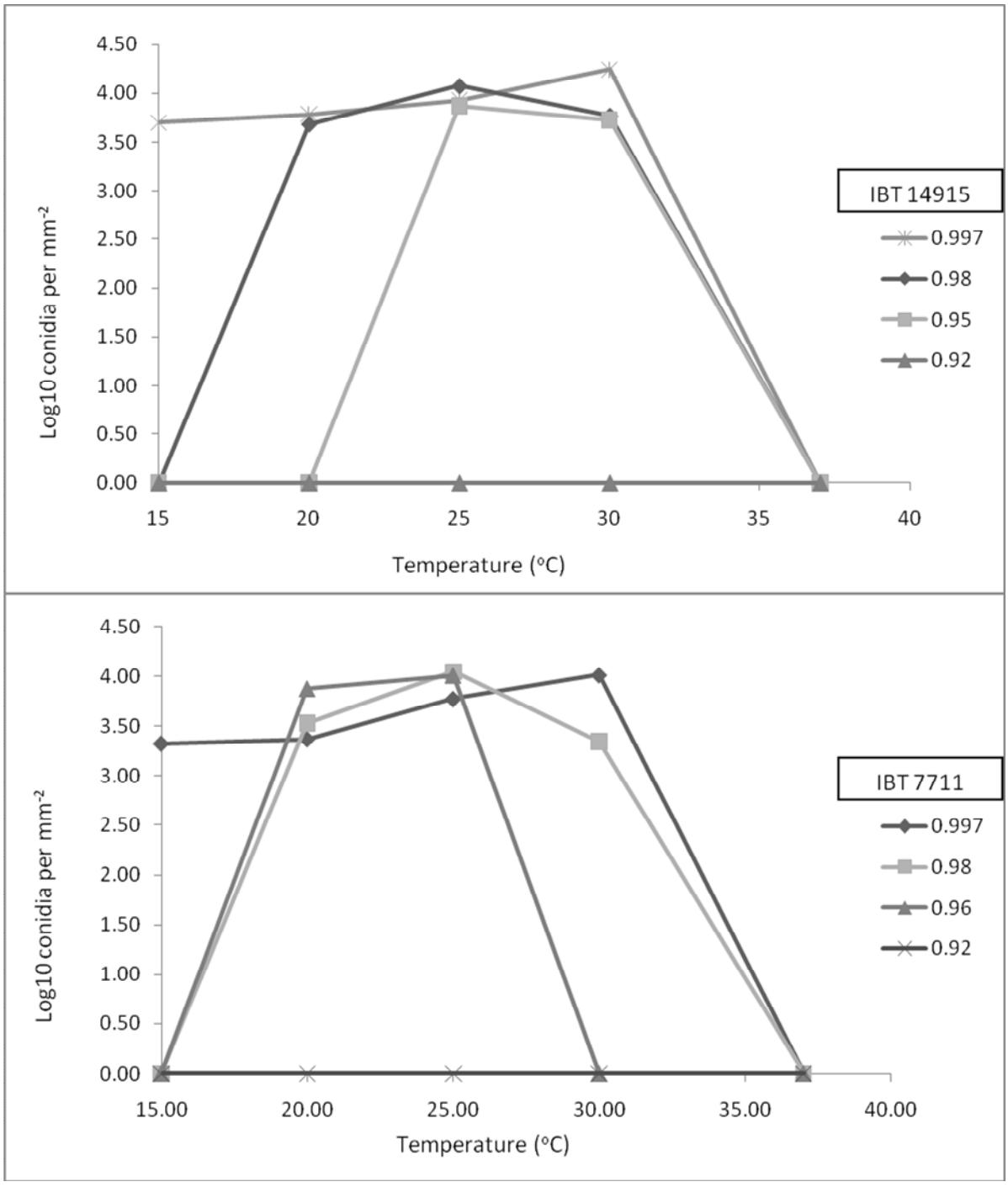


Figure 6. Comparison of sporulation (log_{10} spores mm^2) of (a) *S. chartarum* (IBT 7711) and (b) *S. chartarum* (IBT 14915) when grown on PDA over a range of water activity x temperature conditions after 14 days incubation.