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Reduced water activity during sporogenesis in selected penicillia: impact on spore quality

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Heat resistance, germination and sensitivity to selected antimicrobials was investigated using *Penicillium roqueforti*, *Penicillium aurantiogriseum* and *Penicillium viridicatum* spores, produced on potato dextrose agar (PDA) adjusted to 0.88 a_W using either glycerol or NaCl. Results indicated that spores produced on media without a_W adjustment (0.99 a_W; control) appeared more heat resistant (55°C, 10 min) compared to those produced at 0.88 a_W (treatment). For treatment spores only, heat resistance was observed to decrease further when initially incubated (25°C, 48 h) in sterile, distilled water. All treatment spores were more sensitive to benzoate and sorbate but more resistant to cycloheximide. The time taken for germination, especially for *P. roqueforti* spores appeared to vary depending on the a_W and solute used during spore production. © 1999 Canadian Institute of Food Science and Technology. Published by Elsevier Science Ltd. All rights reserved

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INTRODUCTION

The relationship of water to microorganisms is of special importance to the food industry. The fact that each microorganism has a minimum water activity (a_W) requirement below which growth and/or toxin is inhibited, is both well recognized and used in formulations for the preservation of foods (Corry, 1987; Chirife and Favetto, 1992). Although reduction of water activity in foods by either dehydration, concentration or addition of solutes is efficient in eliminating or reducing bacterial spoilage, filamentous fungi may be more difficult to control.

Many fungi capable of food spoilage and/or mycotoxin production, including penicillia, are well adapted to grow at water activities below 0.90 at which point most spoilage bacteria are inhibited (Beuchat, 1983). In addition, due to their ability to sporulate and disperse widely, they are frequent contaminants of agricultural and food industry products (Richard-Molard *et al.*, 1985). Sporulation represents a vital phase in their life cycle and not only provides a mechanism for survival in marginal environments but also culminates with the formation of reproductive structures which are more resistant than their vegetative forms to most types of physical and chemical stresses.

Studies pertaining to stress resistance (including heat treatment and preservatives) have indicated that nutritional and environmental conditions prevailing during spore formation may exert a profound influence. For example, the nutrient composition and temperature employed during spore formation have been shown to influence their viability (Darby and Mandels, 1955), heat resistance (Conner and Beuchat, 1987*a*,*b*; Beuchat, 1988) and chemistry (Jackson & Schisler, 1992).

Several studies have examined the influence of a_W on spores with regards to heating, recovery and sporulation (Corry, 1974; Doyle and Marth, 1975*a*,*b*; Beuchat, 1981; Inch and Trinci, 1987). However, little information is available regarding the role of water activity during sporulation and its effect on spore quality. This information may be considered important since fungal spores in foods, present as a result of sporulation and or contamination, may be expected to be formed under diverse cultural conditions. Therefore, within any one food

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product identical spore isolates could exhibit varying properties. The purpose of this investigation was to examine the effects of reduced a_W during spore formation with regards to various spore qualities including heat resistance which are important to the food industry.

METHODS AND MATERIALS

Organisms and maintenance

Penicillium roqueforti, *Penicillium aurantiogriseum* and *Penicillium viridicatum* were obtained from the plant pathology laboratory, Agriculture and Agri-Food Canada, (Winnipeg, MB). All penicillia were grown (5 days, 22°C) and maintained (5°C) on potato dextrose agar slants (PDA; Difco, Detroit, MI). Spore inocula were prepared by washing the resultant growth from slants with sterile, distilled water containing 0.05% (v/v) Tween 80 (*ca* 5 ml).

Spore production

The basal medium (control medium) used for spore production was PDA (pH 5.5, 0.99 a_W, basal PDA) adjusted with either glycerol or sodium chloride (analytical grade) to obtain an a_W of 0.88. The appropriate glycerol and NaCl concentrations were calculated according to the data of Chirife *et al.* (1980) and Robinson and Stokes (1959), respectively.

The final water activities of the poured, sterilized media (Table 1.0) were measured using agar plugs with a dew point water activity meter (CS-I Water Activity System, Decagon, Pullman, WA).

Petri plates (100×15 mm) containing 15 ml medium were surface inoculated (0.1 ml), allowed to dry in a laminar air flow hood for *ca* 30 min and placed in sealed, relative humidity chambers maintained at 22°C; the environmental relative humidity within each chamber was controlled by using appropriate glycerol and salt solutions (0.88 a_W). Spores produced at 0.88 a_W (treatment) were incubated for an additional 10 day period following their first appearance (Table 1). Spores produced at 0.99 a_W (control) were incubated for a total of 10 days.

Spores were collected by flooding the surface of the plates with sterile distilled water containing 0.05% (v/v) Tween 80 and gently dislodged using a sterile glass rod. The crude spore suspensions were filtered through glass wool, contained in Pasteur pipettes (14.3 cm long), centrifuged (20 min, 2000 g, 4°C) and washed twice with distilled water (*ca* 25 ml).

Heat resistance, hydration and microscopy

Spore suspensions, harvested and filtered as previously described, were enumerated using a haemocytometer and standardized (*ca* 10^5 ml^{-1}) using sterile distilled water. Aliquots (1 ml) were dispensed into a of series screw-capped test-tubes (12 mm×75 mm) and shaken using a wrist-action shaker (140 rpm, 25°C). Maintenance of spores in water (hydration) during shaking was carried out for 0, 24 and 48 h. Microscopic observations of spores during hydration was carried out on wet mounts using phase optics (×400).

At 0, 24, and 48 h, 0.1 ml aliquots (non-heated) were serially diluted using sterile distilled water and surface plated (0.1 ml) in duplicate using basal PDA. Incubation was at 22°C for 5 days. At each corresponding time period the remainder of the tube contents was positioned in a thermostatically controlled water bath (55°C) such that the surface of the suspension was *ca* 2 cm below the level of the circulating water. Samples were withdrawn after 10 min of heat treatment and chilled immediately on ice. Aliquots (0.1 ml) were surface plated in duplicate using basal PDA. Survivors were enumerated after incubation for 7 days at 22°C. A Duncan's multiple range test (SAS Institute, Cary, NC.) was used to determine significance between non-heated and heated survivors.

Table 1. Final water activities and incubation protocol used for sporulation

Spore crops	Solute	a_w[a]	Lag time[b] for sporulation (d)	Total incubation time (d)
Treatment				
P. roqueforti	Glycerol	0.885	7	17
P. roqueforti	Sodium chloride	0.889	6	16
P. aurantiogriseum	Glycerol	0.885	5	15
P. aurantiogriseum	Sodium chloride	0.882	5	15
P. viridicatum	Glycerol	0.885	6	16
P. viridicatum	Sodium chloride	0.889	7	17
Control	None (basal PDA)	0.995	—	10

[a]Average of three determinations. Individual determinations did not differ by more than ± 0.003.
[b]First appearance of spores.

Minimum inhibitory concentration (MIC)

Sterile and tempered (48–50°C) basal PDA was adjusted to a pH of either 4.2 or 4.8 with tartaric acid (10%) and supplemented with either sodium benzoate or potassium sorbate (Sigma-Aldrich Canada Ltd., Mississauga, ON., analytical grade; filter sterilized, 0.22 μm). Final concentrations of 50, 250, 500, 750, 1000, 1500 and 2000 ppm were prepared for each preservative. Petri plates (100×15 mm) containing the preservative-supplemented media (15 ml) were surface inoculated in duplicate with standardized and filtered spore suspensions of *P. roqueforti* (10^5/ml, 0.1 ml) and incubated at 22°C for 5 days. The entire protocol was repeated for *P. aurantiogriseum* The MIC was determined as the lowest preservative concentration which inhibited growth. Initial MIC values, once established, were further refined using 50 ppm decrements.

The MIC for cycloheximide (Sigma Chemical Corp., St. Louis, MO) was established in a similar fashion using final concentrations of 5, 25, 50, 100 and 200 ppm; however, PDA with a pH of 5.5 was used. Initial MIC values, once established, were further refined using 10 ppm decrements.

Germination time

Agar plugs (0.8 cm diameter; *ca* 3 mm thick) prepared from basal PDA using a sterile cork borer were inoculated with spore suspensions (*ca* 50 μl; 10^8 ml^{-1}) and affixed to the underside of petri dish lids via adhesion (inoculated surface facing up). Following the addition of *ca* 5 ml of distilled water to the bottom of each dish, the lids were replaced. The dishes containing the agar plugs were incubated at 22°C.

To determine the germination time, T_{10} (time necessary for 10% of the spores to germinate) spore plugs were periodically viewed directly through the Petri dish lid using light microscopy (×400). One hundred spores from each crop were randomly examined on each of two agar plugs. Spores were considered germinated when the length of the germ-tube was equal to or larger than one-half of the spore diameter (Paul *et al*., 1992). All trials were performed in duplicate.

RESULTS

Final water activity levels and incubation protocols used to produce the spore crops are presented in Table 1. *P. viridicatum* did not sporulate sufficiently on media at 0.88 a_W, therefore in some experiments its presence was excluded.

P. roqueforti and *P. aurantiogriseum spores* produced at 0.99 a_W were not significantly ($p > 0.05$) affected by the heat treatment at 55°C for 10 min (Tables 2 and 3). Extending the hydration time to 48 h prior to heat treatment also had no significant ($p > 0.05$) effect on their heat sensitivity. In contrast when produced at 0.88 a_W a significant ($p \leq 0.05$) reduction in survivor levels was observed following heating. Hydration of spores especially for 48 h, prior to heat treatment, resulted in further survivor reductions. In the case of *P. aurantiogriseum* for example, increasing the time of hydration from 0 to 48 h resulted in an additional *ca* 1 $\log_{10}$ decrease in survivors (Table 3).

Overall, spores of *P. viridicatum* appeared the most heat sensitive (Table 4). In addition it was observed that decreasing the a_W of the sporulation medium resulted in lower survival levels following heat treatment. However, no clear trend with regards to survivor levels and hydration time could be established.

The MIC values of potassium sorbate and sodium benzoate for *P. roqueforti* and *P. aurantiogriseum* are presented in Table 5. Overall, spores produced at 0.88 a_W were significantly ($p > 0.05$) more sensitive to both sodium benzoate and potassium sorbate.

At pH 4.8, benzoate appeared to have a relatively low inhibitory effect on all *P. roqueforti* spore crops, requiring in excess of 2000 ppm; therefore in order to increase the effectiveness of sodium benzoate, the pH was reduced to 4.2. At pH 4.2, the sensitivity of *P. roqueforti* control spores to sorbate and benzoate appeared similar (MIC = 400 ppm). In contrast, treatment spores appeared more sensitive to sorbate than to benzoate, particularly when glycerol was used as the controlling solute. *P. aurantiogriseum* control spores were significantly ($p > 0.05$) more sensitive to sorbate (MIC = 400 ppm) than to benzoate (MIC = 700 ppm). The sensitivity to either preservative intensified for spores produced at reduced water activity, regardless of solute.

The MIC of cycloheximide for *P. roqueforti* and *P. aurantiogriseum* spores is presented in Table 6. Treatment spores were significantly more resistant to cycloheximide than the controls. However, only treatment spores of *P. aurantiogriseum* showed a differential response to the antimicrobial, based on solute type.

Germination times for the penicillia spores are presented in Table 7. Overall, no clear pattern was evident with regards to germination time and a_W or solute. *P. roqueforti* treatment spores produced with either glycerol or NaCl exhibited shorter germination times than those produced at 0.99 a_W by 5.5 and 4 h, respectively. Spores of *P. viridicatum* and *P. roqueforti* produced with glycerol exhibited the longest (12 h) and shortest (6.5 h) germination times, respectively.

DISCUSSION

Survival of fungal spores is known to be influenced by various factors including age (Doyle and Marth, 1975*a*; Conner and Beuchat, 1987*a*), temperature (Beuchat,

Table 2. Influence of hydration on the survival of *P. roqueforti* spores following heat treatment (55°C, 10 min)

Sporulation a_W/solute	Time of hydration (h)	Survivors ($\log_{10}$ CFU/ml)[c]		
		Non-heated	Heated	Difference following heat treatment[d]
0.99/none	0	$4.74 \pm 0.01^{a}{}_{a}$	$4.74 \pm 0.19^{a}{}_{a}$	0.0
	24	$4.73 \pm 0.02^{a}{}_{a}$	$4.69 \pm 0.05^{a}{}_{a}$	0.04
	48	$4.70 \pm 0.24^{a}{}_{a}$	$4.66 \pm 0.09^{a}{}_{a}$	0.04
0.88/NaCl	0	$5.11 \pm 0.10^{a}{}_{a}$	$4.69 \pm 0.03^{a}{}_{a}$	0.42
	24	$5.04 \pm 0.04^{a}{}_{a}$	$4.69 \pm 0.02^{a}{}_{b}$	0.35
	48	$5.01 \pm 0.03^{a}{}_{a}$	$4.44 \pm 0.17^{b}{}_{b}$	0.57
0.88/glycerol	0	$4.83 \pm 0.13^{a}{}_{a}$	$3.96 \pm 0.38^{a}{}_{a}$	0.13
	24	$4.66 \pm 0.09^{a}{}_{b}$	$3.94 \pm 0.33^{a}{}_{b}$	0.28
	48	$4.49 \pm 0.21^{b}{}_{a}$	$3.85 \pm 0.24^{a}{}_{b}$	0.64

[a,b]Means followed by a different superscript within a column for a specified a_W/solute are significantly different ($p < 0.05$); means followed by a different subscript within a row are significantly different ($p \leq 0.05$).
[c]Values represent the means ± s.d., $n = 4$.
[d]Control-treatment.

Table 3. Influence of hydration on the survival of *P. aurantiogriseum* spores following heat treatment (55°C, 10 min)

Sporulation a_W/solute	Time of hydration (h)	Survivors ($\log_{10}$ CFU/ml)[c]		
		Non-heated	Heated	Difference following heat treatment[d]
0.99/none	0	$4.31 \pm 0.01^{a}{}_{a}$	$4.28 \pm 0.04^{a}{}_{a}$	0.03
	24	$4.30 \pm 0.04^{a}{}_{a}$	$4.24 \pm 0.03^{a}{}_{a}$	0.06
	48	$4.32 \pm 0.12^{a}{}_{a}$	$4.25 \pm 0.08^{a}{}_{a}$	0.07
0.88/NaCl	0	$4.22 \pm 0.03^{a}{}_{a}$	$3.95 \pm 0.02^{a}{}_{b}$	0.27
	24	$3.93 \pm 0.13^{a}{}_{a}$	$3.29 \pm 0.10^{b}{}_{b}$	0.64
	48	$3.81 \pm 0.10^{b}{}_{a}$	$2.84 \pm 0.10^{b}{}_{b}$	0.77
0.88/glycerol	0	$3.88 \pm 0.01^{a}{}_{a}$	$3.53 \pm 0.07^{a}{}_{b}$	0.35
	24	$3.78 \pm 0.10^{ab}{}_{a}$	$2.66 \pm 0.09^{b}{}_{b}$	1.12
	48	$3.68 \pm 0.12^{b}{}_{a}$	$2.22 \pm 0.30^{c}{}_{b}$	1.46

[a,b]Means followed by a different superscript within a column for a specified a_W/solute are significantly different ($p \leq 0.05$); means followed by a different subscript within a row are significantly different ($p \leq 0.05$).
[c]Values represent the means ± s.d., $n = 4$.
[d]Control-treatment.

Table 4. Influence of hydration on the survival of *P. viridicatum* spores following heat treatment (55°C, 10 min)

Sporulation a_W/solute	Time of hydration (h)	Survivors ($\log_{10}$ CFU/ml)[c]		
		Non-heated	Heated	Difference following heat treatment[d]
0.99/none	0	$5.22 \pm 0.01^{a}{}_{a}$	$4.95 \pm 0.07^{a}{}_{b}$	0.27
	24	$5.20 \pm 0.05^{a}{}_{a}$	$4.89 \pm 0.11^{a}{}_{b}$	0.31
	48	$5.11 \pm 0.09^{a}{}_{a}$	$4.83 \pm 0.05^{a}{}_{b}$	0.28
0.88/NaCl	0	$3.79 \pm 0.16^{a}{}_{a}$	$2.54 \pm 0.34^{a}{}_{b}$	1.25
	24	$3.76 \pm 0.07^{a}{}_{a}$	$2.24 \pm 0.28^{a}{}_{b}$	1.52
	48	$3.50 \pm 0.09^{b}{}_{a}$	$2.35 \pm 0.26^{a}{}_{b}$	1.15
0.88/glycerol	0	$5.17 \pm 0.08^{a}{}_{a}$	$3.45 \pm 0.21^{a}{}_{b}$	1.72
	24	$5.10 \pm 0.04^{a}{}_{a}$	$3.54 \pm 0.07^{a}{}_{b}$	1.56
	48	$4.96 \pm 0.13^{b}{}_{a}$	$3.47 \pm 0.12^{a}{}_{b}$	1.49

[a,b]Means followed by a different superscript within a column for a specified a_W/solute are significantly different ($p \leq 0.05$); means followed by a different subscript within a row are significantly different ($p \leq 0.05$).
[c]Values represent the means ± s.d., $n = 4$.
[d]Control-treatment.

Table 5. Minimum inhibitory concentration (MIC) of benzoate and sorbate for penicillia spores

pH of assay medium	Organism	Sporulation a_W/solute	MIC (ppm)[c]	
			Sorbate	Benzoate
4.8	*P. roqueforti*	0.99/none	1000[a]	>2000
		0.88/NaCl	800[b]	>2000
		0.88/glycerol	800[b]	>2000
4.2	*P. roqueforti*	0.99/none	400[a]	400[a]
		0.88/NaCl	300[b]	350[b]
		0.88/glycerol	250[c]	350[b]
4.8	*P. aurantiogriseum*	0.99/none	400[a]	700[a]
		0.88/NaCl	300[b]	500[b]
		0.88/glycerol	300[b]	500[b]

[a,b]For each organism at a specified pH, values followed by a different superscript within a column are significantly different ($p \leq 0.05$).
[c]Results are expressed as means of two trials each performed in duplicate.

Table 6. Minimum inhibitory concentration (MIC) of cycloheximide for penicillia spores

Organism	Sporulation a_W/medium[d]	MIC (ppm)[e]
P. roqueforti	0.99/none	40[a]
	0.88/NaCl	80[b]
	0.88/glycerol	80[b]
P. aurantiogriseum	0.99/none	70[a]
	0.88/NaCl	140[b]
	0.88/glycerol	180[c]

[a,b,c]For each organism, means followed by a different superscript within a column are significantly different ($p \leq 0.05$).
[d]pH = 5.5.
[e]Results are expressed as means of three trials each performed in duplicate.

Table 7. Germination time for penicillia spores

Organism	Sporulation a_W/solute	Germination time (h)[a]
P. roqueforti	0.99/none	12[b]
	0.88/glycerol	6.5
	0.88/NaCl	8
P. aurantiogriseum	0.99/none	8
	0.88/glycerol	9
	0.88/NaCl	8.5
P. viridicatum	0.99/none	10
	0.88/glycerol	12
	0.88/NaCl	10.5

[a]Time necessary for 10% of spore populations to germinate; spore germination was evaluated on basal PDA at 22°C.
[b]Results are expressed as means of duplicate determinations. Each determination examined 100 spores for germination progress.

1988; Palop *et al.*, 1996), composition of sporulation medium (Lubieniecki-von Schelhorn, 1973; Doyle and Marth, 1975*a*; Su and Beuchat, 1984; Beuchat, 1988) and nature of the heating menstrum including a_w and relative humidity (Doyle and Marth, 1975*b*; Conner and Beuchat, 1987*b*; Beuchat, 1988). In this study the list of contributing factors to impact on the heat survival of spores was broadened to include the a_W of the sporulation medium. Specifically, when *P. aurantiogriseum*, *P. viridicatum* and *P. roqueforti* were sporulated on reduced a_W media, survivor levels following heat treatment were reduced significantly ($p > 0.05$) compared to those produced on 0.99 a_W media. Also, the survivor levels of spores produced at reduced water activity with glycerol appeared to decrease the most.

Water activity is one of the most important parameters influencing the growth of fungi and has been investigated extensively (Beuchat, 1983, 1987; Pitt, 1989). However, studies pertaining to relevant characteristics of fungal spores produced at reduced water activity are relatively scarce. In one of the few studies which examined this relationship, Jakobsen and Murrell (1977) reported that reducing the a_W in the sporulation medium had no impact on the heat resistance of resulting bacterial spores of *Bacillus cereus*. Aside from the fact that fungi are eucaryotes, and therefore, might be expected to behave differently, it is possible that structural alterations or imperfections incurred during sporogenesis at reduced a_W contributed to increased spore permeation of nutrients, heat or moisture. A possible consequence of this action could be increased spore germinability or circumvented control mechanisms which inhibit germination such as the release of self-inhibitors. In support of this explanation was the observation that some of the treatment spores, when initially hydrated, appeared non-hyaline. Upon further hydration some spores darkened, and in the case of those produced with glycerol, many appeared swollen while others developed germ-tubes and or appeared cracked, suggesting initiation of germ-tubes. In contrast, control spores appeared hyaline before and after hydration. Clearly, spore darkening, swelling and germ-tube emergence are signs of germination progress (Cotter, 1975; Dute *et al.*, 1989). Coincidentally, treatment spores produced with glycerol, which exhibited the most advanced signs of germination progress, also displayed the greatest decrease in survivor levels following heating. An opposite effect was shown by control spores. It is possible, therefore, that the decrease in survivors observed following heating was due to the presence of spores which became more heat-sensitive as a result of germination progress (Hashimoto, 1991). Increasing the time of hydration for some spore crops/solutes may have resulted in continued germination progress. Since all spore crops were harvested, cleaned and concentrated in a similar fashion, carry-over of nutrients from the sporulation medium seems doubtful. Alternatively it is

possible some of the spores produced at reduced water activity had not fully matured and as such might be more heat sensitive and or structurally deficient. Although spore maturity for the various crops was not assessed (resistance to staining) at the time of harvest, the germination times exhibited by the controls and treatments were fairly similar. This in itself could indicate that spore maturity was not an overriding issue.

Similarly, the susceptibility of treatment spores, to either sorbate or benzoate may also attest to the presence of a germinated and/or injured state. Since germinated or injured spores would be more sensitive to the action of antimicrobials ostensibly due to increased permeability, it is logical that lower concentrations would be required to cause inhibition. If permeability were a factor for injured spores it could indicate alterations in structural components including the spore wall. Since the MIC for sorbate using *P. roqueforti* spores, produced with sodium chloride and glycerol, was shown to be significantly ($p > 0.05$) different, it may suggest that this antimicrobial may have multiple sites and/or mechanisms of action separate from inhibition by lowered pH. Axiomatically, this would infer multiple sites of injury with *P. roqueforti* spores when produced at diminished water activity.

Whereas lower concentrations of preservatives were required to inhibit growth of spores produced at reduced a_w, an opposite effect was observed with cycloheximide. Treatment spores required from 2 to 2.5 times more cycloheximide for inhibition compared to control spores. Cycloheximide is a glutarimide antibiotic that inhibits protein synthesis in eucaryotes (Sullia and Griffin, 1977). Its site of action has been associated primarily with the 60 S ribosomal subunit and it is frequently used to study metabolic processes (Coddington, 1977; Evans and Evans, 1980). Although it is well recognized that protein synthesis occurs during fungal spore germination (Beilby and Kidby, 1982) and that cycloheximide blocks emergence of germ-tubes, its action on dormant (hyaline) spores is not well characterized. Assuming protein synthesis in dormant spores is minimal or lower than in germinated spores, the levels of cycloheximide required for protein synthesis inhibition would be expected to be correspondingly lower. Alternatively, reduced entry into the spore (selective permeability), deactivation of the antibiotic perhaps via binding or modification in the target site could account for the MIC pattern (Cooke and Whipps, 1993).

In conclusion, the results of this study indicate that some penicillia spores including *P. roqueforti*, when produced under reduced a_w conditions, may exhibit alterations in their gemination rate when exposed to subsequent optimum environments such as water. This may lead to a rapid reduction in survival following a relatively mild heat treatment. The possibility of injured but not germinated spores contributing to this phenomenon is currently under investigation. In addition, the rate of spore maturation as a function of sporulation a_W will be further examined. From a food preservation perspective it appears that some spores formed under marginal a_w are less likely to survive thermal treatment and may be more susceptible to certain chemical preservatives. Sporulation of individual fungi on foodstuffs, especially when exposed to varied climatic conditions/ and or storage conditions over a protracted time, may therefore culminate in the development of several spore populations. Individually these populations may exhibit varied properties which should be taken into account when explaining results obtained using laboratory cultured fungi.

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