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Dormancy, activation and viability of *Rhizopus oligosporus* sporangiospores

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Abstract

Interruption of dormancy to improve viability of *Rhizopus oligosporus* sporangiospores is crucial for the application of stored starter cultures for fungal (tempe) production. We aimed to assess the extent of dormancy and factors that could result in activation. Whereas heat treatments were unsuccessful, Malt Extract Broth (MEB) showed to be a good activation medium, with 80% of dormant spores being activated as measured by fluorescence microscopy using a fluorescent marker, compared with 11% with the control. Peptone and yeast extract but not glucose played an important role in activating dormant spores. Metabolically active (fluorescent) and swollen spores, followed by germ tubes were obtained after activation in MEB for 25 min., 2 and 4 h, respectively, at 37 °C. Simultaneously, some interesting transitions took place. Dormant spores represent 85–90% of the total spores at harvest and after drying. Their number decreased to 21–32% after activation with MEB with a concomitant increase of metabolically active spores. As a result of storage, some dormancy was lost, yielding an increase of active spores from 11.2% at harvest to 28.8% after 3 months storage. Levels of active spores were well correlated with their viability. By activation of dormant spores, their viability increased; levels of viable and active spores were maximum in 1 month old starter (61.7% and 75.9% of total spores, respectively) but gradually decreased with concomitant increase of the number of dead spores.

Keywords: Inoculum; Dormancy; Viability; *Rhizopus*; Spores; Activation; Fluorescent markers

1. Introduction

The development of fungal spores can be arbitrarily distinguished into several stages: formation, maturation, dormancy, after-ripening, activation and germination (Griffin, 1994). Dormancy is a common strategy to survive unfavourable external conditions. To become germinable after dormancy, many (fungal) spores require an after-ripening period (e.g., a cold period) and/or activation treatment. The latter may include thermal, chemical (detergents, organic acids, and amino acids, etc.), or light activation (Sussman and Halvorson, 1966; Griffin, 1994).

For example, *Phycomyces blakesleeanus* spores do not germinate in a suitable culture medium unless they are activated by one of a range of treatments, such as

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heating for 3 min at 44 °C (Halbsguth and Rudolph, 1959; Van Laere et al., 1980), γ radiation (Van Assche et al., 1977), dithionite treatment (Van Assche et al., 1978), or treatments with acetate, azide and ammonia (Van Laere et al., 1980). Likewise, *n*-alcohols and high pressure supported the heat activation of *P. blakesleeanus* (Thevelein et al., 1979) and of *Neurospora tetrasperma* ascospores (Belmans et al., 1983). During activation, glycerol formation was observed in *P. blakesleeanus* spores (Van Schaftingen and Van Laere, 1985). Heat treatment was related to trehalase activity in dormant and activated spores of *P. blakesleeanus* (Van Assche et al., 1972; Van Assche and Carlier, 1975).

Glucose was involved in the initiation of germination of *Mucor racemosus* sporangiospores (Tripp and Paznokas, 1982a). Furthermore, glucose induced trehalase activity and trehalose mobilization during early germination of *P. blakesleeanus* spores (Thevelein et al., 1983). The availability of glucose also affected events during germination of *Syncephalastrum racemosum* sporangiospores (Hobot and Gull, 1977).

Whereas amino acids and endogenous protein stimulated germination of *M. racemosus* sporangiospores (Tripp and Paznokas, 1981, 1982b), no single amino acid was as effective as glucose or peptone at triggering germination. So it was suggested that glucose may trigger germination by signalling the breakdown of endogenous protein reserves, while the subsequent increase of free amino acids may be the dormancy-breaking factor.

Earlier studies on the germination of *Rhizopus oligosporus* sporangiospores (Medwid and Grant, 1984) revealed that two phases: I (swelling) and II (germ tube protrusion), could be distinguished. Initial swelling during phase I occurred only in the presence of a suitable carbohydrate, while subsequent production of germ tubes during phase II required exogenous sources of both carbon and nitrogen. It was also shown (Breeuwer et al., 1997) that germination of *R. oligosporus* sporangiospores is prevented by nonanoic acid, a fungal self-inhibitor. Despite this knowledge, there is a lack of understanding relating to the dormancy and activation of the tempe-mould *R. oligosporus*. In this paper, we present physical and nutritional conditions affecting the activation and germination and the accompanying morphological changes of *R. oligosporus*. We demonstrate shifts in physiological categories (i.e., dormant, metabolically active, viable and dead) of *R. oligosporus* sporangiospores in during storage.

Like in other fungal spores, dormancy occurs in sporangiospores of *R. oligosporus*, especially during storage time; dormancy may well be one of reasons for the limited shelf-life of tempe starter cultures as were reported by several authors, e.g. 4 months at 25–30 °C (Tanuwidjaja and Roestamsjah, 1985), or 2.5 months (Tunçel et al., 1989). In powdered tempe starters we can distinguish three categories by fluorescence microscopy, namely: metabolically active (green fluorescence with carboxyfluoresceindiacetate), dead (red fluorescence with propidium iodide) and dormant (no fluorescence). In rice-based spore powders, typically more than 90% of spores were dormant and 5–6% are metabolically active (Thanh and Nout, 2002). Many, but not all, metabolically

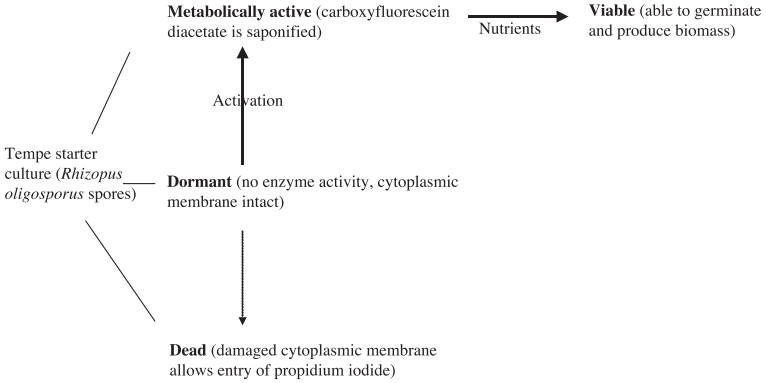

Fig. 1. Physiological categories of *R. oligosporus* sporangiospores.

active spores are viable, i.e. able to germinate and produce colonies of biomass. To facilitate the discussion, the relation between these categories is illustrated in Fig. 1.

2. Materials and methods

2.1. Fluorescent probes and media: cFDA

5-(and-6)-carboxyfluorescein diacetate (Molecular Probes Europe, Leiden, The Netherlands), 10 mg/ml acetone, (cFDA 0.22 mM) was used as a fluorescent marker for metabolically active spores (see also Sections 2.3 and 2.5 below). PI: Propidium iodide 95–98% (TLC) (Sigma, St. Louis, USA) 1- mg/ml distilled water, (30 μM) was used as a fluorescent marker for dead spores (see also Sections 2.3 and 2.5 below). RBCC: Rose-Bengal chloramphenicol Agar Base, Oxoid, CM 549 with addition of 0.2 g/l Rose Bengal (Fluka, Switzerland) was used for viability tests (see Section 2.6). MEA: Malt extract agar (malt extract 30 g/l, mycological peptone 5 g/l, agar 15 g/l), Oxoid, CM 59 was used in the preparation of tempe starter (Section 2.2) and for viability tests (Section 2.6). MEB: malt extract broth (malt extract 17 g/l, mycological peptone 3 g/l), Oxoid, CM 57 was used for activation of spores (Section 2.3). CDM: Czapek-Dox liquid medium Oxoid, CM 95 containing sodium nitrate 2 g/l, potassium chloride 0.5 g/l, magnesium glycerophosphate 0.5 g/l, ferrous sulphate 0.01 g/l, potassium sulphate 0.35 g/l and sucrose 30 g/l was used for activation of spores (Section 2.3). PPS: Peptone physiological salt solution containing neutralised bacteriological peptone 1 g/l (Oxoid, L34) and NaCl 8.5 g/l was used as a diluant for viability tests (Section 2.6).

2.2. Preparation of tempe starter

Tempe starter was prepared as described earlier (Thanh and Nout, 2002). Briefly, the procedure was as follows: *R. oligosporus* LU 575 (NRRL 5905) was grown on MEA plates during 1 week at 30 °C, and spores were harvested by washing with sterile water. The spore suspension was diluted to approximately 10^5 spores per ml. This suspension "A" was used as inoculum for substrate used to prepare tempe starter. Polished broken rice was steam-sterilized, allowed to cool and sterilized ammonium sulfate and sterilized H_2SO_4 were added to adjust the pH to 4, this is the optimal pH for spore germination of *R. oligosporus* (Medwid and Grant, 1984; Breeuwer et al., 1997). The sterile broken rice (50 g) was inoculated and thoroughly mixed with 0.4 ml of spore suspension "A". The inoculated rice was incubated at 40 °C for 2 h, followed by incubation for 4 days at 30 °C. The mouldy mass was broken up and the entire mass of substrate, mycelium and spores was dried at 42 °C for 48 h in a forced-air drying cabinet. Only crushing by sterile pestle and mortar, but no fine grinding was used in order to avoid misjudging between spores and rice particles with similar size and shape. The starter powder was stored in screw-cap glass tubes, protected from light, in a silicagel desiccator at 25 °C, as these conditions were considered to be more representative of practical usage than storage at low temperature. Moreover it was shown that spores survive better at ambient (25–30 °C) temperature than at 5 °C (Thanh and Nout, 2002). The samples were analyzed in triplicate after a defined period of storage.

2.3. Activation of dormant spores

Sporangiospores in crushed rice powder were diluted with sterile water to suspensions (approximately 10^6 spores/ml), and were washed twice by centrifugation at $13{,}000 \times g$ for 3 min in an Eppendorf centrifuge. After decanting, the spores in pellets were re-suspended in phosphate buffer (K_2HPO_4 50 mM, adjusted to pH 4.0 with citric acid 50 mM), CDM, and MEB, respectively. Subsequently, the suspensions were either plated on RBCC and MEA to quantify viable spores (see Section 2.6), metabolically active or dead spores (see Section 2.5). At each sampling time point during the activation process, spore suspensions were stained as described in Section 2.5.

2.4. Total number of spores

One gram of crushed rice-based spore starter was placed in 99 ml of sterile distilled water containing 0.1% (v/v) Tween 80. The suspension of spores was carried out by vigorous agitation and filtration with a Millipore membrane filter [(fluorassure); Chem filter 15, REF: 100-C2003-01; Chemunex]. Spore suspen-

sions were diluted as appropriate and counted using a Bürker-Türk counting chamber. Microscopic counts were also made similarly to determine fluorescent spores, as described in Section 2.5.

2.5. Fluorescent counts of metabolically active and dead spores

Spore suspensions were washed twice by centrifugation at 13,000 × *g* for 3 min in an Eppendorf centrifuge in phosphate buffer (K_2HPO_4 50 mM, adjusted to pH 4.0 with citric acid 50 mM). Subsequently, the suspensions were incubated for 20 min in the presence of cFDA and PI at 40 °C. They were then put on ice and counted in a Bürker-Türk counting chamber by fluorescence microscopy, with an Axioskop epifluorescence microscope equipped with a 50-W mercury arc lamp, a fluorescein isothiocyanate filter set (excitation wavelength 450–490 nm; emission wavelength >520 nm), an × 100 1.3 numerical-aperture Plan-Neofluar objective lens, and a camera (Carl Zeiss, Oberkochen, Germany). Fluorescent, non-fluorescent and spores showing germ tubes were counted. Their values were presented in decade log units and percent of total spores. Fluorescent spores (cFDA) were considered as metabolically active, or dead in case of PI fluorescence; non-fluorescent spores were considered as dormant; spores with germ tubes were considered as germinated spores when the extension of the germ tube was to a length equal to one-half the diameter of the spores (Medwid and Grant, 1984).

2.6. Viability of spores

Viable spores were determined as colony forming units by surface-plating triplicate 0.1-ml aliquots of decimal dilution series in PPS, on RBCC and MEA. After incubation at 37 °C for 12 h (MEA) or 24 h (RBCC), colonies were counted.

2.7. Statistical analysis

Experiments were in duplicate, analyses in triplicate. The results were reported as means of triplicates with standard deviation using unrelated t test to test significance of differences.

3. Results and discussion

3.1. Effect of heat and nutrients on activation

In Table 1, the effects of incubating 2-month old rice-grown as well as fresh MEA-grown spores of *R. oligosporus*, in MEB and buffer are shown. MEB has a significant activation effect as shown by higher numbers of metabolically active (cFDA fluorescent) spores. Attempts to activate spores by heat treatments at at 50 and 60 °C were unsuccessful. This is in

Table 1
Effect of heat treatments on metabolic activation of fresh and stored spores of *R. oligosporus*

Activation treatment	Metabolically active[1] spores of *R. oligosporus* grown on rice and stored for 2 months at 25 °C		Metabolically active spores of *R. oligosporus* grown on MEA for 4 days, at 37 °C	
	log *N*/g dry wt rice powder Avg. ± S.D.	% of total number of spores[2]	log *N*/ml suspension Avg. ± S.D.	% of total number of spores[2]
MEB[3] (37 °C, 4 h)	$8.57^{a} \pm 0.01$	79.4	$8.68^{a} \pm 0.01$	93.3
Buffer[4] (37 °C, 4 h) (Control)	$7.7^{b} \pm 0.04$	10.7	$8.5^{b} \pm 0.04$	61.7
Buffer[4] (50 °C, 10 min)	$7.48^{c} \pm 0.08$	6.5		
Buffer[4] (60 °C, 3 min)	$7.46^{c} \pm 0.04$	6.2	$8.46^{b} \pm 0.23$	56.2
Buffer[4] 60 °C, 10 min)	$7.37^{c} \pm 0.06$	5.0		

Data reported as means of triplicates with standard deviation. In each column, data with the same indicators are not significantly different ($p \leq 5\%$, one-tail unrelated *t*-test).

[1] Fluorescent with cFDA.

[2] Log total spores = 8.71.

[3] Malt Extract Broth, Oxoid CM 57.

[4] Buffer was prepared by K_2HPO_4·50 mM adjusted to pH 4.0 with citric acid 50 mM.

contrast with the very successful activation of *P. blakesleeanus* spores after such heat treatment (Halbsguth and Rudolph, 1959). We conclude that heat activation is not suitable for activation of dormant sporangiospores of *R. oligosporus*.

Table 2 presents data on the effects of control buffer, CDM (Czapek-Dox liquid medium), and MEB (Malt Extract Broth). Incubation in CDM did not result in appreciable activation (low number of fluorescent metabolically active spores). This suggests that its main components, sodium nitrate and sucrose, play no role as activating compounds. The number of fluorescent, i.e. active spores increased strongly by incubation in MEB at 37 °C. In an earlier study (Breeuwer et al., 1997) it was observed that the majority of freshly harvested spores had become fluorescent after 3 h incubation at 37 °C in MEB. Our data in Table 2 show that activation with MEB proceeds very quickly. We found that MEB is suitable for activation of dormant spores, even after prolonged storage of 2 months. This suggests that problems relating to limited shelf-life reported earlier (Tanuwidjaja and Roestamsjah, 1985; Tunçel et al., 1989), might have been caused by dormancy and could have been overcome by activation under appropriate conditions. The results of the control in Table 1 show that the number of active spores in fresh MEA-grown spores (61.7%) was much higher than that of rice-grown spores stored for 2 months (10.7%) and when activated in MEB, fresh spores can be activated up to 93%. This means that not only stored spores, but also fresh spores benefit from MEB activation.

Table 2
Effect of incubation time in activation solutions on the metabolic activation of rice-grown spores of *R. oligosporus* stored for 2 months at 25 °C

Incubation time	Buffer[1] (control)		CDM[2]		MEB[3]	
	log *N*/g dry wt[4]	% of total[5]	log *N*/g dry wt[4]	% of total[5]	log *N*/g dry wt[4]	% of total[5]
5 min[6]	7.75	12.0	7.48	6.5	8.52	70.8
2 h[6]	7.72	11.2	7.65	9.6	8.54	74.1
4 h[6]	7.51	6.9	7.74	11.8	8.58	81.3
6 h[6]	7.53	7.2	7.84	14.8	8.57	79.4
8 h[6]	7.47	6.3	7.80	13.5	8.55	75.9

[1] Buffer: K_2HPO_4·50 mM adjusted to pH 4.0 with citric acid 50 mM.
[2] Czapek-Dox medium, Oxoid CM 95.
[3] Malt Extract Broth, Oxoid CM 57.
[4] *N* = cFDA fluorescent spores; dry wt: dry weight of sample.
[5] Log total spores = 8.67.
[6] Measured after 20 min for fluorescent staining at 40 °C in buffer[1].

The mechanistic base for dormancy of *R. oligosporus* is not known. In *Aspergillus oryzae*, it has been shown (Horikoshi and Iida, 1964) that spore coats contain higher levels of glucosamine and protein; spore walls of *Rhizopus stolonifer* are multilayered (Hawker and Abbott, 1963) and superficial lipid on the asexual spores of *R. stolonifer* prevents wetting (Fisher et al., 1972). Such physico-chemical conformations of the spore surface could prevent nutrient uptake and activation. Considering the rapid activation of dormant spores of *R. oligosporus* as shown in Table 2, we conclude that its dormancy is not caused by such factors, but rather by deficiency of certain nutrients.

We also tested whether MEB may still be limited in its concentrations of activating component(s), by carrying out the activation experiments of Table 2 with MEB (normal strength) to which D-Glucose (0.5%), NH_4Cl (0.1%), and KH_2PO4 (0.15%) were added, and with 1.5-fold concentrated MEB. Similar numbers of fluorescent and germinated spores were found (data not shown) which makes us conclude that the levels in MEB are adequate to activate dormant spores.

3.2. Activation, germination and viability as affected by exposure to MEB

The activation, swelling and germination sequence followed by mycelial growth of *R. oligosporus* sporangiospores is shown in Table 3. The number of activated spores quickly exceeded nine decade log units (42% of total spores) already after 5 min of exposure to MEB, whereas the maximum was reached after 6 h (70% of total spores). Spore swelling, the first visible phase of germination, had started between 20 min and 2 h. Next, the emergence of germ tubes followed after 4 h, with 8.99 decade log unit increasing to 9.27 decade log unit (55% of total spores) after 6 h. However, after 8 h the outgrowth of germ tubes caused such entwinement that counting was not reliable anymore. Our results agree with earlier reports (Ekundayo and Carlile, 1964; Medwid and Grant, 1984; Breeuwer et al., 1997) that germination pro-

Table 3
Activation, germination and viability of rice-grown spores of *R. oligosporus* stored for 3 months at 25 °C after exposure to Malt Extract Broth at 37 °C

Time	Active spores[a]		Germinated spores[b]		Viable spores[c]	
	log *N*/g dry wt[d]	% of total[e]	log *N*/g dry wt[f]	% of total[e]	log CFU/g dry wt[f]	% of total[e]
0 min[g]	0	0	0	0		
5 min[g]	9.15	41.7	0	0		
10 min[g]	9.16	42.7	0	0		
15 min[g]	9.19	45.7	0	0		
20 min[g]	9.21	47.9	0	0	8.63	12.6
2 h[g]	9.25	52.5	0 (swollen)	0	8.96	26.9
4 h[g]	9.32	61.7	8.99	28.8	9.28	56.2
6 h[g]	9.37	69.2	9.27	55.0	9.16	42.7
8 h[g]	Not countable[h]		Not countable[h]			

[a] Fluorescent with cFDA.
[b] Showing germ-tubes.
[c] Producing colonies on MEA and RBCC.
[d] *N* = fluorescent spores.
[e] Log total spores = 9.53.
[f] Dry wt = dry weight of spore powder.
[g] Measured after 20 min for fluorescent staining at 40 °C in buffer (K_2HPO_4·50 mM adjusted to pH 4.0 with citric acid 50 mM).
[h] Caused by mycelial entwinement.

ceeded through two distinguishable phases, viz. phase 1 the enlargement of the sporangiospores and phase 2 the emergence of a germ tube. Table 3 shows that the number of viable spores increased from 8.63 log units (12.6% of total) after 20 min activation, to its highest value of 9.28 log units (56.2% of total) after 4 h and then decreased slightly. We expect this decrease to be an artefact caused by mycelial entwinement and physical damage to germ tubes during surface plating; both will decrease the number of colonies to some extent. After 4 h we obtained higher levels of viability than visible germ tubes probably because the spores that had swollen started to form germ tubes during plating on MEA medium. This assumption is supported by the increase of germinated spores between 4 and 6 h.

3.3. Effect of some carbon and nitrogen sources on viability of stored (11 months) spores

As shown above, MEB containing peptone and maltose, is a good activation medium. The effect of complex carbon and nitrogen sources on germination and colony formation (viability) of dormant spores is shown in Table 4 for (1) MEB as a control, (2) glucose 2%, (3) peptone 1%, (4) yeast extract 1%, (5) casamino acids 1%, and (6) combined glucose and peptone. Without incubation or after an incubation during 4 h at 37 °C of the dormant spores, the activated spores were spread-plated on the same solutions to which 1% w/v Agar Bacteriological (Oxoid L11) had been added. Colonies were counted after 12 h. The data show the effects of activation

Table 4
Effect of some carbon and nitrogen sources on viability of rice-grown spores of *R. oligosporus* stored for 11 months at 25 °C

Activation solution	Without activation		Activation[1] at 37 °C, 4 h	
	log CFU/g Avg. ± S.D.	% of total[2]	log CFU/g Avg. ± S.D.	% of total[2]
1. MEB[3]	$8.53^{a} \pm 0.03$	36.3	$8.63^{a} \pm 0.05$	45.7
2. Glucose[4] (2%)	$< 7.0^{c}$	< 1.1	$< 7.0^{c}$	< 1.1
3. Peptone[5] (1%)	$8.51^{a} \pm 0.04$	34.7	$8.63^{a} \pm 0.04$	45.7
4. Yeast extract[6] (1%)	$8.52^{a} \pm 0.06$	35.5	$8.59^{a} \pm 0.05$	41.7
5. Casamino acids[7] (1%)	$7.96^{b} \pm 0.05$	12.0	$8.25^{b} \pm 0.06$	19.1
6. Glucose[4] (2%) + peptone[5] (1 %)	$8.49^{a} \pm 0.04$	33.1	$8.59^{a} \pm 0.04$	41.7

Data reported as means of triplicates with standard deviation. In each column, data having the same indicators are not significantly different ($p \leq 5\%$, one-tail unrelated *t*-test).
[1] Spores were incubated in activation solution.
[2] log total spores = 8.97.
[3] MEB: Malt Extract Broth, Oxoid CM 57.
[4] D(+)-Glucose-Monohydrate, Merck, Art. 1.08342.
[5] Peptone aus Casein, Merck, Art. 7213.
[6] Yeast Extract, Oxoid L21.
[7] Bacto vitamin-free casamino acids, Difco, Art. 0288-01.

(incubation in activation solution) and of individual components. On glucose alone, viability was very low. Activation in casamino acids increased viability from 12.0% to 19.1% but the viability was significantly lower than in MEB, peptone, yeast extract and glucose + peptone which were not significantly different. It was reported (Ekundayo and Carlile, 1964) that the initiation of germination of *R. arrhizus* sporangiospores required the presence of glucose or fructose and that germinating spores were transferred to a medium lacking glucose, swelling soon ceased. Glucose was also essential for initiation of spore swelling and germ tube emergence in *S. racemosum* (Hobot and Gull, 1977); both quickly ceased when glucose-grown cultures shifted down to non-permissive glucose-free medium. Our data also show that only glucose is insufficient to allow colony formation. Obviously, additional nitrogen and mineral sources will be required for germination and colony formation. In early studies on the slime mold *Dictyostelium discoideum* (Cotter and Raper, 1966) it was noted that the spores were constitutively dormant and did not release myxamoeba if merely suspended in buffer; however, each spore released a simple myxamoeba if peptone were added to the buffer. When the component parts of peptones were tested (exclusive of peptides), the amino acids were mainly responsible for germination whereas vitamins, salts and glucose had no effect. Our data suggest a similar response by *R. oligosporus*. It was interesting to note that even after 11 months of storage, the viability of spores activated by MEB was still rather high (45.7% of total). This is partly due to the fact that the moulded rice was not finely milled since we observed that fine grinding gives considerable losses of viability (Thanh and Nout, 2002).

3.4. Dormancy, activation and viability of spores during a production and storage trial

We studied metabolic activity and viability from production up to 16 months storage of rice-based spore concentrate by measuring total number of spores, metabolically active spores, dormant spores, viable spores, and dead spores. Fig. 2 shows that at harvest most spores (85.1%) are in the dormant stage, and only 11.2% are active. Of the dormant spores, more than 60% could be activated. Activation treatment did not increase the viability of freshly harvested spores; we assume that they were too sensitive to the staining temperature (40 °C) or they were too young. After mild drying, a general but

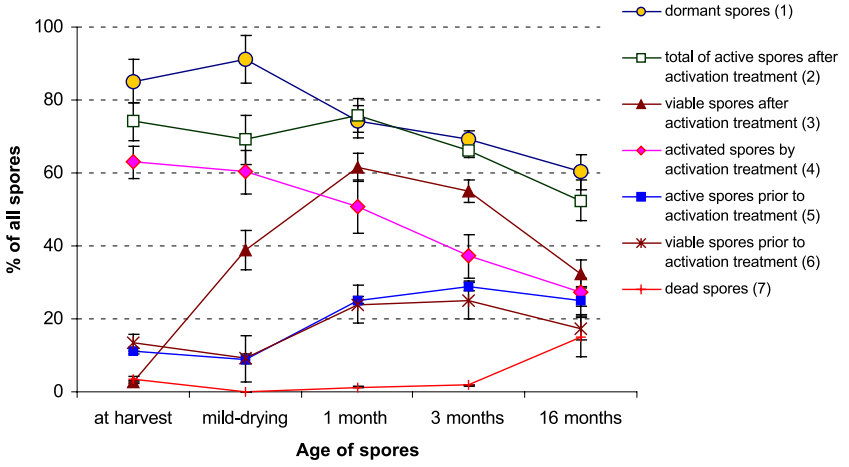

Fig. 2. Activity, dormancy, and viability of *R. oligosporus* sporangiospores in a rice-based spore concentrate: (1) spores lacking enzyme activity, but with intact cytoplasmic membrane; (2) sum of active spores prior to activation treatment and activated spores; (3) sum of viable spores prior to activation treatment and increased viability by activation treatment; (4) spores activated from dormancy by activation treatment; (5) metabolically active spores without activation treatment; (6) viability without activation treatment; (7) spores with damaged cytoplasmic membrane allowing entry of propidium iodide.

slight decrease of active and viable spores was observed. As noticed earlier (Thanh and Nout, 2002) this must be caused by the drying temperature of 42 °C and the loss of moisture which renders rehydration more difficult. We found however, that the viability of dried spores could be enhanced considerably by activation.

After 1 month storage and thereafter, no dead germ tubes were observed anymore, indicating that the spores were maturing, stabilizing their structure and possibly recovering from the drying treatment. After 1-month storage, viability after activation was highest at 8.74 log units (61.7% of total), and decreased gradually during further storage to 32.4% after 16 months. This is in conformity with earlier data (Rusmin and Ko, 1974; Wang et al., 1975) showing that the germination percentage of processed spores decreased rapidly during the early storage period. After 3 months of storage, the number of active spores had increased to 8.41 log unit (28.8%), with concomitant decrease of dormant spores. This would suggest that during storage, some dormant spores woke up, i.e. changed to active spores. After 16 months of storage, a considerable number of stored spores had died (8.13 log units, or 15.1% of total), and the number of viable spores had strongly decreased to 8.2 log unit (17.38%), whereas activation was not very effective any longer. The data after 16 months indicate that dormant spores could still be activated but that active spores could hardly germinate anymore.

In conclusion, we found that the majority of sporangiospores of *R. oligosporus* in rice-based starter are in a dormant stage. During storage, some of the dormant spores become active. The numbers of active and viable spores correlate well. Most dormant spores can be activated by MEB, peptone or yeast extract, with concomitant increase of viable spores.

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