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Spore swelling and germination as a bioassay for the rapid screening of crude biological extracts for antifungal activity

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ABSTRACT

Screening for bioactivity is commonly performed *in vivo* in a bioassay purposefully designed for revealing a defined bioactivity (e.g. fungicide or antibacterial activity). This allows the testing of many crude extracts. In the present work a new method (bioassay) targeting spore swelling and germination to assess antifungal susceptibility is developed and evaluated. Traditionally, antifungal activity has been investigated using disk diffusion assays or micro-well plates. Inhibition is measured as a function of radial growth, inhibition zone or turbidity. The construction of a bioassay composed of germinating fungal spores bears the prospect of being a more rapid method, allowing more extracts to be screened within a shorter time frame. It can also be used to reveal antifungal action at an early state in the prospecting process. Suppression of spore swelling provides early indication of inhibitory potential and the type of swelling curve produced might indicate the mechanism of fungistasis. A strain of *Absidia glauca* Hagem served as model organism. A Beckman Coulter Multiziser™ 3 particle analyser was applied for the determination of bioactivity and investigation of the sporangiospores. Inhibition was standardized against two known fungicides (sorbic and benzoic acid). Four biological extract solvents were also tested; where DMSO was found to be the best candidate as extract solvent in the assay. Inhibition was investigated as changes in volumes of the germinating spores using germination as endpoint target. The new bioassay was found to be a simple and rapid method for detection of antifungal activity of extracts.

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1. Introduction

In our search for bioactive compounds from marine microorganisms, i.e. bioprospecting, we needed a rapid and accurate method as a primary screen of extracts to find fungicidal compounds, as well as for their activity guided fractionation and isolation. The search for new fungicides is a major challenge to current research in mycotic diseases and other area of fungal infections, where bioprospecting of marine microorganism can be of great aid.

The whole-cell bioassay technique is the classical method still in use to detect antifungal compounds because it targets the whole organism. Methods based on the increasing understanding of cell biology and new biotechnological techniques are replacing the non-specific assays with assays using mechanism-based screens directed at specific biochemical targets (Hertzberg, 1993; Gupte et al., 2002; Riedlinger et al., 2004; Shu, 1998). Bioassays with fungi are generally using vegetative growing cultures exposed to extracts. Assays for antifungal screening can roughly be divided into plate dish diffusion assays, turbidity assays (Langvad, 1999) and methods using dry weight as biomass measure of growth (Zulpa et al., 2003). Several other approaches exist designed for more special purposes like aromatic chambers (Begum et al., 1999). Plate dish diffusion assays are however the most common. Growth is estimated by measuring colonial mean radii or inhibition zone compared to controls. One variety is the paper disc method using extracts applied to paper discs placed onto the agar surface in a Petri dish containing spores/CFU (Houdai et al., 2004; Mahakhant, 1998; Soltani et al., 2005). Another technique is the plate method, which consists of agar medium (in a Petri dish) mixed with extracts and inoculated at its center with an agar plug cut from actively growing plates of the fungus (Begum et al., 1999; Demule et al., 1991). In the plate well method cell or spore suspensions (CFU) are mixed with agar medium before plating in Petri dishes, where after wells are stamped in the agar. Extracts are then added to the wells (Kellam et al., 1988). Another method, originating from the field of antibacterial susceptibility testing, is the stable gradient technology marketed as the Etest (Cormican and Pfaller, 1996; Serrano et al., 2003). The Etest is a plastic strip which establishes a continuous gradient of the test compound in the surrounding agar. Finally, automatic image analysis is a promising method that has been developed for evaluating the viability and germination characteristics of fungal spores (Paul et al., 1993). The

Abbreviations: CFU, Colony-forming unit; DMSO, Dimethyl sulfoxide; DW, Dry weight; ESZ, Electrical sensing zone method; H, Hydrophilic extracts; L, Lipophilic extracts; MEX, Malt extract medium; MIC, Minimum inhibitory concentration; NCCLS, National Committee for Clinical Laboratory Standards.

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method offers a number of advantages being rapid, accurate and consistent. Essential in all such assays are the use of controls (blanks and extraction solvents) and standardization to known fungicides, and also the use of different concentrations of extracts. Standardisation of assay parameters such as incubation time and inoculum size is also important elements. Since these methods involve growth, inoculums of spores or mycelium does not need to be high, and are often in the range of $1.0*10^4$–$1.0*10^6$ units per ml. Most often suspensions are prepared of broken mycelium, and concentrations given as CFU/ml measured by spectrophotometric methods (Espinel-Ingroff, 1991). Determining MIC endpoint criteria and the effect of inoculum size on MICs are also important (Espinel-Ingroff et al., 1995; Gehrt et al., 1995). Antifungal susceptibility testing has been limited by a lack of reproducibility and uncertain clinical relevance. This research field is now rapidly evolving with development of standardized reference methods; the microdilution broth method and the broth macrodilution method (NCCLS, 2002). Both estimate MICs after a period of 24–48 h of incubation, or even longer, and have formed the basis for development of a variety of antifungal susceptibility test methods.

We here present a new bioassay for the rapid screening of crude extracts, using swelling and germination of fungal spores and the Coulter particle analyzing system as a means of measurement to investigate fungal susceptibility.

2. Materials and methods

2.1. Preliminary research and tests

To reach the final form of the bioassay all the elements of the assay were thoroughly tested. This involved developing and optimizing methods for growing the fungal model organism, and harvesting and storing the sporangiospores. Furthermore experiments and observations gave the necessary understanding of spore behaviour such as swelling and germination. Based on these tests all parameters of the final bioassay, like inoculum size, test volumes, spore concentrations, incubation conditions and analytical methods to measure inhibition of swelling and germination were determined. The assembled bioassay was then tested, evaluated and optimized using known fungicides. Much of the following materials and methods are therefore a result of this preliminary research.

2.2. Fungal isolate

A strain of *Absidia glauca* Hagem from the culture collection at the Department of Biology, University of Bergen, was used. It consists of two mating types designated + and −. Only the − mating type was used for the bioassay.

2.3. Maintenance of fungal stock cultures

Stocks were prepared every third or fourth month by stamping agar plates containing growing mycelia with a sterile hollow cork borer, producing circular bits from the agar plate whit a diameter of about 0.5 cm. They were stored suspended in 4 ml sterile distilled water in cryo-tubes kept at 4 °C until needed.

2.4. Fungal growth

A. glauca was grown on malt extract agar medium (MEX) in Petri dishes in an incubator at 22.0 ± 0.5 °C for 14 days. The dishes were incubated upside-down to avoid evaporation and accumulation of moisture on the inside of the lid. Inocula were kept at a relative constant size. Spores were germinated in liquid MEX medium.

2.5. Spore harvesting

Spores were harvested when the cultures were fully sporulated; which was achieved after 14 days of incubation. The spores were released from the sporangia by flooding the Petri dishes with 10 ml sterile distilled water (4 °C) and then gently rubbing the culture with a sterile bent glass rod. The subsequent spore suspension was roughly filtered through washed and sterilized cotton positioned in a funnel into a 50 ml Erlenmeyer flask. This procedure was repeated once, and the cotton finally washed with 5 ml sterile distilled water.

2.6. Preparation of spore suspensions

The obtained spore suspensions were transferred directly to 50 ml Nunc tubes after harvesting, and centrifuged repeatedly 3 times for 5 min at 1000 rpm at 4 °C (Beckman J2-HS Centrifuge). In between centrifugation, the supernatant was removed and the pellet re-suspended in 10 ml sterile distilled water containing 0.1% (v/v) Tween-80, using a vortex shaker for 30 s (MS1 Minishaker, IKA® Works, INC). This washed the spores free of any debris and remaining medium. Finally, the spores were re-suspended in 5 ml sterile distilled water and shaken for another 30 s before storage. This produced a high-density spore suspension of more than $2.0*10^8$ spores/ml per Petri dish. All fluids used to harvest and suspend spores, were cooled to 4 °C before use to preserve and keep the spores from initiating the swelling phase.

2.7. Storage of spores

Suspensions were stored in a refrigerator at 4 °C in darkness until needed, for a maximum of 7 days after harvest. All subsequent handling of the suspension was initiated by shaking the tube for at least 30 s using the vortex shaker, followed by sonication in a sonicating bath (Ultrasonic cleaner, Branson 200) for another 5 min to homogenize and separate the spores completely (performed twice in this order). The spore suspension was shaken for a mininum of 30 s before any use during experiments to assure that spores did not cling or become a sediment, and was at all times before introduction to the MEX medium kept on ice to stay cooled down. The concentration of the suspension was determined and adjusted to $1.0*10^8$ spores/ml, serving as starting point for all subsequent work.

2.8. Spore counting and sizing

For all investigations and measurements of the sporangiospores, a Beckman Coulter Multiziser™ 3 was applied. A 0.9% solution (w/v) of NaCl was used as electrolyte. Spore numbers were in addition determined and verified using a Thoma hemocytometer.

2.9. Dry weight (DW)

Dry weight was used to determine the biomass of spores. The spores were collected on washed, dried (70 °C for 24 h, and then kept in a desiccator containing a moisture absorber until needed) and pre-weighted (Mettler Toledo MT5 microbalance) filters by suction filtration. Samples were then washed twice with distilled water (approximately isotonic to the MEX medium) to remove growth medium between the spores and in the filter. Filters with spores were then dried at 70 °C for 24 h, and cooled in the desiccator for a minimum of 2 h until stable weight at room temperature.

2.10. Filtration of experimental liquids

All experimental liquids (e.g. electrolyte, distilled water, DW and medium) were filtered through a layer consisting of a glass micro fibre filter (Whatman GF/C, pore size 1.2 μm) overlaying a membrane filter

(Millipore polycarbonate, pore size 0.2 µm). This reduced the noise level in the Coulter Counter to a minimum. Filtration of MEX and DW were done without the membrane filter. All filtrations were performed using a water jet pump (pressure approximately 0.8 Bar).

2.11. Bioassay

All experiments were carried out under the same standardized incubation conditions. *A. glauca* spore cultures were germinated in 50 ml Erlenmeyer flasks submerged in a temperature-controlled shaking water bath. Cultures were kept at 27.0 ± 0.5 °C, and shaken at moderate speed. Total culture volume was always 5.0 ml. Main assay ingredient was 4.0 ml growth medium (MEX). Spore suspensions were prepared as described above, and always comprised 0.5 ml of the 5.0 ml assay volume. This gave a 10-fold dilution of the suspension of $1.0*10^8$ spores/ml, to give the inoculum a concentration of about $1.0*10^7$ spores/ml. The remaining 0.5 ml consisted either of Milli-Q water (in controls and general tests on spore behaviour), a fungicide or a biological extraction solvent. Controls were always run in each experiment. Cultures were sealed with aluminium foil. When constant temperature was reached, the experiments were started by adding the spores to the assay mixture in the flasks, and incubating for 6 h. Before sampling for measurements in the Coulter Counter the cultures were thoroughly homogenized. Degree of inhibition was estimated from the following formula giving percentage of inhibition:

$$\text{Inhibition}(\%) = \frac{\text{Control}\,(\Delta\,\text{vol.}) - \text{Treated}\,(\Delta\,\text{vol.})}{\text{Control}\,(\Delta\,\text{vol.})} \times 100$$

Treated means cultures treated with known fungicides or extraction solvents. Δvol. = average increase in spore volume during the 6 h experiment.

2.12. Standardized swelling and germination

Experiments performed to investigate and standardize spore swelling and germination were done according to the bioassay described above. A spore was defined to be germinated when the length of the germination tube was half the diameter of the spore. Standardization of swelling and germination processes were performed on newly harvested and untreated spores, i.e. without centrifugation, washing and re-suspending in Tween-80. Spores were directly transferred after harvest to the bioassay. Measurements were performed immediately after their addition to the medium, and three parallel cultures were run simultaneously. Samples were taken every 30 min from each culture for 6 h. Harvesting and transfers to the bioassay took about 10–15 min at best before spores were measured in the Coulter Counter. The immediate response of suspending the spores in water, i.e. passive swelling, was therefore investigated. Temperature optimum for the swelling and germination was not investigated, but was based on results from another study of the same strain by Frøyen (1975).

2.13. Storage of spores

The viability of untreated spores was tested during 14 days of storage in a refrigerator at 4.0 °C. All handling and preparation of spores were as previously described. Viability was determined as growth at day 1, 2, 3, 6, 8, 11 and 14. Samples were at these days removed from the stored spore suspension and incubated in MEX under standard bioassay conditions, and three parallels were run simultaneously. The Coulter Counter was applied to detect any changes in swelling and germination patterns during the first 6–8 h. After 24 h, the cultures were added 100 µl paraformaldehyde to prevent further growth. They were then harvested, and DW of the total culture volume was used to determine the increase in biomass. The spore suspensions were also investigated in the light microscope to reveal any morphological changes.

2.14. Effects of spore concentrations in the bioassay

To examine if the inoculum concentration would have any effects on the viability of the spores, 9 different spore concentrations were tested. DW of un-swollen spores was determined. The spore concentration gradient was logarithmic; $5.0*10^7$, $2.5*10^7$, $1.25*10^7$, $6.25*10^6$, $3.125*10^6$, $1.56*10^6$, $7.81*10^5$, $3.9*10^5$ and $1.95*10^5$ spores/ml. Three parallels were run for each concentration. All cultures were started simultaneously, incubated for 24 h before growth was terminated with paraformaldehyde and DW obtained. An estimate of specific growth after 24 h was obtained by dividing accumulated biomass by the spore concentration. Swelling and germination was monitored using the Coulter Counter.

2.15. Paraformaldehyde as a fixative

The use of a fixative to fix spores would greatly enhance the method by postpone spore counting and volume determinations. For such use, it was necessary to investigate whether paraformaldehyde influenced spore volumes. The method described by Throndsen (1978) was used preparing the fixative. The effect on spore volumes was investigated by adding the paraformaldehyde when the spores had swollen for 2, 4 and 6 h. Volumes were determined 30 min after the addition of paraformaldehyde, and also after 3 days to investigate the persistence of fixation.

2.16. Standardization with known fungicides

To be able to interpret and express screening results, and to investigate inhibiting properties on swelling spores, two well-known fungicides were applied to standardize antifungal susceptibility. The agents used were the potassium salt of sorbic acid (Fluka Chemika 85520, Assay >99%) and the sodium salt of benzoic acid (Norsk medisinaldepot, Natriumbenzoat). Both are preservatives widely used in the food industry. A 1.0% stock solution (w/v) of each was prepared and stored at 4 °C until needed. The standardization covered the effect range from zero to full inhibition. Inhibition was measured after 2, 4, 6 and 24 h of swelling and growth in the presence of the compounds.

2.17. Extract solvent control assays

Crude biological extracts are prepared for bioassay by extracting *Hydrophilic* (H) and *Lipophilic* (L) compounds basically according to the method used by Patterson et al. (1991). Control assays were therefore performed to determine possible effects on the spores by two solvents used for lipophilic extracts, namely ethanol (98%, Arcus AS) and DMSO (SIGMA min. 99.9%). Each solvent was diluted to give 7 different assay concentrations (w/v) in a gradient (0.1%, 0.5%, 1.0%, 2.5%, 5.0%, 7.5% and 10.0%). All preparations and growth conditions were as previously described. Measurements were taken at the start of the experiment, and then again after 2, 4, 6 and 24 h of incubation to determine the persistence of inhibition. Acetone and methanol was briefly investigated as candidates besides DMSO and ethanol. These were tested in two concentrations (1.0% and 5.0%) at time 0, 6 and 24 h after application.

3. Results

3.1. Swelling and germination

A. glauca sporangiospores began swelling immediately when introduced to the growth medium. No lag period was observed. No passive swelling was seen when spores were incubated in distilled

water only, indicating that water and optimal temperature alone is not sufficient to trigger swelling. Swelling was observed as a logarithmic increase of volume with time (Fig. 1). Germination was initiated after approximately 5–6 h after introduction to the growth medium, whereas swelling would impede. One or more germination tubes were present in each spore. Growth then proceeded by rapid extension of mycelium. During swelling the spores increased their volume from approximately 30 μm^3 at start to more than 350 μm^3 when germinating. Swelling measured in the Coulter Counter showed a smooth size distribution till spores entered the germination phase, whereas size measurement thereafter were greatly influenced by the presence of germination tubes. The hydrophilic spores also increased their buoyancy during swelling, from being denser than water to become buoyant when germinating. If not thoroughly homogenized, spores would easily cling and be interpreted as one particle when measured in the Coulter Counter. It was therefore absolutely mandatory to thoroughly treat the spore suspensions by sonication and blending. The use of Tween-80 during spore harvesting also diminished this problem to some extent besides removing surface tension. By additional diluting and blending in electrolyte when measured in the Coulter Counter, it was regarded that this problem was reduced to a minimum.

3.2. Storage of spores

It was necessary to store the spores for an efficient use of the screening method, as growing and harvesting spores are time consuming. This would also make the experimental aspects more flexible. Firstly elements that could initiate spore swelling had to be eliminated. This was done already in the harvesting through washing the spores thoroughly and removing the remains of the growth medium. Concentrating the spores in distilled water and keeping them cold at all stages was the next step before storage in refrigerator at 4.0 °C. The experiment further established the viability of the stored spores. Observations indicated that a small fraction of the spores germinated after about 6–7 days of storage. Counts in the hemocytometer demonstrated that this was valid for less than 0.5–1.0% of the spores, which was considered not constituting an error. Storing spores in distilled water with Tween-80 had more germinating spores than storage without this compound, and the observation raised the question whether Tween-80 acted as a nutrient source. It was therefore decided not to use Tween during storage. The greater portion of spores stayed dormant throughout the experimental period. No passive swelling was observed during 14 days of storage. All spores swelled immediately after exposure to MEX, and no difference in swelling or germination patterns were found due to time of storage. DW measurements after an incubation period of 24 h were used to investigate the viability of the spores, if affected but not visible during swelling and germination. If the spores accumulated less biomass after 24 h of growth, this could mean that their viability was weakened due to the storage, and could possibly increase sensibility to a fungicide in screens. As can be interpreted from the results (Fig. 2), spores can be stored to some extent without any decrease of viability. Nevertheless a limit was set to a maximum of 5 days of storage to be within a safe margin and due to uncertainties whether they would be increasingly sensitive to inhibition of extracts or fungicides during longer periods of storage.

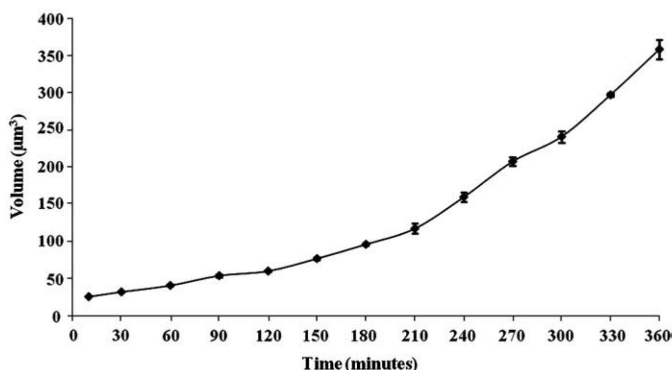

Fig. 1. Standardized swelling of *A. glauca* sporangiospores, shown as increase in volume. Average of three parallels.

3.3. The effect of spore concentration on the germination

It was necessary to investigate if the concentrations of spores had any effects on the swelling and germination, hence also on the assay. No difference between inoculum size and time of germination or spore volumes was observed in the Coulter Counter. Growth measured as accumulated biomass after a 24 hour incubation period, decreased with all increasing spore concentrations (Fig. 3), most probably due to the exhaustion of nutrients in the MEX medium long after spores had germinated. As long as the medium would not be limiting during swelling and germination, i.e. during 6 h after addition of spores to the growth medium, and spores did not inhibit each other at these high concentrations, we hold that high spore concentrations can be used in the bioassay. Based on these experiments the spore density was $1.0*10^7$ spores/ml in all subsequent tests.

3.4. Paraformaldehyde as a preservative

Paraformaldehyde was briefly investigated as a candidate to preserve spores when sampled during tests to ease work efforts. Spores could then be preserved for later investigations. As can be read in

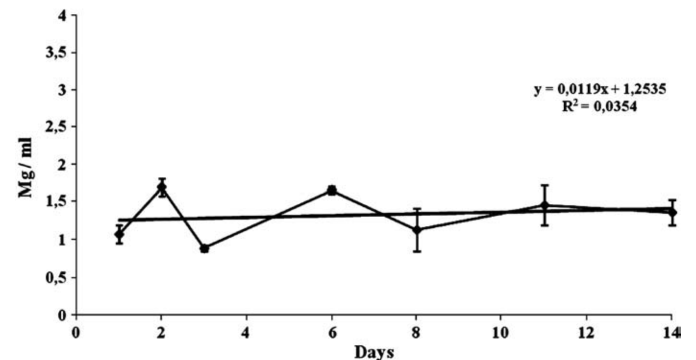

Fig. 2. Increase in biomass of whole assay cultures after 24 h of growth measured after different times of storage over a period of 14 days.

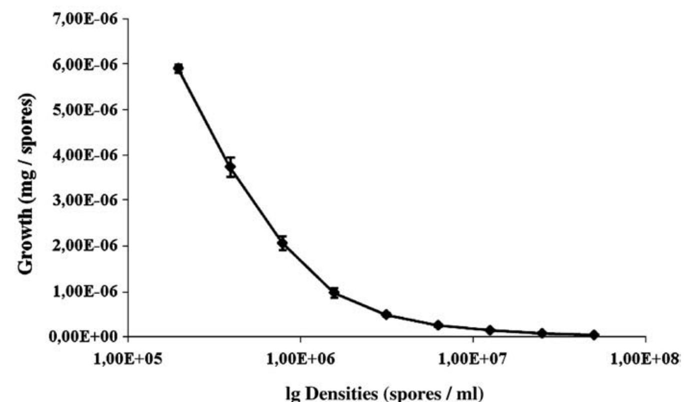

Fig. 3. Increase in the weight of single spores after 24 h incubation in growth medium, as a function of spore density.

Table 1
Formaldehyde as a preservative.

Swelling period:	2 h	4 h	6 h	Average
30 min after addition	0.0%	18.8%	11.8%	10.2%
3 days after addition	48.4%	46.9%	50.7%	48.5%

Effects of paraformaldehyde on swelling spores recorded as percent shrinking of volume.

Table 1, formaldehyde greatly affected spore volumes, and therefore was not an option for such use. Nevertheless it showed an interesting trend between time of addition (exposure to spores in different phases of the swelling process) and time of storage before sizing. The percentage of shrinking differed and was most profound after 4 h of swelling. After 3 days, all spores had shrunk regardless of swelling phase by 50%. Even though these results further complicate the use of the preservative, it stresses the need to thoroughly examine all parameters in a bioassay to avoid uncertainties.

3.5. Inhibition with known fungicides

Standardization of the bioassay with known fungicides was performed using sorbic and benzoic acid. Sorbic acid concentrations from 0.005%–0.4% (w/v), and benzoic acid concentrations from 0.005%–0.9% (w/v) covered the range from no inhibition to full inhibition of germination. Benzoic acid was a weaker inhibitor than sorbic acid. Figs. 4–6 illustrate the type of swelling curve typically produced by fungicides; in this case by sorbic acid. As spores were exposed to increasing fungicide concentrations, an inhibition of germination was first observed. Spores swelled, but did not germinate. Spores would then attain lower volumes during swelling with increasing fungicide concentrations, until swelling was not initiated. Based on these results two endpoint targets were identified; the MIC preventing germination (≥10% inhibition) and the MIC preventing swelling (≥90% inhibition). Inhibition also proved to be persistent when measured again at 24 h compared to inhibition after 6 h of swelling (Fig. 6). At high fungicide concentrations, it was clear that some degree of passive swelling was initiated as spore volumes increased slightly to a certain point independent of the fungicide concentration. This was best observed when spores were treated with benzoic acid. When inhibition was measured and compared after 2, 4 and 6 h after exposure, time-dependent inhibition patterns emerged (Fig. 5). Spores were more inhibited early in their swelling phase than after 4 and 6 h, probably caused by metabolic adaptation to the fungicide compound. This demonstrates that inhibition measured before untreated spores normally would have reached germination, might give inaccurate estimates, and further suggests that time of sampling is critical and must be at the time when most viable spores would have been allowed to germinate, to obtain the best results reflecting the true inhibition. Minimum inhibitory concentrations (MIC) are listed in Table 2.

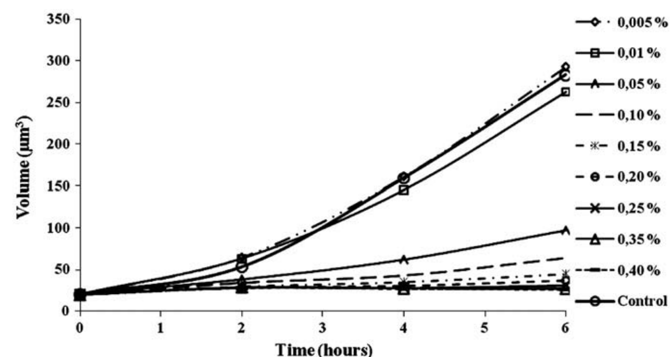

Fig. 4. Effect of different concentrations of sorbic acid on spore volumes measured after 0, 2, 4 and 6 h.

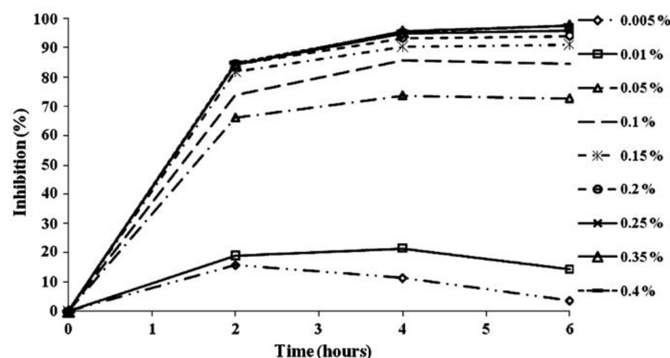

Fig. 5. Inhibition measured after 2, 4 and 6 h.

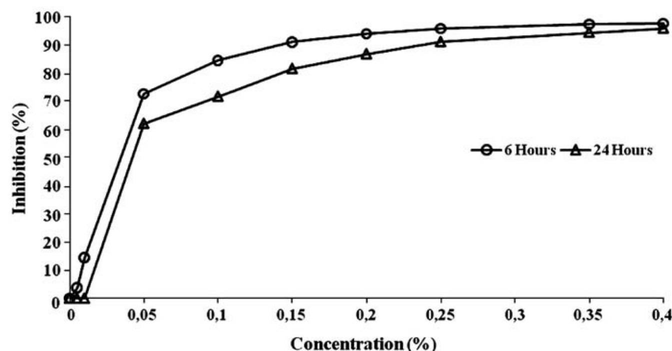

Fig. 6. Percent inhibition of spore swelling by sorbic acid after 6 and 24 h.

3.6. Extract solvent control assays

The possible effects of ethanol and DMSO on the spores had to be determined. Spores were not inhibited by concentrations below 1.0% (v/v) ethanol (Fig. 7). Inhibition by DMSO was first observed in concentrations of 2.5% (v/v) and above (Fig. 8). No significant reduction of the inhibition was observed after 24 h, as also observed in the two fungicide assays. Based on these results, it is clear that DMSO is a better choice as extraction solvent in the assay compared to ethanol. Estimated MIC values at different endpoints can be read in Table 2. Acetone and methanol were briefly investigated as candidates beside DMSO and ethanol. Acetone inhibited spores slightly more than ethanol, whereas methanol inhibited within the same range as DMSO.

3.7. Inhibition endpoint criteria

Based on the findings reported above, it was decided to set 6 h as the end of all assays. This would allow all unaffected spores to germinate. Weak inhibition of germination is difficult to estimate

Table 2
MIC (minimum inhibitory concentrations): mM.

Endpoint MIC:	≥90% (S)	50%	≥10% (G)	ΔG % (G/S*100)
Sorbic acid	9	2	0.5	5.7%
Benzoic acid	34	27	8	22.2%
Ethanol	1368	695	261	19.1%
DMSO	947	589	384	40.5%

Compared MICs of different substances investigated. Concentrations (mM) of tested compounds to prevent S = swelling and G = germination. Endpoints of 90% inhibition or more prevent spores from swelling, whereas an endpoint criterion of 10% or more prevents spores from germinating (not from swelling). ΔG estimates the percentage of the total concentration preventing swelling that is needed to prevent only germination.

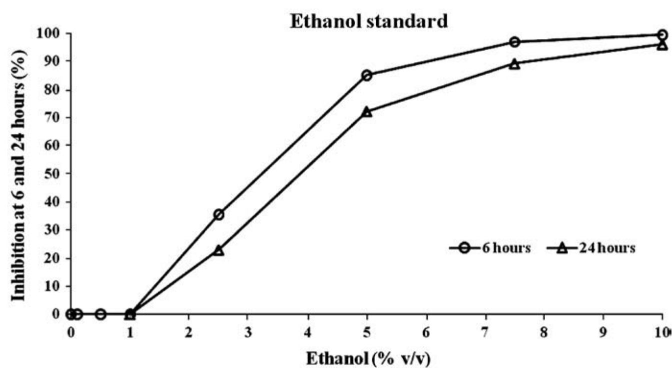

Fig. 7. Standardized inhibition by ethanol. Inhibition is measured after 6 and 24 h of exposure to swelling spores.

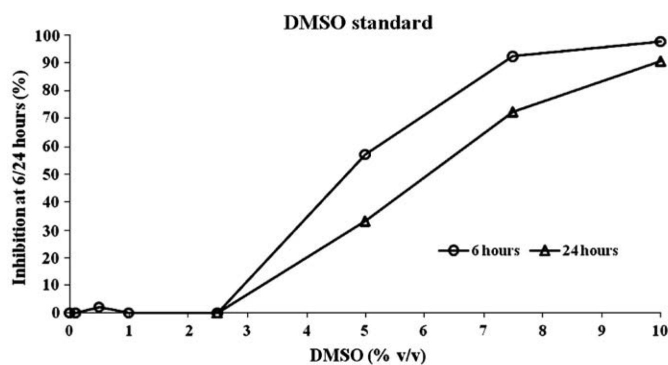

Fig. 8. Standardized inhibition by DMSO. Inhibition is measured after 6 and 24 h of exposure to swelling spores.

accurately. This is caused by uncertainties regarding spore volumes when they germinate, compared to controls, due to the formation of germ tubes. It was therefore decided to use 10% inhibition as an endpoint criterion for germination (all germination is completely inhibited), and 90% as endpoint for swelling. Table 2 summarizes estimated endpoint minimum inhibitory concentrations (MICs) for the two fungicides applied, and for the extraction solvents tested. Values which gave 50% inhibition are also included. They are used to compare results and to evaluate each substance in their strength to prevent germination (G) and swelling (S).

4. Discussion and conclusion

It is necessary for the method to effectively harvest spores intact and viable, and to preserve them intact until they are used in the bioassay. Therefore, a proper method was developed for this purpose, and it was investigated whether storage of spores was possible and if time of storage would affect viability or affect properties (e.g. passive swelling) related to the bioassay. Tween-80 was employed as a surface-active agent (0.1% v/v) to remove surface tensions in the suspension and to reduce clustering of spores, but only during harvesting. As our results indicated, Tween might act as a nutrient source triggering swelling. Both Tween-20 and -80 are widely recognized as appropriate surface-active agents, also by The National Committee for Clinical Laboratory Standards (NCCLS, 2002), but no standardized amounts of these agents have been employed in most of the reports published until now. This might however be of great importance, as it has been found that significantly higher MICs were associated with higher Tween concentrations and that Tween apparently interfered with the action of some antifungals (Parker et al., 1966; Gomez-Lopez et al., 2005). This emphasizes the need to investigate antagonistic effects in bioassays using such agents.

Spore densities has to be kept at a certain standardized level. It was therefore considered necessary to include a gradient of different spore concentrations into the method to ensure that the medium used would not be limiting during swelling and germination, but also to investigate if any sporostatic factors were present. Spores of many fungi germinate poorly or not at all in a dense suspension or when crowded upon a surface (Carlile, 1994). In many fungi self-inhibition has been traced to inhibitory substances given off by the spores themselves, but in others competition for key substances such as oxygen or nutrients may be a factor (Cochrane, 1958; Macko et al., 1976). Another scenario that also would affect the screening if present is *Quorum sensing*. Growth patterns resembling this have been reported for spores of *A. glauca* in earlier research on the same strain (Frøyen, 1975).

Degree of resistance is determined by the lowest concentration of inhibitor required to prevent growth, the minimum inhibitory concentration (MIC). Determination of susceptibility in an assay composed of swelling spores can be determined in two ways, by no swelling or no germ tube formation (both preventing growth). Spores undergo a period of both passive and active swelling, and metabolic changes and responses to the external environment before they finally germinate. MICs of swelling can be difficult to estimate accurately, as long as it is unclear what mechanisms lay behind the initiation of swelling, or whether spores afterwards first swell mainly by a passive mode (i.e. uptake of water and no metabolic growth). MICs will also be higher than those required to prevent germination (Table 2). Changes in spores are measured as increase of volume in a matter of hours prior to germination, and it is assumed that when germ tubes emerge growth resembles that of the mycelium, but often occurs at an exponential rate after the onset of germination (Cochrane, 1958). Germ tube elongation can also be used as an indicator of the growth status, but hyphal growth cannot be measured accurately using the ESZ method. The main problem complicating this is that germinated spores weave into each other. Using germination as endpoint target therefore seems to be the best option for this method. It also seems to be necessary to determine inhibition when most untreated spores would have been allowed to germinate. As can be interpreted from Fig. 5, inhibition measured earlier tends to differ from values measured after 6 h, being higher for low concentrations and lower for the higher ones. This was especially apparent for benzoic acid, and can be explained by metabolic adaptations when growth environments possess harmful compounds. It is likely to assume that these trends will be more obvious when inhibition is characterized by reversible changes within the spores, whereas irreversible changes will be seen as a more instant inhibition (e.g. sorbic acid), and can possibly be used in the preliminary interpretation of inhibition patterns of an antifungal screen. Parker (1971) and McCafferty (1970) investigated spores of several species. Chemical preservatives were found to differ in their effects; either reducing the rate of swelling of germinating spores, or increasing the time before swelling was started and thus extending the lag period. The way spores are inhibited may well give leading information, as different fungistatic action may inhibit the spores at different stages or disrupt the swelling and germination by other means (dEnfert, 1997). These results support that suppression of spore swelling provides early indication of preservative potential of a given compound and the type of swelling curve produced indicates the mechanism of fungistasis.

The volume of swelling spores increased exponential with time (Fig. 1), and was assumed to reflect growth (i.e. metabolic activity) best. It is worth noting that inhibition could also be estimated as a function of the diameter of the swelling spores. But this would in turn lead to different inhibition values compared to those estimated from volumes obtained from the same sample, as the growth diameter is linear with time. It is therefore essential to standardize this approach to estimate inhibition on swelling spores.

The use of Coulter Counter as a method to investigate spores and detect changes in swelling and germination has been proposed in earlier studies, first reported by Barnes and Parker (1966), and then later as a candidate for investigations on fungal and bacterial spore developments, and for the screening of preservatives and detection of antifungal activities (Barnes and Parker, 1967; Barnes and Parker, 1968; McCafferty, 1970; McRobbie et al., 1972; McRobbie and Parker, 1975; Parker, 1971). Several of these have given background information for the development of this method.

The spore swelling assay presented here in this work are superior to most assays when it comes to the experimental time required, allowing many extracts to be screened within a short time frame, and has an advantage in that regard. The approach is simple and reliable. Spores can easily be harvested and stored, and after standardizing against known fungicides of bioprospective relevance antifungal effects from crude biological extracts can be screened for. This approach also makes it easy to control incubation conditions for spores, and to operate within standardized assay conditions. Bioassay parameters such as volumes, incubation time and inoculums are easily adapted, optimised and standardized for different fungal species. Keeping culture volumes small avoids extensive use of large quantities of spore suspensions and biological extracts. This is necessary in the larger screens. The method, however, seems to be limited to the swelling phase. Further improvements of the fungal spore assay can be achieved by using a fixative that would terminate growth and at the same time preserve spores fully intact without volumetric changes. This would greatly increase work efficiency, both by increasing the amount of extracts run simultaneously and by making screens less dependent on strict time bound measures for accurate and reproducible results. An alternative to formaldehyde might be Lactophenol cotton blue (Paul et al., 1993). Further optimising the work by automatic measurements like image analysing can increase the number of screens and decrease work. Several different fungal species also need to be tested within the terms of the present bioassay in this work, to further standardize the method and its application as a fungal susceptibility method for a variety of fungal species. When screening for highly bioactive compounds, spore assays should also be tested against relevant medical compounds in use today.

It is concluded that the fungal spore bioassay is a worthy candidate for screening for antifungal effects. To discover new and maintaining an array of effective fungicides is critical in resistance management. This new method can contribute to the challenges of discovering and developing new drugs, and at the same time bears the prospect of being rapid and allowing the fungistatic action of fungicides to be assessed early in the developing phase.

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