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1-Octen-3-ol inhibits conidia germination of *Penicillium paneum* despite of mild effects on membrane permeability, respiration, intracellular pH, and changes the protein composition

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Abstract

1-Octen-3-ol is a volatile germination self-inhibitor produced by *Penicillium paneum* that blocks the germination process. The size of conidia treated with 1-octen-3-ol was similar to that of freshly harvested conidia. Exposure to 1-octen-3-ol resulted in staining of 10–20% of the conidia with PI and TOTO, fluorescent DNA probes that cannot enter cells with an intact membrane, whereas only 3–5% of non-treated conidia were stained. Addition of 1-octen-3-ol to germinating conidia resulted in transient dissipation of the pH gradient. From this, we conclude that slight permeabilisation of the fungal membrane occurs in the presence of the inhibitor. Two-dimensional gel electrophoresis analysis of protein patterns revealed striking differences between non-germinated conidia, germinated conidia and 1-octen-3-ol-treated conidia. In conclusion, 1-octen-3-ol has mild effects on the plasma membrane, but interferes with essential metabolic processes, such as swelling and germination of the conidia, but in a reversible manner.

Keywords: *Penicillium paneum*; Self-inhibitor; Germination; 1-octen-3-ol; Fungal membrane; Proteins

1. Introduction

Fungal spores are dispersed in different ways, namely through the air, the soil or the water. In this process, living organisms such as insects, seeds and plants can be used as vehicles of fungal transport. Fungal spores can be divided into two main groups; dispersal spores or survival spores. The dispersal spores are often released in massive numbers and germinate under favorable environmental conditions of temperature, pH, light and nutrients. Examples of dispersal spores are the conidia formed by many genera of *Penicillium* and *Aspergillus*, fungi that are extremely important for man with respect to the food industry. These are dominant fungi present in the air and they colonise and spoil food in enormous quantities. Survival spores are characterised by a much longer period of dormancy, which can be imposed by restricted environmental conditions such as nutrient exhaustion, inappropriate temperature and host resistance for fungal germination. During dormancy, the metabolic rate of spores is much lower than that of vegetative cells and germination is prevented [1]. Some fungi have very special requirements for breaking the dormancy of spores, including heat [2] or high pressure treatment [3].

Fungi produce substances that inhibit premature germination of spores before dispersion. These compounds, called self-inhibitors, act at very high spore

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densities; a phenomenon designated as the crowding effect. Self inhibitors are volatile or non-volatile and have been characterised in different fungal genera including *Puccinia*, *Uromyces*, *Colletotrichum*, *Dictyostelium*, *Fusarium* and *Aspergillus* [4–6]. Volatile compounds formed by fungi have been evaluated in chemotaxonomic studies, indicators of food spoilage and can have biological effects on growth of other species of fungi [7,8] or even of other microorganisms [9]. It is clear that the effects of these compounds must be reversible, as the inhibition must be released once the spores are dispersed and have experienced suitable germination conditions. New insights into the mechanisms of this reversible inhibition may lead to new knowledge about germination and to novel, mild methods of food preservation. The volatile compound 1-octen-3-ol is one of the most important flavour components found in mushrooms and is used as a spoilage indicator in stored cereals [10–12]. Conidia of *P. paneum* at high concentrations (10^9 conidia ml^{-1}) produce 1-octen-3-ol which functions as a self-inhibitor. The compound inhibits swelling (isotropic growth), prevents germ tube formation (polarised growth) and induces microcycle conidiation [7]. The detailed pathway involved in its production is not known, but 1-octen-3-ol is a product of the oxidative breakdown of linoleic acid [13–16].

The aim of this study was to identify the specific changes occurring to spores in the presence of the self-inhibitor 1-octen-3-ol, and attempt to correlate these with a mechanism by which this compound is able to inhibit spore germination. Its impact on different cellular parameters, including membrane integrity, intracellular pH, respiration and protein composition, was investigated.

2. Materials and methods

2.1. Preparation of conidial suspensions

Penicillium paneum CBS 114483 was initially classified as *P. roqueforti* LU 510, but then reclassified as *P. paneum* on the basis of morphological and molecular data [7,17]. This fungus was grown on malt extract agar medium (MEA, CM59, Oxoid, Hampshire, England) at 25 °C for a maximum of seven days. A spore suspension was prepared by adding 9 ml of peptone physiological salt solution (8.5 g l^{-1} NaCl with 1 g l^{-1} bacteriological peptone (Oxoid, Hampshire, England) + 0.1% Tween 80) to the fungal culture. Suspensions were prepared by collecting spores from 25 culture tubes, filtering through a 17 μm nylon membrane and centrifugation at 4000*g* for 3 min. Conidia were resuspended in 2 ml of malt extract broth (MEB) medium (CM57, Oxoid, Hampshire, England), adjusted to pH 4.0 with lactic acid. Spore concentrations of 10^9–10^6 conidia ml^{-1} were adjusted using a Bürker Türk haemocytometer.

2.2. Chemicals and probes

The chemicals used in this study were: 1-octen-3-ol (Janssen, Geel, Belgium); TOTO 1,19 (4,4,7,7-tetramethyl-4,7-diazaundecamethyl-ene)-bis-4-[3-methyl-2,3dihydro(benzo-1,3-oxazole)-2-methylidene]-1-(39-trimethylammoniumpropyl)-pyridinium tetraiodide; PI (propidium iodide) and cFDA-SE (5- (and 6)-carboxyfluoresceindiacetate succinimidyl ester) (Molecular Probes Europe BV, Leiden, The Netherlands). The protease inhibitor cocktail appropriate for fungal extracts was purchased from Sigma Chemical, St. Louis, USA.

2.3. Location of germination self-inhibitor

To investigate if the self-inhibitor compound produced by *P. paneum* could be washed from the spores, 1 ml of spore suspension with a high concentration of conidia (10^9 ml^{-1}) was centrifuged every 10 min at 5000*g* for 2 min at 25 °C, and the pellet containing the spores was resuspended in 1 ml of MEB pH 4.0. Washing was repeated during a period of 4 h. As a control, a spore suspension of 10^9 conidia ml^{-1} was centrifuged and resuspended in the same medium, i.e., the obtained supernatant. Another control was the spore suspension at 10^6 conidia ml^{-1} in the presence of 4 mM of 1-octen-3-ol, incubated for 4 h at 25 °C, without washing. After the washing experiment, all the samples were incubated at 25 °C for 22 h and the percentage of spore germination was determined by light microscopy. One hundred conidia were analysed for each treatment with an Olympus Optical CO microscope (1000× magnification), BX40 (Tokyo, Japan) and the spore was considered germinated when a conidium produced a germ tube. The experiment was performed in triplicate.

2.4. Measurement of respiration

Experiments were performed in 4 ml MEB, pH 4.0 in a stirred, water-jacketed vessel. The oxygen concentration was recorded continuously with an oxygen electrode (YSI model 5300 biological oxygen monitor, Yellow Springs Instruments Inc., Yellow Springs, USA). The electrode was calibrated in distilled water and in MEB, pH 4.0. The spore suspensions had a density of 10^7 conidia ml^{-1}. This amount of spores was needed to detect notable oxygen consumption. After temperature equilibration for 2 min, 0.1 ml of *P. paneum* suspension was added and measured after incubation for 10 min. Freshly harvested conidia and conidia at the stage of isotropic growth, after incubation of 2 h (swelling stage) at 25 °C, were used. Subsequently, 156 μl of 1-octen-3-ol at 40 mM was added to give a

final concentration of 4 mM and its effect on respiration was assessed after 10 min. Two independent experiments were carried out.

2.5. Size determination of conidia

The size of conidia was determined using a ProtoCOL System micrometer in the microscope (Synbiosis, Cambrigde, UK). A conidial suspension of *P. paneum* with a concentration of 4×10^6 conidia ml^{-1} in MEB pH 4.0 was prepared as described before. The size of conidia was measured at different developmental stages: directly after harvesting and after incubation for 2 h (swelling stage) and 4 h (germ tube formation) at 25 °C, and also in the absence (MEB) and presence of 4 mM 1-octen-3-ol. At least 100 conidia were measured. The results are the means of three independent experiments.

2.6. Fluorescence labelling of conidia

To assess whether 1-octen-3-ol affected membrane integrity, spore suspensions were incubated for 2 h with 4 mM of 1-octen-3-ol at 25 °C and stained with either TOTO or PI. A stock solution of 100 μl of TOTO ml^{-1} was prepared in dimethyl sulfoxide (DMSO) and a stock solution with PI (1 mg of PI ml^{-1}) was prepared in distilled water. Both solutions were stored at 4 °C in the refrigerator. To label conidia with TOTO, spore suspensions of 10^6 conidia ml^{-1} were incubated with 1 μM TOTO at 30 °C for 10 min and subsequently washed once with KP_i-buffer (pH 7.0). To label conidia with PI, spore suspensions of 10^6 conidia ml^{-1} were incubated with 10 μM PI at 30 °C for 10 min and subsequently washed once with KP_i pH 7.0. As controls, heat-killed conidia (90 °C for 10 min) and freshly harvested conidia were used. For analysis of internal pH, conidia were labelled with cFDA-SE (20 μM in 50 mM KP_i, buffer pH 7.0) for 1 h at 35 °C [18]. Subsequently, conidia were washed and the pellets were kept in 50 mM KP_i buffer, pH 7.0 with glucose (10 mM) for 30 min at 30 °C to eliminate non-conjugated cFSE. The efficiency of the fluorescent labelling of the spores was observed by upright Zeiss Axiophot fluorescence microscope 1000W Xenon monochrometer (VisiTech International Limited, Sunderland, UK).

2.7. Immobilisation of conidia and image analysis

The fluorescence ratio image microscopy (FRIM) system was equipped with an upright Zeiss Axiophot fluorescence microscope 1000W Xenon monochrometer. Conidia were suspended in 50 mM potassium citrate phosphate buffer pH 4.0 (CKP_i 4), and immobilised on glass slides as described earlier [18]. For this, glass slides were treated with a Fe (III) solution that resulted in adhesion of cells. Immobilised spores were studied inside a perfusion chamber through which solutions were pumped. Two solutions, MEB pH 4.0 and 1-octen-3-ol in MEB pH 4.0, diluted five times in distilled water, were used. Ratio imaging was initiated at time zero of perfusion of a solution through the chamber. The emission light was collected by Plan Neofluar (n.a. = 1.3) 100× oil objective, a 500–560 nm dichroic mirror, and a 515 nm emission bandpass filter. Emission was collected with an intensified CCD Camera (VisiTech International Limited, Sunderland, UK) and images were acquired by the image processing unit (X-Windows on an interactive UNIX system) equipped with the Quanticell 900 software package from Applied Imaging (Joyce Loebl Instruments, Sunderland, UK). The resolution of the images was 256×256 pixels during studies on germination and the effect of 1-octen-3-ol. The fluorescence intensity and the ratio values of the pixels in the images were expressed on a scale from 0 to 4096. The time interval between acquisition of images at 490 and 470 nm, adjusted at 20 nm bandwidth, was 5800 ms. The images were saved as Tiff files, which were imported to Photopaint to improve the images for contrast enhancement and Coreldraw 9 was used to process the images (Corel, Ontario, Canada). For analysis, micrographs of randomly selected labelled conidia were used using the computational drawing procedure on the computer screen.

2.8. Calibration of pH_{in}

To construct a calibration curve of labelled conidia with cFDA-SE, pH-dependent fluorescence was determined in vitro (in buffer) and inside the permeabilised conidia (in vivo) at pH values ranging from pH 4.0 to 9.0. For this, fluorescence intensity at 490 nm was divided by fluorescence intensity at 470 nm ($R_{490/470}$) for each pixel of a spore image. The buffers used were 50 mM potassium citrate phosphate buffer from pH 4.0 to 7.0 and 50 mM sodium borate from pH 8.0 to 9.0. For in vitro calibration, cFDA-SE (20 μM final concentration) was used in the different buffers that were adjusted to various pH values with NaOH. In order to equilibrate the pH of the buffer (pH_{ex}) and the interior of the spores (pH_{in}), ethanol (63%, v/v) was added to the labelled spores to permeabilise the membrane. This is designated as the in vivo treatment [19]. The suspension was incubated for 30 min at 30 °C and, after centrifugation, the spores were resuspended in buffer. In each experiment at least 13 spores were analysed with two repetitions. The calibration curve was fitted according to a four parameter sigmoid function $y = a + b/\{1 + \exp[-(x - c)/d\}$ and the parameters a–d were determined. The pH_{in} was calculated using the formula $pH_{in} = -[\ln((b - (\text{ratio} - a))/(\text{ratio} - a)) \times d] + c$.

2.9. Effect of 1-octen-3-ol on pH_{in}

The pH_{in} was analysed on labelled immobilised conidia in a perfusion chamber. The measurements started at time 0 of perfusion with 5-fold diluted MEB and after 20 min of perfusion 4 mM of 1-octen-3-ol in MEB was added. $Ratio_{(490/470)}$ imaging was initiated at time 0 of perfusion and the images were recorded with intervals of 10–20 min for a period of 120 and at 420 min.

2.10. Protein extraction

Conidial suspensions with a density of 9.0×10^6 conidia ml^{-1} were collected from six tubes of *P. paneum*, grown on MEA at 25 °C for 5 days, in a peptone physiological salt solution as described above. Freshly harvested conidia, conidia incubated for 5 h in MEB pH 4.0 and conidia incubated for 5 h in the presence of 4 mM of 1-octen-3-ol were used for protein extraction. Incubation was done at 25 °C, with rotation shaking at 140 rpm, and the spores were centrifuged at 4000*g* for 4 min. A volume of 15 µl of protease inhibitor was added to 0.2 g of the pellet (wet weight) resuspended in 1 ml^{-1} of potassium phosphate buffer, KP_i pH 7.0, after which 0.5 ml of conidia suspension was added to 0.8 g of zirconium beads (diameter, 1 mm; Biospec Products). Total cellular proteins were extracted from the conidial suspensions using a bead beater (B. Braun Biotech International, Melsungen, Germany), with four treatments of 3 min, after each homogenising treatment and the preparations were cooled on ice [20]. The preparations were centrifuged at 13,000*g* for 5 min at 4 °C and the supernatant was collected and centrifuged twice. The supernatant containing the cellular proteins was used for two-dimensional gel electrophoresis (2D-E). The protein content of the conidial extract was determined using the bicinchoninic acid assay (Sigma, St. Louis, MO) with bovine serum albumin as the standard and equal amounts of protein were applied on the gel.

2.11. Protein analysis by 2D-E

2D-E was performed as previously described [20] using a Pharmacia 2D-E system (Pharmacia Biotech, Uppsala, Sweden). Equivalent amounts of protein (30 µg of protein) were used for each gel. The cell free extract was treated with an equal volume of lysis solution (9 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 3–10 l [Pharmacia Biotech.], 2% Triton X-100, 6 mM Pefabloc SC [Merck, Darmstadt, Germany]). These preparations were kept on ice and one volume of sample solution (8 M urea, 2% 2-mercaptoethanol, 2% IPG buffer 3–10 l, 0.5% Triton X-100, a few grains of bromophenol blue) was added to the protein solution. The total volume was loaded on the acidic end of a first-dimension IEF gel with a pI range of 4–7 (Immobiline Dry Strips; Pharmacia Biotech). For the second-dimension, homogeneous sodium dodecyl sulphate (SDS) 12–14% polyacrylamide gels (PAGE) (ExcelGel; Pharmacia Biotech) were used. Two molecular weight markers were used, with band sizes of 97, 66, 45, 30, 20, and 14 kDa (Pharmacia Biotech, Sweden) and of 210, 125, 101, 56.2, 35.8, 29, 21.1, and 6.9 kDa (BioRad, Richmond, USA), respectively. The gels were silver-stained and analysed [21]. Representative gels obtained from triplicate experiments are shown below.

3. Results

3.1. Association of the self-inhibitor with the conidium

Spore suspensions with a concentration of 10^6 conidia ml^{-1} in MEB exhibited 100% germination after incubation for 22 h, whereas only 4 ± 1% germination was observed in the presence of 4 mM 1-octen-3-ol (data not shown). Spore suspensions under crowding conditions (10^9 conidia ml^{-1}) that were successively washed every 10 min in MEB (pH 4.0) only showed 1 ± 0.6% germination after 22 h. These data suggest that the self-inhibitor is continuously produced by the conidia and/or that it is very strongly associated with the conidia.

3.2. Effect of 1-octen-3-ol on respiration of conidia

The respiration of conidia at a concentration of 10^7 conidia ml^{-1} increased 39 ± 3% during a period of 2 h ranging from harvesting of the cells to the swelling stage. Freshly harvested conidia in the presence of 4 mM of 1-octen-3-ol exhibited a reduction in respiration of 12 ± 1% compared to conidia in MEB alone. The same experiment was done with spores at the swelling stage (2 h after harvesting) and a reduction of respiration of 5 ± 0% was observed in the presence of the inhibitor. These results indicate that spore respiration is slightly affected by 1-octen-3-ol.

3.3. Effect of 1-octen-3-ol on isotropic growth

The size of the conidia was measured at different stages of germination in the absence or presence of 1-octen-3-ol (Fig. 1). Untreated *P. paneum* conidia were less than 6 µm in size (average of all cells 4.4 ± 1.3 µm) at the beginning of the experiment. After incubation in MEB for 2 h, average cell size was 6.5 ± 1.8 µm, and the majority of the cells, namely 74 ± 9% of the conidia, had increased in size ranging from 6 to 9 µm. In the presence of 1-octen-3-ol for 2 h, the majority of the conidia, namely 78 ± 3% of the cell population, was smaller than 6 µm, with an average size of 5.5 ± 0.7 µm. When conidia were incubated for 4 h in MEB, 81 ± 9% of

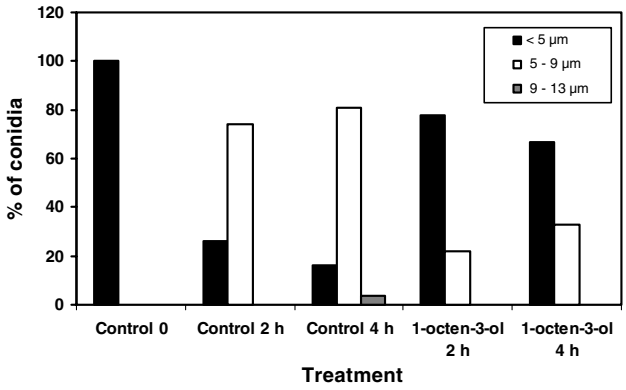

Fig. 1. Sizes of conidia (µm) of *P. paneum*; freshly harvested (control), after incubation for 2 and 4 h at 25 °C in MEB pH 4.0, and in the presence of 4 mM 1-octen-3-ol. Percentages of conidia with sizes of <6 µm (■), between 6 and 9 µm (□), and between 10 and 13 µm (▩) are shown.

the population was between 6 and 9 µm in size and 3.5 ± 2.1% had a size between 10 and 13 µm. In the presence of 1-octen-3-ol for 4 h, 67 ± 21% of cell population was smaller than 6 µm, with an average of 5.5 ± 1.3 µm in size. Conidia bigger than 7 µm in size were not observed at all in the presence of the inhibitor and germ tube formation was blocked (data not shown).

3.4. Assessment of membrane integrity

In order to determine if treatment with 4 mM 1-octen-3-ol for 2 h had an effect on membrane permeability, conidia were labelled with the fluorescent probes TOTO or PI. The results revealed that exposure to 1-octen-3-ol resulted in slight permeabilisation of the spore membrane. The number of fluorescent conidia, i.e., with increased membrane permeability as judged by labelling with TOTO, was 10 ± 5% in the presence of 1-octen-3-ol, 79 ± 14% in heated-treated (10 min, 90 °C) conidia and 5 ± 5% with freshly harvested conidia (Fig. 2). Staining with PI showed 20 ± 1% fluorescent conidia after treatment with 1-octen-3-ol, 94 ± 2% staining after heat-treatment and only 3 ± 2% was staining with untreated conidia. These results show that 1-octen-3-ol has a permeabilising effect on the conidial membrane. Notably, with 1-octen-3-ol not all conidia were affected and the staining efficiency was significantly lower than that of the heat treatment, although higher than untreated conidia.

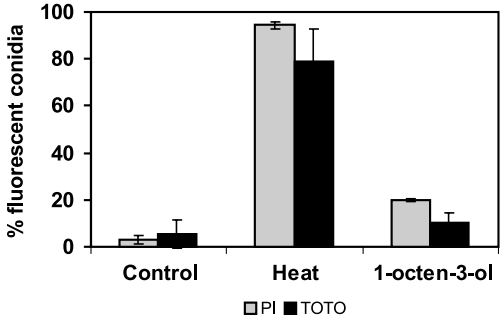

Fig. 2. Effect of 4 mM 1-octen-3-ol on fluorescent staining of *P. paneum* conidia. Conidia incubated in MEB, heat-treated at 90 °C for 10 min and exposed to 1-octen-3-ol for 2 h at 25 °C, were labelled with fluorescent probes. Percentage of fluorescent PI- (▩) and TOTO- (■) labelled conidia, monitored by epifluorescence microscopy.

3.5. 1-Octen-3-ol has a transient effect on the pH gradient

Calibration curves with fluorescence ratios of cFDA-SE at different pH values both in vitro and in vivo are shown in Fig. 3. There was a clear difference between $R_{490/470}$ values when the probe was in buffer alone (in

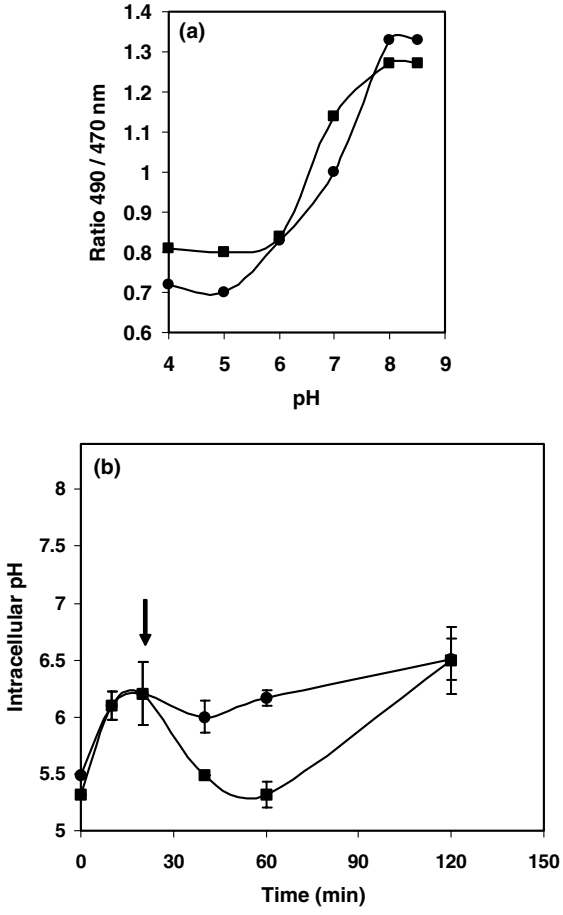

Fig. 3. (a) Relationship between the pH and the fluorescence ratio (490/470 nm) of cFSE in vitro (▩, buffer) and inside conidia (●). With conidia, pH_{in} and pH_{ex} were equilibrated by incubation with 63% (v/v) ethanol. The buffers used were 50 mM potassium citrate phosphate (pH 5.0–7.0) and 50 mM sodium borate (pH 8.0–8.5) (b) Immobilised cFDA-SE labelled conidia were perfused at 25 °C in MEB 5× diluted in distilled water pH 4.0. The pH_{in} of conidia of *P. paneum* in MEB (●), and in the presence of 4 mM 1-octen-3-ol (■) are shown. The arrow indicates the time at which perfusion with 1-octen-3-ol started. The ratio values are averages based on analysis of approximately 13 conidia and the experiment was performed in duplicate (b).

vitro) or inside conidia that were permeabilised with ethanol (in the literature designated as in vivo). The fluorescence ratio values of the probe inside permeabilised conidia were in most cases lower than values in buffer. Permeabilised conidia showed a larger range in which the internal pH could be measured, namely between pH 5 and pH 8 (Fig. 3(a)). The internal pH of viable conidia was calculated at different external pH values (6.0–8.5) and ranged from pH 6.4 to 7.0 (data not shown). The pH_{in} of *P. paneum* conidia was pH 5.4 ± 0.1 in diluted MEB at pH 4.0. During initial stages of germination an increase of the internal pH to 6.2 was observed within 20 min. Then, the internal pH increased only slightly to 6.5 during a period of 90 min (Fig. 3(b)). In the presence of 4 mM of 1-octen-3-ol added after 20 min of perfusion, the pH_{in} decreased from 6.2 to pH 5.3 within 40 min. Thereafter, a gradual increase in pH_{in} was observed in approximately 60 min finally reaching pH_{in} 6.2 again. This suggests that 1-octen-3-ol has a transient effect on the pH gradient, resulting in lowering of the pH_{in} of conidia during the germination phase.

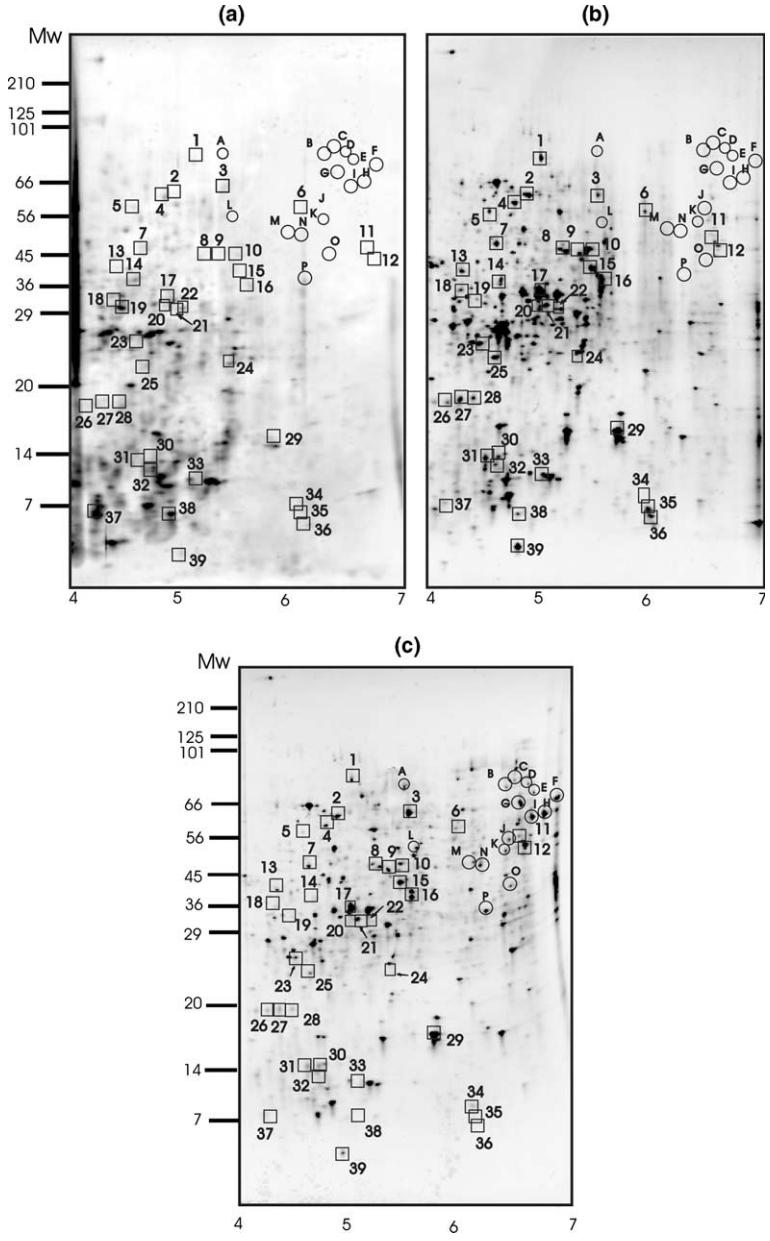

Fig. 4. Effect of 1-octen-3-ol on protein profiles of *P. paneum* conidia. Two-dimensional gel electrophoresis of cell free extracts of freshly harvested conidia (a), and germinated conidia incubated for 5 h at 25 °C (b), and conidia incubated in the presence of 4 mM 1-octen-3-ol at 25 °C for 5 h (c), using a pI ranging from 4 to 7.

3.6. Protein profiles of non-germinated, germinated and 1-octen-3-ol-treated conidia

Protein profiles of cell free extracts of non-germinated conidia, germinated conidia (5 h) and 1-octen-3-ol-treated-conidia (5 h) were analysed using two-dimensional gel-electrophoresis. For each treatment three very similar gels were studied and one representative gel (Fig. 4(a)) shows the protein profiles from freshly harvested non-germinated conidia. Protein profiles of germinated conidia incubated for 5 h in MEB are represented in Fig. 4(b) and proteins of conidia which were kept for 5 h in the presence of the self-inhibitor, 1-octen-3-ol are presented in Fig. 4(c). Conidia in the presence of 1-octen-3-ol showed reduced swelling and had not formed germ tubes, while 35% of conidia had germinated after incubation for 5 h in MEB in the absence of the inhibitor (data not shown). Selected proteins were labelled with numbers 1, 2, 3, etc., or letters, A, B, C, etc. Numbers indicate all proteins specific for untreated conidia, and letters are used for new proteins that appeared in treated conidia. Freshly harvested conidia showed many proteins of low molecular mass (the majority of the proteins were smaller than 40 kDa (Fig. 4(a)) and low pI (4.0–5.5). After incubation for 5 h in MEB many proteins appeared (Table 1, Fig. 4(b)). Proteins extracted from these conidia showed a broader pI range and more proteins with a higher molecular weight, with most proteins between 20 and 66 kDa (Fig. 4(b). Conidia maintained in the presence of 1-octen-3-ol showed a very different protein pattern, with the majority of proteins carrying a molecular weight between 20 and 90 kDa and a pI ranging from 4 to 7 (Fig. 4(c)). Comparisons were made among protein patterns under these three different conditions by marking specific proteins. Gels were analysed and substantial differences in protein profiles are listed in Table 1. Eight proteins (18, 20, 22, 33, 34, 35, 36, and 38) that were not present in fresh conidia appeared in conidia during germination (including swelling and germ tube formation), while they were not found in conidia treated with 1-octen-3-ol. Another 26 proteins were observed in both germinated and 1-octen-3-ol-treated conidia, although their levels were different in treated conidia, 23 decreasing and 3 proteins showing increased levels in treated conidia (Table 1). Five proteins (14, 19, 24, 32, and 37) that were present in fresh conidia and in conidia during germination were not found in treated conidia. Notably, a group of 16 proteins was induced in the presence of 1-octen-3-ol (designated as A–P). All these proteins clustered together in an area with a relative high molecular weight and pI.

Table 1
Comparison of proteins patterns among non-germinated, germinated conidia in MEB and 1-octen-3-ol-treated conidia incubated 5 h at 25 °C

Proteins	Non-germinated spores	Germinated spores	
		5 h	5 h with 1-octen-3-ol
18, 20, 22, 33, 34, 35, 36, 38	–	+	–
A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P	–	–	+
14, 19, 24, 32, 37	+	+	–
1, 2, 4, 5, 6, 7, 8, 9, 10, 13, 15, 16, 17, 21, 23, 25, 26, 27, 28, 29, 30, 31, 39	–	+	+[a]
3, 11, 12	–	+	+[b]

[a] Proteins that appeared at lower levels in treated conidia.
[b] Proteins that appeared at higher levels in treated conidia.

4. Discussion

A wide range of fungi exhibit the so-called crowding effect, which is the phenomenon that germination is inhibited at high spore concentrations. They range from the class of Zygomycetes (*Rhizopus*) to Ascomycetes, including the genera *Aspergillus* and *Penicillium*. However, information about the physiological state of the spores during crowding effects is scarce. In the present study, we investigated different possible target sites of the self-inhibitor 1-octen-3-ol produced by *P. paneum*. At a low concentration this compound inhibits germ tube formation on conidia and also the size of the spores is equivalent to untreated spores, which confirms that this compound is active during early stages of germination.

A slight inhibitory effect of 1-octen-3-ol on oxygen consumption was observed in freshly harvested and swollen conidia. A stronger effect was observed in the case of an unidentified self-inhibitor of *Geotrichum candidum* arthrospores in suspensions with 10^7–10^8 spores ml^{-1}, and here oxygen consumption decreased 12-fold [22].

Two different membrane impermeable fluorescent DNA-binding dyes, TOTO and PI, were tested to evaluate membrane permeability in the presence of 1-octen-3-ol. Both were able to stain 10–20% of conidia-treated with 1-octen-3-ol, 79–90% of heated killed conidia and 3–5% of untreated conidia. These data indicate a small membrane permeabilising effect of the compound. TOTO has also been used as an indicator of viability of *Listeria monocytogenes* cells [23]. PI has been used as an indicator of cell death of *Bifidobacterium* cells during bile salt stress [24], for assessment of viability of fungal spores in combination with flow cytometry [25] and for assessment of membrane integrity of *Oenococcus oeni* cells after exposure to ethanol [26]. Very recent data in fungal spores of *Rhizopus oligosporus* have shown that TOTO is even a more reliable predictor of cell death than PI [27].

Exposure to 1-octen-3-ol was also observed to affect the intracellular pH of germinating conidia. At the onset of germination *P. paneum* conidia had an intracellular pH of 5.4. During the first stage of germination (swelling) an increase in intracellular pH to 6.2 was observed. When 1-octen-3-ol was added, the internal pH of the spore dropped to pH 5.4, but subsequently the pH_{in} was restored to 6.2 within 60 min at pH_{ex} of 4.0. This recovery of pH_{in} indicated that 1-octen-3-ol treated-conidia had sufficient energy to pump out H^+ of the cell and that ATPase activity was not affected. *Fusarium culmorum* incubated with nonanoic acid (1 mM) was shown to display similar behaviour, the intracellular pH fluctuating between 5.4 and 6.5 during a period of 90 min at an extracellular pH of 4.0 (Chitarra et al., unpublished results). Sporangiospores of *Rhizopus oligospores* exposed to sublethal doses of nonanoic acid (1 mM) show a similar restoration phenomenon [28].

Since, the function of the self-inhibitor is to prevent premature germination of the spore and it acts during situations of high cell density it is vital that this compound inhibits germination in a reversible manner. When the compound is removed from the cell or is degraded its inhibitory effect is alleviated and germination is initiated [29]. Our results indicate that several effects of 1-octen-3-ol on conidia are mild and transient, which is a prerequisite for its action as a self-inhibitor.

Analysis of protein patterns, however, revealed striking differences between freshly harvested conidia, germinated conidia and 1-octen-3-ol-treated conidia. Although the treated conidia had not formed germ tubes, this protein pattern was very different from that of fresh conidia. Eight proteins that were absent from the non-germinated conidia were present during germination (swelling and germ tube) yet were not found in conidia exposed to 1-octen-3-ol. Presumably, these proteins play a role in isotropic growth or germ tube formation, which is inhibited or blocked upon exposure to 1-octen-3-ol, respectively. Sixteen proteins were unique for 1-octen-3-ol-treated conidia. The nature of the proteins whose levels were affected or that were unique in 1-octen-3-ol treated-conidia is unknown. Identification of these proteins may add further to our knowledge of the action of 1-octen-3-ol, or the possibility of the proteins to be interrelated in the mechanisms of the germination, such as the involvement in the glyoxylate cycle. In *Colletotrichum graminicola*, physiological and biochemical studies have been done with mycosporines, which may be acting on the spore via an effect on the glyoxilate cycle, which plays an important role in the conversion of lipids into carbohydrates [30]. It was reported that the cytoplasm of ungerminated conidia was filled with lipid droplets that disappeared in association with the enlargement of vacuoles during germination [31].

Taken together, these results suggest that 1-octen-3-ol has a mild systemic effect on the developing conidial cell, with large consequences for its protein composition. This shows that 1-octen-3-ol has an important effect on the expression of sets of proteins and as such is a factor that is involved in development of cells, as hormones do. The mode of action of self-inhibitors of rust fungi such as *Uromyces phaseoli* and *Puccinia graminis*, namely methylcisferulate and methyl 3,4 dimethoxycinnamate, may be entirely different and focused on one cell feature namely to prevent digestion of the pore plug where germination begins and therefore blocking the release of the germ tube from the urediospores [32].

We must realise that the crowding effect in solutions is not the same as self-inhibition of conidia that are present in chains on conidiophores in the fungal culture. The effect of 1-octen-3-ol under these conditions may be different, while the cells are not present in a complex medium as malt extract. Additional effects of 1-octen-3-ol are observed on fungal structures as microcycle conidiation [7], the formation of a new conidium on a germ tube and it has an effect on vegetative growth of hyphae [7]. This observations done in our study provide more evidence that 1-octen-3-ol is a compound that acts as a fungal hormone, while it has a large effect on the expression of a set of proteins in fungal cells. Earlier studies have indicated that the co-product of 1-octen-3-ol during linoleic acid degradation, ODA, has effects on fungal development in *Agaricus bisporus* [33,34] and linoleic acid derivatives play a role in sporulation phenomena in *Emericella nidulans* [35]. Hormone-like factors may also play a role in the regulation of the development of filamentous bacteria, for example, A-factor control of secondary metabolism and cellular differentiation in *Streptomyces griseus* [36].

Detailed insight into the fungal germination process and the action of self-inhibitory substances may contribute to more efficient control of fungal infection and food spoilage. 1-Octen-3-ol has a broad spectrum of action against different genera of fungi [7], but is already an important flavour compound in mushrooms. This indicates that the toxicity of the compound is relatively low.

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