Chitosan permeabilizes the plasma membrane and kills cells of *Neurospora crassa* in an energy dependent manner

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**A B S T R A C T**

Chitosan has been reported to inhibit spore germination and mycelial growth in plant pathogens, but its mode of antifungal action is poorly understood. Following chitosan treatment, we characterized plasma membrane permeabilization, and cell death and lysis in the experimental model, *Neurospora crassa*. Rhodamine-labeled chitosan was used to show that chitosan is internalized by fungal cells. Cell viability stains and the calcium reporter, aequorin, were used to monitor plasma membrane permeabilization and cell death. Chitosan permeabilization of the fungal plasma membrane and its uptake into fungal cells was found to be energy dependent but not to involve endocytosis. Different cell types (conidia, germ tubes and vegetative hyphae) exhibited differential sensitivity to chitosan with ungerminated conidia being the most sensitive.

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1. Introduction

Chitosan is a polymer of β-1,4-glucosamine subunits, and is a partly deacetylated form of chitin (Rabea et al., 2003). It is not toxic to mammals (Dodane and Vilivalam, 1998; Lee et al., 2004) and elicits plant defense mechanisms (Ait Barka et al., 2004; Benhamou et al., 1994; Lafontaine and Benhamou, 1996; Trotel-Aziz et al., 2006). Chitosan displays antibiotic activity against bacteria (Liu et al., 2001, 2004; Tikhonov et al., 2006) and fungi (Bautista-Banos et al., 2006; Bell et al., 1998; Laflamme et al., 1999; Palma-Guerrero et al., 2008; Park et al., 2002; Plascencia-Jatomea et al., 2003), and has been reported to damage the plasma membranes of both bacteria (Liu et al., 2004) and the yeast *Saccharomyces cerevisiae* (Zakrzewska et al., 2005). The deletion of genes encoding proteins that are involved in maintaining plasma membrane integrity was found to increase the sensitivity to chitosan (Zakrzewska et al., 2007). Plasma membrane damage has also been suggested to explain the fungicidal effects of chitosan on filamentous fungi (El Ghaouth et al., 1992; Laflamme et al., 1999). The permeabilization of the plasma membrane by chitosan has been proposed to be caused by the interaction of the positive amino groups of chitosan with the negative charges on phospholipids (Liu et al., 2004). Recently, rhodamine-labeled chitosan was found to be taken up by conidia of plant pathogenic and nematophagous fungi in an energy dependent manner and not by passive diffusion (Palma-Guerrero et al., 2008). Previously, the endocytic marker FM4-64 had been shown to be internalized by conidia of *Magnaporthe grisea* in an energy dependent manner (Atkinson et al., 2002), which suggests that chitosan may also be endocytically internalized.

The aim of the present study was first, to test the hypothesis that chitosan is endocytically internalized by fungal cells, and second, to obtain further insights into the mode of antifungal action by chitosan. For this purpose we used *Neurospora crassa*, a species in which endocytosis has been previously analyzed (Fischer-Parton et al., 2000; Read and Hickey, 2001; Read and Kalkman, 2003) and which is particularly amenable to live-cell analysis using confocal microscopy (e.g. Hickey et al., 2005). Our results show that chitosan enters and kills cells after plasma membrane permeabilization by an unknown energy dependent mechanism that does not involve endocytosis. We have also found that conidia, germ tubes, and vegetative hyphae exhibit differential sensitivity to the fungicidal effects of chitosan.

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2. Materials and methods

2.1. Strains and culture conditions

The *N. crassa* wild-type strain 74-OR23-IVA (FGSC #2489), and the *N. crassa* H1-GFP strain (N2283, Freitag et al., 2004) were grown and maintained on solid Vogel's agar medium (Davis, 2000). A transformed strain of *N. crassa* expressing codon-optimized aequorin (strain #272, Fungal Cell Biology Group, University of Edinburgh, UK; Nelson et al., 2004; Zelter, 2004) was stored as a stock culture on solid Vogel's agar medium containing 200 μg ml⁻¹ hygromycin B, but was grown on solid Vogel's medium lacking hygromycin B for the production of the conidia used in luminometry experiments.

2.2. Chemicals and dyes

Chitosan (T8s) with a molecular weight of 70 KDa and exhibiting 79.6% deacetylation was obtained from Marine BioProducts GmbH (Bremerhaven, Germany). Chitosan was dissolved in 0.25 mol l⁻¹ HCl and the pH adjusted to 5.6 with 1 mol l⁻¹ NaOH. The resulting solution was dialyzed for salt removal, and the dialyzed chitosan was autoclaved at 120 °C for 20 min (Palma-Guerrero et al., 2008).

Rhodamine-labeled chitosan (rhodamine-T8s chitosan) was kindly provided by Dr. V. Tikhonov (Laboratory of Physiologically Active Biopolymers, A.N. Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, Moscow, Russia). After dialysis, the labeled chitosan was filtered through 3 KDa ultra-membranes in an Amicon Cell (Omega, Pall Corporation, Ann Arbor, MI, USA) and sterilized by filtration through a 0.2 μm pore-size syringe filter (Albet, Barcelona, Spain).

Sodium azide and the cell viability dyes, fluorescein diacetate (FDA) and propidium iodide (PI), were obtained from Sigma (St. Louis, MO, USA). The membrane selective fluorescent dye, FM4-64, was obtained from Invitrogen (Eugene, OR, USA). Stock solutions of FDA (1 mg ml⁻¹ in acetone), PI (1 mg ml⁻¹ in H₂O) and FM4-64 (10 mg ml⁻¹ in DMSO) were all diluted in distilled H₂O and then added to the conidial inocula at final concentrations of 5 μg ml⁻¹, 5 μg ml⁻¹, and 5 μM, respectively.

2.3. Quantitation of conidial germination, conidial lysis and CAT fusion

Conidia were collected from 4 to 5 day old wild type cultures by adding 1 ml sterile distilled water and removing the conidial suspension with a pipette. The conidia were counted using a hemocytometer, diluted with a haemocytometer, and immediately used in the bioassays. Conidial germination, conidial lysis and conidial anastomosis tube (CAT) fusion assays were carried out in 8-well slide culture chambers (Nalge Nunc International, Rochester, NY). Each well was filled with 200 μl of conidial suspension at a final concentration of 10⁶ conidia ml⁻¹ in potato dextrose broth (PDB) (Becton Dickinson and Company, Sparks, MD, USA) with chitosan at the appropriate final concentration. PDB (2.5 g l⁻¹) was used for the conidial germination and conidial lysis assays. PDB was replaced with liquid Vogel's medium (Davis, 2000) diluted 100 times for the CAT fusion assay. After incubation at 24 °C in continuous light, the number of non-lysed conidia were counted (for conidial lysis assays) or the percentage conidia that had not lysed and had germinated were quantified (for conidial germination assays). Percentage germination was defined as the percentage of conidia possessing one or more germ tubes and/or CATs. A 20× dry or a 60× water immersion plan apo objective with differential interference contrast (DIC) optics on an inverted TE2000E microscope (Nikon, Kingston-Upon-Thames, United Kingdom) was used in the germination and lysis assays. CAT fusion was only assessed with the 60× objective on the inverted microscope and CAT fusion was quantified as the percentage of conidia/conidial germlings involved in fusion. Three wells per treatment, with ten fields of view per well (each containing 100–300 conidia), were assessed for each of the three experimental assays, and each experiment was performed twice.

2.4. Monitoring rhodamine-chitosan internalization

Conidia were collected as described in Section 2.3 and were treated with 0.1 mg ml⁻¹ rhodamine-chitosan for different times at room temperature (~22 °C). The conidia were washed three times with distilled water by centrifugation at 19,300g for 5 min to remove unbound rhodamine-chitosan. The resultant conidial suspension was then dispensed as 30–50 μl droplets onto glass coverslips, and conidia were immobilized by the inverted agar block method (Hickey et al., 2005) and imaged by confocal microscopy (Section 2.8) with a 100× oil immersion objective.

2.5. Staining with fluorescent probes

Staining of conidia with FM4-64 or PI was carried out by adding FM4-64 (at a final concentration of 5 μM) or PI (at a final concentration of 5 μg ml⁻¹) to a conidial suspension prepared as described in Section 2.3. For the chitosan treatment, the conidia (to which FM4-64 or PI had been added) were treated with chitosan at a final concentration of 0.1 mg ml⁻¹. The conidial suspensions were inoculated, immobilized and imaged as described in Section 2.4.

2.6. Cell viability tests

A combination of FDA and PI was used to check cell viability after conidia and conidial germlings were treated with chitosan. Wells of the 8-welled culture chambers (Section 2.3) were filled with 100 μl of conidial suspension at a final concentration (after dye addition) of 10⁶ conidia ml⁻¹. FDA and PI were added to the conidial suspension at a final concentration of 5 μg ml⁻¹. Once the culture chamber was mounted on the confocal microscope stage, 100 μl of chitosan was added at a final concentration of 0.1 mg ml⁻¹. Samples were immediately imaged using a 40× dry or a 60× water immersion objective, and images were captured every 30 s for 12 min. For the germling viability tests, 100 μl of conidial suspension in 2.5 g l⁻¹ PDB was first incubated in each well of the 8-welled culture chamber for 6 h at 24 °C in continuous light. FDA, PI and chitosan were added as described in Section 2.5. Ungerminated conidia and 6 h old conidial germlings were imaged by confocal microscopy (Section 2.8) with a 20× dry or a 60× water immersion plan apo objective, and an image was captured every min for 1 h. The percentages of live and dead conidia or germlings were recorded at each time point and the percentage of live cells was determined. Three wells per treatment were recorded (100–300 conidia per field of view, 10 fields of view per well) and the experiment was performed twice.

FM4-64 was used to check the viability of vegetative hyphae after chitosan treatment. The wells containing 100 μl conidial suspension at a final concentration of 10⁶ conidia ml⁻¹ in 2.5 g l⁻¹ PDB were incubated for 18 h at 24 °C in continuous light. FM4-64 was added to the vegetative hyphae at a final concentration of 5 μM. Chitosan was added at a final concentration of 0.1 mg ml⁻¹ and samples were immediately imaged by confocal microscopy (Section 2.8) with a 20× objective, and an image captured every min for 1 h.

2.7. Inhibition of energy dependent processes

Sodium azide and low temperature (4 °C) were used to inhibit ATP production and thus energy dependent processes (Atkinson et al., 2002; Hoffmann and Mendgen, 1998). Conidia or conidial germlings
were either pre-treated for 30 min with 75 mM sodium azide or kept at 4 °C for 30 min, before treating them with rhodamine-chitosan, chitosan, the fluorescent dyes, or a combination of chitosan and the dyes. Pre-treatment with 70% ethanol for 30 min was used to kill conidia to assess rhodamine-chitosan uptake by dead cells.

2.8. Confocal microscopy

Confocal laser scanning microscopy was performed using a Radiance 2100 system, equipped with argon ion and green HeNe lasers (Bio-Rad Microscience), which was mounted on a Nikon TE2000U Eclipse inverted microscope. Rhodamine-chitosan was imaged with excitation at 543 nm and fluorescence detection at >560 nm. FM4-64 was imaged by excitation at 488 nm and fluorescence detection at >560 nm. When rhodamine-chitosan and FM4-64 were combined and imaged simultaneously, excitation was at 543 nm and fluorescence detection at 560–620 nm (for rhodamine-chitosan) and >650 nm (for FM4-64). FDA and PI were detected simultaneously using excitation at 488 nm and detection at 500–530 nm (FDA) and >560 nm (PI). GFP and PI were detected simultaneously with the same filter settings used for FDA and PI. The laser intensity and laser scanning were reduced to a minimum to reduce phototoxic effects. Simultaneous DIC images were captured with a transmitted light detector. Kalman filtering (n = 1 or n = 2) was used to improve the signal-to-noise ratio of images. Images were captured with a laser scan speed of 166 lines per sec at a resolution of 512 by 512 pixels. Confocal images were captured with Lasersharp 2000 software (version 5.1; Bio-Rad Microscience) and processed using ImageJ (version 1.38x for Mac Os; freeware) and Adobe Photoshop (version 10.0; Adobe Systems, Inc.) software.

2.9. Luminoimetry

10⁶ conidia ml⁻¹ of the strain expressing codon-optimized aequorin (Nelson et al., 2004; Zelter, 2004) were incubated in 2.5 g l⁻¹ PDB medium with 0.68 mM CaCl₂ (Sigma, St. Louis, MO, USA) and 10 µM coelenterazine (Biosynth, Staat, Switzerland) added. The coelenterazine was initially dissolved in methanol and subsequently diluted in 2.5 g l⁻¹ PDB (Nelson et al., 2004). 90 µl of the conidial suspension was dispensed into each of the individual flat-bottomed wells of a white 96-well plate (Fisher scientific, Loughborough, Leicestershire, UK). Each plate was covered with aluminum foil and incubated: (a) at 4 °C for 6 h in the dark (for the analysis of ungerminated conidia), (b) at 24 °C in the dark for 6 h (for the analysis of conidial germlings), or (c) at 24 °C in the dark for 18 h (for the analysis of vegetative hyphae). Luminoimetry was performed using a Berthold LB96P plate luminometer equipped with two injectors. In some experiments, 100 µl chitosan, diluted to 0.2 mg ml⁻¹ in 2.5 g l⁻¹ PDB with 0.68 mM CaCl₂, was injected into wells containing the fungal material (the final concentration of chitosan was 0.1 mg ml⁻¹). Control injections omitted the chitosan. In other experiments, 10 µl sodium azide (at a final concentration of 75 mM) was pipetted into the wells containing fungal material 30 min before the injection of the chitosan. Controls for these experiments involved adding 10 µl of distilled H₂O per well. Measurements from six wells for each treatment were performed in each experiment, and each experiment was performed three times.

3. Results

3.1. Chitosan inhibits conidial germination and conidial germling fusion

To assess the effects of different concentrations of chitosan on conidial germination and conidial germling fusion via conidial anastomosis tubes (CATs) (Roca et al., 2005; Read et al., in press), liquid Vogel’s medium (the standard growth medium used for N. crassa, Davis, 2000), was initially used. However, the addition of chitosan was found to cause visible precipitation in Vogel’s medium when the medium was used at its normal concentration. An alternative medium, potato dextrose broth (PDB), in which chitosan-induced precipitation was not evident, was used. After 6 h at 24 °C in PDB 2.5 g l⁻¹, 98.8% (sD ± 0.1) of untreated conidia had germinated (Fig. 1A). Conidial germination was completely inhibited at chitosan concentrations of 0.01 or 0.1 mg ml⁻¹ and none of these conidia germinated after 24 h. After 6 h, conidia treated with 0.01 or 0.1 mg ml⁻¹ chitosan were shrunken and highly vacuolated (Fig. 1B). Treatment with lower concentrations of chitosan showed that the 50% inhibitory concentration (IC₅₀) of chitosan for conidial germination in PDB was ~7.5 µg ml⁻¹.

Unexpectedly we discovered that CAT fusion was greatly inhibited in PDB and only 4.0% sD ± 1.26 of conidial germings went on to fuse. To assess the effects of chitosan on CAT fusion we diluted Vogel’s medium 100 times to a concentration at which no signs of chitosan precipitation were observed (not shown). After 6 h at 24 °C in 100 x-diluted Vogel’s medium, 95.0% (sD ± 0.3) of untreated conidia had germinated (produced a germ tube and/or a CAT) and 48.0% (sD ± 1.2) of conidial germings were involved in CAT fusion (Fig. 1C). The IC₅₀ values of chitosan for conidial germination and CAT fusion in this medium were both ~0.25 µg ml⁻¹ and thus neither process was more sensitive to chitosan than the other.

3.2. Rhodamine-labeled chitosan uptake is energy dependent but not by endocytosis

To monitor the internalization of chitosan by living cells, chitosan fluorescently labeled with rhodamine was used. Rhodamine-chitosan was detected inside conidia within 30 min of addition (Fig. 2A). Treatment of conidia of M. grisea with the mitochondrial ATP synthetase inhibitor sodium azide, or incubation at low temperature (4 °C), were previously shown to inhibit energy dependent internalization of endocytic marker dyes (Atkinson et al., 2002). Both treatments were found to inhibit the uptake of rhodamine-chitosan by conidia of N. crassa. Consequently, rhodamine-chitosan fluorescence was only detected bound to what seemed to be the conidial cell wall (Fig. 2B and C). However, the localization of rhodamine-chitosan in the putative cell wall was non-uniform around conidia (Figs. 2A-C, and 3B). Conidia that had taken up rhodamine-chitosan looked unhealthy because their cytoplasm had a ‘granular’ and partially vacuolated appearance when imaged with DIC optics (Fig. 2A). This contrasted with untreated conidia in which the cytoplasm had a ‘smoother’ appearance (Fig. 2D). The conidia that had been subjected to the inhibitory treatments all had a similar healthy appearance to the untreated conidia (Fig. 2B-D). When rhodamine-chitosan was applied to conidia that had been killed with 70% ethanol, fluorescence was detected within the conidia, and the DIC image shows that the cytoplasm of the killed cells was granular, similar to rhodamine-chitosan-treated conidia (Fig. 2E). The pattern of rhodamine-chitosan localization in the killed conidia (Fig. 2E) was different from that in the untreated living conidia (Fig. 2A).

To determine if rhodamine-chitosan was entering cells by endocytosis, its uptake was compared with that of the endocytic marker FM4-64 (Fischer-Parton et al., 2000; Hickey et al., 2005). Rhodamine-chitosan and FM4-64 showed markedly different patterns of internalization. Five minutes after applying FM4-64, fluorescence was detected in the conidial plasma membrane and in small endosome-like structures. Between 15 and 60 min more organelles, including larger ones, became increasingly stained (Fig. 3A). This staining pattern is similar to the sequential staining of

Fig. 1. (A) Conidial germlings in 0.25% PDB after 6 h at 24 °C in continuous light. Untreated control without chitosan. Note that although a number of germ tubes are touching each other, none of the conidial germlings have undergone fusion. (B) Dead conidia after treatment with 0.1 mg ml⁻¹ chitosan in 0.25% PDB for 6 h at 24 °C in continuous light. (C) Conidial germlings in 0.01% Vogel’s medium after 6 h at 24 °C in continuous light. Note that a number of conidial germlings have fused (asterisks) by means of conidial anastomosis tubes. Bar = 10 μm.

Fig. 2. Conidia treated with 0.1 mg ml⁻¹ rhodamine-chitosan for 30 min. Fluorescence images on the left and DIC images of same conidia on the right. (A) Conidia without any pre-treatment. (B) Conidia pre-treated with 75 mM sodium azide for 30 min. (C) Conidia pre-treated at 4 °C for 30 min. (D) Conidia to which rhodamine-chitosan has not been added. (E) Dead conidia after treatment with 70% ethanol for 30 min. Note that rhodamine-chitosan has not been taken up by conidia after pre-treatments that inhibit ATP production (B and C). Bar = 5 μm.
3.3. Chitosan permeabilizes cells in an ATP-dependent manner

The non-specific localization of rhodamine labeled chitosan within conidia (Fig. 3B) suggests that chitosan itself permeabilizes the plasma membrane allowing it to enter the cytoplasm. To confirm this, experiments were performed with unlabeled chitosan in combination with the endocytic marker dye, FM4-64 (Fischer-Parton et al., 2000; Hickey et al., 2005). FM4-64 uptake by conidia was initially shown to be energy dependent by being blocked by sodium azide treatment (Fig. 4C) or incubation at 4 °C (Fig. 4E), as previously reported for conidia of M. grisea (Atkinson et al., 2002). Treatment with unlabeled chitosan for 30 min, however, produced a different pattern of FM4-64 staining (Fig. 4B) compared with the untreated control (Fig. 4A). FM4-64 staining after chitosan treatment was much more intense suggesting that the dye had diffused into the cells and bound non-selectively to membranous organelles. This pattern of FM4-64 staining has been described previously in permeabilized fungal cells (Read and Roca, 2006). Sodium azide treatment or incubation at 4 °C prevented FM4-64 uptake by chitosan-treated-conidia (Fig. 4D and F) indicating that chitosan induced permeabilization of the plasma membrane is ATP dependent.

3.4. Chitosan induces rapid Ca$^{2+}$ uptake by conidia and conidial germlings

N. crassa, like other eukaryotic cells, normally maintains its cytosolic free Ca$^{2+}$ ([Ca$^{2+}$]$_{i}$) concentration at a very low level (typically 50–100 nM) (Miller et al., 1990). This contrasts markedly with the extracellular free Ca$^{2+}$ concentration which, in the media used in the experiments, is several orders of magnitude higher. Eukaryotic cells are able to maintain an extremely steep free Ca$^{2+}$ gradient across the plasma membrane by means of a tightly regulated [Ca$^{2+}$]$_{i}$ homeostatic mechanism involving Ca$^{2+}$ being actively transported up the Ca$^{2+}$ gradient out of the cytosol either into the extracellular medium and/or into intracellular Ca$^{2+}$ storage organelles. Permeabilization of the plasma membrane can dramatically upset [Ca$^{2+}$]$_{i}$ homeostasis in the cell resulting in the [Ca$^{2+}$]$_{i}$ increasing. To analyze the influence of chitosan on membrane permeabilization we measured changes in luminescence in a transformant expressing the bioluminescent [Ca$^{2+}$]$_{i}$ reporter, aequorin (Nelson et al., 2004; Zelter, 2004). However, in these experiments we did not convert the aequorin luminescence into [Ca$^{2+}$]$_{i}$ concentration because the extremely high levels of aequorin light emission following treatment with 0.1 mg ml$^{-1}$ chitosan reflected increases in [Ca$^{2+}$]$_{i}$ concentration (>10 μM) that were too high to measure with normal aequorin (Cobbold and Lee, 1991).

Previously it has been found that ungerminated conidia of N. crassa contain much lower levels of aequorin compared with 6 h old germlings and 18 h old vegetative hyphae (Marris, 2007). This is the reason why the amount of light emission (measured in relative light units [RLUs]) is so low in Fig. 5A compared with Fig. 5B and C. A common and easily applied stimulus to fungal cells is an individual well of a multiwell inside the luminometer is mechanical perturbation. This involves injecting onto the cells in each well 100 μl of growth medium which is iso-osmotic to the medium that the cells are incubated in (Nelson et al., 2004). When the conidial germlings and vegetative hyphae were stimulated in this way, a small transient increase in aequorin luminescence (amplitude = 1400–2200 RLUs) which reached its maximum amplitude within 2 s of stimulation (Fig. 5B and C). In contrast, no significant change in luminescence, and thus no response, was observed when the ungerminated conidia were subjected to mechanical perturbation (Fig. 5A). The transient increase in [Ca$^{2+}$]$_{i}$ in conidial germlings and vegetative hyphae (Fig. 5B and C) will be part of the response when injecting the chitosan solution onto these cells.

![Fig. 3](image-url). Comparison of a time course of staining with 5 μM FM4-64 (A) with the uptake of 0.1 mg ml$^{-1}$ rhodamine-chitosan uptake (B) by conidia. Note that FM4-64 and rhodamine-chitosan exhibit different patterns of localization within the conidia. Bar = 5 μm.
Treating conidia and conidial germlings with 0.1 mg ml\(^{-1}\) chitosan resulted in very large transient increases in aequorin luminescence (Fig. 5A and B). With the vegetative hyphae, however, a very different result was obtained. In this case, there was an initial transient increase in aequorin luminescence (probably mainly a result of mechanical stimulation) which was followed by a much longer transient increase in aequorin luminescence (Fig. 5C). These results indicate that chitosan significantly permeabilizes the plasma membranes of both conidia and conidial germlings causing rapid Ca\(^{2+}\) uptake but that the permeabilization of vegetative hyphae occurs much more slowly and to a lesser extent.

Pre-treating all three cell types with 75 mM sodium azide in each case resulted in very significant inhibition of the increases in aequorin luminescence following treatment with 0.1 mg ml\(^{-1}\) chitosan (Fig. 5A–C). With ungerminated conidia, no significant increase in luminescence could be detected following azide treatment (Fig. 5A). With conidial germlings and vegetative hyphae treated with sodium azide, however, there was an initial transient increase in aequorin luminescence (again probably mainly a result of mechanical stimulation), which was followed by a much longer transient increase in aequorin luminescence. This longer luminescence transient had a larger maximum amplitude in conidial germlings compared with vegetative hyphae. However, direct comparison of the luminescence measured with the different cell types was not possible because the amount of aequorin synthesized per unit weight of the conidial germlings and vegetative hyphae was not determined. These results indicate that: (a) the rapid Ca\(^{2+}\) uptake in conidial germlings resulting from chitosan injection is mostly ATP dependent but that azide induces a secondary increase in Ca\(^{2+}\) uptake that is not ATP dependent and (b) the longer luminescent transient induced by chitosan in vegetative hyphae is mostly ATP dependent.

3.5. The rate of cell death is cell type specific

A combination of fluorescein diacetate (FDA) and propidium iodide (PI) was used to assess cell viability after applying chitosan. FDA is taken up by living cells and is hydrolyzed to fluorescein, which fluoresces green. PI is taken up by dead cells with damaged plasma membranes and fluoresces red (Oparka and Read, 1994; Hickey et al., 2005). By simultaneously imaging cells in the presence of FDA and PI after applying chitosan it was possible to assess which cells were living (green) and which were dead (red), and determine the time required by chitosan to kill these cells (Figs. 6–8).

Four minutes after applying 0.1 mg ml\(^{-1}\) chitosan to a conidial suspension, 99.15% of the conidia were dead (Fig. 8). Most of the...
Fig. 5. Luminometry of strain expressing recombinant aequorin subjected to mechanical stimulation alone (control), treatment with 0.1 mg ml⁻¹ chitosan on its own, and treatment with 0.1 mg ml⁻¹ chitosan after pre-treatment for 30 min with 75 mM sodium azide. (A) Conidia. (B) 6 h old conidial germlings. (C) 18 h old vegetative hyphae. Values ± standard errors. Arrows indicate the points in time when mechanical perturbation by injection was applied.

Fig. 6. Chitosan-induced cell death at different times following treatment with 0.1 mg ml⁻¹ chitosan. Living cells stained green with 5 μg ml⁻¹ FDA and dead cells stained red with 5 μg ml⁻¹ PI. Fluorescence images on the left and DIC images of same conidia on the right. (A) Conidia after 1 min. (B) Conidia after 3 min. (C) Conidia after 7 min. (D) Conidial germlings after 1 min. (E) Conidial germlings after 35 min. (F) Conidial germlings after 50 min. Bar = 5 μm for (A–C). Bar = 10 μm for (D–F).
conidia died between 1.5 and 4 min (Figs. 6A–C, 7B and 8A). This pattern of cell death was completely inhibited after sodium azide pre-treatment (Fig. 8). When 0.1 mg ml\(^{-1}\) chitosan was applied to conidial germlings most of them died between 35 and 45 min (Figs. 6D–F and 8B). Cell death was inhibited by sodium azide pre-treatment, but not completely (Fig. 8B).

Problems and inconsistent results were encountered when using FDA and PI with vegetative hyphae. We therefore used FM4-64 instead because it has been shown to cause markedly more intense staining of dead than of living hyphae (Read and Roca, 2006). When 0.1 mg ml\(^{-1}\) chitosan was applied to vegetative hyphae they started to die \(\sim 20\) min after application and by 40 min they were all dead. Cell death thus occurred over a similar time period to germlings treated with chitosan.

To monitor nuclear breakdown by chitosan, a strain in which H1-GFP was targeted to the nuclei was used in combination with PI. The disappearance of green H1-GFP nuclear fluorescence and appearance of red PI fluorescence was detected just after plasma membrane permeabilization occurred and was mostly complete within \(\sim 3\) min (Fig. 7).

### 3.6. Chitosan causes conidial lysis

During the analysis of the effects of chitosan on conidial germination we observed that the number of conidia at the beginning of an experiment seemed to be lower than at the end of it. When we quantified this we discovered that chitosan caused a concentration-dependent lysis of conidia. The IC\(_{50}\) for conidial lysis was \(\sim 0.0075\) mg ml\(^{-1}\) chitosan (Fig. 9).

### 4. Discussion

We analyzed the mode of fungicidal action of chitosan on fungal cells using the experimental model, \(N.\ crassa\), and made a number of novel findings. First, we showed that chitosan permeabilizes the fungal plasma membrane and is internalized by fungal cells. Both of these processes were found to be ATP dependent.
Second, chitosan was found to cause the lysis of fungal spores. Finally we showed that different cell types (conidia, germ tubes and vegetative hyphae) exhibit differential sensitivity to chitosan.

Our previous results suggested that fluorescently labeled chitosan requires ATP to enter conidia of the plant-pathogenic fungus *Fusarium oxysporum* F.sp. *radicis-lycopersici* and the nematophagous fungus *Pochonia chlamydospora* (Palma-Guerrero et al., 2008). This finding has been confirmed in the present study with conidia and conidial germings of *N. crassa* by using two standard treatments to inhibit ATP production: (a) sodium azide and (b) low temperature (4 °C) (Atkinson et al., 2002; Hoffmann and Mendgen, 1998). Thus ATP seems to be required for chitosan uptake by filamentous fungi. Furthermore, we have shown that ATP-dependent uptake is required to kill fungal cells.

We first tested the hypothesis that chitosan enters conidia by the energy dependent process of endocytosis. It is now clear that endocytosis is an important part of the vesicle trafficking network in filamentous fungi where it serves a number of important roles (Read and Kalkman, 2003; Fuchs and Steinberg, 2005; Peñalva, 2005; Fuchs et al., 2006; Steinberg, 2007; Sánchez-Ferrero and Peñalva, 2008; Taheri-Talesh et al., 2008; Upadhya and Shaw, 2008). Much of the evidence for endocytosis in filamentous fungi has involved the use of the endocytic marker dye, FM4-64, and this has been extensively tested with cells of *N. crassa* by using two standard treatments to inhibit ATP production: (a) sodium azide and (b) low temperature (4 °C) (Atkinson et al., 2002; Hoffmann and Mendgen, 1998). Thus ATP seems to be required for chitosan uptake by filamentous fungi. Furthermore, we have shown that ATP-dependent uptake is required to kill fungal cells.

The significance of endocytosis in filamentous fungi has involved the use of the endocytic marker dye, FM4-64, and this has been extensively tested with cells of *N. crassa* (Fischer-Parton et al., 2000; Hickey et al., 2005; Araujo-Palomares et al., 2007; Riquelme et al., 2007), although not previously with conidia of this species. Nevertheless, the endocytic internalization of FM4-64 has been well characterized during conidial germination in the plant pathogen, *M. grisea* (Atkinson et al., 2002). In our study we showed that FM4-64 is internalized in an ATP-dependent manner by conidia of *N. crassa* which is consistent with it being internalized by endocytosis. However, the differences in localization of rhodamine-chitosan and FM4-64 indicate that chitosan is not internalized by endocytosis in filamentous fungi.

Using *N. crassa* expressing recombinant aequorin, chitosan was found to cause permeabilization of the plasma membrane to external Ca\(^{2+}\) within two seconds. This resulted in a very large and rapid Ca\(^{2+}\) uptake into conidia and conidial germings, although the kinetics of Ca\(^{2+}\) uptake by these two cell types was different. In contrast, Ca\(^{2+}\) uptake by vegetative hyphae was less rapid and a lower amount of Ca\(^{2+}\) was taken up. The pattern of chitosan-induced Ca\(^{2+}\) uptake is thus cell type specific. The experiments with the sodium azide pre-treatment to inhibit ATP formation showed that the rapid Ca\(^{2+}\) uptake by conidia following chitosan treatment seemed to be completely ATP dependent, and mostly ATP dependent in conidial germings and vegetative hyphae. The sodium azide pre-treatment of conidial germings induced a secondary increase in Ca\(^{2+}\) uptake that is not ATP dependent. It is possible that this relates to the permeabilization of a subpopulation of germings which were shown to die in response to chitosan + azide treatment (Fig. 8B). The longer luminescent transient induced by chitosan in vegetative hyphae was mostly ATP dependent. Calibration of the [Ca\(^{2+}\)]c was not possible in the fungal cells because the [Ca\(^{2+}\)]c changes were so large they were out of the dynamic range that can be measured with normal aequorin (Cobbold and Lee, 1991).

Although our results show that exogenous chitosan causes very rapid, ATP dependent permeabilization of the plasma membrane, it is not clear how endogenous ATP is required for this process to occur. One possibility is that after chitosan interacts with membrane components, ATP binds to a molecule (e.g. lipid or protein) on the cytoplasmic side of the plasma membrane, causing a conformational change which allows the formation of pores that traverse the plasma membrane. The binding of ATP to a number of proteins have been found to cause conformational changes which result in regulatory activity (Traut, 1994). Cell viability tests using FDA and PI that are based on the esterase activity of intact living cells and detection of plasma membrane damage, respectively, showed that chitosan killed conidia in less than four minutes, conidial germings within 35–45 min, and vegetative hyphae within 40 min. Nuclear destruction, using a *N. crassa* H1-GFP transformant, was found to occur within ~3 min, and some conidia were found to lyse in the presence of chitosan. Neither chitosan-induced nuclear destruction nor cell lysis have been previously demonstrated in filamentous fungi. The lower sensitivity to chitosan of germings and hyphae than conidia may relate to differences in the composition of their plasma membranes. For example, different amounts of ergosterol have been found in different fungal cell types (Alvarez et al., 2007; Martin and Konopka, 2004; Van Leeuwen et al., 2008).

CAT fusion was found to be similarly sensitive to chitosan (the IC\(_{50}\) in Vogel’s medium diluted 100 times was ~0.25 µg ml\(^{-1}\)). However, during the course of this study we made the interesting observation that CAT fusion is very much reduced when conidia were germinated in PDB. We have also found that hyphal fusion in the mature colony is very reduced or possibly absent when *N. crassa* is grown on solid potato dextrose agar medium (Huang, L-C and Read, N.D., unpublished data). At this stage it is not clear whether PDB contains an inhibitor or Vogel’s medium contains a stimulator of CAT and hyphal fusion. Nevertheless, the ability to inhibit hyphal fusion from occurring by simply growing the fungus on PDB medium will provide a very useful experimental tool to analyze the roles of CAT and hyphal fusion in the future, and will complement the use of hyphal fusion mutants in these studies (Read et al., in press).

This paper helps our understanding of the mode of action of chitosan, which has great potential as a fungicide (Rabea et al., 2003). Further work is in progress to elucidate the mechanism by which chitosan permeabilizes the plasma membranes of filamentous fungi.

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