Research Article

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Nitric-oxide-mediated cell death is triggered by chitosan in *Fusarium eumartii* spores

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Abstract

BACKGROUND: The genus *Fusarium* comprises a heterogeneous group of fungi important for agriculture. *Fusarium solani* f. sp. *eumartii* (*F. eumartii*), historically considered to be a fungal pathogen of potato, has also been associated with tomato disease. Currently, chitosan and its derivatives have been receiving more attention as environmentally friendly antimicrobial compounds in sustainable practices. The aim of the present work was to characterize downstream events associated with the mode of action of chitosan, including nitrosative reactive species, in order to identify new biomarkers of its cytotoxic action.

RESULTS: Data indicated that chitosan-mediated nitric oxide (NO) production might lead to conidial death, concomitant with the strong reduction in fungal pathogenicity in tomato plants. Following chitosan applications, a notably dose-dependent reduction in conidial viability was demonstrated in *F. eumartii*. Thereafter, the infectivity of chitosan-treated spores was tested by a bioassay using tomato seedlings.

CONCLUSION: All these data highlight NO valuable properties as a quantitative and qualitative biomarker of cytotoxic action of chitosan in conidial cells. In addition, these findings place the chitosan assayed here as a fungicide with a high potential of application in sustainable horticultural practices.

Supporting information may be found in the online version of this article.

Keywords: chitosan; fungicide; *Fusarium solani* f. sp. *eumartii*; nitric oxide

1 INTRODUCTION

Many species of the genus *Fusarium*, including *Fusarium oxysporum* and *Fusarium solani*, cause severe pre- and post-harvest damage to agriculture worldwide.[1] *Fusarium solani* f. sp. *eumartii* (*F. eumartii*) is one of the most economically important post-harvest diseases of potato tubers, producing reddish brown mottling symptoms between leaf veins and dry rot in potato tubers.[2] Although *F. eumartii* has historically been considered to be a potato (*Solanum tuberosum*) pathogen, it is also found in tomato plants (*Solanum lycopersicum*).[3] The ability of one of the potato isolates to form heterokaryons with the tomato isolates is further evidence that the same fungus may cause diseases in tomato and potato plants. Isolates from both these plants have also been pathogenic in other Solanaceae species.[3] In general, the use of toxic fungicides to control Fusarium diseases is a current practice in agriculture. However, chemical applications cause hazards to human health and the environment. Alternatively, chitosan and chitosan derivatives are well accepted as being non-toxic to humans and environmentally friendly compounds for agricultural uses.[4–8] Chitin, a homopolymer of β-(1-4)-linked *N*-acetyl-D-glucosamine, is one of the most abundant and renewable natural polymers. It is commonly found in the exoskeletons or cuticles of many invertebrates and in the cell walls of most fungi. Chitosan is a deacetylated derivative of the chitin, manufactured either by enzymatic or non-enzymatic procedures. Each protocol for chitosan preparations may result in differences in chemical traits.[9] The mode of action of chitosan is rather variable on different biological systems. Occasionally, each specific cell type might have a different response to chitosan. The antimicrobial properties of chitosan in *F. solani* have been previously assayed.[10,11] However, nowadays the characterisation of downstream events mediated by chitosan in fungal cells is an emerging issue. In the present work, the antimicrobial properties of chitosan obtained from shrimp waste under an optimised procedure are described. It is demonstrated that nitric oxide (NO) production and NO-mediated cell death are downstream events mediated by chitosan in *F. eumartii* spores. In sum, it is proposed that endogenous NO could represent a convenient and easily measurable

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biomarker of chitosan-mediated cytotoxicity in spores of fungal pathogens.

2 MATERIALS AND METHODS

2.1 Biological material

Fusarium solani f. sp. *eumartii* (*F. eumartii*) was obtained from Estación Experimental Agropecuaria (EEA) INTA, Balcarce, Argentina. The fungus was grown in solid potato dextrose agar (PDA) medium at 25 °C in darkness. Spores were collected from eight-day-old cultures by suspension in sterile water. Conidia were counted using a haemocytometer, diluted to the appropriate concentration and used for the different assays. Tomato (*Solanum lycopersicum*) cv. Ace 55 VF seeds were obtained from commercial supply.

2.2 Isolation and chemical characterisation of chitosan

Chitin was isolated from shrimp (*Pleoticus müller*i) waste. Chitosan was prepared by heterogeneous deacetylation of chitin with 50% (w/w) NaOH. For biopolymer characterisation, moisture and ash contents were determined at 100–105 °C and 500–505 °C respectively. The degree of deacetylation (DD) was obtained using FT-IR spectroscopy (Nicolet iS10 FT-IR spectrometer; Thermo Fisher Scientific, Waltham, MA) with samples in the form of KBr at a ratio of 1:2. The chemical properties of the chitosan used in this study were 70 kDa, DD 78%, viscosity 80 mPa s, 6.7% (w/v) moisture and 0.67% (w/v) ash. A stock solution of 10 mg mL^{-1} of chitosan was prepared in 1% (w/v) acetic acid, pH 5.5, adjusted with 1 M of KOH and sterilised by autoclaving. The viscosity of 1% (w/v) chitosan in 1% (v/v) acetic acid solution was measured with a Brookfield model DV-IV+ viscometer (Brookfield, Middleboro, MA) with spindle 21.

2.3 Measurements of endogenous NO production in *F. eumartii* spores

Determination of endogenous NO production was monitored by incubating 10^7 spores mL^{-1} in 20 mM of HEPES-NaOH, pH 7.5, with different concentrations of chitosan in a final volume of 100 µL at 25 °C for 4 h. Controls corresponded to the equivalent concentration of acetic acid used to prepare the chitosan solution. One hour before the end of the treatments, samples were added with 10 µM of 3-amino, 4-aminomethyl-2,7-difluorofluorescein diacetate (DAF-FM DA) (San Diego, CA) and incubated at 25 °C in darkness. Fluorescence of cells was visualised by fluorescence microscopy with an excitation filter of 485 nm and a barrier filter of 515 nm and bright field microscopy in an Eclipse E200 (Nikon, Tokyo, Japan) microscope. The production of green fluorescence under these conditions was due to NO. For quantification of NO production, fluorometric measurements were performed in a Fluoroskan Acsent microwell fluorometer (Thermo Electron Company, Vantaa, Finland) using Chroma (Chroma Technology Corp., Rockingham, VT) filters D480-40 and D525-30 for excitation and emission respectively. Samples were pipetted into wells of a Greiner 96-well plate, and after treatments the microwell plate was immediately transferred to the fluorometer for measurement. Fluorescence of each individual well was measured every 4 min at 25 °C over 2 h. All experiments were performed in triplicate.

2.4 Measurements of endogenous hydrogen peroxide

Detection of hydrogen peroxide (H_2O_2) was performed by an endogenous peroxidase-dependent histochemical staining procedure using 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich, St Louis, MO), as described by Thordal-Christensen *et al.*,[12] with minor modifications. DAB polymerises instantly and locally as soon as it comes into contact with H_2O_2 in the presence of peroxidase. It produces an insoluble coloured complex in contact with H_2O_2. Briefly, spores at a final concentration of 1.5×10^5 spores mL^{-1} were incubated with different concentrations of chitosan or, alternatively, acetic acid solution (as control) at 25 °C for 4 h. Then, 0.5 mg mL^{-1} of DAB was added in each tube and incubated for an additional 1 h before rinsing. For H_2O_2 determination, spores were observed under an Eclipse E200 (Nikon) light microscope.

2.5 Cell viability assay

Cell viability was determined by propidium iodide (PI) exclusion.[13] PI is used as a DNA stain to evaluate cell viability or DNA content. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20–30-fold. PI (Sigma-Aldrich) was added in each well to a final concentration of 120 µM containing 10^7 spores mL^{-1} and kept at room temperature for 4 h in darkness. Spores were observed in an Eclipse E200 microscope (Nikon) equipped with an epifluorescence unit and a G-2E/C filter set containing an excitation filter at 540/25 nm, a suppressor filter at 630/60 nm and a dichroic mirror at 565 nm.

2.6 Analysis of fungicidal activity on *F. eumartii* cells

Samples containing 2×10^2 spores mL^{-1} were incubated with different concentrations of chitosan or acetic acid solution (as control) in a final volume of 50 µL. Samples were mixed, incubated at 25 °C for 4 h in darkness and then spread on PDA plates. After 3 days, colonies were counted. The number of colony-forming units (CFUs) was calculated in each sample.

2.7 Measurements of conidial germination

To assay the effect of chitosan on the germination of spores of *F. eumartii*, *in vitro* bioassays were performed as described by Mendieta *et al.*[14] Test samples containing 0.75×10^3 spores mL^{-1} in 50 mM sodium acetate buffer, pH 5.2, 4% (w/v) sucrose and different concentrations of chitosan or, alternatively, diluted acetic acid solution were incubated at 25 °C for 16 h in darkness in a final volume of 25 µL. Spores were observed under an Eclipse E200 light microscope (Nikon) by using a haematocytometer. Conidia were considered to be germinated when the germ tube length was longer than one-half of the conidial length. The IC_{50}, which is the concentration needed to reduce spore germination by 50%, was determined.

2.8 *F. eumartii* hyphal growth assay

For *in vitro* antifungal activity assays, petri dishes containing PDA media were supplemented with different concentrations of chitosan or acetic acid solution. A 0.5 cm diameter disc of an actively growing culture of *F. eumartii* on PDA was placed mycelium side down in the middle of a petri dish containing fresh PDA medium and then sealed with parafilm. Plates were incubated at 25 °C in darkness. After 3 days, radial growth of fungal mycelia was quantified. The IC_{50} was also determined.

2.9 *In planta* bioassays

To evaluate the infectivity of chitosan-treated spores, experiments were performed using tomato seedlings grown on Murashige and Skoog (MS) medium[15] containing 0.8% (w/v) agar. Bioassays were performed as follows. (i) Seven-day-old tomato seedlings

germinated in MS plates were infected with 10^7 spores mL^{-1} treated with 10 μg mL^{-1} of chitosan or, alternatively, with diluted acetic acid solution at 25 °C for 4 h in darkness. To achieve uniform inoculation, the spore suspension was dispensed into petri dishes until seedlings were completely submerged. Then, seedlings were exposed to conidial cells with gentle mixing for 10 min. After the inoculum was discarded, petri dishes containing inoculated seedlings were sealed with parafilm and incubated at 25 °C for 5 days. Alternatively, (ii) four-day-old tomato seedlings germinated in MS plates at 25 °C in darkness were placed on MS-agar plates supplemented with 10 μg mL^{-1} of chitosan or with the corresponding diluted acetic acid solution. Then, eight-day-old seedlings were infected with 10^7 spores mL^{-1} as described above. The symptoms in cotyledons were observed after 5 days of inoculation. Approximately 15 seedlings were analysed in each experiment.

2.10 Measurements of residual fungal inoculum

The residual fungal inoculum present in cotyledons was analysed at the end of each treatment. To eliminate epiphytic bacteria prior to sampling, seedlings were surface sterilised with 70% (v/v) ethanol for 1 min. A quantity of 50 mg of green tissue was washed twice with sterile distilled water and then homogenised in 500 μL of cold and sterile distilled water using a pestle in sterile microcentrifuge tubes. The homogenates were vortexed for 5 s, serially diluted 10 times and cultured on PDA medium plates supplemented with 100 μg mL^{-1} of ampicillin. Plates were placed at 25 °C for 2 days in darkness. For each dilution, the number of CFUs was counted. The lesion area in tomato cotyledons was measured using the image-processing software ImageJ (NIH, Bethesda, MD) and related to 100% as the total leaf area.

2.11 Western blot assays

For western blot assays, total proteins from 50 mg of fresh weight from tomato cotyledons were extracted in sample buffer. Protein samples were boiled for 5 min and loaded on 12% (w/v) SDS-PAGE. Proteins were transferred onto nitrocellulose using a semi-dry blotter (Novex; Invitrogen, Carlsbad, CA). Immunodetection was performed as described by Turner[16] using polyclonal antibodies raised against chitinase[17] or cytosolic ascorbate peroxidase (APX) (Agrisera, Vännäs, Sweden). The blots were allowed to react with goat antirabbit antibody conjugated with alkaline phosphatase (Sigma-Aldrich) and revealed with BCIP/NBT according to procedures recommended by the manufacturer. Equal amounts of fresh weight (3 mg) were loaded in each lane of the gel.

2.12 Statistical analysis

The values shown in each figure are mean values ± SD of at least three experiments. The data were subjected to analysis of *t*-test or variance (one-way ANOVA), and post hoc comparisons were done with Tukey's multiple range test at $P < 0.05$ level. SigmaStat 3.1 was used as the statistical software program.

3 RESULTS

3.1 Chitosan triggers endogenous NO and cell permeabilisation in *F. eumartii* spores

As NO is a well-characterised molecule acting as a downstream signal in a wide band of physiological processes, *in vivo* fluorescence assays were used to measure endogenous NO in *F. eumartii* spores. Firstly, conidia were treated with chitosan for 4 h and subsequently incubated with DAF-FM DA. This is a cell-permeable fluorescent probe for the detection of NO. DAF-FM DA permeates well into living cells and is rapidly transformed into water-soluble DAF-FM by cytosolic esterases, where the dye can remain for a long time. Figure 1A (upper panel) shows that chitosan triggered NO production in conidial cells. However, when chitosan-treated spores were preincubated with the NO-specific scavenger 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), fluorescence was clearly reduced. In this experiment, the effect of the widely used NO scavenger cPTIO was assayed as confirmation that DAF-FM fluorescence was due to endogenous NO.[18] According to Akaike and Maeda,[19] cPTIO is a stable organic

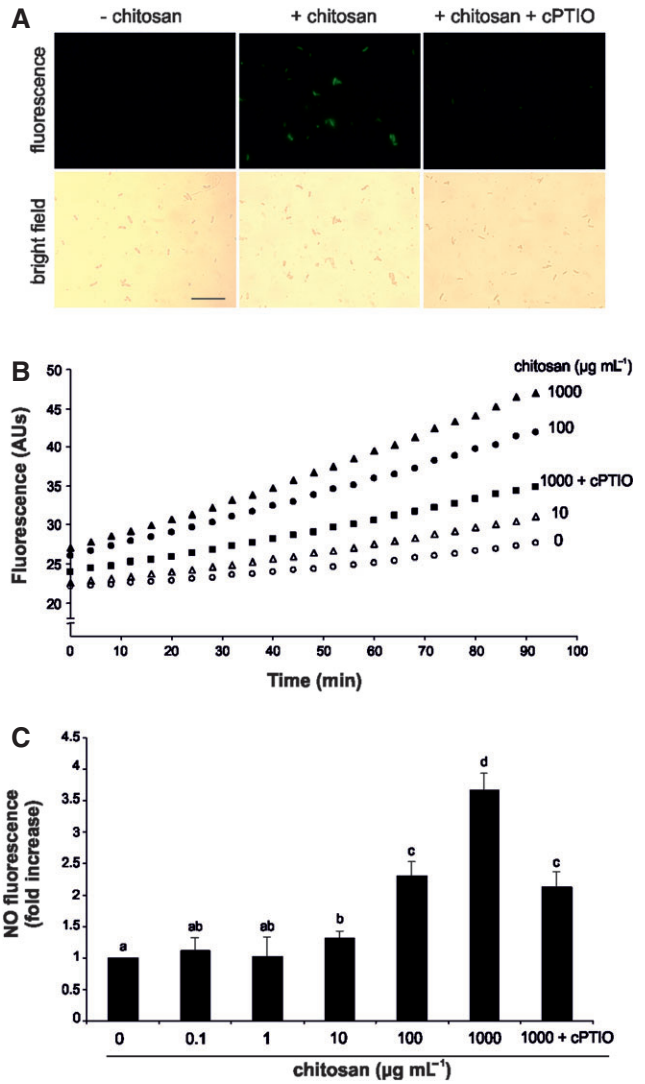

Figure 1. Chitosan induces NO production in *F. eumartii* spores. Fungal spore suspension was exposed to chitosan for 4 h and incubated with the NO-specific fluorescent probe DAF-FM DA. (A) NO production is visualised as green fluorescence. Pictures were taken after control solution treatment and 1 mg mL^{-1} of chitosan or 1 mg mL^{-1} of chitosan combined with 1 mM of cPTIO treatment, and show general phenomena representative of at least five individual experiments. A bright field image for each treatment is shown below the fluorescent image. Bar: 100 μM. (B-C) Dose response curves of NO production. Fluorescence was determined in a fluorometer. (B) Data are expressed as arbitrary units (AUs). A representative graph of five independent experiments is shown. (C) Data are expressed as the fold increase with respect to the control. Different letters indicate statistically significant differences (Tukey's test, $P < 0.05$). Error bars denote SD ($n = 5$).

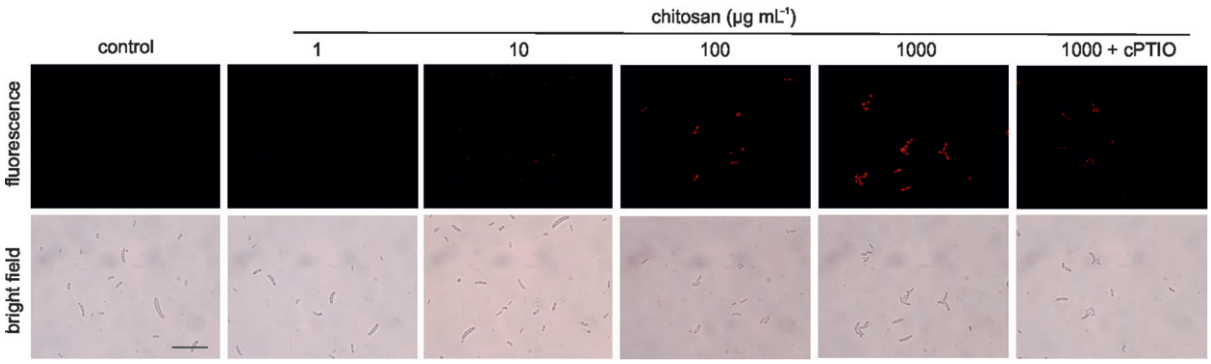

Figure 2. Chitosan treatment affects *F. eumartii* cell viability. Fungal spore suspension was incubated with different concentrations of chitosan for 4 h and loaded with propidium iodide. Membrane permeabilisation is visualised as red fluorescence. Pictures show general phenomena representative of at least five individual experiments. A bright field image for each treatment is shown below the fluorescent image. Bar: 100 μM.

radical and oxidises the NO molecule to form different NO radicals. Previously, different cPTIO concentrations had been assayed, with 1 mM of cPTIO established as a proper dose (data not shown). This approach was followed in a quantitative chitosan dose–response analysis using a fluorometer. As shown in Fig. 1B, fluorescence indicating NO production was measured during a 90 min period. The accumulation of NO was rapid and sustained over at least the period of analysed time. It was clearly observed at chitosan concentrations ranging from 10 to 1000 μg mL^{-1} (Figs 1B and C). At concentrations lower than 10 μg mL^{-1} there was no statistical increase in DAF-FM DA fluorescence, probably owing to the sensitivity of the method (Fig. 1C). As nitrosative burst is an event that frequently leads to cell death, PI staining was used to assess cell viability in chitosan-treated cells. PI is only taken up by cells that have damaged plasma membranes, and the red fluorescence is a consequence of DNA–dye binding. As shown in Fig. 2, an increase in red fluorescence was observed in chitosan-treated spores. At 10 μg mL^{-1} of chitosan, a slight red fluorescence was initially detected and went on increasing at higher concentrations of chitosan (≥100 μg mL^{-1} and up to 1000 μg mL^{-1}). The NO scavenger cPTIO, in combination with chitosan, caused a significant reduction in fluorescence intensity, suggesting that NO could be part of downstream signalling leading to chitosan-mediated cell death in *F. eumartii* spores. In order to validate the loss of cell viability, endogenous H_2O_2 following chitosan treatments was monitored in conidial cells by using DAB staining. Chitosan-treated spores developed a strong reddish-brown pellet, indicating H_2O_2 accumulation (supporting information Fig. S1). The generation of H_2O_2 gradually increased according to higher chitosan doses. At 2.5 mg mL^{-1} of chitosan, 95% of spores were stained, evidencing a drastic oxidative action of chitosan in *F. eumartii* spores.

3.2 Chitosan reduces conidial germination and hyphal growth

To assess the fungicidal action, *F. eumartii* spores were incubated with 10 μg mL^{-1} of chitosan at 25 °C for 4 h and then plated on chitosan-free PDA medium for 3 days. Figure 3A shows that incubation with 10 μg mL^{-1} of chitosan caused a reduction of 95% in the number of CFUs, indicating a very high sensitivity of conidia to chitosan action. Additionally, a dose–response analysis of germination was recorded after addition of chitosan to spore suspensions (Fig. 3B). It caused a dose-dependent (0.1 and 250 μg mL^{-1} of chitosan) decrease in spore germination. The calculated IC_{50} value of chitosan for conidial germination was 3.9 μg mL^{-1}. Furthermore, chitosan action was also assayed in hyphal

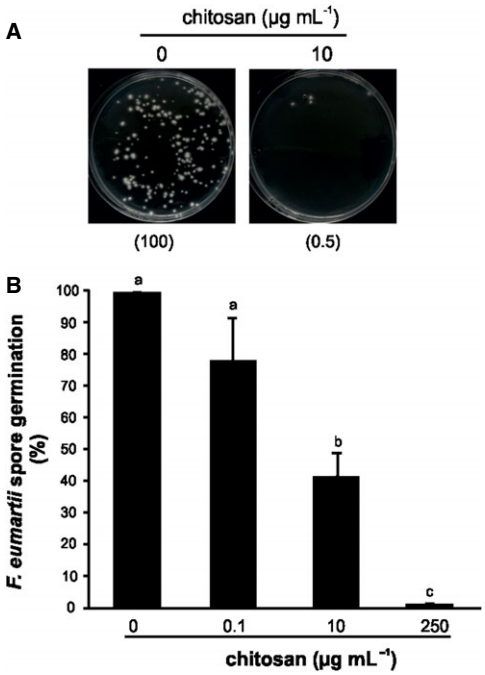

Figure 3. Lethal effect of chitosan on *F. eumartii* spores. (A) Fungal spore suspension was incubated with 10 μg mL^{-1} of chitosan for 4 h and then spread on plates containing PDA medium. Pictures were taken at 3 days and show general phenomena representative of at least three individual experiments. Colonies were counted, and data are expressed as the percentage of colony-forming units (CFUs) with respect to the control plates (above photographs). (B) Fungal spore suspension was incubated with different concentrations of chitosan at 25 °C. After 16 h, the number of germinated spores was measured. Values were expressed as percentage of control treatment. Different letters indicate statistically significant differences (Tukey's test, $P < 0.05$). Error bars denote SD ($n = 3$).

growth. The addition of chitosan at concentrations >0.5 mg mL^{-1} and up to 5 mg mL^{-1} to fungal culture agar media caused a clear reduction in biomass, evidenced by a decrease in radial hyphal growth (supporting information Fig. S2). A strong inhibition of radial growth occurred at concentrations of ≥1 mg mL^{-1}, with an estimated IC_{50} value of 2 mg mL^{-1} of chitosan.

3.3 Chitosan counteracts fungal disease in tomato seedlings

To evaluate *in planta* action of chitosan-treated spores, bioassays were performed according to Uppalapati *et al.*[20] in tomato

seedlings. Seven-day-old seedlings grown on MS agar plates were inoculated by flooding the plates with spore suspensions previously treated with 10 μg mL^{-1} of chitosan for 4 h. As expected, *F. eumartii* spores caused necrotic damage and wilting symptoms in cotyledons of tomato seedlings (Fig. 4A). However, in the case of chitosan-treated spores, necrotic symptoms were rarely observed. As compared with the control, a reduction of approximately 90% in infected area was observed (Fig. 4B). In order to validate whether the reduced incidence of the disease corresponded to less fungal inoculum, the residual spores present in cotyledons were quantified. For these assays, infected cotyledons from either chitosan-treated or non-treated spores were homogenised, and serial dilutions were plated onto petri dishes containing PDA medium. After 2 days, the number of CFUs was measured in each plate. A reduction of 83% in the CFU value was calculated in extracts from seedlings inoculated with chitosan-treated spores compared with the control (Fig. 4C).

Owing to the well-known properties of chitosan as an elicitor of the plant defence mechanism against biotic stress, an additional experiment was performed. Tomato seedlings were pretreated with 10 μg mL^{-1} of chitosan for 4 days and then inoculated with *F. eumartii* spores. The lesion area and the fungal inoculum remaining in the chitosan-treated tissue were significantly reduced compared with non-treated seedlings (Fig. 5). Therefore, as defence marker proteins, the levels of antioxidant enzyme, ascorbate peroxidase (APX) protein and chitinase were measured in tomato seedlings by western blots. At 10 μg mL^{-1} of chitosan, the level of both these protein markers remained almost unchanged in treated and non-treated seedlings (Fig. 5D).

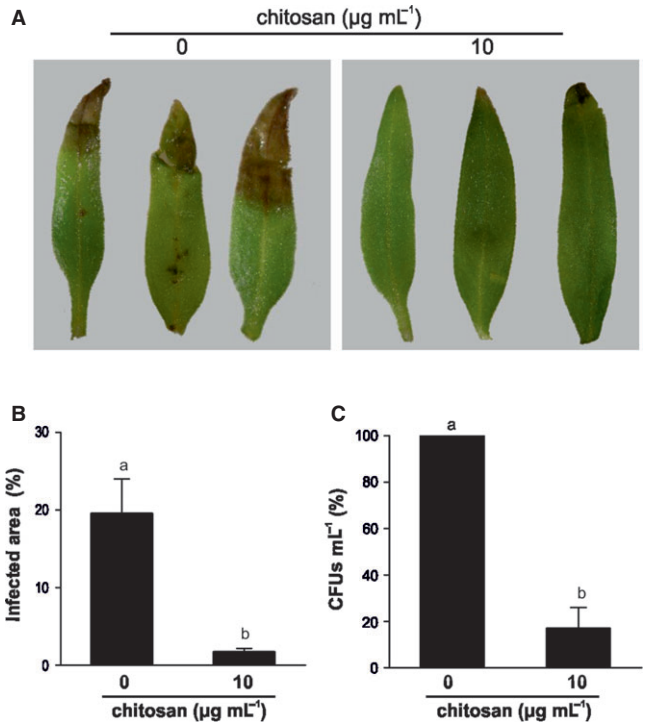

Figure 4. Chitosan-treated spores have reduced ability to infect tomato seedlings. Fungal spore suspension was exposed to 10 μg mL^{-1} of chitosan for 4 h and then used to inoculate seven-day-old tomato seedlings grown in MS agar plates. (A) Pictures were taken at 5 days post-inoculation and show general phenomena representative of at least three individual experiments. (B) Quantification of the lesion area. Data are expressed as percentage of total cotyledon area. (C) Quantification of remaining fungal inoculum in tomato seedlings after 5 days post-inoculation. Data are expressed as percentage of control treatment. Different letters indicate statistically significant differences (*t*-test, $P < 0.001$). Error bars denote SD ($n = 3$).

4 DISCUSSION

In this study it has been shown that chitosan stimulates endogenous NO production in *F. eumartii* spores, leading to the loss of cell viability soon after treatment. This fact was revealed by DAF-FM DA fluorescence emission measured in chitosan-stimulated spores, as well as by the reduction in this fluorescence caused by the NO scavenger. As fluorescence changes are not always indicative of NO production but may also reflect NO oxidation and/or production of other DAF-reactive compounds, endogenous NO was confirmed by the use of the NO scavenger cPTIO.[21] NO is an unstable gas and a multifunctional molecule involved in many physiological processes in phylogenetically distant species.[22] Most research has shown that NO is a component of the signalling pathways remarkably similar to those found in animals and plants (recently reviewed by Mur *et al.*[23]). However, evidence for NO as a signal molecule in fungi is much more recent.[24] In the present study, the authors demonstrated that NO production and conidial viability are influenced by chitosan in *F. eumartii* spores. The observation that chitosan had a powerful cytotoxicity against both conidia and hyphae in *F. eumartii* made it possible to suggest it might exert its strong fungicidal action through a nitrosative/oxidative burst. In general, it has been proved that NO might have opposite functions, acting as a toxic or preventive molecule.[25] In the case of fungi, NO has been implied in the germination of conidia in *Colletotrichum coccodes*[26] and during plant infection in *Magnaporthe oryzae*.[24] It also participates in the cross-talk between the necrotrophic pathogen *Botrytis cinerea* and the plant host.[27] At higher concentration, but probably because of the sensitivity of the method, the cytotoxic action of chitosan was validated by the increase in H_2O_2 accumulation in conidial cells.[28] Thus, its killing activities could be potentiated by H_2O_2, depending on chitosan doses and timing.[29] In other cases, H_2O_2 generation can occur via the superoxide, and NO may react with this radical to form peroxynitrite, which in turn influences cellular damage.[30] Although nitrosative and oxidative species have short half-lives, endogenous accumulation of NO and H_2O_2 could be proposed as early-outcome predictors of cytotoxicity in fungal spores. The imbalance of oxidative/nitrosative species can cause damage to cellular constituents such as DNA, proteins and lipids, being responsible for several damage and degenerative processes in cells.[31] Therefore, the accuracy and short turnaround time make endogenous NO a promising qualitative and quantitative biomarker to assess the fungicidal and cytotoxic activity of chitosan.

Interestingly, the chitosan concentrations needed to reach sublethal doses in *F. eumartii* spores were significantly lower than those previously reported for other chitosans.[32,33] This represents a valuable property and gives additional value to the chitosan assayed here, conferring upon it a great potential for use as a fungicide. Interestingly, hyphae proved to be sensitive to chitosan, with an IC_{50} value of 2.2 mg mL^{-1}, but conidial germination registered an IC_{50} value much lower (3.9 μg mL^{-1}), indicating a cell-specific sensitivity to chitosan. Coincidently, *F. oxysporum* conidial germination has been shown to be more sensitive than hyphal growth to chitosan.[5,32,34,35] Differential sensitivity of spores and hyphae to chitosan has also been described in *Neurospora*

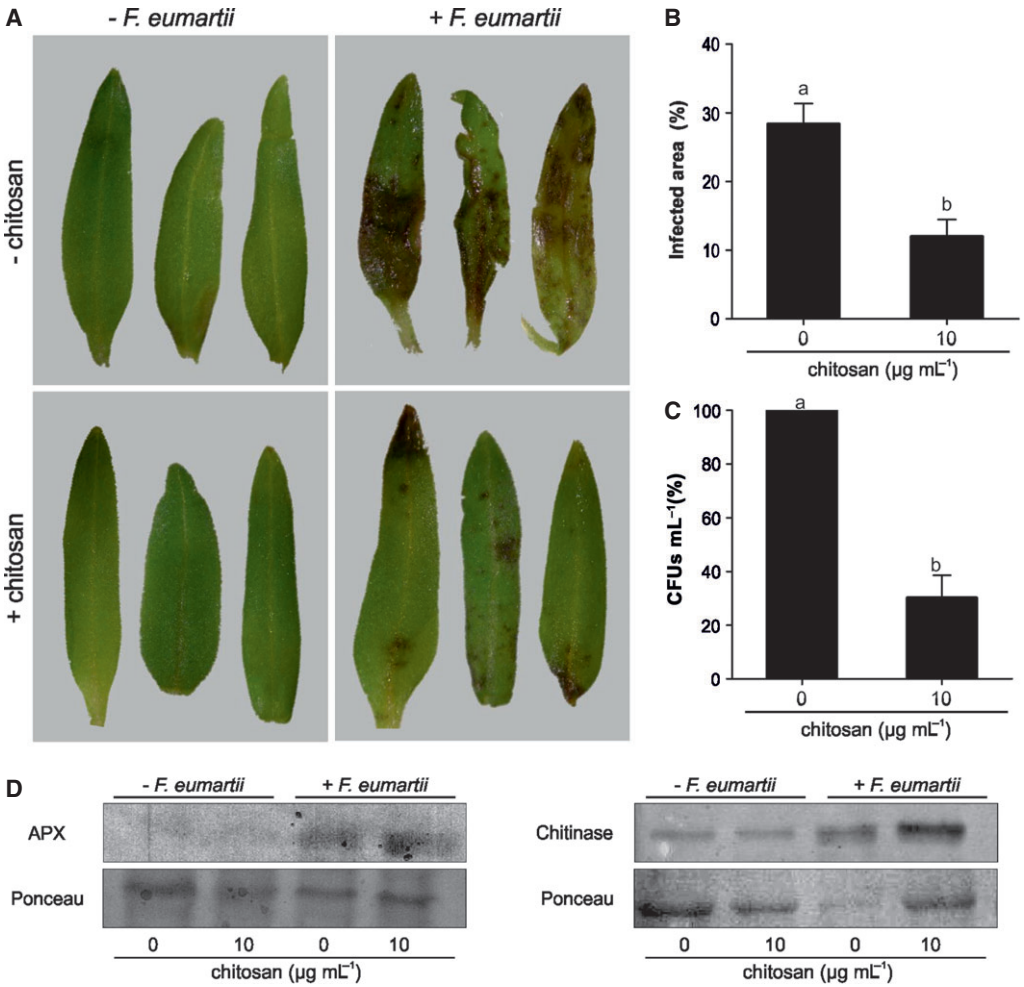

Figure 5. Chitosan treatment counteracts *F. eumartii* infection in tomato seedlings. Four-day-old tomato seedlings grown on MS agar plates were transferred to MS agar containing 10 µg mL^{-1} of chitosan and incubated for 4 days in the growth chamber. Inoculations were performed by immersing seedlings in 10^7 fungal spore suspension. (A) Pictures were taken at 5 days post-inoculation and show general phenomena representative of at least three individual experiments A representative experiment is shown. (B) Quantification of the lesion area. Data are expressed as percentage of total cotyledon area. Different letters indicate statistically significant differences (*t*-test, $P < 0.001$). Error bars denote SD ($n = 3$). (C) Quantification of *F. eumartii* remaining inoculum in tomato seedlings at 5 days post-inoculation. Data are expressed as percentage of control treatment. Different letters indicate statistically significant differences (*t*-test, $P < 0.001$). Error bars denote SD ($n = 3$). (D) Western blot analysis using the anti-APX or the anti-chitinase antibodies (left and right panels respectively). Ponceau stain of the nitrocellulose membrane was used as the loading control.

crassa.[36] Thereafter it was hypothesised that chitosan may act on *F. eumartii* cells, depending on the lipid composition of the target cell membranes. The disruption of cell membranes and the action on the synthesis of hydrolytic fungal enzymes have been proposed as chitosan-mediated mechanisms.[5,37] Palma Guerrero *et al.*[38] reported that the cell membranes of chitosan-sensitive fungi have more polyunsaturated fatty acids than chitosan-resistant fungi, suggesting that cell permeabilisation by chitosan may be dependent on membrane fluidity. A well-defined lipid system, such as liposomes, may be one of the first approaches to understanding the possible effects of target membrane composition. In this same direction, the phytopathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 has also shown sensitivity to the chitosan assayed here, which makes it even more interesting for its application in crop fields.[7]

Finally, the identification of predictive biomarkers in fungal cells would enable better selection of cytotoxic compounds and provide a promising issue, with emphasis on their putative uses in the prevention of fungal diseases in plants.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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