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Chitosan as Source for Pesticide Formulations

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Abstract

Late blight and wilt caused by the oomycete, *Phytophthora infestans*, and the fungus, *Fusarium solani* f. sp. *eumartii*, respectively, are severe diseases in Solanaceae crops worldwide. Although traditional approaches to control plant diseases have mainly relied on toxic chemical compounds, current studies are focused to identify more sustainable options. Finding alternatives, a low molecular weight chitosan (LMWCh) obtained from biomass of Argentine Sea's crustaceans was assayed. In an attempt to characterize the action of LMWCh alone or in combination with the synthetic fungicide Mancozeb, the antimicrobial properties of LMWCh were assayed. In a side-by-side comparison with the SYTOX Green nucleic acid stain and the nitric oxide-specific probe, diamino fluorescein-FM diacetate (DAF-FM DA), yielded a similar tendency, revealing LMWCh-mediated cell death. The efficacy of LMWCh, Mancozeb, and the mixture LMWCh-Mancozeb was in turn tested. A synergistic effect in the reduction of *F. eumartii* spore germination was measured in the presence of subinhibitory doses of 0.025 mg ml⁻¹ LMWCh and 0.008 mg ml⁻¹ Mancozeb. This mixture was efficient to increase the effectiveness of the single treatments in protecting against biotic stress judged by a drastic reduction of lesion area in *P. infestans*-inoculated tissues and activation of the potato defense responses.

Keywords: chitosan, *Fusarium* f. sp. *Eumartii*, Mancozeb, *Phytophthora infestans*, potato, tomato

1. Introduction

The Solanaceae species potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum*) are important horticulture crops. The oomycete, *Phytophthora infestans* (Mont.) de Bary and the fungus, *Fusarium solani* (Mart.) Sacc. f. sp. *eumartii* (Carp.) Snyder & Hansen, isolate 3122 (*F. eumartii*) are potentially pathogens in both Solanaceae species [1]. Late blight and fusariosis caused by *P. infestans* and *F. eumartii*, respectively, are frequently controlled with toxic and chemical fungicides. Mancozeb is a broad-spectrum contact fungicide commonly used to control early and late blights, rusts, downy mildews, and black spots, including *Fusarium* wilt [2]. In potato, Mancozeb treatments have been used by application rates of 2 and 4 kg ha⁻¹ [3]. In the US, approximately 3.4 million kg of Mancozeb is applied annually in agriculture. The Mancozeb breakdown metabolite, ethylene thiourea (ETU) is an industrial contaminant [4]. ETU has been shown to produce tumors, birth defects, cell mutations, and thyroid effects in human and animals.

Despite the valuable contribution of Mancozeb to control plant diseases, alternatives and more sustainable options are still hot topics in phytopathology. In this sense, chitosan has been proved as a nontoxic and environmental-friendly compound for agricultural uses [5]. Chitosan is a linear polysaccharide composed of randomly distributed β -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. This polymer is obtained from chitin which is commonly isolated from the crustacean exoskeletons by enzymatic or nonenzymatic procedures. Chitosan of high and low molecular weights has been found to be differentially effective against fungal diseases [5, 6]. The mode of action of chitosan is rather variable, depending on its biological target and interacting lipids in the cell plasma membrane [7, 8]. Chitosan in combination with different chemicals (e.g., antioxidants, saccharine, essential oil) has been successful for the control of foliar diseases in cucumber, cantaloupe, pepper, and tomato [9]. Low-level copper and chitosan have been provided to confer protection against late blight in potato [10].

This work provides data from *in vitro* and *in vivo* studies revealing insights into the water-soluble chitosan, low molecular weight chitosan (LMWCh) action in phytopathogens and plants. An effective action of LMWCh in combination with suboptimal doses of Mancozeb for the control of late blight was demonstrated. Additional field trials could also provide knowledge on its efficiency and environmental implications.

2. Materials and methods

2.1. Isolation and characterization of chitosan

Chitin was isolated from shells waste of the shrimp *Pleoticus mülleri* from Argentine seacoast. Chitosan was prepared by heterogeneous deacetylation of chitin with 19 M NaOH. The water-soluble LMWCh (2.764 kDa) with a degree of deacetylation (DD) of 68% was prepared from chitosan by oxidative degradation with 1 M H₂O₂ under microwave irradiation (700 W) for 4 min. The molecular mass was estimated by using Mark-Houwink-Kuhn-Sakurada's equation [11]. The sample contained 13.3% moisture and 2.60% ash content [12].

2.2. Biological materials

Solanum tuberosum L. var. Spunta plants were grown to the seven-leaf stage under greenhouse conditions at 18°C with 16:8 h light/dark cycles in a growth chamber. It has been described as a susceptible genotype to *P. infestans* [13]. Expanded leaves from the third to the sixth node of potato plants were excised at the stem [14] and used for detached leaflet assays. *P. infestans* race R2 R3 R6 R7 R9, mating type A2 was cultivated as described by Andreu et al. [15], Andreu et al. [16], and Lobato et al. [17]. *F. eumartii* was obtained from Agricultural Experimental Station, INTA, Balcarce (Argentina). The fungus was grown as described by Terrile et al. [18].

2.3. Chemical treatments and inoculations

Commercial Mancozeb (800 g kg⁻¹) WP (Dow AgroSciences, Argentina) was used at 8 mg ml⁻¹ (8000 ppm) or at 0.08 mg ml⁻¹ and 0.008 mg ml⁻¹ in aqueous solution. Each solution was sprayed with a hand-held spray separately, or in combination with 0.25 mg ml⁻¹ LMWCh on detached leaflets placed in Petri dishes. Controls were sprayed with distilled water. After being sprayed, leaflets were incubated at 18°C with light intensity of 120 μmol photons m⁻² s⁻¹ with 16:8 h light/dark cycles in a growth chamber. Each treatment combination was tested in a series of three independent trials.

After chemical treatments on detached leaflets, 10 μl of spore suspension containing 2 × 10⁴ spores ml⁻¹ was infiltrated in the center of the abaxial side of each lateral leaflets using a 1 ml needleless syringe. Petri dishes with leaflets were sealed with parafilm to maintain 90–100% relative humidity and incubated at 18°C. After 5 days, diameter of lesion area was measured in each leaflet using the image-processing software ImageJ (NIH, Maryland, USA).

2.4. *P. infestans* sporangium germination assay

The sporangium germination assay was conducted as described by Mendieta et al. [19].

2.5. Measurements of *F. eumartii* spore germination

Determination of *F. eumartii* spore germination was performed as described by Mendieta et al. [19].

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

Determination of endogenous NO production was monitored by incubating 10⁷ spores ml⁻¹ in 20 mM of HEPES-NaOH, pH 7.5, with different concentrations of LMWCh or LMWCh in combination with NO-specific scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO, 1 mM) as described in Terrile et al. [18].

2.7. Cell death stain and fluorescence microscopy

F. eumartii spore suspension (2 × 10⁵ spores ml⁻¹) was incubated with different concentrations of LMWCh for 16 h and 100% relative humidity at 25°C in the dark. SYTOX Green probe

(Molecular Probes, Thermo Fisher Scientific, USA) was added at a final concentration of 1 mM, and detection of SYTOX Green uptake was done after 30 min of incubation with a Nikon Eclipse E200 fluorescence microscope equipped with a B-2A Fluorescein filter set. SYTOX[®] Green nucleic acid stain is a green-fluorescent nuclear and chromosome counterstain that penetrates the compromised membranes characteristic of dead cells.

2.8. Protein extraction and western blot assay

Total proteins from potato leaflets were extracted as described by Terrile et al. [18]. Protein samples were boiled for 5 min and running on SDS-PAGE 12% polyacrylamide gels according to the method of Laemmli [20]. Proteins were transferred onto nitrocellulose using a semi-dry blotter (Invitrogen, USA) [21]. Immunodetection was performed using polyclonal antibodies raised against chitinase [22].

2.9. Statistical analysis

Treatments were established in a randomized complete block design, typically with four to seven treatments in each trial. The values shown in each figure are mean values \pm SD of at least three experiments. Data were subjected to analysis of 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. Limpel's formula as described by Richer [23] was used to determine synergistic interactions between LMWCh and Mancozeb. This formula corresponds to $E_e = X + Y - (XY \cdot 10^{-2})$, in which E_e is the expected effect from additive response of two treatments, and X and Y are the percentages of inhibition of germination relative to each agent used alone. Thus, if the combination of the two agents produces any value of inhibition of germination greater than E_e , the synergism does exist [24].

3. Results

3.1. LMWCh exerts antimicrobial action on *P. infestans* and *F. eumartii*.

The high water solubility and easy handling for agricultural application make LMWCh an attractive compound to deepen on its properties in the control of plant diseases. In this work, we hypothesized that LMWCh exerts protection against biotic stress in Solanaceae species, and in turn, it can be used in combination with reduced doses of Mancozeb (hundred times less than recommended dosage) for utilization in putative safer formulations. With the intention to move in that direction, we characterized the antimicrobial properties of LMWCh on *P. infestans* and *F. eumartii* as phytopathogen models of potato and tomato, respectively. LMWCh exerted inhibitory activity on germination of both *P. infestans* and *F. eumartii* in a dose-dependent mode (**Figure 1**). The estimated IC_{50} value (the concentration needed to inhibit half of the maximum spore germination) was $IC_{50} = 0.1 \mu\text{g ml}^{-1}$ LMWCh and $IC_{50} = 130 \mu\text{g ml}^{-1}$ LMWCh for *P. infestans* and *F. eumartii*, respectively.

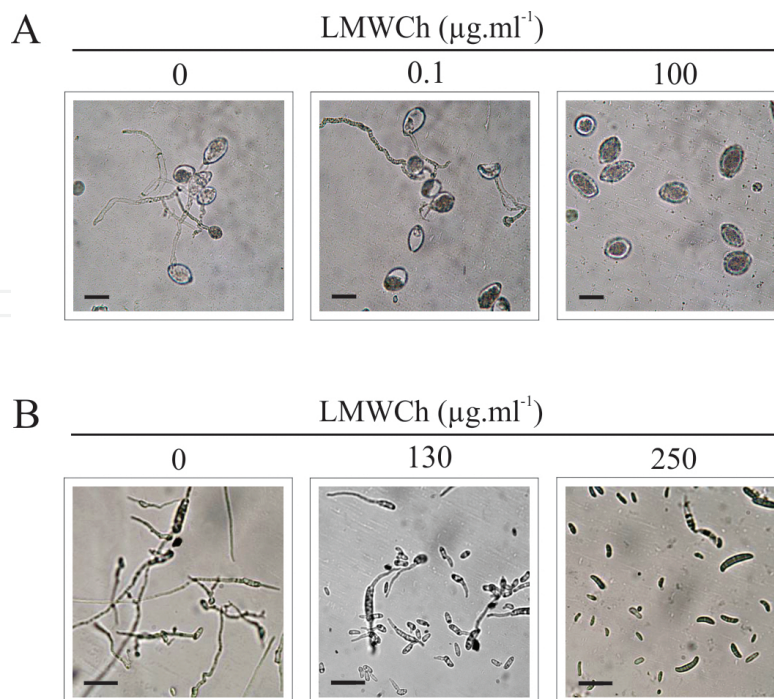


Figure 1. LMWCh affects germination of reproductive structures in phytopathogenic microorganisms. *P. infestans* sporangia (A) and *F. eumartii* spores (B) were incubated with different concentrations of LMWCh for 16 h and 100% relative humidity at 18°C in the dark. Scale bar: 30 µm (A) and 25 µm (B). Values are the mean (±SD) of three independent experiments.

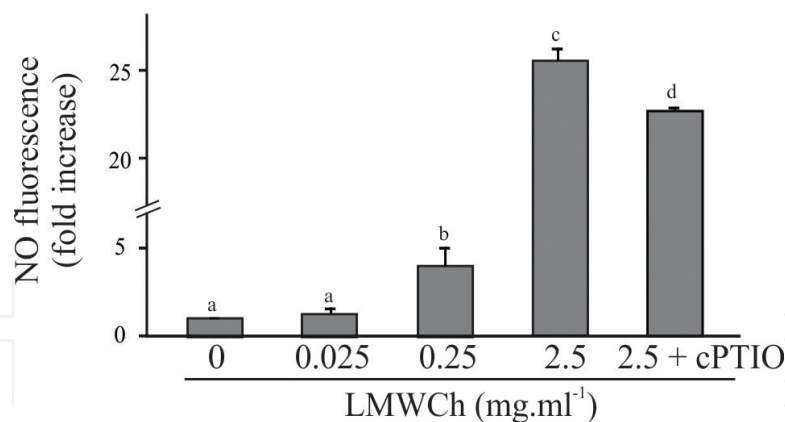


Figure 2. LMWCh induces NO production in *F. eumartii* spores. Spore suspension was incubated with different concentrations of LMWCh in combination with the NO scavenger, cPTIO for 16 h. Then, the suspension was loaded with the NO-specific fluorescent probe DAF-FM DA, and the fluorescence was determined in a fluorometer. Data are expressed as the fold increase with respect to the control. Values are the mean (±SD) of at least three independent experiments. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$).

In turn, due to the easy handle and reproducibility, *F. eumartii* was used to characterize LMWCh-mediated biological action. NO-specific fluorophore diaminofluorescein-FM diacetate (DAF-FM DA) was assayed to compare endogenous NO production in LMWCh- and non-treated spores as a biomarker of cytotoxicity (**Figure 2**) [18]. Exogenous LMWCh application

ranging from 0.25 to 2.5 mg ml⁻¹ induced NO production indicating a dose-dependent LMWCh-mediated effect on *F. eumartii* spores. Since DAF-FM DA fluorescence was significantly reduced in the presence of the NO scavenger, cPTIO, we propose that endogenous NO production is a downstream event upon LMWCh treatment in fungal spores.

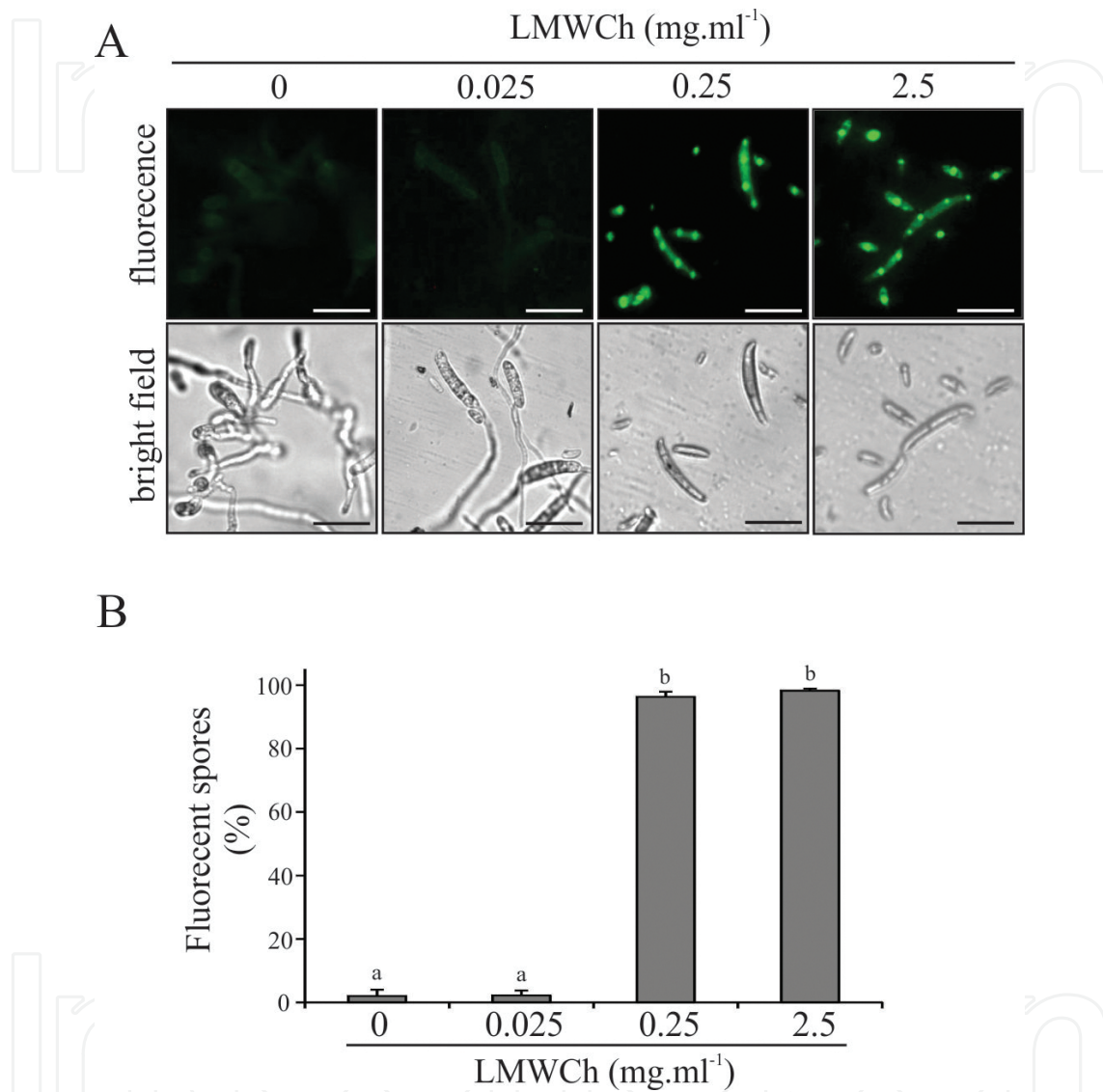


Figure 3. LMWCh treatment induces cell death in *F. eumartii* spores. Fungal spores were exposed to different concentrations of LMWCh for 16 h and then incubated with the fluorescent probe SYTOX Green. (A) Dead cell was visualized as green fluorescence. Pictures show general phenomena representative of at least three individual experiments. A bright field image for each treatment is shown below fluorescent images. Scale bar: 25 μ m. (B) Quantification of fluorescent spores. Values are the mean (\pm SD) of at least three independent experiments. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$).

SYTOX Green dye is often used to distinguish between live and dead cells. The fluorescence emission of SYTOX Green stain was measured in the presence of increasing concentrations of LMWCh. A substantial enhancement was measured in 0.25 and 2.5 mg ml⁻¹ LMWCh-treated spores (Figure 3).

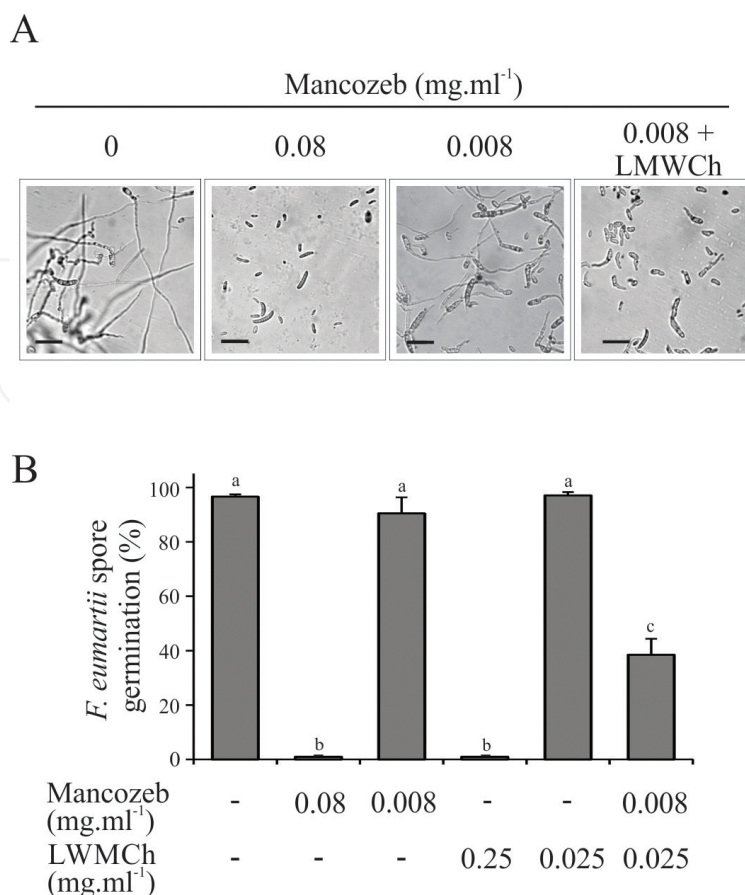


Figure 4. LMWCh plus Mancozeb have a synergistic action on *F. eumartii* spore germination. *F. eumartii* spores were incubated with different concentrations of LMWCh, Mancozeb, or a combination of both, for 16 h and 100% relative humidity in the dark. (A) Pictures show general phenomena representative of at least three individual experiments. Scale bar: 25 μ m. (B) Quantification of spore germination. Values are the mean (\pm SD) of at least three independent experiments. Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$).

Then, LMWCh at 0.025 and 0.25 mg ml⁻¹ and Mancozeb at 10⁻¹ and 10⁻² dilutions from the recommended field dosage were tested on fungal spore germination (**Figure 4**). Compared with control, the combination of suboptimal doses of 0.025 mg ml⁻¹ LMWCh and 0.008 mg ml⁻¹ Mancozeb had a remarkable inhibitory activity on spore germination (**Figure 4**). The treatment with the combined solution resulted in a synergistic effect in the reduction of spore germination. According to Limpel's formula, the E_c value calculated for percent reduction of germinated spores using LMWCh and Mancozeb alone and their combination was 61.51%. Thus, we proposed that the mixture of LMWCh and Mancozeb exerted a synergistic effect on germination of *F. eumartii* spores.

3.2. LMWCh potentiates Mancozeb effect and protects against late blight in potato

The effect of LMWCh and Mancozeb treatments, alone or in combination, to control late blight in potato was tested. Meanwhile, single treatments revealed no significant differences, and the combined treatment of 0.25 mg ml⁻¹ LMWCh and 0.08 mg ml⁻¹ Mancozeb evidenced a reduction of at least sixfold in the late blight symptoms on potato leaflets (**Figure 5**).

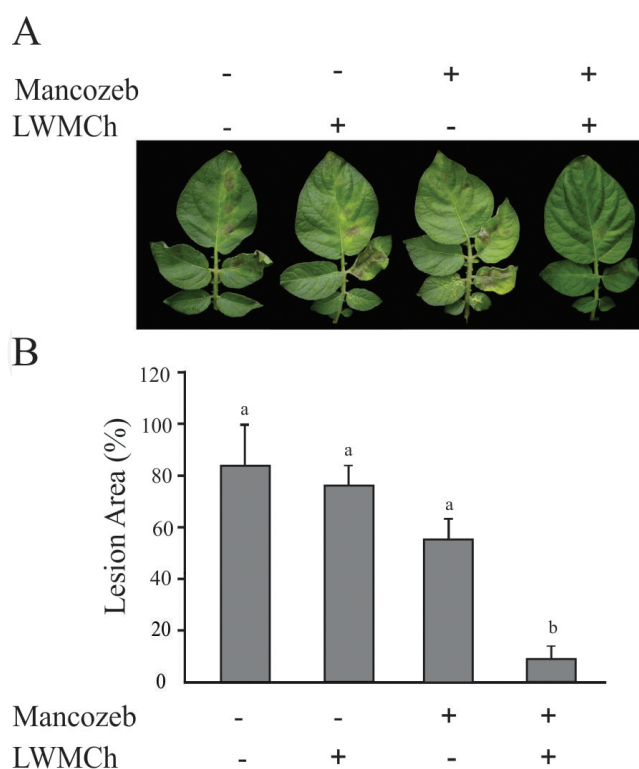


Figure 5. LMWCh and Mancozeb protect plant from microbial attack. Potato leaves were pretreated with 0.25 mg ml^{-1} LMWCh, 0.08 mg ml^{-1} Mancozeb or a combined solution of LMWCh and Mancozeb and then inoculated with *P. infestans*. (A) Representative images from leaflets at 5 days upon *P. infestans* inoculation. (B) Quantification of lesion area; 100% represents the total area in each leaflet. Values are the mean (\pm SD) of at least three independent experiments. Different letters indicate statistically significant differences (Tukey's test, $p < 0.001$).

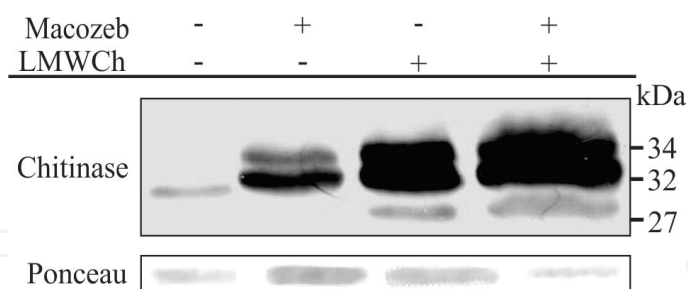


Figure 6. LMWCh and Mancozeb activate plant defense responses. Potato leaves were pretreated with 0.25 mg ml^{-1} LMWCh, 0.08 mg ml^{-1} Mancozeb, or the combined solution of LMWCh and Mancozeb. Western blot analysis was assayed with antichitinase antibody (upper panel). Ponceau staining was used as loading control (lower panel). Picture is representative of two independent experiments.

With the aim to provide broader evidence on LMWCh-mediated mechanism, we assayed its elicitor properties in potato plants (**Figure 6**). Chitinases, the well-known pathogenesis-related proteins used as defense markers, are constitutively expressed at low levels, but highly induced by biotic stresses in potato leaves [22, 25]. Meanwhile, the 32 kDa chitinase was only detected at very low level in control; it moderately increased in Mancozeb-treated leaflets and was remarkably high in LMWCh-treated leaflets. Otherwise, the highest levels of chitinase isoforms

were detected in potato leaflets upon LMWCh and Mancozeb treatment. Particularly, in the presence of LMWCh, the level of a third 27 kDa isoform was increased, suggesting that specific defense proteins are elicited by LMWCh in potato tissues (**Figure 6**).

4. Discussion

LMWCh was effective as an antimicrobial compound on spores from both *P. infestans* and *F. eumartii* under assayed conditions. As spores were treated with LMWCh, the SYTOX Green signal went up. The fact that NO production has been postulated as stress signals and is detected in LMWCh-treated spores allowed us to suggest that it probably downstream modulates cell toxicity in *F. eumartii* [18]. The combination of LMWCh and Mancozeb had a synergistic effect in reducing spore germination and also remarkably induced chitinase accumulation in potato leaflets. The sensitivity to LMWCh as judged by its estimated IC₅₀ values on the reduction of germination was higher in the oomycete than in fungal spores. The inhibition of *P. infestans* [26] and fungal growth by chitosan has been extensively demonstrated [7, 18, 27–29]. Thus, the positive effect of LMWCh on the control of late blight might derive from the combination of its antimicrobial and eliciting properties.

In this work, we demonstrated that the addition of LMWCh to an ineffective Mancozeb dose became a highly effective treatment to control late blight. The synergistic effect of LMWCh and commercially synthetic fungicides has been showed on *Botrytis cinerea*, *Alternaria brassicicola*, and *Muocor piriformis* [30]. The combination of chitosan and ethanol in reduced doses showed an effective control of gray mold [31]; however, the mechanism of action has not still been elucidated. Due to the intrinsic polycationic chemistry of chitosan, we speculated that LMWCh might modify cell membrane permeability [32] and in turn facilitate the uptake of Mancozeb when both compounds are applied simultaneously, on plant tissues. In our work, consistent with the less damage, a potentiated accumulation of three chitinase isoforms was detected on potato leaflets, allowing us to suggest that LMWCh and Mancozeb might activate natural defense mechanisms and maximize the known action of chitosan as plant elicitor [33]. Similar accumulation patterns were also obtained in tomato seedlings subjected to identical treatments (data not shown). In addition to chitinases, the host defense responses elicited by chitosan might include glucanase activation, cell wall lignifications, phytoalexin biosynthesis, and generation of reactive oxygen species, among others [33]. Majid et al. [34] demonstrated that Mancozeb induces antioxidant compounds in *Cassia angustifolia* Vahl. Considering that the antioxidant metabolism is also activated during the plant defense response and adding the fact that chitosan is a suitable candidate to enhance antioxidative enzymes activities [35], we also speculated that summative antioxidant activity might be exerted by Mancozeb and LMWCh on the plant tissue. Since at least two degraded metabolites, ETU and ethylene urea from Mancozeb, have been found on potato tissue [36], we cannot rule out that Mancozeb derivatives also had action on potato defense mechanisms. It has also been accepted that the risk of *P. infestans* resistance against Mancozeb is rather low [37]. Thus, chitosan applied in combination with Mancozeb could repre-

sent a useful strategy with additional advantages within environmental-friendly plant protection strategies.

5. Conclusion

In summary, water-soluble LMWCh is an effective antimicrobial compound on spores from both *P. infestans* and *F. eumartii*. NO is a downstream stress signal mediating LMWCh cytotoxicity in spores. In addition, the combination of LMWCh and the synthetic fungicide Mancozeb showed an effective control late blight in potato leaflets. However, major studies are needed on new agro-inputs and environmental science to provide the conditions for the use of more eco-friendly pesticide formulations in plant crops.

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