TOMATO-*Fusarium oxysporum* INTERACTIONS: II-CHITOSAN AND MSB INDUCED RESISTANCE AGAINST FOL IN YOUNG TOMATO PLANTS

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ABSTRACT. Chitosan, chitosan hydrolysates and menadione sodium bisulphite (MSB) were applied to soil and sprayed on tomato plants in different sets of experiments, to evaluate their combined effects on disease development in tomato plants. In young plants, the three products were effective protecting plants against the disease, foliage sprays showing the best results with chitosan hydrolysates and MSB (0.25 + 0.05 $g.L^{-1}$ respectively), suggesting that the induction of systemic resistance plays a major role as defense mechanism of tomato against *Fusarium oxysporum f.sp lycopersicii* (FOL) attack. Several enzymatic mechanisms related with host defense were measured in plants treated with elicitors and inoculated with FOL as markers for disease resistance.

Key words: chitosan, *Fusarium oxysporum*, fungal elicitors, defensive mechanism, PR-proteins

RESUMEN. Quitosana, hidrolizados de quitosana y menadiona bisulfito de sodio (MBS), fueron aplicados al suelo y asperjados sobre plantas de tomate en diferentes experimentos, para evaluar su efecto combinado sobre el desarrollo de la enfermedad. Los tres productos protegieron con efectividad las plantas contra la enfermedad, mostrando los mejores resultados la aspersión al follaje con hidrolizados de quitosana y MBS (0.25 + 0.05 $g.L^{-1}$ respectivamente), sugiriendo que la inducción de resistencia sistémica juega un papel importante como mecanismo de defensa del tomate contra el ataque de *Fusarium oxysporum f.sp lycopersicii* (FOL). Se midieron algunos mecanismos enzimáticos relacionados con la defensa en plantas tratadas con elicitores e inoculadas con FOL como marcadores de resistencia a la enfermedad.

Palabras clave: quitosana, *Fusarium oxysporum,* elicitores fúngicos, mecanismo defensivo, proteínas-PR

INTRODUCTION

The response linked with microbial diseases involves the recognition by plant cells of a potential pathogen (1). Recognition results in a series of localized as well as systemic responses classified into three classes according to their temporal and spatial pattern of occurrence (2). The first class comprises immediate plant defense responses that involve the hypersensitive response (HR) (3). The second line of defense involves the biosynthesis of phytoalexins and pathogenesis-related proteins (PR) (4). The third line of defense that can occur in many plant pathogen interactions is triggered in the non-infected part of the plants, and is called systemic acquired resistance (SAR) (5). In a general model, elicitors fall into two categories depending on their source: exogenous and endogenous signals.

Today, increasing expectations are emerging in the area of plant disease management for new strategies that have the potential to be efficient, reliable and safe for the environment. The process of plant immunization or induced systemic resistance (SAR) has received increasing attention and has been abundantly documented (6). SAR provides plant protection against a broad spectrum of pathogens for up to several months (7). Chitosan derived from *Fusarium* cell walls or from crab shell chitin induces the accumulation of phytoalexins and induces resistance to *Fusarium solani* in pea tissues. Both the polymer and its hydrolysates protect bean and tobacco against pathogenic viruses (8). Part of its potential as biocontrol agents could be based on its inhibitory activity to a number of pathogenic fungi (9). However, its practical use should take into account the structure and stability of chitosan and its hydrolysates in solution. Polymer chains self associate in aqueous solution (10).

MSB is a derivative of vitamin K_3, which levels of free IAA act in plants raising the endogenous IAA and might play a central role in several host-pathogen interactions. The buildup of IAA nearby the sites of pathogen ingress constitutes one of the main host factors that determine plant resistance to *Fusarium wilt* (11). *Fusarium oxysporum f. sp. lycopersicii* (FOL) and *Fusarium oxysporum* Schlechtend: Fr. *f.sp. radicis-lycopersicii* (FORL) are the causal agents of *Fusarium wilt* of tomato. Since 1995, the Oligosaccharins Laboratory at INCA has manufactured discrete amounts of chitosan, with the objective of developing practical uses of this polymer in ecological agriculture.

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MATERIALS AND METHODS

Fungus and plant culture, growth conditions and inoculation. An isolate of FOL known to be virulent on tomato *(Lycopersicon esculentum* Mill; var. A321) was found in the culture collection of the Laboratory of Oligosaccharins at INCA. It was grown on a liquid Coon medium at 25 °C for 15 days. Then, the mycelium was gently disrupted, filtered through cheesecloth and the resultant solution was adjusted to 5 x 10^7 spores.mL^{-1}.

Tomato plants were grown from germinated seeds in vermiculite in a growth chamber with a photoperiod of 18 h light (9400 lux, 26-28°C) and 6 h dark (24°C). Three weeks after germination (approximately 10 cm high, the first two fully expanded leaves) the plants were sprayed until run-off with elicitors, stored 24 h on distilled water and transferred into individual pots of soil. The soil amended with chitosan was thoroughly mixed for two hours prior to placing 250 cm^3 of each treated soil into 7-cm plastic pots. The presence of zeolite and vermiculite allowed chitosan to be distributed more or less uniformly. Half of plants previously treated with elicitors received 3 mL of spore suspension as inoculum, nearby the roots, 48 h after planted in soil. Ten days after inoculation, the symptoms of wilting in the aerial parts were observed, and the invasion score of roots was measured, using the following scale of 0-5 degrees:

0: foliage or not damaged root
1: wilting or damaged root (20%)
2: wilting or damaged root (40%)
3: wilting or damaged root (60%)
4: wilting or damaged root (80%)
5: wilting or damaged root (100%). Plant death

Elicitor preparation. Lobster shell chitosan was prepared in a 40 L reactor in the Laboratory of Oligosaccharins at INCA, from lobster chitin manufactured by “Mario Muñoz” Laboratory, from the Public Health Ministry of Cuba. Alimuniar and Zainuddin reported the procedure used, with some modifications (12). Chitosan hydrolysates were obtained as described by Pombo (13).

For experimental use, chitosan was dissolved in 0.25 N HCl under continuous stirring and the pH was adjusted to 5.6 using 1 N NaOH. The chitosan hydrolysates was dialyzed against 2 x 20 vol of deionized water and stored at -20°C until use. MSB was kindly supplied by Dr. Andrés Borges, INPA, Canary Islands, and was dissolved in slightly acidulated deionized water. Before applied by spraying, Tween 80 was added to elicitor solution at a final concentration of 0.01 %. A control was sprayed with water plus Tween 80 at the same concentration.

Treatments

- Control
- Chitosan 500 ppm soil
- Chitosan hydrolysates 250 ppm foliage spray
- MSB 100 foliage spray
- Chitosan 500 ppm soil + Chitosan hydrolysates 250 ppm foliage spray
- Chitosan 500 ppm + MSB 100 ppm foliage spray
- Chitosan hydrolysates + MSB 100 ppm foliage spray

Statistical analysis. The results of each experiment were analyzed by ANOVA and, when significative differences were detected, each mean was discriminated with letters applying Duncan test at 5% probability. Each experiment was performed twice, with three or five replicates per treatment.

Extraction of tissues and enzyme assays. All plants for each treatment were frozen in liquid nitrogen and homogenized in TRIS-HCl 200mM + EDTA 0.1 mM + ß mercaptoethanol 14 mM buffer, pH=8.0. Homogenates were centrifuged at 4000 g for 20 min. The supernatants were used to assay PAL, ß 1,3 glucanase, peroxidase enzymes, and measuring protein content with Coomassie Brilliant blue with BSA as standard.

PAL activity was determined by measuring the production of trans-cinnamic acid from L-phenylalanine spectrophotometrically (290 nm), and peroxidase activity was determined by measuring the increase in absorbance (Abs sample-Abs control min^{-1}, 470 nm, 25ºC) (14). β 1,3 glucanase activity was measured using reduced laminarin as substrate, following the protocol described by Paz-Lago and Gutiérrez (15). All activities were determined 24 and 72 h postelicitor treatment, with inoculation and without it.

RESULTS AND DISCUSSION

The degree of root invasion and fouler wilting were measured ten days later. These results are shown in Table I. Keeping in mind the self-association between polymer chains of chitosan, it was dissolved, adjusted in pH value and added to soil, in order to avoid possible decrease in its biological activity. The soil amended with chitosan was thoroughly mixed during two hours and placed in pots. One day later, the pots were planted with young sprayed or untreated tomato plants. On the contrary, chitosan oligomers are stable in aqueous solution, therefore, chitosan hydrolysates ready for use were stored as concentrated solutions at -20ºC, until they were applied to foliage.

Table I. Effect of chitosan, chitosan hydrolysates and MSB on infection development by FOL in young tomato plants ten days after inoculation

Treatments	Invasion score	Wilting
Control	4.80 e	4.60 e
Chitosan 500 ppm soil	3.00 d	3.80 d
Chitosan hydrolysates 250 ppm foliage spray	2.20 c	1.90 c
MSB 100 ppm foliage spray	1.70 bc	1.50 bc
Chitosan 500 ppm substrate+hydrolysates 250 ppm foliage spray	1.30 b	1.10 b
Chitosan 500 ppm soil+ MSB 100 ppm foliage spray	1.20 b	0.90 b
Chitosan hydrolysates 250 ppm+ MSB 500 ppm foliage spray	0.30 a	0.10 a
	Esx= 0.01	Esx= 0.01

Values with common letter did not differ as Duncan test at 5 % probability

All the treatments with elicitors resulted in less disease development than occurred in non-treated plants. Control plants showed severe symptoms of root invasion and wilting ten days after root inoculation with spores of FOL. Most of them were dead (approximately 75 %) three weeks after inoculation. Chitosan applied to soil showed low levels of protection, perhaps due to rapid disappearance of chitosan from soil caused by soil ¨microflora¨, joined with the low dose of chitosan employed (500 ppm substrate, composed by soil, zeolite and vermiculite in a ratio 1:1:1). The fact that spraying chitosan hydrolysates at 250 ppm gave better protection than chitosan treatment to soil further supports this point of view. On the other hand, spraying MSB presented better protection than chitosan hydrolysates by visual inspection, but without significative differences between both treatments.

The combination of chitosan treatment to soil with foliage spraying either with chitosan hydrolysates or MSB gave even better protective effects against FOL attack. However, it appears that biological behavior is due to additive rather than synergistic effect of elicitor combination, if compared values of invasion score and wilting from each individual treatment with its combination. Mixing chitosan oligomers and MSB, applied together as foliage spray, exhibited a healthy appearance with practically absence of visible leaf symptoms. Twenty-one days after inoculation, more than 90 % plants sprayed with both mixed elicitors remain free of symptoms. Although any synergistic interaction must be experimentally evaluated using different doses of each elicitor (16), it seems that these elicitors showed synergism in the protective effects against FOL attack on tomato plant, as judged by the results presented in Table I. This result further supports the importance of induced systemic resistance as the most potent mechanism by means of which plant defended themselves against microbial diseases.

Besides its protective effects against FOL attack, the plant treated with MSB showed slightly better overall growth and height, compared with the other treatments (data not shown) three weeks after spraying. The size of pots could not permit the pursuit of this effect, probably related with the endogenous IAA levels on treated tomato plants (17).

Conceivably, the process of inducing protective effects in young tomato plants against FOL attack should be preceded to the activation of several defensive mechanisms provoked by elicitor treatment. In line with this thought, some enzymatic activities were measured in young plants, 24 and 72 hours after treatment with elicitors. Half of the treated ones was inoculated and the other half was not. The enzymatic defense mechanism selected to be measured is likely to be involved in host reactions to chitosan treatments and related with induced systemic resistance in tomato plant against *Fusarium wilt* (18). The most representative results are shown in Table II. Neither elicitor treatment nor FOL inoculation influenced phenylalanine amonialyase (PAL) activity, in contrast with a previous report, working with *Fusarium oxysporum f. sp. radicis lycopersicii* chitosan from crab shell chitin in susceptible tomato plants. Perhaps the results from Benhamou (18) were recorded in root tissues; however, in this case the enzymatic activities were measured in the whole aerial parts of the plants. On the other hand, PAL activity is more related to localized defensive responses to pathogen attack rather than the systemic acquired resistance (3) induced by elicitors.

Both peroxidase and β 1,3 glucanase activities did respond to both stimuli: elicitor treatment and FOL inoculation. Some stimulation of peroxidase activity may be recorded 72 hours after inoculation, coinciding with Benhamou (18). In contrast, fungal inoculation decreased β 1,3-glucanase activity. This enzyme plays a principal role as defense mechanism against fungal attack in most plant species, directly connected with the structural relevance of β 1,3 glucans in the wall architecture of fungi.

Table II. Effect of chitosan, chitosan hydrolysates and MSB applied to soil and spraying on foliage respectively, on the activation of several defense mechanisms

Treatments	β 1,3 glucanase activity (µmol/glucose.h^{-1}.mg^{-1}protein) 24 h	Peroxidase activity (U. mg^{-1} protein) 72 h	PAL activity (µmol.cinamic acid.h^{-1}.mg^{-1} protein) 24 h
Control H_2O	2.09 e	3.88 d	0.20 a
Control FOL	0.90 f	4.93 c	0.21 a
Chitosan 500 ppm soil	4.98 d	7.40 b	0.18 a
Chitosan 500 ppm soil+chitosan hydrolysates 250 ppm foliage spray	5.78 c	6.72 b	0.20 a
Chitosan hydrolysates 250 ppm+MSB 100 ppm foliage spray	7.45 a	12.17 a	0.25 a
Chitosan 500 ppm soil-inoculated	4.81 d	5.50 c	0.15 a
Chitosan 500 ppm soil+chitosan hydrolysates 250 ppm foliage spray--inoculated	5.40 c	6.90 b	0.19 a
Chitosan hydrolysates 250 ppm+MSB 100 ppm foliage spray--inoculated	6.80 b	12.40 a	0.20 a
	Esx= 0.1	Esx= 0.1	Esx= 0.05

Values with common letter did not differ as Duncan test at 5% probability

In general, the β 1,3-glucanase activity increased by elicitor treatment and slightly decreased due to FOL inoculation. It appears that this enzyme is related with induced resistance to FOL, because the elicitor treatment with more pronounced protective effects showed also higher levels of β 1,3-glucanase activity. This behavior was simultaneously reflected by peroxidase activity; in spite of the effect of inoculated elicitor, treated plants did not show significative differences with elicitor treated and inoculated plants, on peroxidase activity. In conclusion, results of the experiments shown in this paper extend the results of Benhamou (19, 20) to the interaction of FOL with susceptible tomato plants. The induced resistance of tomato plants was found to be associated with increase in peroxidase and β 1,3 glucanase activities.

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