TOMATO-*Fusarium oxysporum* INTERACTIONS: I- CHITOSAN AND MSB EFFECTIVELY INHIBITS FUNGAL GROWTH

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ABSTRACT. Chitosan and menadione sodium bisulphite (MSB) were applied as agar amendment and seed coating to test the combined effects of them on *Fusarium oxysporum f.sp. lycopersicii* (FOL) *in vitro* growth and disease development of tomato plant seeds. In agar amendment, chitosan combined with MSB (chitosan 1.0 mg.mL^{-1}+ MSB 0.1 mg.mL^{-1}) was found to significantly inhibit FOL growth. Only chitosan as agar amendment and seed coating was effective, it diminishing disease occurrence in emerging roots from tomato seeds.

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

RESUMEN. Quitosana y menadiona bisulfito de sodio (MBS) fueron aplicados como enmendantes y recubrimiento de semilla, para probar su efecto combinado sobre el crecimiento *in vitro* de *Fusarium oxysporum f.sp. lycopersicii* (FOL) y el desarrollo de la enfermedad en semillas de plantas de tomate. En agar enmendante, la quitosana combinada con el MBS (quitosana 1.0 mg.mL^{-1} + MBS 0.1 mg.mL^{-1}) inhibió significativamente el crecimiento de FOL. Solamente la quitosana como enmendante y como recubrimiento a la semilla fue efectiva, disminuyendo la incidencia de la enfermedad en las raíces emergentes de las semillas de tomate.

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

INTRODUCTION

The past two decades have been considerable advances in our understanding of the mechanism associated with plant resistance. Elicitors defined as extracellular signal compound involved in non-host recognition and triggering defense reactions can bring about the induction of defensive mechanism in plants. In a general model, elicitors fall into two categories depending on their source: exogenous and endogenous signals. Exogenous elicitors can be considered the primary signals in plant-pathogen interaction (1). The exogenous elicitors vary widely in their chemical nature. Proteins, oligosaccharides, glycoproteins, polysaccharides and derivatives have been identified as elicitors. Elicitors that are basic structural constituents of a pathogen surface or excreted products of its metabolism are likely to be involved in the activation of defense responses directly related with the establishment of the three levels of basic resistance to phytopathogenic microorganisms (2, 3). Evidence from recent studies indicates that some of the characterised elicitors have the potential of becoming a new class of biocontrol agents in agriculture and post-harvest storage (4, 5).

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Chitosan (ß-1, 4-linked glucosamine polymer) was found to be involved in *Fusarium solani*-pea interaction and subsequently has been shown to influence other plant-pathogenic interaction, activating a multitude of biological defensive related processes in plant tissues (4, 6). In tomato, chitosan oligomers are a potent inducer of proteinase inhibitor accumulation (7), and sprayed on tomato leaves protect susceptible cultivars against *Alternaria solani* (8). Menadione sodium bisulphite (MSB) is a water soluble derivative of vitamin K_3 with promising properties as plant growth regulator, which acts raising the endogenous level of free IAA in plants when it is sprayed at 10^{-5} M (9). IAA might play a central role in several host-pathogen interactions. In the last years, MSB has been validated by researchers at INPA, Canary Islands, as immunising fungicide against fungal and viral diseases in several crops (10, 11) as well as growth regulator compound (12) under laboratory and field conditions. The mode of action of this compound might be related with the release of endogenous elicitors from plant cell walls (13, 14). The question about possible synergism between chitosan and MSB is open. Fungal and endogenous elicitors act synergistically in the induction of defensive mechanism in plants (15).

Fusarium oxysporum f.sp. lycopersicii (FOL) and *Fusarium oxysporum Schlechtend f.sp. radicis-lycopersicii* (FORL) are the causal agents of *Fusarium wilt* of tomato. In Cuba, the second form species has not been identified, but FOL causes occasionally serious losses in field and hydroponics, in spite of the existence of several tomato

cultivars with resistance to FOL (16). Since 1995, the Laboratory of Oligosaccharins at INCA has manufactured discrete amounts of chitosan from lobster chitin, using the procedures of Alimuniar and Zainuddin (17), modified by Pombo *et al*. (18), with the objective of developing practical uses of this polymer in ecological agriculture. The results from Benhamou's group and the availability of MSB and low cost chitosan stimulate us to search similar successful approach in the interaction of susceptible tomato cultivars with FOL.

MATERIALS AND METHODS

Fungus and plant culture, growth conditions and inoculation. An isolate of FOL known as 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}.

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 (17) reported the proceeding used. 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. MSB was kindly supplied by Dr. Andrés Borges, IPNA, Canary Islands, and was dissolved in slightly acidulated deionised water .

Experimental design on petri dishes and seed treatment. Tomato seeds (cv. A321, highly susceptible to FOL) were surface sterilised by immersion in 2% sodium hypochlorite, thoroughly rinsed in sterile distilled water and immersed into each of the chitosan solution (pH 5.6) at different concentrations. After gently stirring for 15 min, the wetted seeds were air-dried and kept in a dryer until use. Seeds treated with 0.05 % NaCl and dissolved in distilled water were used as controls. Ten seeds were placed at 0.7 cm around a disk of FOL mycelium deposited on PDA-agar 1.5 % in sterile petri dishes. The agar medium was amended with chitosan and MSB at different concentrations, depending on the experiment (*in vitro* activity against FOL or effect of seed coating on FOL infection).

Statistical analysis. The results of each experiment were analysed 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.

RESULTS AND DISCUSSION

Two experiments were undertaken to determine the effect of chitosan and MSB as agar amendment and as seed coating on the susceptibility of tomato seedlings to FOL attack and the inhibition of *in vitro* fungal growth. Table I shows the effect of both elicitors alone and combined, on the mycelia growth of FOL at the third and fifth post-inoculation days. Addition of chitosan to the agar medium led to severe inhibition to FOL growth. This effect was less while diminishing chitosan concentration from 1 $mg.mL^{-1}$ to 0.5 $mg.mL^{-1}$, but the inhibitory effect was clear compared to control. It was surprising to us the inhibitory effect of MSB to FOL growth, which seems it is not depending upon the concentration employed. Why MSB exerts this influence on fungal growth is not yet known.

Table I. Results of *in vitro* mycelia growth of *Fusarium oxysporum f.sp. lycopersicii* on PDA medium at the third and fifth post inoculation days

Treatments	Growth (cm) 3th day	5th day
1-Chitosan 1.0 $mg.mL^{-1}$	2.25 bc	2.50 b
2-Chitosan 0.75 $mg.mL^{-1}$	2.40 c	2.55 c
3-Chitosan 0.50 $mg.mL^{-1}$	2.50 c	2.80 c
4-Chitosan 0.50 $mg.mL^{-1}$+MSB.050 $mg.mL^{-1}$	2.05 b	2.35 b
5-MSB 0.050 $mg.mL^{-1}$	2.85 d	3.35 b
6-MSB 0.100 $mg.mL^{-1}$	2.75 d	3.50 d
7-Chitosan 1.0 $mg.mL^{-1}$+MSB 0.050 $mg.mL^{-1}$	1.40 a	2.30 a
8-Chitosan 1.0 $mg.mL^{-1}$+MSB 0.1 $mg.mL^{-1}$	1.26 a	2.02 a
9-Control PDA	3.50 e	5.40 e
	Esx = 0.05	Esx =0.05

Values with common letters did not differ as Duncan test at 5 % probability. The mean radial growth was measured from three petri dishes at each treatment

Table I and Figure 1 have also shown that mixing chitosan and MSB enhances the inhibitory activity of both elicitors (treatments 4, 7 and 8) compared with the influence of each individually assayed compound. Chitosan induces marked morphological changes and structural alterations on fungal cells (3). Perhaps both compounds act on different target sites interfering the *in vitro* growth and development of this pathogen; therefore, mixing the elicitors is more effective in halting fungus growth. Further work will be necessary to elucidate, at ultrastructural level, the inhibitory effect of MSB alone and mixed with chitosan, on *in vitro* FOL growth and development.

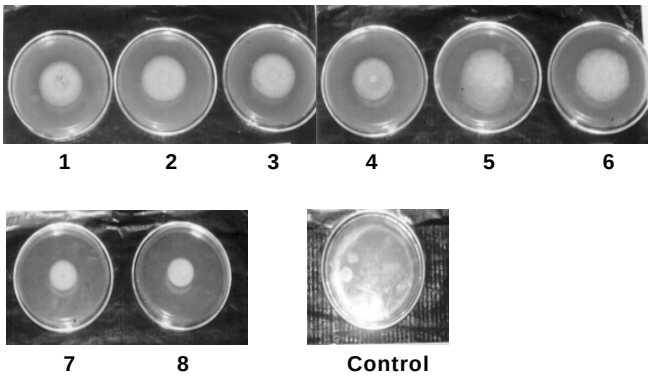

Figure 1: Inhibitory effect of lobster chitosan and MSB on *in vitro* mycelia growth of FOL (Treatments are the same as Table I, five days after inoculation)

Table II has shown the effect of elicitors tested as seed coating and as agar amendment, on the number of damaged roots per petri dish, four and seven days after inoculation. In control plates (untreated seed and agar), the fungus grew extensively and infects the greatest number of roots. However, it seems it does not interfere the normal germination of untreated seeds. In contrast, seed coating with MSB resulted in fungal growth on the surface of several seeds and a retardation of seedling emergence, possibly due to inhibitory effect of the little bit high doses of MSB used on seed germination (treatments 5 and 6). Thus, it is supposed that MSB has been metabolized in an unknown manner by seeds, like a toxic compound. This can explain fungus growth on the surface of seeds, in spite of the growth inhibitory activity of MSB on fungal development. This result emphasizes the need of testing each elicitor according with its structure and proposed mode of action. The efficacy of MSB probably needs its perception by photosynthetically active tissues to reach its target and induce the IAA build-up, linked with the protective effect of this compound against microbial diseases in plants.

Table II. Effect of chitosan and MSB on the number of root lesions induced by FOL infection in emerging tomato roots

Treatment	Number of damaged roots (4 days)	(7 days)
1-Chitosan 0.5 mg.mL^{-1} agar amendment	2.00 bc	3.40 bc
2-Chitosan 1.0 mg.mL^{-1} seed coating	2.60 cd	3.75 cd
3-Chitosan 1.0 mg.mL^{-1} agar amendment	1.60 bc	2.95 b
4-Chitosan 1.0 mg.mL^{-1} agar + 0.5 mg.mL^{-1} seed coating	1.40 b	2.75 b
5-MSB 0.050 mg.mL^{-1} seed coating	3.60 e	5.80 e
6-MSB 0.100 mg.mL^{-1} seed coating	3.20 e	6.30 e
7-Control (untreated seed and agar)	4.80 f	7.50 f
8-Chitosan 1.0 mg.mL^{-1} agar and seed coating	0.33 a	1.85 a
	Esx = 0.01	Esx = 0.01

Values with common letter did not differ as Duncan test at 5 % probability. Number of damaged roots from 10 seeds for each petri dish with three replicates per treatment.

In plates containing chitosan-coated seeds and chitosan-treated agar, the number of damaged roots was lower compared with control. However, this protective effect was greater applying chitosan to agar than coating seeds with chitosan, at 1.0 mg.mL^{-1}. Effective protection against FOL attack was observed with a combination of seed coating and agar amendment with chitosan, in a similar behavior as previously reported (19). Seven days after seed germination, very few symptoms of root attack were seen, it confirming the fact that chitosan, besides interfering directly with fungal growth, can induce defensive responses in emerging plant tissues. However, a little bit delay in germination of seeds was seen in treatment 8, perhaps due to the high doses of chitosan both included in agar and as seed coating or any impurities contained in our chitosan samples. Therefore, in subsequent experiments, a dose of 0.5 mg.kg^{-1} substrate is chosen, to avoid any detrimental side effect on plant growth and development.

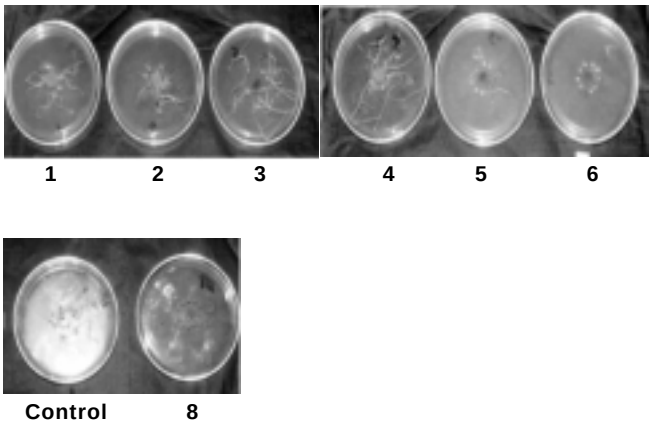

Figure 2. Influence of chitosan and MSB assayed as agar amendment and as seed coating on FOL infection in emerging tomato roots (Treatments are the same as table II, seven days after inoculation)

Interestingly, besides the evidence provides additional support that natural host defense mechanism may be manipulated to produce fungal resistant plants, it should be noted the synergistic effects between known fungal elicitors (chitosan) and MSB, a compound proposed to be involved in the releasing of endogenous elicitors from plant cell walls. The drastic increase in protection and the relative low concentration of chitosan hydrolisates and MSB that provoke the protective effect could constitute novel findings that might have profound influence on the future integration of biological inducers of systemic acquired resistance with the integrated pest management (IPM) (20).

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REFERENCES

1. Ebel, J. and Cosio, E. G. Elicitors of plant defense responses. *International Review of Cytology*, 1994, vol. 148, p. 1-36.
2. Hahlbrock, K., *et al*. Oligopeptide elicitor-mediated defense gene activation in cultured parsley cells. *Proceedings of the National Academy of Science*, 1995, vol. 92, p. 4150-4157.
3. Benhamou, N. Elicitor-induced resistance in tomato plants against fungal pathogens: ultrastructure and cytochemistry of the induced response. *Scanning Microscopy*, 1995, vol. 9, no. 3, p. 861-880.
4. El-Ghaouth, A., *et al*. Chitosan induces systemic resistance against *Phytium aphanidermatum* in greenhouse grown cucumber. *Phytopathology,* 1994, vol. 84, no. 9, p. 1120-1127.
5. El-Ghaouth, A. and Wilson, C. L. Biologically-based technologies for the control of postharvest diseases. *Postharvest News and Information*, 1995, vol. 6, no. 1, p. 5-11.

6. Benhamou, N. and Thierault, G. Treatment with chitosan enhances resistance of tomato plants to the crown and root rot pathogen, *Fusarium oxysporum f.sp. radicis-lycopersicii. Physiological Molecular Plant Pathology,* 1992, vol. 41, p. 33-52.
7. Doares, S. H., *et al*. Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. *Proceedings of the National Academy of Science*, 1995, vol. 92, p. 4095-4098.
8. Ge,Y. and Li, D. A molecular study on tomato induced resistance to *Alternaria solani*. In: International Congress of Molecular Plant-Microbe Interaction (8:1996, Jul. 16-19 : Knoxville), 1996, p. 14-19.
9. RamaRao, A.V., *et al*. Menadione sodium bisulphite: a promising plant growth regulator. *Plant Growth Regulation*, 1985, vol. 3, p. 111-118.
10. Borges, A. P. and Fernández, M. F. Report from the Superior Council of Scientific Research on the product PARAM-1 and their use in banana fields in the Canary Islands. 77, 1996.
11. Borges, A. P. and Fernandez, M. F. Compositions in order to induce resistance to pathogens and plagues in plants. Their application. Patent 95 ES-9500522, 1995a.
12. Borges, A. P. and Fernández, M. F. Compositions in order to stimulate the advance of the floral development in the cultivations of bananas. Their application. Patent 95 ES-9500523, 1995b.
13. Wakabayashi, K., Sakurai, N. and Kuraishi, S. Differential effects of auxin on molecular weight distribution of xiloglucans in cell wall of outer and inner tissues from segments of dark grown squash (*Cucurbita máxima* Dutch) hypocotyls. *Plant Physiology*, 1991, vol. 95, p. 1070-1076.
14. Reyes de la Cruz, H., *et al*. Defense elicitors and auxin share some biochemical responses. In: National Congress and Joint Mexico-US Symposium on Plant Biochemistry and Molecular Biology (8,2 L: 1998 March 15-18: Guanajuato), 1998.
15. Davis, D. A., *et al*. Enhancement of phytoalexin accumulation in cultured plant cells by oxalate. *Phytochemistry*, 1992, vol. 31, no. 5, p. 1603-1607.
17. Alimuniar, A. and Zainuddin, R. An economical technique for producing chitosan. In: Proceedings from the International Conference on Chitosan (6: 1994 Aug. 16-19 : Poland), 1994.
16. Armas, G. de, Díaz, T. and Hernández, J. C. Mejoramiento para la resistencia ante enfermedades de importancia económica en el cultivo del tomate (*Lycopersicon esculentum* Mill) en Cuba. In: Evento científico "Producción de cultivos en condiciones tropicales". Instituto de Investigaciones "Liliana Dimitrova" (1997: Nov. 24-29:La Habana), 1997.
18. Pombo, R., *et al*. Antifungal activity of lobster chitosan on growth of two phythogenic fungi. In: Congress of Cell Wall (7: 1995 Sep. 22-29: Santiago de Compostela), 1995.
19. Benhamou, N., Lafontaine, P., Nicole, J. and Nicole, M. Induction of systemic resistance to Fusarium crown and root rot in tomato plants by seed treatment with chitosan. *Phytopathology*, 1994, vol. 84, no. 12, p. 1432-1444.
20. Ye, X. S., Strobel, N. E. and Kúc, J. A. Induced systemic resistance (ISR). Activation of natural mechanism as part of integrated pest management (IPM). In: Novel Approaches to Integrated Pest Management. R. Reuveni eds. Florida. Lewis Publishers, 1995, p. 95-113.

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