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ANTIFUNGAL ACTIVITY OF CHITOSAN AND ONE OF ITS HYDROLYSATES ON *Pyricularia grisea,* Sacc. FUNGUS

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ABSTRACT. Chitosan and its derivatives considerably reduce mycelial growth of several fungi with a higher inhibitory effect in high concentrations. The *in vitro* fungal and fungicidal effect of chitosan and chitosan hydrolysate was analyzed on *Pyricularia grisea* fungus. The experiment was carried out in the Microbiology Laboratory from the Crop Physiology and Biochemistry Department, at the National Institute of Agricultural Sciences (INCA). Results showed that the two products reduce the radial growth of fungus mycelia, with significant differences between the treatments. The best concentrations were 1000 mg.L^{-1} for chitosan, as well as 500 and 100 mg.L^{-1} for chitosan hydrolysate, due to the full inhibition observed in all evaluations. The biocide effect of these concentrations was also proved.

Key words: chitosan, antifungal properties, *Magnaporthe grisea*, Pyricularia

RESUMEN. La quitosana y sus derivados reducen marcadamente el crecimiento micelial de algunos hongos con un mayor efecto inhibitorio a elevadas concentraciones. El efecto fungistático y fungicida *in vitro* de la quitosana y el hidrolizado de quitosana fue investigado sobre el hongo *Pyricularia grisea*. El experimento se realizó en el laboratorio de Microbiología del Departamento de Fisiología y Bioquímica Vegetal del Instituto Nacional de Ciencias Agrícolas (INCA). Se encontró que ambos principios activos reducen el crecimiento radial del micelio del hongo con diferencias significativas entre los tratamientos. Las mejores concentraciones utilizadas fueron 1000 mg.L^{-1} para la quitosana, 500 y 1000 mg.L^{-1} para el hidrolizado de quitosana, pues en todas las evaluaciones realizadas se observó total inhibición. Además, se demostró el efecto biocida de estas concentraciones.

Palabras clave: quitosana, propiedades antimicósicas, *Magnaporthe grisea*, Pyricularia

INTRODUCTION

Fungal diseases are among the main causes of low yields in rice (*Oriza sativa,* L.) crop. The highest degree of importance, not only in Latin America but also in the rest of the world, has been historically assigned to Rice Blast, a disease caused by *Pyricularia grisea* Sacc. fungus, due to its destructive capacity which reaches up to 80 % (1, 2) under favorable conditions.

Varietal resistance, some agronomical measures, as well as chemical control are within the most commonly used methods to fight against such a disease, the chemical control being the most frequently used one, mainly by means of systemic fungicides like Hinosan, Fuji One, Silvacur Combi EC30, and Kitazin CE 48 (3). However, the Technical Pattern of Rice is always providing new names of stronger fungicides with unstable efficiency to control this disease; then, they are more harmful to the man and environment, besides being more expensive. Therefore, much work is done nowadays, with the purpose of finding new ecologically bioactive products in order to control rice blast.

Chitosan, a linear β-(1,4)-glucosamine polymer, is an important cell wall component of some phytopathogenic fungi. It can be obtained by the alkaline desacetylation of chitin, another cell wall component of fungi and arthropod exoesqueletons, which is the most prevailing mucopolysaccharide in nature.

In recent times, this bioproduct has caught much attention from researchers due to its dual function: it influences directly the development of some phytopathogenic fungi, such as *Rhizopus stolonifer*, *Botrytis cinerca* and *Rhizoctonia solani* (4, 5, 6, 7, 8, 9), also it stimulates some defense mechanisms in plants (10, 11). Due to these properties, together with its natural, biocompatible, and fully innocuous polysaccharide character (12), chitosan is considered the perfect substitute of the harmful phytosanitary compounds for the environment.

Up to now, there is only one work, published in 1989 (13), about the use of chitosan with high and low molecular weights, hydrolyzed with a polymerization degree of 7, to control rice blast. None of the products inhibited fungus mycelial growth; however, there is no previous work published on this matter in Cuba. Thus, the objective of this work is to analyze the influence of chitosan and acid chitosan hydrolysate on a *Pyricularia grisea* strain.

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MATERIALS AND METHODS

Fungistatic activity test. Chitosan, with a degree of acetilation of 63 %, was obtained in the Oligosaccharin Laboratory from the Crop Physiology and Biochemistry Department of the National Institute of Agricultural Sciences (INCA). It was dissolved in a watery acetic acid (HAc) 2 % (v/v) solution and its pH adjusted to 5.7, using potassium hydroxide (KOH) to 2M (14). On the other hand, chitosan hydrolysate (HQ), with a degree of polymerization between 2 and 7, was achieved by means of acidic hydrolysis of chitosan with HCl.

Pyricularia grisea strain was isolated at "Los Palacios" Rice Research Station, from leaf samples collected in the areas of "Los Palacios" Rice Agroindustrial Complex (CAI). The fungus was then grown in a culture medium recommended for this pathogen (1), in petri dishes, with a pH of 6, at a temperature between 26 and 28°C, alternating light and darkness. Chitosan, as well as chitosan hydrolysate were added to the culture medium, so that expected concentration of each product was obtained, after sterilizing them separately in autoclave, during 15 minutes at 121°C. The following treatments were applied:

- Control
- Chitosan 100 mg.L^{-1} (Q-100)
- Chitosan 250 mg.L^{-1} (Q-250)
- Chitosan 500 mg.L^{-1} (Q-500)
- Chitosan 1000 mg.L^{-1} (Q-1000)
- Chitosan hydrolysate 100 mg.L^{-1} (HQ-100)
- Chitosan hydrolysate 250 mg.L^{-1} (HQ-250)
- Chitosan hydrolysate 500 mg.L^{-1} (HQ-500)
- Chitosan hydrolysate 1000 mg.L^{-1} (HQ-1000)

20 mL from each treatment were poured in petri dishes and inoculated with an 0.8-cm-diameter mycelial disk.

Fungal activity evaluations were made the third, fifth, seventh, ninth, and eleventh days after inoculation, where growth was determined by measuring the diameter of each colony.

For analyzing results, a randomized complete design with three repetitions per treatment was used. The experiment was carried out during 2000-2001 and repeated four times each year. Results were very similar when averaging the four treatments of each year. There were not significant differences among the repetitions of the same treatment, so proving the repeatability of results. Data were transformed by arcsen $\sqrt{\%}$ formula and a single variance analysis was made to them. Means of the treatments were compared according to Duncan's multiple range test, with a significance level of 0.05.

Biocide activity test. This test was applied to those cases in which a total mycelial growth inhibition was observed. Mycelial disks were inoculated in fresh medium, according to the following treatments:

- Control
- Mycelial disk of Q-1000 mg.L^{-1} + culture medium
- Mycelial disk of HQ- 500 mg.L^{-1} + culture medium
- Mycelial disk of HQ- 100 mg.L^{-1} + culture medium

Growth was evaluated at the third, fifth, seventh, ninth, and eleventh days after inoculation, with three repetitions per treatment and results were analyzed by the same statistical treatment.

RESULTS AND DISCUSSION

Fungistatic activity. In the case of Q-63, considerable differences were observed in all the treatments with respect to the control and, when using 1000 mg.L^{-1} concentration, there was a marked fungus radial growth inhibition in all evaluations (Figure 1). Results are similar to the information provided by a study (15) using other fungal species.

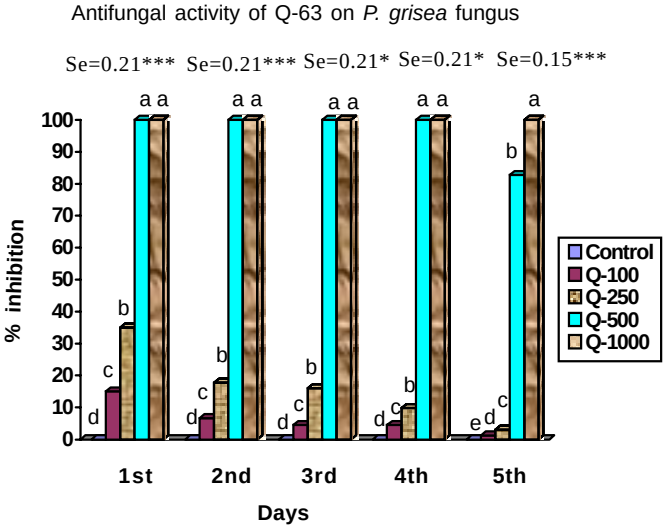

Figure 1. Effect of different Q-63 concentrations on the mycelial growth of *P. grisea* fungus

As to the first four evaluations, it is appropriate to highlight that 500 mg.L^{-1} treatment as well as 1000 mg.L^{-1} kept 100 % inhibition. Therefore, differences between them were not significant, as they were with other treatments; however, in the last evaluation, inhibition had decreased to 82.7 % which implied great differences among all treatments. On the other hand, inhibition percentages in 100 and 250 mg.L^{-1} concentrations were decreasing with the course of the experiment, keeping significant differences between them and the control, in all evaluations.

These results could be due to the fact that at low concentrations of the product, no lethal effects on the fungus but static are recorded; in other words, the microorganism is able to adapt itself to the presence of the product in the culture medium. On the other hand, the concentrations used are relatively low and they do not seem to be directly effective against the fungus. In this sense, some researchers have proved that at the concentrations from 1000 (14,16) to 6000 mg.L^{-1} (15), there is a high inhibition on some fungi. Similar results were observed with the use of HQ (Figure 2); at high concentrations, there was a high inhibition percentage of the fungus mycelium as well.

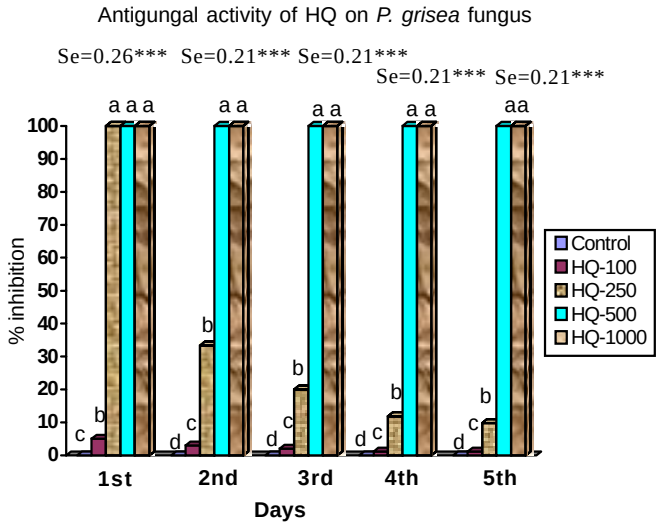

Figure 2. Effect of different HQ concentrations on the mycelial growth of *P. grisea* fungus

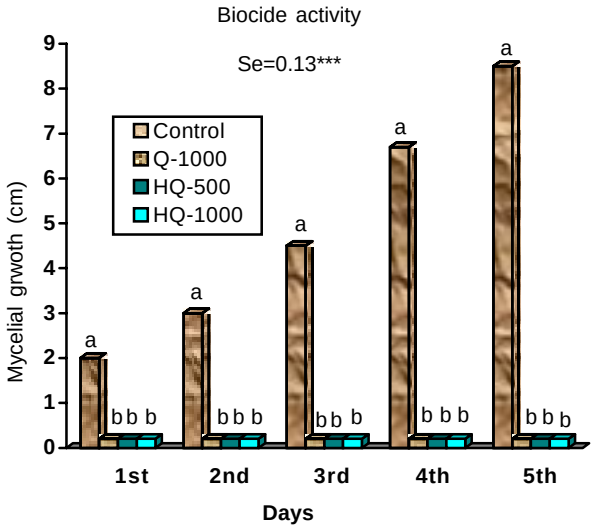

Figure 3. Biocide effect of Q-63 and HQ products on *P. grisea* fungus

In all the evaluations, 500 and 1000 mg.L^{-1} concentrations showed 100 % inhibition. That is why differences between them were not considerable, as they were in respect to the rest of the treatments.

The same percentage was shown by 250 mg.L^{-1} treatment in the first evaluation whereas in the rest, its inhibiting action on the fungus was decreasing until it fell down to 9.8 %, when the last evaluation was made. As to 100 mg.L^{-1} treatment, in spite of showing significant differences with respect to the control, its inhibition percentage was very low in all evaluations: from 12.85 to 6.30 %. Due to these results, it can be stated in general that 100 and 250 mg.L^{-1} concentrations in both products, did not totally affect fungus growth; however, 500 mg.L^{-1} in Q-63 did affect it until the ninth day, and at the eleventh day the fungus began to grow.

Other studies made by using chitosan and other phytopathogenic fungi (12) proved that hypha cell ultraestructure was not totally affected, but the hyphae were dying; therefore, they would continue growing and developing while exposed or adapted to fresh media.

Biocide activity. Results of biocide activity are shown in Figure 3. In this case, all evaluations of the control treatment showed a normal mycelial growth in time, compared to mycelia from treatments, which had a total inhibition. Therefore, in the latter treatments, products had an antifungal and a fungitoxic activity on the pathogen used in the experiment. In other words, the hyphae and therefore the fungus died radically at these concentrations and under such conditions.

On the other hand, chitosan hydrolysate with 500 and 1000 mg.L^{-1} doses showed a fungicidal activity. However, in the case of chitosan, this activity was observed only when using 1000 mg.L^{-1} doses. This result could be related to molecular size, because chitosan oligomers with a polymerization degree of 7 show a higher antifungal activity (15).

It is important to note that the way by which chitosan and its derivatives perform such an activity is not elucidated. There are two hypotheses to explain chitosan way of acting. According to the first one, chitosan activity can be related to an increment on membrane permeability, due to an interaction with phospholipid groups present in it (13, 14). Consequently, there is a cell disruption provoking amino acid and protein escape (17). According to the second hypothesis, chitosan is able to enter fungus cells and interact with DNA, causing a disorder of its structure, as well as inhibiting RNAm and protein syntheses. Then, the fungus stops growing.

Results are very promising and confirm that chitosan and its hydrolysates have potentialities to become active principles for biopesticide formulation and can be used in the integrated management of pests and diseases.

REFERENCES

1. Fabregat, M. Caracterización de patotipos de *Pyricularia grisea* en la región occidental. [Tesis de grado], CENSA, 1984.
2. Cárdenas, R. M. Caracterización morfoagronómico de aislados monospóricos de *Pyricularia grisea*. [Tesis de grado], ISCAH, 1999.
3. Cordero, V. /*et al*./. Manejo integrado de *P. grisea* Sacc. en el cultivo del arroz en Cuba. En: Libro Resumen del Encuentro Internacional del Arroz. (1:2001 jun. 9-10:La Habana).
4. El Ghaouth, A.; Arul, J.; Grenier, J. and Asselin, A. Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. *Phytopathology*, 1992, vol. 82, p. 398-402.
5. Roller, S. and Covill, N. The antifungal properties of chitosan in laboratory media and apple juice. *Int. J. Food Microbiol*., 1999, vol. 47, p. 67-77.
6. Pombo, R. Relación estructura actividad de la quitosana y sus hidrolizados solubles sobre algunos hongos fitopatógenos. [Tesis de Maestría], ISAAC, 1996.

7. Nápoles, M. C., Cabrera, J. C.; Cabrera, G. and Varela, M. Efecto de diferentes polisacáridos sobre el crecimiento de algunos hongos fitopatógenos. *Cultivos Tropicales*, 1997, vol. 18, no. 3, p. 27-29.
8. Benhamou, N.; Kloepper, J. W. and Tuzun, S. Induction of resistance against Fusarium wilt of tomato by combination of chitosan with endophytic bacterial strain: ultraestructure and cytochemistry of the host response. *Plant*, 1998, vol. 209, p. 153-168.
9. Parra, Y.; Ramírez, M. A. and Rodríguez, G. Efecto de la quitina y sus derivados en el crecimiento *in vitro* del hongo *Rhizoctonia solani*. *Cultivos Tropicales*, 2002, vol. 23, no. 2, p. 73-75.
10. Rhoades, J. and Roller, S. Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. *Applied and Environmental Microbiology*, 2000, vol. 66, no. 1, p. 80-86.
11. Ramírez, M. A.; Cabrera, G.; Gutiérrez, A. and Rodríguez, T. Metodología de obtención de quitosana a bajas temperaturas a partir de quitina de langosta. *Cultivos Tropicales*, 2000, vol. 21, no. 1, p. 79-82.
12. Laflamme, P.; Benhamou, N.; Bussieres, G. and Dessureault, M. Differential effect of chitosan on root rot fungal pathogens in forest nurseries. *Can. J. Bot*., 1999, vol. 77, p. 1460-1468.
13. Hirano, S. and Nagao, N. Effects of chitosan, pectic acid, lysozyme, and chitinase on the growth of several phytopathogens. *Agric. Biol. Chem*., 1989, vol. 53, no. 11, p. 3065-3066.
14. Leuba, J. L.; Stossel, P. Chitosan and other polyamines: Antifungal activity and interaction with biological membranes. In Chitin in nature and technology. New York:Plenum Press, 1986. 222 p.
15. Kendra, D. F. and Hadwiger, L. A. Characterization of smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisun sativum*. *Exp. Mycol*., 1984, vol. 8, p. 276-281.
16. Ben Shalom, N. and Patt, D. Composition and method for controlling fungal disease in plants. United States Patent, No. 5965 545, October 12, 1999.
17. Kauss, H.; Jeblick, W. and Domard, A. The degrees of polymerization and N-acetilation of chitosan determine its ability elicit callose formation in suspension cells and protoplasts of *Catharanthus roseus*. *Plant*, 1989, vol. 178, p. 385-392.

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