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RESEARCH

Effect of chitosans of different molecular masses on the fungus *Curvularia lunata*

Yosleidy Valle Fernández[1], ✍Aida Tania Rodríguez Pedroso[1], Miguel Ángel Ramírez Arrebato[1], Juan José Reyes Pérez[2], Luis Guillermo Hernández Montiel[3], Rubén Cruz Pérez[4]

[1] Unidad Científico Tecnológica de Base Los Palacios, Instituto Nacional de Ciencias Agrícolas, INCA Carretera La Francia Km 1½, Los Palacios Pinar del Río, CP 22900, Cuba
[2] Quevedo State Technical University. Av. Quito. Km 1.5 vía a Santo Domingo. Quevedo, Los Ríos, Ecuador
[3] Northwest Biological Research Center S.C., National Polytechnic Institute No. 195 Colonia Playa Palo de Santa Rita Sur, La Paz, Baja California Sur, México
[4] Empresa Agroindustrial de Granos Los Palacios Calle 26 No.1920 entre 19 y 21 Los Palacios, Pinar del Río, Cuba
✍atania@inca.edu.cu

ABSTRACT

Chitosan is a biopolymer that has different applications in agriculture as it is biostimulant, antifungal, inducer of resistance, among others. In this work, it was evaluated the direct effect of chitosans of different molecular masses on the fungus *Curvularia lunata*, a pathogen that affects rice cultivation. For them, three chitosans were used, of low, medium and high molecular mass, at the 1, 2 and 3g/L concentrations. In vitro studies were carried out, determining the percentage of inhibition of mycelial growth, as well as the presence of spores, if these were viable, and if the chitosan affected the germination of the spores. The results showed that the higher the concentration of chitosans of different molecular mass, the higher the percentage of inhibition of the mycelial growth of the fungus. Furthermore, the low molecular mass chitosan increased the production of spores at the highest concentration of the bioproduct (3 g/L) used.

Keywords: spores, inhibition, antifungal, rice, bioproduct, germination, concentration

RESUMEN

Efecto de quitosanos de diferente masa molecular en el hongo *Curvularia lunata*. El quitosano es un biopolímero con varias aplicaciones en la agricultura, como bioestulante, antifúngico, inductor de Resistencia, entre otras. En este trabajo se evaluó el efecto directo de quitosanos de tres masas moleculares sobre el hongo *Curvularia lunata*, patógeno que afecta a los cultivos de arroz. Para ello, se ensayaron quitosanos tres masas moleculares (baja, media y alta), a concentraciones de 1, 2 y 3g/L. Se desarrollaron ensayos in vitro para determinar el porcentaje de inhibición en el crecimiento del micelio, así como la presencia de esporas, y de ser viables, cómo afectaron los quitosanos a su germinación. Los resultados mostraron que los quitosanos a su mayor concentración tuvieron el mayor porcentaje de inhibición sobre el crecimiento del micelio fúngico. Además, el quitosano de la menor masa molecular incrementó la producción de esporas a la mayor concentración (3 g/L).

Palabras clave: esporas, inhibición, antifúngico, arroz, bioproducto, germinación, concentración

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Introduction

Diseases are one of the main causes of low yields in rice cultivation [1]. Around 80 diseases caused by abiotic and biotic factors affecting this crop in the world have been described [2]. Of these, seven are of economic consideration in our country: six caused by fungi and one by virus. They are: *Pyricularia grisea* (SACC), *Rhizoctonia solani* (KUHN), *Sarocladium oryzae* (Sawada), *Helminthosporium oryzae* Bredade (HAA), *Rhynchosporium oryzae* (HAS), Fungal complex and the no less important White leaf rice virus [3].

The staining of the grain produced by the fungal complex, where the following fungi have been registered with greater frequency: *Bipolaris oryzae*, *Phyllasticta* sp., *Gerlachia oryzae*, *Alternaria padwickii* (Ganguly) M. B. Ellis, *Curvularia* sp., *Pyricularia grisea* Sacc, *Cercospora oryzae* Miyake, *Sarocladium oryzae* (Sawada) W. Gams and D. Hawkswort and bacteria *Pseudomonas* sp. and *Erwinia* sp., [4]. This disease negatively affects yield components by producing high percentage of vane, affecting germination between 26 and 41 %, as well as the vigor and size of the seedlings; decreases the number of grains per panicle, the mass of the grains up to 40 %, and the filling in 30 % [5]. On the other hand, it demerits the quality of the seed, because it reduces the number of whole grains, increases both the brittle grains in the mill process and the gypsum and abnormal coloring. In addition, in the fields of seed production, the incidence of this disease requires the discard of productive lots [6].

1. Hernández-Rodríguez A, Rives-Rodríguez N, Acebo-Guerrero Y, Díaz-de la Osa A, Heydrich-Pérez M, Divan-Baldani VL. Potencialidades de las bacterias diazotróficas asociativas en la promoción del crecimiento vegetal y el control de *Pyricularia grisea* (Sacc) en el cultivo del arroz (*Oryza sativa* L.). Rev Prot Veg. 2014;29(1):1-10.

2. Almaguer M, Rojas TI, Hernández A. Perspectivas de los estudios aeromicológicos para la protección del cultivo del arroz. Rev Prot Veg. 2008;23(3):137-43.

3. Pérez L, Cordero V, Fabret L. Eficacia del azoxystrobin y diferentes triazoles en el control en campo de las principales enfermedades fúngicas del arroz en Cuba. Centro Agrícola. 2009;36(2):15-23.

Among the measures applied for the control of the disease is the use of treated seeds, phytotechnical management according to the type of intensity of the disease and chemical treatment. Despite the latter is the most used, however, the products applied are highly toxic, environmentalpollutants and very expensive. That is why our team is working on the search for new alternatives that allow a better management, in which bio-friendly products are used with the environment. Within these is the chitosan, a compound found in the exoskeleton of crustaceans such as lobsters, shrimps, crabs, etc. [7]. It has the potential to inhibit the mycelial growth of numerous phytopathogenic fungi and to stimulate the defense mechanisms of plants [8-12].

Consequently, this work was aimed to evaluate the antifungal activity in vitro of chitosan of different molecular masses on mycelial growth, of the fungus *Curvularia lunata*.

Materials and methods

Chitosans

Three samples of commercial chitosan (Sigma-Aldrich, USA) with different molecular mass were used: low molecular mass chitosan (LMMC) of 121.6 kDa, medium molecular mass chitosan (MMMC) of 152.6 kDa and high molecular mass chitosan (HMMC) of 211.6 kDa, respectively, all three with a degree of deacetylation of 90 %. The samples were dissolved separately in 1 % (v / v) acetic acid solution and the acidity was adjusted to a pH 5.6 using 2 M sodium hydroxide solution.

Fungus strain and culture conditions

The isolation used was *C. lunata* (CL-02) from the ceparium of the Plant Protection Laboratory from Los Palacios Base Scientific Technological Unit, located in the municipality of Los Palacios, Pinar del Río, Cuba. The fungus was cultivated in potato-dextrose-agar medium (PDA) in Petri dishes, at 26 to 28 ºC, with 16 h light and 8 h of darkness alternation. Chitosans and the culture medium were sterilized separately in autoclave at 121 ºC for 15 min. Then, they were combined to obtain the following treatments: 1, 2 and 3 g/L and a control with only PDA. In Petri dishes, 10 mL were added to the culture medium containing each treatment and were inoculated with a 5 mm *C. lunata* mycelium disc.

The evaluations of the diameters of the colonies formed were made manually with a graduated scale, every 24 h for 5 days and compared with the growth of the fungus in the control medium (without chitosan), and the percentage of inhibition on the colony (Equation 1).

$$\text{Radial inhibition (\%)} = \frac{(Tc - Tt)}{Tc} \times 100$$

Where:

Tc: average radio of the control colony.

Tt: radio of the colony in medium with chitosan.

The mycelial growth inhibition results were analyzed by a completely randomized design with 5 repetitions per treatment, using the NCSS 2001 program for the analysis of variance. Means were compared with the Tukey multiple range test ($p \leq 0.05$).

Spore germination assay

At the end of the experiment, the presence of spores and their germination capacity in the different treatments were evaluated. To this end, 10 mL of sterile distilled water was added to each plate, scraped with a glass rod; 1 mL was taken per treatment, and the presence of spores was observed under an optical microscope.

Three milliliters of the suspension were taken from treatments with the presence of spores and added to a plate containing PDA culture medium, which were incubated at 25 ºC for 4 h. Subsequently, using the optical microscope (Novel), 50 spores (germinated and non-germinated) were counted from each treatment, determining the number of spores germinated per plate every 1 h. A spore was considered germinated when the germ tube doubled the diameter of the spore [13].

Effect of chitosan on germination of spores

The spore suspension was obtained from a culture of *C. lunata* after 10 days of incubation. An aliquot of 0.5 mL was distributed in the PDA containing chitosan (1, 2 and 3 g/L) in a Petri dish. The control treatment did not contain chitosan. The plates were incubated for 72 h and the germinated spores were counted.

Results and discussion

The inhibitory effect of LMMC, MMMC and HMMC on the fungus Curvularia lunata was tested in vitro. The results are shown in figure 1.

The percentage of inhibition on the *C. lunata* fungus of the three chitosans at the concentrations used was significantly higher as the concentration increased; being the highest concentration 3 g/L. It is important to highlight that 72 h seems to be the period of adaptation and recognition of the pathogen to the presence of the biocomposite and from this time its effect on the fungus under study is defined. Pabón-Baquero *et al*. [14] also observed an inhibition of mycelial growth in 98.7 % in an isolate of *C. lunata* obtained from a *Jatropha curcas* seed with respect to the control when applying a chitosan of low molecular mass at a concentration of 4 g/L. Also, Rivero *et al*. [4] evaluated the antifungal activity of a high molecular mass chitosan on a *C. lunata* strain isolated from rice grains, observing that chitosan concentration increased the greater the inhibition of mycelial growth.

Other authors [15] studied the influence of the molecular mass of chitosan and its concentration but on the fungus *Aspergillus niger*, determining that the chitosan of low molecular mass at the highest concentration applied had a strong antifungal activity on this pathogen. Like Rodriguez *et al*. [16] proved that a chitosan of medium molecular mass (152.6 kDa) at a concentration of 4 g/L achieved greater inhibitory effect on the fungus *B. oryzae*. On the other hand, Hengiun *et al*. [17] detected an antifungal activity of 75 % of the high and low molecular mass chitosans on the *Botryotina fuckeliana* pathogen at a concentration of 2 g/L.

Micrographs of the mycelium were observed through the optical microscope with the 10× objective of the control hyphae and those treated at 72 h (Figure 2).The hyphae of the control treatment are further

4. Rivero-González D, Cruz-Triana A, Martínez-Coca B, Ramírez-Arrebato MA, Rodríguez-Pedroso AT. Actividad antifúngica in vitro de la quitosana Sigma frente a hongos fitopatógenos causantes del manchado del grano en el cultivo del arroz (*Oryza sativa* L.). Fitosanidad. 2009;13(2):101-7.

5. Pinciroli M, Sisterna MN, Bezus R, Vidal AA. Manchado del grano de arroz: efecto de la fertilización nitrogenada. Rev Fac Agron. 2003;105(2):11.

6. Ibrahim E, Abao El-Dahab MS. Seed discoloration and their effect on seedling growth of Egyptian hybrid rice. Res J Seed Sci. 2014;7(3):63-74.

7. Maghsoodi V, Yaghmel S, Beigi SM. Influence of different nitrogen sources on amount of chitosan production by *Aspergillus niger* in solid state fermentation. Iran J Chem Chem Eng. 2008;27:47-52.

8. Ayala A, Colina M, Molina J, Vargas J, Rincón D, Medina J, *et al*. Evaluación de la actividad antifúngica del quitosano contra el hongo *Mycosphaerella fijiensis* morelet que produce la Sigatoka negra que ataca el plátano. Rev Iberoamer Polímeros. 2014;15(6):312-38.

9. Bhattacharya A. Fungicidal potential of chitosan against phytopathogenic *Fusarium*. J Exp Biol Agric Sci. 2013;1(4):260-3.

10. Rodríguez AT, Ramírez MA, Cárdenas RM, Hernández AN, Velázquez MG, Bautista S. Induction of defense response of *Oryza sativa* L. against *Pyricularia* (Cooke) Sacc. by treating seeds with chitosan and hydrolyzed chitosan. Pest Biochem Physiol. 2007;89:206-15.

11. Xing K, Xiao Z, Xue P, Sheng Q. Chitosan antimicrobial and eliciting properties for pest control in agriculture: a review. Agron Sustain Dev. 2015;35(2):569-88.

12. Ramírez-Arrebato MA, Rodríguez-Pedroso AT, Bautista-Baños S, Ventura-Zapata E. Chapter 4. Chitosan Protection from Rice Diseases. In: Bautista-Baños S (editor). Chitosan in the Preservation of Agricultural Commodities. Oxford: Oxford Academic Press; 2016. p. 115-26.

13. Paul GC, Kent CA, Thomas CR.. Viability testing and characterization of germination of fungal spores by automatic image analysis. Biotechnol Bioeng. 1993;42:11-23.

14. Pabón-Baquero D, Velázquez-del Valle M, Evangelista-Lozano S, León-Rodríguez R, Hernández-Luzardo AN. Efectos del quitosano en hongos fitopatógenos y en la germinación de semillas de *Jatropha curcas* L. Rev Chapingo Serie Cienc Forest Ambiente. 2015;XXI(3):241-53.

15. Xiao-Fang L, Xiao-Qiang F, Sheng Y, Ting-Pu W, Zhong-Xing S. Chitosan on antifungal activity against *Aspergillus niger*. Iranian Polymer J. 2008;17(11):843-52.

16. Rodríguez-Pedroso AT, Plascencia-Jatomea M, Bautista-Baños S, Cortez Rocha MO, Ramírez Arrebato MA. Actividad antifúngica *in vitro* de quitosanos sobre patógeno del arroz. Acta Agron. 2016;65(2):169-74.

17. Hengjun J, Zhimin S, Ruixiu J, Wang X, Jianying H. Effect of chitosan as an antifungal and preservative agent on postharvest Blueberr. J Food Qual. 2016;39(5):516-23.

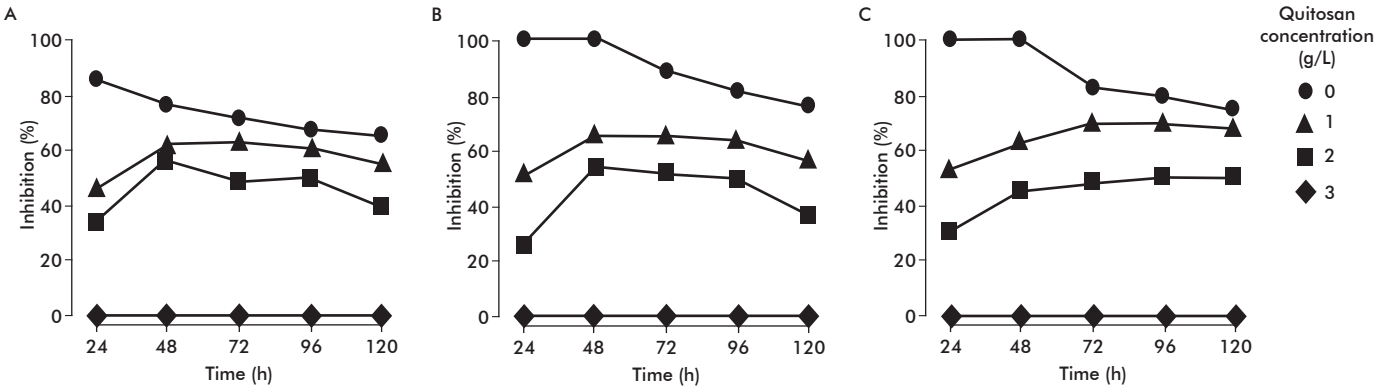

Figure 1. Effect of chitosan with different molecular masses and at different concentrations (1, 2 and 3 g/L) on the percentage of inhibition of mycelial growth of the fungus, *Curvularia lunata* during a 120 h incubation period. A) Treatment with low molecular mass chitosan, 121.6 kDa. B) Treatment with medium mass molecular chitosan, 152.6 kDa. C) Treatment with high mass molecular chitosan, 211.6 kDa. Numerical values stand for standard error of the mean.

apart and no spores are visible. However, the mycelial hyphae treated with the LMMC and at the concentration of 3 g/L are more agglomerated, clustered and more spore areas were observed.

Some authors have pointed out that chitosan stimulates sporulation. The formation of spores of *Alternaria alternata* in the presence of chitosan at concentrations of 0.1 and 0.5 g/L was significantly higher than control without chitosan [18]. It was also indicated that high sporulation may have been due to a stress response induced by this polymer [18].

Similar results were observed by Rodríguez *et al*. [16] with the fungus *B. oryzae* at the concentration of 3 g/L with LMMC. The mycelium of the fungus grew in the medium treated with chitosan, characterized by a high production of spores and damage to aerial hyphae.

Evaluation of spore germination

Spore germination from the mycelium treated with chitosan is shown in figure 3. As can be seen, all the spores had germinated at 4 h and there were no deformations in the germ tubes. So it demonstrates that the spores produced as defense mechanism of the fungus were germination viable spores. Significantly, there were no previous reports on germination of *C. lunata* spores from mycelium treated with chitosans.

Effect of chitosan on germination of spores

The germination of spores of *C. lunata* was completely inhibited in the treatments with either LMMC, MMMC and HMMC at the concentrations used during the first 4 h. This contrasted with the effect seen for the control treatment without chitosan, which already started to emit the germ tube and 90 % of the spores were already germinated at 3 h (Figure 4).

However, at 5 h, all the spores were germinated, the germ tube began to emit the spores that were found in the 3 g/L LMMC. This is the same treatment where the mycelium grew and produced the spores which germinated. Therefore, this corroborated that at that chitosan concentration, the production of spores and its germination were stimulated. In this respect, Pabón-Baquero *et al*. [14] observed that the germination of the spores of *C. lunata* was totally inhibited with a

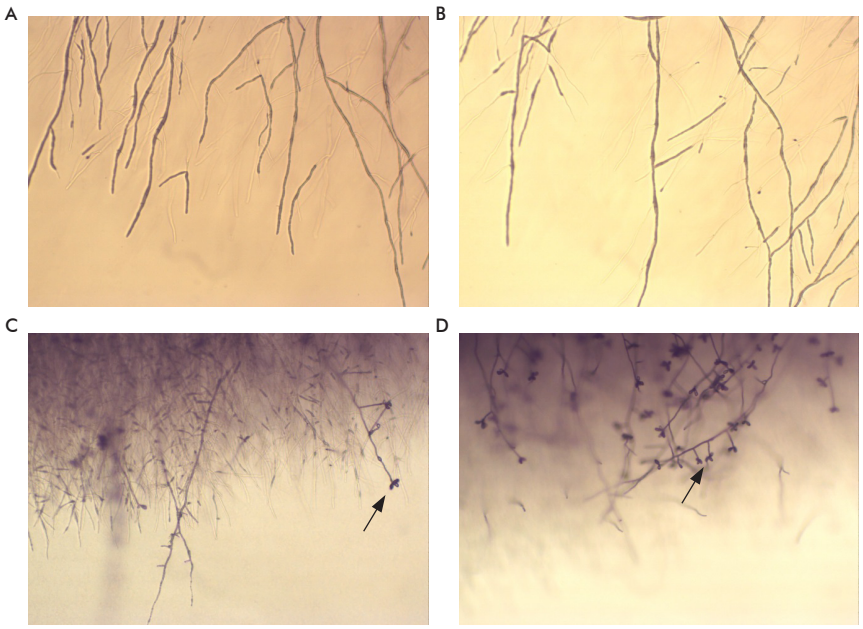

Figure 2. Micrograph of the effect of chitosan on hyphae and the production of spores. A and B) Control treatment without chitosan, showing normal hyphae production. C and D) Treatment with 3 g/L low molecular mass chitosan (121.6 kDa). C) Agglomeration of hyphae (arrow). D) High production of spores (arrow).

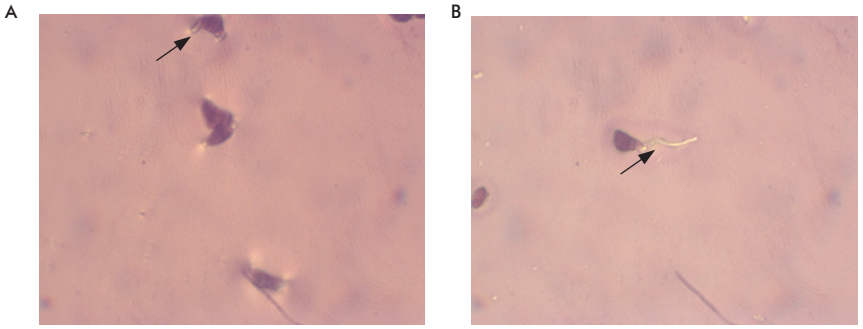

Figure 3. Micrograph of spores germination from the treatment with LMMC at the concentration 3 g/L. A) Start of the germination process at 2 h, 40×. B) Germination of the spores at 4 h, 40×.

LMMC at 2 and 4 g/L, proving the difference in sensitivity to this compound of different strains of the same species.

Hengiun *et al*. [17] evaluated chitosans of high and low molecular mass at 0.4 g/L, showing a complete inhibition of conidial germination on the fungus *Botryotina fuckeliana*. In fact, different structures (spores, germinal tube, vegetative hyphae) of the fungi show sensitivity to chitosan [19-21].

Conclusions

In summary, it can be proposed that chitosans of different molecular masses do not have a fungicidal effect, but they do affect the growth of the *C. lunata* fungus as the chitosan concentration increases. Regarding the production of spores, it was found that the LMMC at 3 g/L achieved a high spore production, while chitosan affected their germination speed. Therefore, LMMC, MMMC and HMMC affect the *C. lunata* fungus. Our results could be used to develop biocompatible fungicided for crop protection in the future..

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

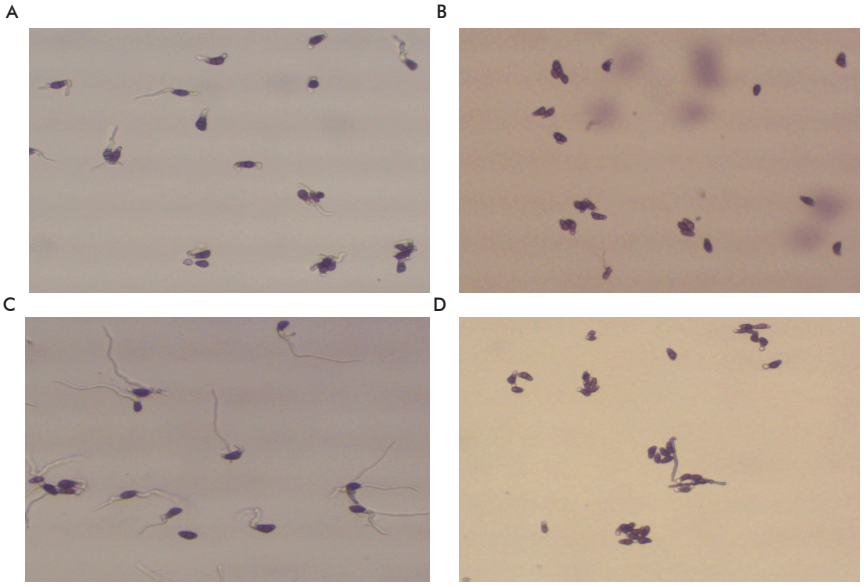

Figure 4. Micrograph of spores germination in media without chitosan (Control) and with chitosan. A) Control at 3 h. B) Treatment with chitosan (low molecular mass chitosan (121.6 kDa). C) Control at 5 h of germination. D) Treatment with low molecular mass chitosan (121.6 kDa) at the concentration of 3 g/L.

18. Bautista-Baños S, Hernández-López M, Bosquez-Molina E. Growth inhibition of selected fungi by chitosan and plant extracts. Mexican J Phytopathol. 2004;22:178-86.

19. Cota-Arriola O, Cortez-Rocha MO, Rosas-Burgos EC, Burgos-Hernández A, López-Franco YL, Plascencia-Jatomea M. Antifungal effect of chitosan on the growth of *Aspergillus parasiticus* and production of aflatoxin B1. Polymer Int. 2011;60(6) :937-44.

20. González-Peña D, Falcón Rodríguez AB, Gómez Izaguirre G, Fernández-Morales A. La quitosana: potencialidades antimicrobianas de los hongos y Oomycetes. Fitosanidad. 2013;17(2):111-5.

21. Silva A, Stamford T, Souza P, Berger L, Leite M, Nascimiento A, *et al*. Antifungal activity of microbiological chitosan and coating treatment on cherry tomato (Solanum lycopersicum var, cerasiforme) to post-harvest protection. Int J Curr Microbiol Applied Sci. 2015;4(9):228-40.

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