Low molecular weight chitosan hydrolyzate inhibits the growth of some phytopathogenic *Ascomycota* fungi

T A Timofeeva, A O Zakurin¹, A V Nezhdanova, B Ts Shagdarova, A A Davlekanova, S E Gaydukova, I V Yakovleva and A M Kamionskaya

Federal Research Centre «Fundamentals of Biotechnology» of the Russian Academy of Sciences, Moscow, 119071, Russian Federation

¹E-mail: arturus@inbox.ru

Abstract. The work studied the effect of low molecular weight chitosan hydrolyzate on the growth and development of phytopathogens *Botrytis cinerea*, *Fusarium solani*, *Alternaria solani*. Chitosan hydrolyzate, when added to potato-glucose agar, inhibits the growth of mycelium *in vitro* of *Botrytis cinerea*, *Fusarium solani*, *Alternaria solani*. Treatment with chitosan hydrolyzate suppresses the development of fusarium on tomato leaves and the development of gray rot on fruits. Further studies of chitosan hydrolyzate effect mechanisms on plants can expand the scope of its application.

1. Introduction

Chitosan is widely known in the world as a biological polymer. It is known that chitosan causes important physiological changes in plants, such as stimulation of growth and differentiation of plant tissues, increases seed germination, enhances the rate of photosynthesis, and acts as a causative agent of the synthesis of secondary plant metabolites [1]. Chitosan also increases the tolerance of plants to water deficiency [2], that is, it helps plants adapt to drought when climatic conditions change.

Chitosan may be involved in the signaling pathway of the biosynthesis of phenolic compounds. It has been shown that chitosan can induce the synthesis of chitinase and chitosanase, which are members of a group of proteins associated with plant pathogenesis (PP). These PP proteins can destroy the cell walls of some phytopathogens and, therefore, can play a role in the defense systems of host plants [3].

The fungicidal activity of chitosan has been previously shown. Chitosan inhibits the development of various fungal pathogens, inhibits spore germination [4], and is also able to penetrate the plasma membrane of some pathogens and lead to cell death [5].

Chitosan forms a protective barrier on the surface of the fruit, while reducing the loss of water and nutrients, preventing gas exchange and preventing the growth of microorganisms that affect the fruit during storage [2].

A number of studies have shown that the properties of chitosan depend on molecular weight [6], chemical structure (degree of deacetylation, degree of polymerization, degree of protonation), as well as on polymer concentration [7].

However, the use of chitosan in agriculture is limited by many factors, including its poor solubility at neutral pH. One of the ways to solve this problem is the use of chitosan depolymerization products. In this work, we studied the possibility of using a low molecular weight chitosan hydrolyzate (HC) as an agent for plant protection.
2. Materials and methods

2.1. Chitosan hydrolyzate
Chitosan hydrolyzate, average molecular weight 33 kDa (lp = 2.1), degree of deacetylation 95%, concentration of the initial solution 10 mg / ml. To obtain a HC solution, hydrolysis of high molecular weight chitosan (1040 kDa) was initially carried out using 10% nitric acid at a temperature of 70 °C for 7 hours, after 24 hours the hydrolyzate was diluted with distilled water and the pH of the solution was adjusted to 5.0–5.2 with ammonia hydrate [8]. The chitosan hydrolyzate was kindly provided by the Biopolymer Engineering Laboratory of the Federal Research Center of Biotechnology, Russian Academy of Sciences.

2.2. Plant material
Cherry tomato plants (Solanum lycopersicum var. Cerasiforme) were grown under the experimental climate control facility in individual containers with a volume of 250 cm³ in universal soil, air temperature 24 °C, natural light with additional lighting to maintain a 16-hour photoperiod.

Ripe tomato fruits were sized and checked for damage or signs of infection. The selected fruits were kept in 0.1% sodium hypochlorite solution for 15 minutes, then thoroughly washed with distilled water and dried in air.

2.3. Pathogens
We used cultures of isolated pathogens Botrytis cinerea, Fusarium solani, Alternaria solani. These species are the causative agents of tomato diseases, causing serious damage to their productivity [9]. Cultures of pathogens were kindly provided by the Laboratory of Biotechnology of Physiologically Active Substances, Federal Research Center of Biotechnology, Russian Academy of Sciences.

Pathogen samples were cultured on potato glucose agar (PGA) at 25 °C. The spore suspension was obtained from the sporulating mycelium of a one-week-old culture using a bacteriological loop and suspended in sterile water. The spore concentration was measured using the Goryaev chamber. The spore suspension was used immediately after preparation.

2.4. Effect of chitosan hydrolyzate on the development of fungal pathogens in vitro
We used potato-glucose agar containing chitosan hydrolyzate in the following concentrations: 6 mg/ml, 4 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml.

The daily culture of the pathogen on solid nutrient medium was cut into discs with a diameter of 8 mm. The inverted disk was placed in the center of a Petri dish with PGA + HC. They were cultured at 25°C in the dark for two weeks. On days 3, 7, 14, the diameter of the mycelium was measured.

2.5. Protective effect of chitosan hydrolyzate on tomato leaves
Four-week-old cherry tomato plants were sprayed with a solution of chitosan hydrolyzate at the following concentrations: 5 mg/ml, 2 mg/ml, 0.2 mg/ml, 0.02 mg/ml until falling drops. Distilled water was used as a control. In each variant of the experiment, 5 plants were used. A day later, the leaves were cut, placed in a humid chamber, and inoculated with 25 drops of 10 μl of F. solani spore suspension at a concentration of 2x10⁵ spores/ml. The samples were cultured for 10 days at a temperature of 25°C under natural light [10].

2.6. Protective effect of chitosan hydrolyzate against gray rot of tomatoes
Tomato fruits were sprayed with a solution of chitosan hydrolyzate at a concentration of 5 mg/ml; as a control, they were sprayed with distilled water. The tomatoes were placed for 24 hours in closed containers with a relative humidity of 95%. To maintain humidity, a vessel containing a saturated KNO₃ solution was placed in the containers.

A day later, the fetuses were wounded with a diameter of 3 mm and a depth of 3 mm with a sterile instrument in the equatorial zone. 10 μl of B. cinerea spore suspension at a concentration of 2x10⁴
spores/ml was applied to the wound. The fruits were placed back in containers and cultured at a temperature of 25 °C and a relative humidity of 95%, in the dark. In each variant of the experiment, two replicates of 10 fetuses were used [11].

3. Results

3.1. Effect of chitosan hydrolyzate on the development of fungal pathogens in vitro
The presence of chitosan hydrolyzate in the culture medium has a negative effect on the development of the studied fungal strains in vitro. The suppression of the mycelium growth rate for F. solani and A. solani was achieved at a concentration of chitosan hydrolyzate of 1 mg/ml, while in order to obtain a similar effect on B. cinerea, the working concentration of chitosan hydrolyzate should be increased to 4 mg/ml (table 1).

| Pathogen               | Concentration of chitosan hydrolyzate, mg/ml | * Average colony diameter, mm | Overgrowth has reached the edges of the Petri dish |
|------------------------|---------------------------------------------|-------------------------------|--------------------------------------------------|
|                        |                                             | 3rd day | 7th day | 14th day | 3rd day | 7th day | 14th day |
| Botrytis cinerea       | 6                                           | 13±0.6 | 47±2.0 | 72±1.5  |         |         |         |
|                        | 4                                           | 22±2.6 | 60±2.0 |         | *       |         |         |
|                        | 2                                           | 27±2.1 | 65±1.5 | *       |         |         |         |
|                        | 1                                           | 31±0.6 | 72±1.5 | *       |         |         |         |
|                        | K                                           | 55±2.1 |         |         |         |         |         |
| Fusarium solani        | 2                                           | 16±0.6 | 40±1.0 | 78±0.6  |         |         |         |
|                        | 1                                           | 17±1.2 | 36±0.6 | 69±1.2  |         |         |         |
|                        | 0.5                                         | 21±1.5 | 49±3.5 |         | *       |         |         |
|                        | 0.1                                         | 26±1.2 | 70±1.2 |         | *       |         |         |
|                        | K                                           | 28±0.6 | 75±1.0 |         |         | *       |         |
| Alternaria solani      | 2                                           | 15±0.6 | 19±0.6 | 25±0.6  |         |         |         |
|                        | 1                                           | 19±1.0 | 28±0.6 | 43±0.6  |         |         |         |
|                        | 0.5                                         | 26±0.6 | 44±1.0 |         | *       |         |         |
|                        | 0.1                                         | 41±1.0 | 78±1.5 |         | *       |         |         |
|                        | K                                           | 45±0.6 |         |         |         |         |         |

The most significant effect on the development of the pathogen of gray rot B. cinerea has an increased concentration of hydrolyzate - 6 mg/ml. On the 3rd day of cultivation, the average diameter of the colony under the influence of this concentration was 4 times less than the control, while the growth of mycelium in the control variants had already reached the edges of the Petri dish.

A similar effect on F. solani and A. solani was observed at concentrations from 0.5 mg/ml to 2 mg/ml. On the 7th day of cultivation, the average diameter of the colonies at concentrations of 1 and 2 mg/ml was more than 1.5 times less than in the control variants of the experiment (figure 1).
Figure 1. Colonies of *Botritis cinerea* (a), *Fusarium solani* (b), *Alternaria solani* (c) on a nutrient medium with chitosan hydrolyzate at various concentrations on days 3, 7 and 14 of cultivation.

3.2. Effect of chitosan hydrolyzate on the development of *F. solani* on tomato leaves
High concentrations of chitosan hydrolyzate solution at concentrations of 2 and 5 mg / ml reduce the infestation of tomato plants with fusarium. Lower concentrations have no protective effect against *F. solani*. It is also worth noting that a single treatment of cherry tomato leaves with chitosan hydrolyzate did not have a noticeable effect on tomato leaves and the general condition of the plant as a whole.

Figure 2. Infestation of *Fusarium solani* tomato leaves on days 6 and 10 after infection. a - tomato leaves without treatment on the 6th day of cultivation. b - tomato leaves treated with chitosan hydrolyzate 5 mg / ml on the 6th day of cultivation.

3.3. Effect of chitosan hydrolyzate on the development of *B. cinerea* on tomato fruits
Chitosan hydrolyzate at a concentration of 5 mg / ml with daily processing of tomato fruits reduces the infestation of *B. cinerea*. On the 10th day after infection, 85% of the fruits in the control variant were
affected by gray rot, while the damage to the fruits treated with chitosan hydrolyzate was only 45% (30% on the 4th day versus 75% in the control).

Figure 3. Infestation of *B. cinerea* tomato fruits depending on the presence of treatment with chitosan hydrolyzate solution. Affected fruits are marked in the photographs: a - tomato fruits treated with chitosan hydrolyzate on the 10th day of cultivation, b - tomato fruits without treatment for 10 days.

4. Conclusion
The low molecular weight chitosan hydrolyzate inhibits the development of *B. cinerea*, *F. solani*, and *A. solani*. It does not cause negative effects on tomato leaves and fruits during processing, while it has a protective effect.

It was shown that the concentration of chitosan hydrolyzate 6 mg/ml inhibits the development of *B. cinerea in vitro*. When processing tomato fruits with chitosan hydrolyzate at a concentration of 5 mg/ml, the incidence of gray rot is also reduced by more than 2 times. Treatment of tomato leaves with the same concentration reduces leaf infestation with fusarium.

To suppress the development of *F. solani* and *A. solani in vitro*, the presence of chitosan hydrolyzate in the nutrient medium at concentrations of 1-2 mg/ml is sufficient.

Further studies of the molecular mechanisms of chitosan hydrolyzate effect on plants will expand the scope of its application.

Acknowledgments
The authors are grateful to the staff of the Biopolymer Engineering Laboratory of the Federal Research Center of Biotechnology, Russian Academy of Sciences, for the provided low-molecular-weight chitosan hydrolyzate for research.

The authors also express their gratitude to the staff of the Laboratory of Biotechnology of Physiologically Active Substances of the Federal Research Center of Biotechnology RAS for the provided isolated cultures of fungal pathogens.

This work was performed using the experimental climate control facility in the institute of Bioengineering, Research Center of Biotechnology, Russian Academy of Sciences.

References
[1] Lopez-Moya F, Escudero N, Zavala-Gonzalez E A, Esteve-Bruna D, Blázquez M A, Alabadi D and Lopez-Llorca L V 2017 *Sci. Rep.* 7(1) 16813
[2] Qiuping Z and Wenshui X 2007 *L.-W. Technol.* 40 404-11
[3] Dixon R A, Harrison M J and Lamb C J 1994 *An. Rev. of Phytopathology* 32 479-510
[4] El Hadrami A, Adam L R, El Hadrami I and Daayf F *Mar Drugs* 2010 8(4) 968-87
[5] Palma-Guerrero J, Huang I C, Jansson H B, Salinas J, Lopez-Llorca L V and Read N D Fungal
Gen Biol. 2009 46 585-94
[6] Popova E V, Domnina N S, Kovalenko N M, Borisova E A, Kolesnikov L E and Tyuterev S L
Bulletin of Plant Protection 2017 3(93) 28-33
[7] Batool M, Asghar R 2013 Eurasia J. of Biosc. 7 69-76
[8] Shagdarova B, Ilyina A, Lopatin S, Kartashov M I, Arslanova L, Dzhavakhiya V and Varlamov V 2018 App. Biochem. and Microbiol. 54 71-5
[9] Stancheva J 2005 Atlas of Crop Diseases vol 1 eds O A Kulinich and L V Shirnina (Sofia, Moscow: Pensoft)
[10] Badawy M and Rabea E 2014 Cellulose 21 10
[11] Sun C, Fu D, Jin L, Chen M, Zheng X and Yu T 2018 Carbohydr. Polym. 199 341–52