THE EFFECT OF CHITOSAN HYDROLYSATE ON THE FUNGI *ALTERNARIA SOLANI, FUSARIUM SOLANI* AND *RHIZOCTONIA SOLANI* IN VITRO

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Abstract

Chitosan hydrolysate was obtained using nitric acid; the prevailing fraction had a molecular weight of 30 kDa and a deacetylation degree of 95%. The effect of chitosan hydrolysate when added to potato dextrose agar (PDA) in different concentrations (0.5, 1, 1.5, 2, 4, 6 and 8 mg/mL) was studied on the growth of the fungi Alternaria solani Sorauer, Fusarium solani (Mart.) Sacc. and Rhizoctonia solani J.G. Kühn. A. solani was the most sensitive to the addition of chitosan hydrolysate to PDA in radial growth experiments. On days 3 and 7 of incubation, the antifungal activity of the phytopathogen growth was 69%-92% and 69%-88%, respectively, in the concentration range of 0.5-2 mg/ml.

Keywords: *chitosan, chitosan hydrolysate,* Alternaria solani, Fusarium solani, Rhizoctonia solani

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1. Introduction

In the world of agriculture, the use of chemical agents for plant protection is constantly increasing. They are used to control phytopathogenic microorganisms and the diseases they cause. The use of such chemicals negatively affects the habitat of humans and animals [1]. Interest in the study and use of compounds with antifungal effects from natural sources has increased markedly [2]. When using biopolymers, it is necessary that they have good solubility, low toxicity and antimicrobial effects on a wide range of pathogenic microorganisms in a short period of contact. Chitosan is an available biological polysaccharide with antimicrobial activity, with good biocompatibility and non-toxicity [3]. Possible mechanisms of action of this biopolymer are as follows: (1) polycationic chitosan interacts with electronegatively charged functional groups on the cell surface and changes their permeability, which leads to leakage of intracellular electrolytes and protein components [4]; (2) chitosan penetrates the fungal cell, chelates trace elements and inhibits the synthesis of messenger RNA (mRNA) and protein [5]. Many authors have attributed the antifungal activity of chitosan to the nonspecific mechanism of pathogen suppression. Inhibition of phytopathogens has been observed at various stages of development, such as mycelial growth, spore formation, spore viability and germination [6]. Studies using microscopy of several fungi treated with chitosan showed mycelial swelling, abnormal shapes, excessive branching and hyphal reduction. [6, 7]. Chitosan primarily affects the cell walls/cell membranes of the microorganism [8]. The antifungal activity of chitosan has been determined against various fungi. According to the literature, the minimum inhibitory concentration (MIC) of chitosan was from 0.01 to 7.75 mg/mL [9].

It is known that the antifungal activity of chitosan depends on its molecular weight (MW), the deacetylation degree (DD), the pH of the solution and the strain of the microorganism. Therefore, the results that relate to the determination of the antifungal activity of chitosan are usually difficult to compare, due to the lack of information in the presented studies on its MW, DD and solubility, which significantly decreases at pH values greater than 4.5.

Many species of representatives of the genera *Fusarium*, *Alternaría* and *Rhizoctonia* are pathogens of plant diseases, including important agricultural crops from the Solanaceae family: potatoes, tomatoes, eggplants, peppers and tobacco. Solanaceae includes about 98 genera and about 2,700 species with a wide variety of habitats and morphologies [10]. Some species of fungi from the genus *Fusarium*, such as *Fusarium oxysporum* and *Fusarium solani*, form white or pinkish mycelium on rotting roots. *F. solani* is known to cause diseases of economically important crops, such as potatoes, tomatoes and peppers. The inhibitory effect of chitosan on the growth of *F. solani* colonies was studied *in vitro*. The inhibition caused by chitosan depended on the concentration. The greatest suppression of the growth of fungal colonies (76.0%) occurred at the chitosan dose of 0.20% [11].

Alternaria solani affects the stems, leaves and fruits/tubers of tomatoes, potatoes, eggplants, bell peppers, hot peppers and other members of the Solanaceae family. Tomato crop losses from alternariasis can reach 78%-90% [12]. The results of the study of the effect of chitosan and nanochitosan on the growth of *A. solani* showed that 0.75, 1.5, 3 and 6 mg/ml led to an increase in the percentage of inhibition of the fungus compared with the control treatment. When using nano-chitosan, the highest percentage of inhibition (93.88%) was achieved at a concentration of 6 mg/ml. Chitosan at the same concentration gave an inhibition percentage of 58.9%. The results showed that the percentage of inhibition increases with increasing concentration [13].

Rhizoctonia solani causes a wide range of diseases in potatoes, cereals, sugar beets, cucumbers and rice. Researchers investigated the effect of chitosan with a MW of 150 kDa

and a DD of 80% on the growth of mycelium when it was added at 0.125%, 0.25%, 0.5% or 1% to potato dextrose agar (PDA). Chitosan inhibited the growth of *R. solani* mycelium directly in a dose-dependent manner [14].

There are many methods of combating phytopathogens, each with disadvantages and advantages. Therefore, there is a need to use modern methods that will effectively combat the spread of pathogens without risk to the environment [15]. The aim of the work was to study the effect of chitosan hydrolysate on the growth of phytopathogens *A. solani*, *F. solani* and *R. solani in vitro.*

2. Materials and Methods

2.1. Preparation Chitosan Hydrolysate

To obtain chitosan hydrolysate, 200 ml of 10% nitric acid was added to 10 g of high-molecular chitosan (Mη 1000 kDa, 85% DD) at room temperature. The resulting suspension was heated to 70°C with stirring. Hydrolysis was carried out for 8 h, then the mixture was kept without stirring for 16 h at 23°C, and the required amount of water was added until the precipitate dissolved. The pH of the solution was adjusted to 5.0-5.4 with an ammonia solution (25%), and the mechanical impurities were removed by filtration [16]. The resulting chitosan hydrolysate was used in experiments.

2.2. Characterisation of Chitosan Hydrolysate

The MW characteristics of chitosan hydrolysate fractions were determined with high-performance gel permeation chromatography by using an S 2100 Sykam chromatograph (Germany) with a column (8 × 300 mm) of PSS NOVEMA Max analytical 1 000 A and a pre-column (8.0x50 mm) [17]. Pullulans were used as standards for column calibration.

The DD of chitosan hydrolysate fractions was determined by proton nuclear magnetic resonance (^{1}H-NMR). The proton spectra were recorded on a Bruker AMX 400 spectrometer (USA). Samples were prepared in deuterated water, and 4,4-dimethyl-4--silapentane-sulfonic acid was used as a standard.

2.3. Phytopathogens

F. solani, *A. solani* and *R. solani* were obtained from the Laboratory of Biotechnology of Physiologically Active Compounds, Institute of Bioengineering, Research Center of Biotechnology RAS. The strains were grown on an agar medium of the following composition (g/l) – potatoes 200, glucose 20 and agar 20 – for 7 days at 25°C. The finished culture was stored for 1 month at 4°C.

2.4. Determination of the Effect of Chitosan Hydrolysate on the Growth of Phytopathogens

The antifungal activity of chitosan hydrolysate was evaluated *in vitro* in relation to the studied fungi according to the a previously described method [18]. Chitosan hydrolysate (pH 5.4) was added to the PDA to reach a concentration of 0.5, 1, 1.5, 2, 4, 6 or 8 mg/ml. The prepared media was then transferred to sterile Petri dishes with a diameter of 70 mm. After solidification of the medium, a disc with an 11-cm diameter with daily cultures of the studied strains was placed in the centre of the test dishes and incubated at 25°C. The fungal growth and development were recorded on day 3, 7 or 14 after the experiment was performed. Antifungal activity was calculated by the formula:

$$\text{Antifungal activity (AA)} = \left(1 - \frac{(De-11)}{Dc}\right) * 100\% \left(1 - \frac{(De-11)}{Dc}\right) * 100\%,$$

where Do is the diameter of the colony in the test (mm), Dk is the diameter of the colony in the control (mm) and 11 mm is the diameter of the disk with daily cultures. Three repetitions of each test were performed, and the arithmetic mean was used for the calculation.

The results are presented as the mean ± standard deviation (SD). The means were compared using the Mann-Whitney U test or Student's t test to determine differences. A p value < 0.05 was considered statistically significant.

3. Results and Discussion

Consistently with most of the previous literature, 'individual' samples of chitosan were used in the experiments. They were obtained using various methods of fractionation of chitosan hydrolysate. In our work, we used a solution of chitosan hydrolysate with a weight-average MW of 30 kDa, obtained as a result of chemical hydrolysis using nitric acid. The DD was 95% (Figure 1).

Previously, Tsai *et al.* [19] used a water-soluble chitosan hydrolysate obtained by enzymatic hydrolysis, consisting of chitosan with a MW of 12.0 kDa and chitooligosaccharides, with a degree of polymerisation from 1 to 8. The authors showed that the 12.0-kDa fraction had high activity at 100 ppm against *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar *typhi* and *Saccharomyces cerevisiae*, while chitooligosaccharides had weaker activity [19].

Antifungal activity was evaluated using the radial growth method on a medium with the addition of chitosan hydrolysate. The most sensitive pathogens and the concentrations of chitosan hydrolysate that inhibit their growth were identified (Table 1).

The phytopathogenic fungus *A. solani* was the most sensitive to the addition of chitosan hydrolysate to the PDA in the radial growth experiments. The content of hydrolysate in the medium in concentrations from 0.5-2 mg/mL significantly inhibited its growth. The colonies were pale golden or olive-brown. The mycelial fungi created a pigmented brown halo around itself during growth. On days 3 and 7 of incubation, the antifungal activity was 69%-92% and 69%-88%, respectively. On day 21, the culture in the experimental variants still did not reach the maximum size, but the colour of the colonies changed to pale golden, probably due to a lack of nutrients. It is worth noting that in the control sample (without the addition of hydrolysate), the mycelium of *A. solani* reached the edges by day 7 (Figure 2).

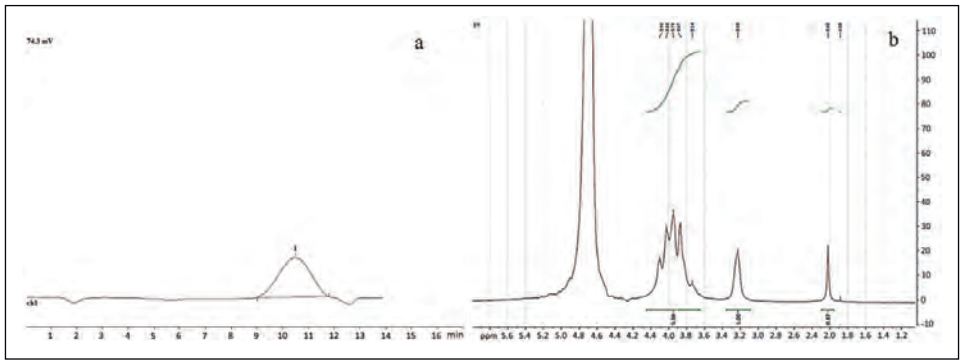

Figure 1. (a) Chromatogram of chitosan hydrolysate weight-average MW of 30 kDa and IP of 2.11 (b) Proton nuclear magnetic resonance spectrum of chitosan hydrolysate with 95% DD

Table 1. Antifungal activity (%) in the growth of phytopathogens on the medium with the addition of various concentrations of chitosan hydrolysate

Phytopathogens	Chitosan hydrolysate concentration in the medium (mg/mL)	Incubation time (days)			
		3	7	14	21
Alternaria solani	0.5	62 ± 0.15	69 ± 0.17	40 ± 0.26	43 ± 0.11
	1.0	88 ± 0.06	85 ± 0.11	67 ± 0.06	59 ± 0.43
	1.5	91 ± 0.10	88 ± 0.15	73 ± 0.06	61 ± 0.15
	2.0	92 ± 0.06	88	78 ± 0.06	64 ± 0.2
Fusarium solani	0.5	62 ± 0.17	50 ± 0.15	*	*
	1.0	60 ± 0.12	42 ± 0.15	*	*
	1.5	63 ± 1.65	42 ± 0.05	*	*
	2.0	61 ± 0.06	43 ± 0.12	*	*
Rhizoctonia solani	1.0	44 ± 1.3	*	*	*
	2.0	55 ± 0.40	*	*	*
	4.0	51 ± 0.15	*	*	*
	6.0	92 ± 0.71	23 ± 1.04	*	*
	8.0	99 ± 0.11	39 ± 1.04	*	*

*No antifungal activity because the colonies reached the edges of the Petri dish

The culture of *F. solani* completely filled the surface of the growth medium with a concentration of chitosan hydrolysate from 0.5 to 2 mg/mL on day 14 of incubation. Chitosan hydrolysate added to the PDA slowed the growth of *F. solani* slightly; on day 7 the antifungal activity was 43%-50% (Figure 3).

The least sensitive culture in relation to the addition of chitosan hydrolysate in the radial growth experiments was the phytopathogenic fungus *R. solani* (Figures 4 and 5). Its mycelium reached the edges of the Petri by day 7 of culture with a concentration of chitosan hydrolysate from 1 to 6 mg/ml. On day 3 of culture, its growth in the presence of 1, 2 or 4 mg/ml chitosan hydrolysate was comparable to the growth of other fungi studied on day 7 of the culture. Starting on day 7, there were no significant differences in the growth of mycelium among the concentrations of the added chitosan hydrolysate, except for 6 and 8 mg/ml (Table 1). The growth rate of *R. solani* slowed down only in the first three days, with the mycelium appearing as an aerial net of milky white colour (Figure 4).

Although the presence of chitosan hydrolysate in the PDA did not affect the growth of the fungus, there was an effect on the morphology of the mycelium starting at day 7 of incubation. The young mycelium was white and with further incubation, the pigmentation of the fungus culture increased. Differences in the colour of colonies are due to differences in the pigments produced by pathogens in the media [20]. On day 7, on Petri dishes with 1, 1.5 or 2 mg/ml of chitosan hydrolysate in the medium, the mycelium was light and different in colour from the control group (felt cords with thin brown branches);

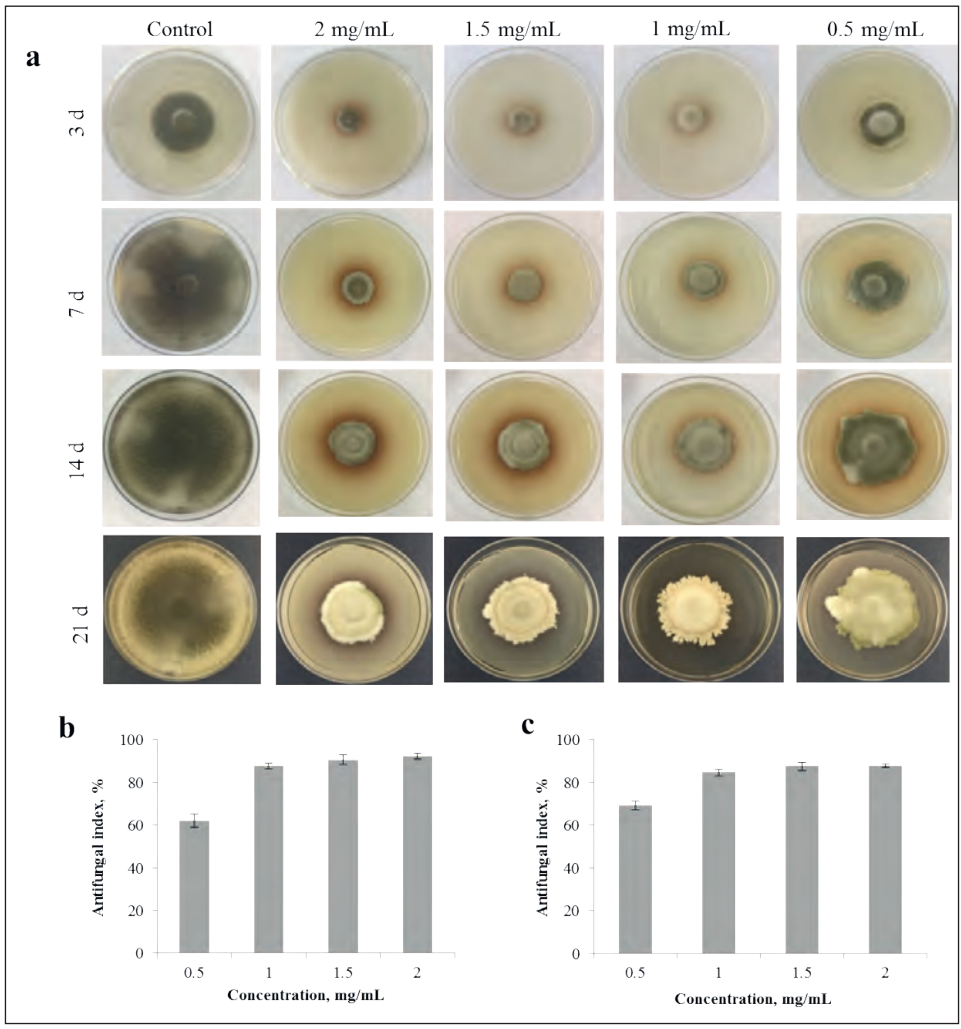

Figure 2. (a) Growth of *Alternaria solani* on potato dextrose agar with different concentrations of chitosan hydrolysate during incubation for 3, 7, 14 and 21 days; antifungal activity of chitosan hydrolysate against *A. solani* on (b) day 3 and (c) day 7

white and small microsclerotia appeared on the mycelium [21]. A similar trend persisted on day 14; in the control group, the fungus had a bright brown colour with a large number of dark brown macrosclerotia [22]. By contrast, when incubated with chitosan hydrolysate, the mycelium was white and had only small inclusions of light brown microsclerotia.

Because the addition of 0.5 to 2 mg/mL chitosan hydrolysate to the PDA did not produce a growth inhibition of *R. solani* similar to that for *A. solani* and *F. solani*, it was increased to 8 mg/mL (Figure 5). On day 3 of incubation with 6 or 8 mg/mL chitosan hydrolysate in the medium, the fungicidal activity was almost 100%, but on day 7 in the same concentrations it did not exceed 50%.

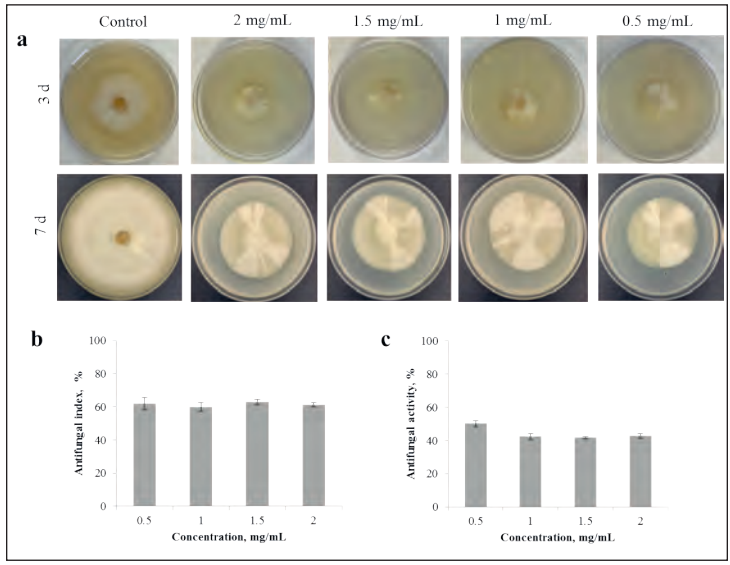

Figure 3. (a) Growth of *Fusarium solani* on potato dextrose agar with different concentrations of chitosan hydrolysate during incubation for 3 and 7 days; antifungal activity of chitosan hydrolysate against *F. solani* on (b day 3 (c) day 7

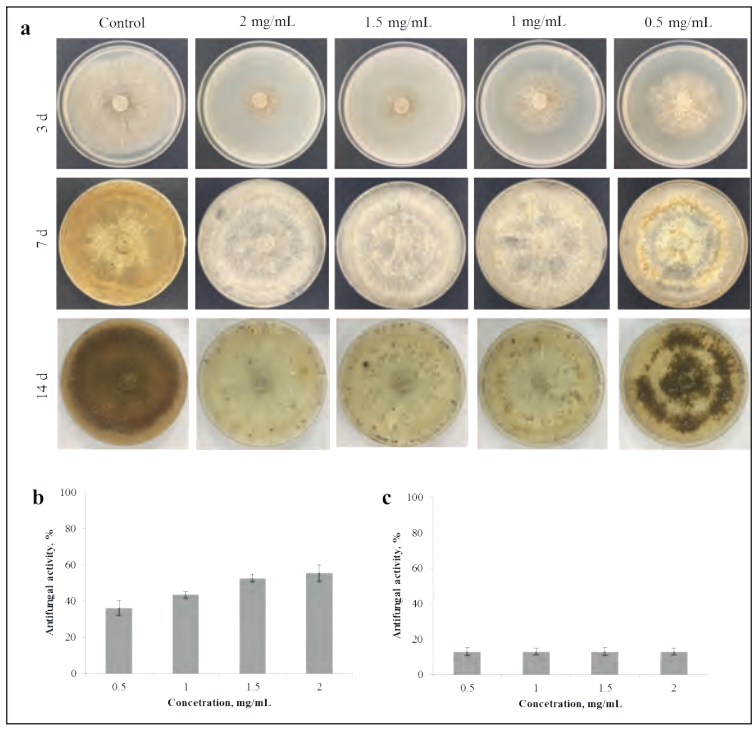

Figure 4. (a) Growth of *Rhizoctonia solani* on potato dextrose agar with chitosan hydrolysate (0.5-2 mg/ml) during incubation for 3, 7 and 14 days; antifungal activity of chitosan hydrolysate against *R. solani* on (b) day 3 and (c) day 7

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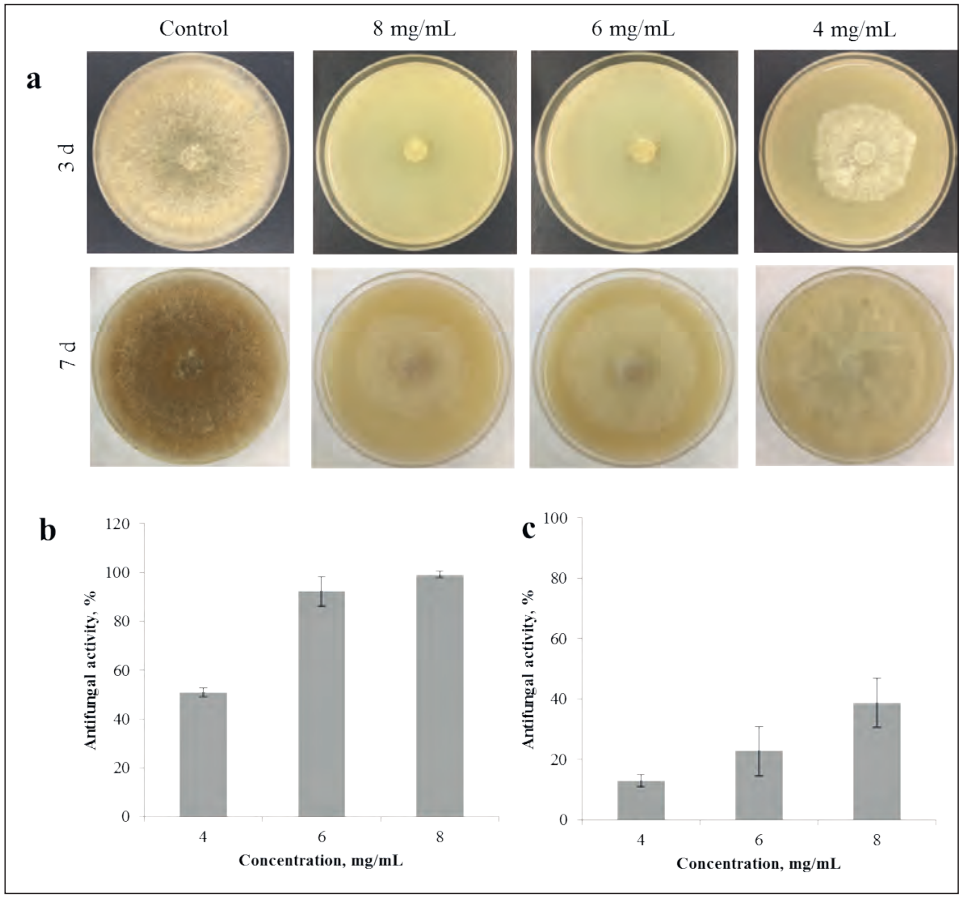

Figure 5. (a) Growth of *Rhizoctonia solani* on potato dextrose agar with chitosan hydrolysate in concentrations (4-8 mg/ml) during incubation for 3 and 7 days; antifungal activity of chitosan hydrolysate against *R. solani* on (b) day 3 and (c) day 7

4. Conclusions

The findings have shown that the addition of 0.5 to 8 mg/mL chitosan hydrolysate to the PDA led to at least a 50% growth inhibition on day 3 of incubation of phytopathogens. The phytopathogenic fungus *A. solani* was the most sensitive to the addition of chitosan hydrolysate to the nutrient medium in radial growth experiments, with antifungal activity of chitosan hydrolysate on day 7 of 69%-88% at concentrations of 0.5-2 mg/ml. *R. solani* was the least sensitive to chitosan hydrolysate, with 23%-39% growth inhibition at concentrations of 6-8 mg/ml.

5. Acknowledgements

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