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Chitosan Compatibility with Microbial Agents for the Management of *Alternaria solani* of potato Crop

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Abstract

Alternaria solani Sorauer is a fungus that causes damage to potato crops. It is characterized by producing black spots and wilting on plants and fruits; synthetic fungicides are the main tools to control this fungus. The present study aimed to determine under laboratory conditions (*in vitro*), the antifungal effect of chitosan against the pathogenic strain of *A. solani* A160, as well as to establish the compatibility between chitosan and the biological control agents *Trichoderma asperellum* Samuels *et al.*, (strain *Ta.* 85) and *Pseudomonas protegens* Ramette *et al.*, (Pf-5). The latter, proposed for the management of early blight of the potato crop in Cuba. The effect of chitosan concentrations (1 and 2%) on the radial mycelial growth of *A. solani* and *T. asperellum* and the sporulation of the latter, as well as the number of the latter, was evaluated by the poisoned culture medium technique. colonies of *P. protegens*. In each experiment, the treatments were compared by analysis of variance and Tukey's test; furthermore, the inhibition percentages were processed according to the proportions comparison test. The results showed that chitosan at the concentrations under study inhibited the mycelial growth of *A. solani*, with a concentration of 2% standing out. Chitosan at a concentration of 1% showed compatibility with biological control agents in the tests carried out. The results show the possibility of incorporating chitosan with these agents, in the control of *A. solani* of the potato crop. These will contribute to the preservation of environmental health.

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Keywords

Trichoderma asperellum, *Pseudomonas protegens*, chitosan, compatibility, *Alternaria solani*.

Introduction

Disease incidence is one of the factors that limits potato crop production in most tropical countries (Pérez and García, 2007). Among these, early blight, caused by the fungus *A.solani*, is one of the most significant foliar diseases of the crop worldwide (Lourenço *et al.*, 2011). This disease is considered of great importance due to its rapid spread and destructive power, as it attacks foliage, stems and tubers (Restrepo *et al.*, 2011; Minag, 2016), which leads to losses of up to 30% of the yield in the world (Minag, 2016; Acuña and Sandoval, 2017; Veitía *et al.*, 2008). The control and management of this pathogen is carried out by the application of synthetic

fungicides (Ahmed, 2017); However, its excessive and continuous use causes problems for human health and the environment, in addition to generating resistance in phytopathogens (Sacsa, 2015 and Sánchez-Bayo and Tennekes, 2015). Therefore, there is a demand from consumers in the production of food free of agrochemicals. The world increasingly encourages the application of different environmentally friendly alternatives to reduce the use of pesticides that affect the ecosystem, and among these, less aggressive methods for the control of *A. solani*. One of the little-explored alternatives is the use of organic products such as chitosan in conjunction with biological control agents (BCA).

Various species of the genera of these BCA have antagonistic capacity against various phytopathogenic fungi of plants in Cuba and worldwide; However, against *Alternaria*, and specifically for early blight control, few are reported. Among these, the isolates of the genus *Trichoderma* spp., *Pseudomonas* spp. from the fluorescent group and polymers such as chitosan (Sánchez-Bayo and Tennekes, 2015; Hanuman and Bindu, 2018; Velásquez *et al.,* 2019). Also, there are few reports on the compatibility of these BCAs with chitosan for the application of consortiums of microorganisms in the control of foliar phytopathogens under Cuban conditions. This is essential for the management of the disease, since the integration of BCA with this biopolymer can improve the effectiveness of control at low doses or in fewer applications of these (Nitu *et al.*, 2016 and Rodríguez-Romero *et al.,* 2019), which would minimize the environmental impact.

Chitosan is a linear biopolymer of glucosamine residues linked by ß (1-4) bonds. Its production is from the exoskeleton of crustaceans, it is also the second most abundant polymer in nature after cellulose, and it is easily obtainable and renewable (Ramírez *et al.,* 2000 and Falcón *et al.,* 2004).

Among its advantages is its importance in agriculture, its potential application is based on its dual quality of inhibiting the in vitro growth of fungi and phytopathogenic bacteria and inducing defense mechanisms in plants (Nitu *et al.,* 2016; Rodríguez-Romero *et al.,* 2019; Waewthongrak *et al.,* 2015).

These uses are mainly due to its high content of amines, which gives it a polycationic nature with a high charge density, in addition to its high molecular mass (Ramírez *et al.,* 2000).

Various authors (Rodríguez-Romero *et al.,* 2019; Maqbool *et al.,* 2010; De Oliveira, 2016) point out that this biopolymer controls diseases caused by phytopathogenic fungi in papaya (*Carica papaya* L.), tomato (*Solanum lycopersicum* L.), banana (*Musa paradisiaca* L.) crops.

Based on these antecedents and in order to contribute to the solution of disease problems in potato cultivation through the integration of different management tactics, the degree of compatibility of the strain *Ta.*85 of *T. asperellum* and the strain Pf-5 of *P. protegens* was evaluated *in vitro* with chitosan, as well as its antifungal effect on the mycelial growth of *A. solani* in potato crops.

Materials and Methods

Origin of microbial cultures and inoculum production

The work was carried out at the Laboratories of Plant Mycology (LMV) and Plant Bacteriology (LBV) of the National Center for Agricultural Health (CENSA) (Latitude 22.991867; Longitude -82.153892), Mayabeque Province, Cuba.

Pathogenic agent

The highly pathogenic isolate of *A. solani* No. 160 (catalog number), belonging to the LMV of CENSA collection of the *Alternaria* spp. was used, previously isolated from potato leaves with typical symptoms of early blight, from the province of Las Tunas.

The mycelium of the fungus was obtaining by preparing discs of 5 mm diameter taken from potato dextrose agar (PDA) Petri plates (90 mm Ø) medium (BIOCEN) pH 5.6 with 7 days of growth at a temperature of 30 ± 1°C and darkness.

As biological control agents, were used the *Ta.* 85 strain of *T. asperellum* from the CENSA LMV Collection and the Pf-5 strain of *P.protegens* from the Collection of Microbial Cultures of the Faculty of Biology of the University of Havana, strains selected integrally for their antagonistic action against *A. solani* in previous studies (Gakegne *et al.,* 2017; Gakegne and Martínez, 2015).

The inoculum of *T. asperellum* was prepared by passage of 5 mm diameter discs of the strain in Petri plates (70

mm Ø) with Malta Agar medium (AM) (BIOCEN) for 72 h at 30 ± 1° C and darkness.

For the bacterial inoculum to be used in the experiment, the strain was prepared in Petri plates containing King B medium (BIOCEN) and incubated for 24 h at 28°C. Subsequently, they were poured in test tubes containing 5 ml of Nutrient Broth (NB) medium (BIOCEN) and incubated in agitation for 48 h in brand shaker (Thys 2) at 28°C and 150 rpm. The final cell suspension was prepared at a concentration of 10^8 $CFUml^{-1}$.

The standard solution of chitosan of 5 g/L was donated by the Laboratory of Plant Physiology of the National Institute of Agricultural Sciences (INCA), from it, by volumetric calculation two solutions were prepared at 1 and 2%.

Antifungal effect of chitosan on the mycelial growth of *A. solani*

In this experiment, potato dextrose agar (PDA) (BIOCEN) pH 5.6 medium was prepared, and mixed with chitosan at the corresponding volumes to obtain biopolymer concentrations at 1 and 2%. They were stirred manually for 5 minutes with a glass spatula to remove the viscosity of the chitosan. They were sterilized in an autoclave at 121 °C for 15 min and then allowed to cool off to 40°C, and poured into Petri plates (Ø 90 mm). After the culture medium was solidified, a 5mm disk of 7days'growth *A. solani* mycelium, taken from the periphery of the colony, was centrally inoculated. The controls consisted of PDA plates without the chitosan solution, inoculated with the pathogenic fungus the same way as the previous plates. The Petri plates were sealed with parafilm and incubated at 30 ± 1°C and dark. Five replicas (Petri plate) were used per treatment. The mycelial growth of *A. solani* was measured with a graduated ruler every 24 h until 72 h. The mycelial growth from the control plates was taken as a reference to determine the antifungal effect of chitosan on the mycelial growth of *A. solani*. The percentage of radial growth inhibition was calculated according to the formula of Abbott *et al.*, (Ciba-Geigy, 1981).

Antifungal effect of chitosan on the mycelial growth of *T. asperellum*

For the carry out this experiment, Petri plates were prepared the same way as in the previous experiment. A 5 mm disk of mycelium of *T. asperellum*, with 72 h growth was taken from the periphery of the colony and centrally inoculated. For the controls, PDA plates were obtained without the chitosan solution, inoculated with the ACB fungus, in the same way as the previous plates. The Petri plates were sealed with parafilm and incubated at 30 ± 1°C and dark. Five replicas (Petri plate) were used per treatment. Mycelial growth of *T. asperellum* was measured with a graduated ruler every 24 hours until 72 hours. The mycelial growth from the control plates was taken as a reference to determine the effect of chitosan on the mycelial growth *T. asperellum*. The effect on radial growth was calculated, according to the formula of Abbott *et al.*, (Ciba-Geigy, 1981).

Effect of chitosan on the Pf-5 strain of *P. protegens*

The culture media to perform this test were prepared in a similar way as described above. From the bacterial suspension previously obtained, 10 µl were inoculated centrally in Petri plates containing PDA and spread evenly with a Drigalsky spatula. The control consisted of Petri plates with PDA medium without the chitosan solution, and inoculated with the bacterial suspension in the same way as the treatment plates. All plates were incubated at 30 ± 1°C. Five replications were used per variant. The number of colonies was evaluated every 24 h until 72 h.To verify the effect of chitosan on the CFU of *P.protegens*, from the chitosan treated plates with the growth of the antagonist, at 72 h a bacterial suspension was prepared by using the colony sweeping method. To this end, 30 ml of sterilized distilled water were added fractionally to each plate. In Petri plates containing PDA, 10 µl of the previously obtained bacterial suspension were inoculated in the center of the plate and spread evenly with a Drigalsky spatula. As a control, plates were inoculated in the same way as the previous ones, but with the suspension of the colonies of the culture medium from the control plates of the previous experiment. All plates were incubated at 30 ± 1°C. Five replications were used per variant. The number of colonies was evaluated every 24 h until 72 h.

Statistical analysis

The data collected for all the experiments were tabulated in Microsoft Excel and processed by simple analysis of variance. Mean comparisons were conducted according to Tukey's multiple range comparison test for a significance level of 95%. The statistical package Info Stat / Professional version 1.1 (Di Rienzo *et al.*, 2016) was used. Percentage data were analyzed using the system for comparison of multiple proportions (COMPAPROP) (Castillo and Miranda, 2014). A

completely randomized design was used for all experiments.

Results and Discussion

Antifungal effect of chitosan on the mycelial growth of *A. solani*

The inhibition of mycelial growth of *A. solani* caused by chitosan at the concentrations tested oscillated from 50.00 to 100%; with significant differences ($p \leq 0.05$) compared to the control at all times of evaluation (Fig. 1). The antifungal effect of chitosan at a concentration of 1% remained constant until 48 h, from the 72 h, its inhibitory effect was slightly greater over the time.

However, the inhibitory effect of chitosan 2% decreased at 48 h and increased again at 72 h (Table 2). Nevertheless, the effect of chitosan at the highest concentration on this pathogenic strain was higher than at the 1% concentration at all times of the evaluation.

These results coincide with those previously obtained by Sánchez-Domínguez *et al.*, (2007) who reported that the inhibition of mycelial growth of *A.alternata* (Fr.) Keissl increased as the concentration of chitosan increased.

Also, with this same pathogen, Rodríguez-Romero *et al.*, (2019) observed an inhibitory effect on mycelial growth between 9 and 33%. On the other hand, the results are similar to those of Guerra-Sánchez-Domínguez *et al.*, (2010) who observed *in vitro* an inhibitory effect on the mycelial growth of *Rhizopus stolonifer* (Ehrenb.:Fr) Vuill at different concentrations of chitosan.

The results of this work did not coincide in part with those of Pedroso *et al.*, (2016) who observed that the high molecular mass chitosan did not present an inhibitory effect in *Bipolaris oryzae* (Breda de Haan) Shoemaker, nonetheless, with the low molecular mass chitosan, the inhibition percentage decreased until it reached the value 0% over the time.

Considering the results obtained in this investigation and the previous ones cited, it could be inferred that the responses are variable, and it depend on the concentration of the polymer, the molecular mass of the chitosan and the pathogenic species.

Antifungal effect of chitosan on the mycelial growth of *T. asperellum*

The two concentrations of chitosan tested showed an inhibitory effect against the strain *Ta.* 85 of *T. asperellum* which oscillated between 3.95 and 100%, with statistically significant differences ($p \leq 0.05$) compared to the control at all times of evaluation. At 24 h, the growth inhibition of the *Ta.* 85 at the concentration of 2% was total; however, in the other evaluations a progressive decrease in said inhibition is observed (Table 2).

Although statistically there were differences in the growth inhibition of the *Trichoderma Ta*. 85 due to the effect of chitosan, in figure 2 it is observed that *Trichoderma* strain *Ta*. 85 grew in both concentrations of the biopolymer. Apparently, it is only manifested a growth delay caused by the effect of chitosan, which shows that as the interaction time goes by, the *Trichoderma* strain achieves some compatibility with the biopolymer, especially at a concentration of 1%. These results confirm what was stated by Woo *et al.*, (2014), the versatility of *Trichoderma* spp. it is extended to its adaptability to different ecological environments or agricultural situations, as well as its compatibility with numerous commonly used plant protection products and other BCA. All these characteristics expand the potential field of application of *Trichoderma* based products in the agricultural market not only as a biofungicide against phytopathogens, but also as a bioinoculant that stimulates the resistance of plants to the biotic and abiotic stress, and increases growth and yield of plants, and improves the agro-ecosystem.

In the consulted literature, no references were found about the compatibility between chitosan and *Trichoderma*. However, there are studies with combined applications of chitosan with *Trichoderma*, with satisfactory results, but without previously knowing whether or not there is compatibility between them. El-Mohamedy *et al.*, (2014) in their research under greenhouse conditions, observed that with the combined application of *T.harzianum* Rifai and chitosan there was a reduction in the incidence and severity of *Fusarium oxysporum* Schlechtend:Fr. F. sp. *radicis-lycopersici* W. R. Jarvis & Shoemaker (Forl) in 66.6 and 47.6%, respectively.

Table.1 Inhibitory effect of chitosan at different concentrations on the mycelial growth of *A. solani*.

Treatments	* Percentage of inhibition of the growth of *A. solani*		
	24 h	**48 h**	**72 h**
Qt1 %	50.00 b	50.00 b	55.32 b
Qt2 %	100.00 a	88.24 a	90.43 a
ESx	0.19	0.70	0.96

Means with different letters in the same column differ significantly (p≤0.05, according to Tukey's multiple range).
Chitosan 1 % = Qt1 %; Chitosan 2 % = Qt2 %
* Analysis of proportions (Wald method for corrections)

Table.2 Inhibitory effect of fungicides at different concentrations on the mycelial growth of *T. asperellum*.

Treatments	* Percentage of inhibition of the growth of *T. asperellum* strain *Ta.* 85		
	24 h	**48 h**	**72 h**
Qt1% +*Ta*.85	50.00 a	23.91 a	3.95 a
Qt2% +*Ta*.85	100.00 b	57.97 b	33.90 b
ESx	0.85	1.28	1.04

Means with different letters in the same column differ significantly (p≤0.05, according to Tukey's multiple range).
Chitosan 1 % = Qt1 %; Chitosan 2 % = Qt2 %
* Analysis of proportions (Wald method for corrections)

Fig.1 Mycelial growth (mm) of *A. solani* in culture medium with chitosan at different concentrations, at 72 h: a: 1% chitosan, b: 2% chitosan, c: Control without chitosan.

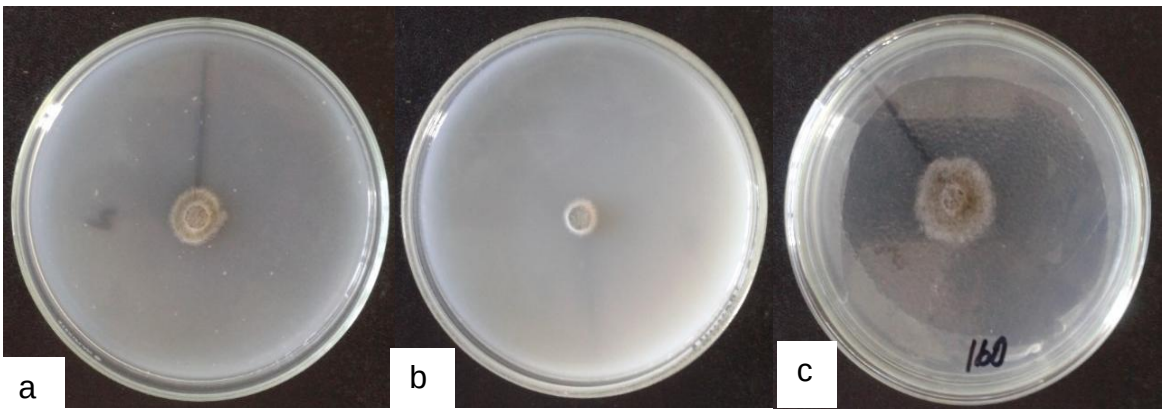


Fig.2 Mycelial growth (mm) of *T. asperellum* in culture medium with chitosan at different concentrations, at 72 h: a: 1% chitosan, b: 2% chitosan, c: Control without chitosan.

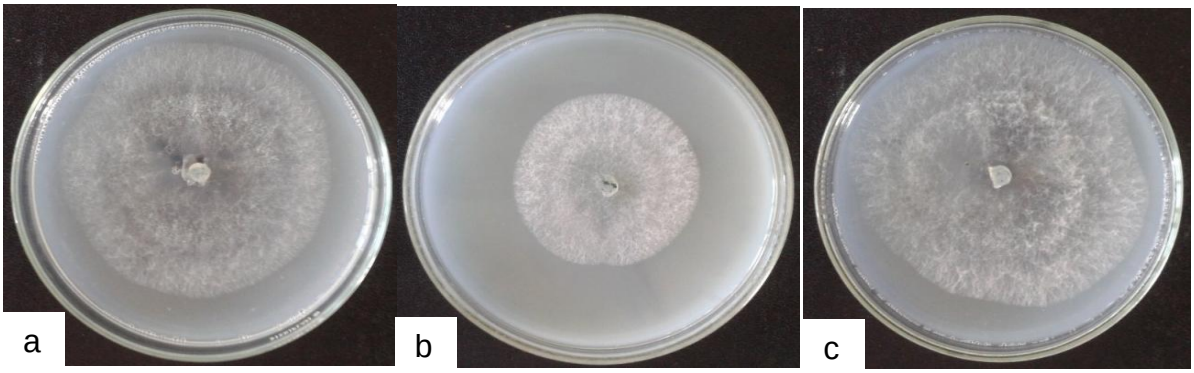


Fig.3 Antibacterial effect of chitosan at different concentrations on the number of colonies of *P. protegens* Pf-5, at 72 h: a: chitosan 1%, b: chitosan 2%, c: Control without chitosan.

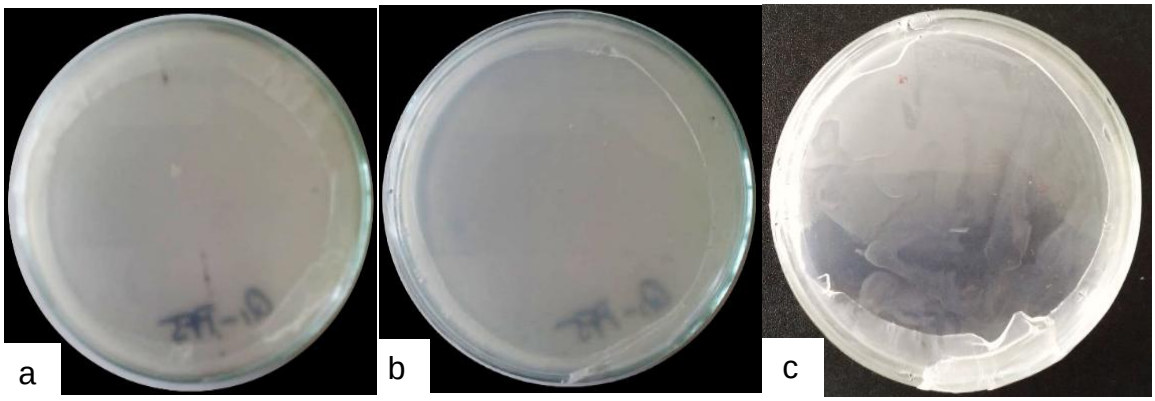
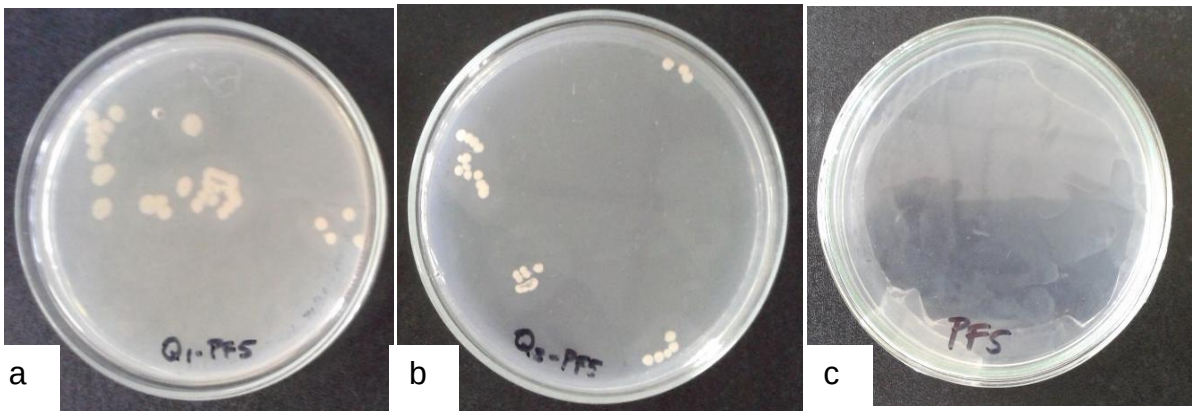


Fig.4 Confirmation of *P.protegens* colony growth in culture medium with chitosan at different concentrations, at 72 h: a: 1% chitosan, b: 2% chitosan, c: Control without chitosan.



Singh and Chittenden (29) demonstrated that the combination of chitosan with *T. harzianum* provided protection against *Ophiostoma piceae* (Münch) Sydow & P. Sydow, a pine pathogen (*Pinus radiata* D. Don).

Effect of chitosan on the Pf-5 strain of *P. protegens*

The number of *P.protegens* colonies in the chitosan treatments at 1 and 2% were uncountable, similar to those of the control without the biopolymer; chitosan does not appear to show any inhibitory effect on this BCA (Fig. 3).

Although in the consulted literature no evidence was found to determine the *in vitro* compatibility between these agents, there are investigations with encouraging results with combination of chitosan and *Pseudomonas* spp. Rodríguez-Romero *et al.,* (2019) observed in their *in vitro* experiments that the use of the mixture of chitosan 1.5% (p / v) + extract of *P. fluorescens* 50% (v / v) inhibited mycelial growth and mycelial growth by 60 and 100%. germination of conidia of *A. alternata*, respectively. Trejo-Raya *et al.,* (30) reported that the combination of *P. fluorescens* extract and chitosan [50–1.5% (v / v)] *in vitro*, had an inhibitory effect on the mycelial growth of *A. altenata* and *F.solani* (Martius) Appel & Wollenweber emend. Snyder & Hansen.

It was found that in the plates with chitosan at 1 and 2%, the PF-5 strain, although it grew in the entire area of the plate, showed inhibition of the number of colonies in the previous experiment with respect to the growth of its control (Fig. 4).

Knowing the compatibility between the selected BCAs and chitosan is of utmost importance, since the form and timing of their application depends on it, in order to avoid a diminution in control of the pathogen under field conditions. In general, the treatments with the polymer, although they show significant differences between

them, they showed that these BCAs present some compatibility to chitosan, and it is increase with the interaction time. These results show that these BCA could be used with this biopolymer at low concentrations, which could amplify the effects of the control in an integral way.

The results obtained leave open the possibility of integrating the BCA under study and chitosan, in the integrated management of *A. solani* in potato cultivation, which must be demonstrated under field conditions.

Chitosan at low concentration was shown to be compatible with the *Ta.* 85 strain of *T. asperellum* and Pf-5 of *P.protegens*, as well as their inhibitory effect on the growth of *A. solani* under *in vitro* conditions.

As the interaction time of biological controls with chitosan increases, its inhibitory effect on these BCA decreases.

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