EFFECT OF CHITOSAN ON THE MYCELIAL GROWTH OF FUSARIUM OXYSPORUM, RHIZOCTONIA SOLANI AND TRICHODERMA SPP.

Amanda Keller Siqueira [1]
Osmar da Rocha Levandoski Junior[2]
Gabriela Aparecida Pereira Bertan[3]
Jeislane Camila Sampietro Klack de Brito[4]
Vanessa Gomes da Silva[5]

RESUMO
Fungicides, often used in conventional farming systems, can harm humans, animals and damage the environment. Chitosan, which is extracted from the crustacean shell, has shown to be a harmless and cheap alternative to fungicides. This study aimed to test the inhibitory effect of chitosan on the mycelial growth of Fusarium oxysporum, Rhizoctonia solani and Trichoderma spp. Different concentrations of chitosan (0, 1, 2, 4, 8 and 16%) were supplemented to 1% acetic acid Potato Dextrose Agar (PDA). The fungi were inoculated in the center of the plates, incubated at 25 °C, with or without a 12-hour photoperiod. Daily measurements were performed in two transverse diameters until the mycelium reached the edges of the Petri dish. Fusarium oxysporum mycelial growth was significantly inhibited by chitosan at concentrations 1, 2, 4, and 8%, on the 4th, 5th and 6th evaluation days, in a 12-hour photoperiod. However, for Rhizoctonia solani, there was no reduction on mycelial growth. Trichoderma spp. presented the greatest mycelial reduction, in both light schemes, in all the concentrations evaluated, on days one, two and three. The results indicate that the mycelial growth of Fusarium oxysporum and Trichoderma spp. were inhibited by chitosan. Although, the combined use of chitosan and Trichoderma spp. as biocontrol agents of phytopathogens is not indicated.
Keys Words: biofungicide; control; fungi; shrimp.

[1] http://orcid.org/0000-0002-1419-8332 - Universidade Estadual de Campinas - UNICAMP. E-mail: kellersiqueira@hotmail.com

[2] http://orcid.org/0000-0002-3883-4778 - Universidade Federal da Fronteira Sul - UFFS.

[3] http://orcid.org/0000-0002-5774-2000 - Universidade Federal da Fronteira Sul - UFFS.

[4] http://orcid.org/0000-0002-0506-1975 - Universidade Federal da Fronteira Sul – UFFS.

[5] http://orcid.org/0000-0002-4609-2778 - Universidade Federal da Fronteira Sul - UFFS.

EFEITO DA QUITOSANA NO CRESCIMENTO MICELIAL DE FUSARIUM OXYSPORUM, RHIZOCTONIA SOLANI E TRICHODERMA SPP.

ABSTRACT
Os fungicidas, frequentemente utilizados em sistemas agrícolas convencionais, podem prejudicar seres humanos, animais e danificar o meio ambiente. A quitosana, extraída da casca do crustáceo, tem se mostrado uma alternativa inofensiva e barata aos fungicidas. Este trabalho teve como objetivo testar o efeito inibitório da quitosana sobre o crescimento micelial de *Fusarium oxysporum*, *Rhizoctonia solani* e *Trichoderma* spp. Diferentes concentrações de quitosana (0, 1, 2, 4, 8 e 16%) foram adicionadas em Batata Dextrose Agar (BDA) preparado em 1% de ácido acético. Os fungos foram inoculados no centro das placas, incubados a 25 °C, com ou sem fotoperíodo de 12 horas. Medições diárias foram realizadas em dois diâmetros transversais até o micélio atingir as bordas da placa de Petri. O crescimento micelial de Fusarium oxysporum foi significativamente inibido pela quitosana nas concentrações 1, 2, 4 e 8%, nos dias 4, 5 e 6 de avaliação, em fotoperíodo de 12 horas. No entanto, para *Rhizoctonia solani* não houve redução no crescimento micelial. *Trichoderma* spp. apresentou a maior redução micelial, em ambos os esquemas de luz, em todas as concentrações avaliadas, nos dias um, dois e três. Os resultados indicam que o crescimento micelial de *Fusarium oxysporum* e *Trichoderma* spp. foram inibidos pela quitosana. Assim, o uso combinado de quitosana e *Trichoderma* spp. como agentes de biocontrole de fitopatógenos não é indicado.
Palavras-chave: biofungicida, camarão, controle, fungos.

1. INTRODUCTION

Phytopathogens are fungi, bacteria, viruses and nematodes that cause disease in plants. Its pathogenic nature is favored by different environmental conditions such as temperature, water availability, humidity, luminosity incidence, amount of oxygen or nutrients (Lazarrotto 2013). Among phytopathogens, fungi are often associated with plant diseases (Poloni et al. 2008).

In recent years, there has been a tendency to look for alternative controls for diseases, aiming at easy and low-cost access and, a similar efficiency to existing control methods, especially to minimize side effects (Mazaro et al. 2009). In this sense, chitosan is a derivative of crustacean shells, which has attracted the

attention of industry and researchers because it has multidimensional potential, ranging from applications in the food area such as in nutrition, but also in gene therapy, biotechnology, drugs and pharmaceuticals, as well as the use in agriculture and environmental protection (Azevedo et al. 2007).

Chitosan has been used as a control tool for *Rhizoctonia* sp. and *Fusarium* sp. (Freddo 2012, Freddo et al. 2014, Mazaro et al. 2009, Mota 2015, Pinto et al. 2010, Rocha, 2015). Both are causal agents of various diseases in crop plants at different stages of development, resulting in severe economic losses by farmers.

Trichoderma spp. is a fungus that participates in the decomposition and mineralization of plant residues, contributing to the availability of nutrients to the plants, besides being considered a biofungicide, reducing up to 100% the chances of pathogenic fungi reaching the culture (Menezes et al. 2009). In that way, *Trichoderma* spp. has been used in the natural control of diseases caused by *Rhizoctonia* sp. and *Fusarium* sp.

The objective of the present study was to evaluate the effect of chitosan on the mycelial development of *Rhizoctonia solani* and *Fusarium oxysporum*, as well as on the mycelial growth of *Trichoderma* spp. used in the biocontrol of phytopathogens.

2. MATERIAL AND METHODS

The experiment was carried out at the Federal University of Fronteira Sul (UFFS), Campus Laranjeiras do Sul (25º24'28''S and 52°24'58''W), Paraná, Brazil, at the Laboratory of Microbiology, between 20/03/2017 and 23/02/2018.

The fungi *Fusarium oxysporum, Rhizoctonia solani* and *Trichoderma* spp. (SisGen A87D2DB) used in the research, are deposited in the library of the Phytopathology Laboratory of UFFS, Campus Laranjeiras do Sul – PR, Brazil.

For the recovery and maintenance of the isolates, these were weekly cultivated in Potato Dextrose Agar (PDA), until the moment of the experiments. The isolates were incubated at 25°C and maintained for seven days.

The chitosan used in the experiments was purchased from Santosflora, Comércio de Ervas Ltda. According to the company, the product was 68% pure and showed no microbial contamination. The purity was verified in the Laboratory of Microbiology by culturing the chitosan in different culture media for fungi (PDA and Sabouraud - Oxoid™) and bacteria (Nutrient Agar, Blood Agar, MacConkey Agar, and EMB Agar - Oxoid™), in aerobic and anaerobic conditions, before the beginning of the experiments.

For the fungal mycelial inhibition tests, Potato Dextrose Agar was prepared in 1.0% acetic acid, with chitosan at concentrations (1, 2, 4, 8 and 16%) as well as control plates (0% chitosan), always in quadruplicates, according to Freddo et al (2014). Plates containing pure PDA were also prepared for growth control of the isolates during the experiments.

Flasks containing PDA, diluted in 1% acetic acid, were autoclaved (121°C for 15 minutes). After this process, the chitosan was added to the culture medium at 45°C, the flasks were then incubated under shaking (3500 rpm) and held at 60°C for 12 hours. Then, the medium was incubated in a water bath at 100°C for one hour and poured into Petri dishes. The plates were kept under ultraviolet light for 15 minutes and then incubated at 35°C for 18 hours for sterility control.

For the mycelial growth inhibition tests, the PDA plates received 10 mm diameter filter paper discs containing mycelium of the fungi *Fusarium oxysporum, Rhizoctonia solani* or *Trichoderma* spp., with seven days of development. Next, the plates were sealed with plastic film and incubated at 25°C, quadruplicates were prepared for each concentration. Two Petri dishes were maintained without photoperiod and the other two in a 12 hour-photoperiod.

Measurements of the mycelial growth were performed daily, in two cross-sectional diameters using a pachymeter, until the isolates in the control plates reached the edges of the Petri dishes.

Data were submitted to analysis of variance, by Tukey's test, and the results expressed as mean, at 5% of significance, using ASSISTAT software version 7.7 BETA (Silva and Azevedo 2009).

3. RESULTS AND DISCUSSION

In the plates without 12-hour photoperiod, a reduction in mycelial growth was obtained from the 4th day at concentrations 1, 2, 4 and 8%, compared to the mycelial growth of *Fusarium oxysporum* from the control plates (0%). The 8% of chitosan plate caused the most expressive reduction in fungal growth, and it could be considered the lowest inhibitory concentration with potential for "in vitro" use (Figure 1).

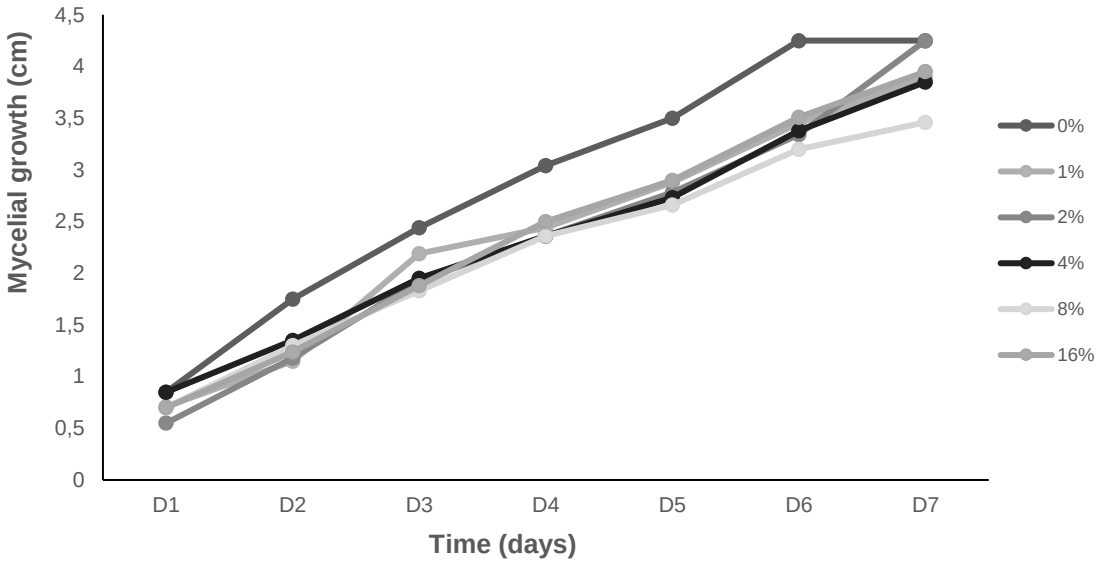

Figure 1. Effect of chitosan (0, 1, 2, 4, 8 and 16%) on the inhibition of the mycelial growth of *Fusarium oxysporum*, without a 12-hour photoperiod, during a seven-day interval (D1 - D7).

When *F. oxysporum* plates were incubated with 12-hour photoperiod, fungal mycelial inhibition was also observed starting on the 4th day at concentrations 1%, 2%, 4% and 8%. On day five and six, the inhibition was remarkable for concentrations 2 and 8%, respectively, when compared to the mycelial growth of *F. oxysporum* in the control plate (0%). In this case, the concentration 2% may be considered the lowest inhibitory concentration with potential for "in vitro" tests (Figure 2).

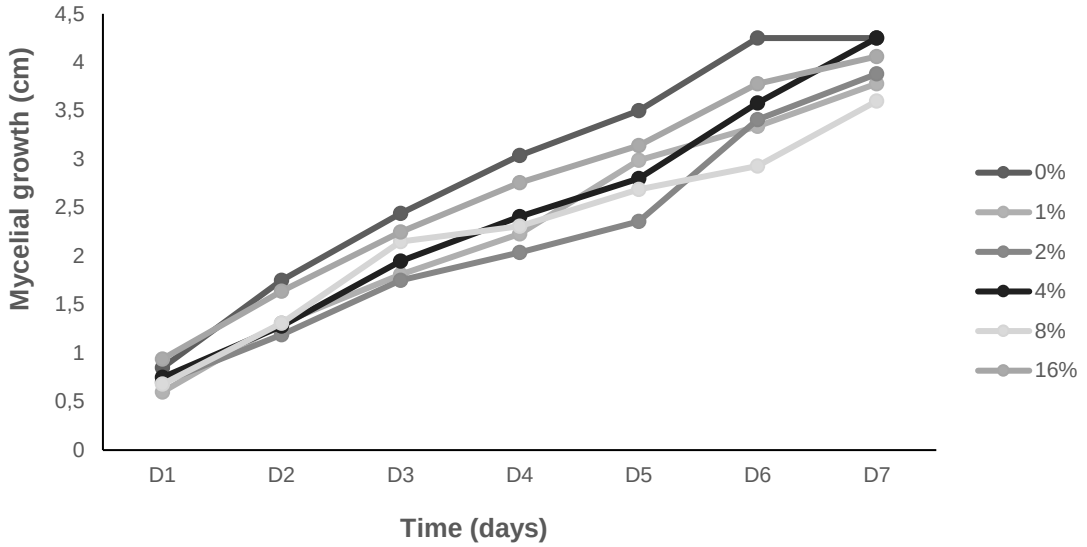

Figure 2. Effect of chitosan (0, 1, 2, 4, 8 and 16%) on the inhibition of the mycelial growth of *Fusarium oxysporum*, with 12-hour photoperiod, during a seven-day interval (D1 - D7).

In general, the effect of chitosan on *F. oxysporum* is neither immediate nor persistent, since its action could be observed from day 4, at concentrations 1, 2, 4 and 8%, and this action was not maintained on the following days.

Differing from our results, Pinto et al. (2010) obtained mixed results during the tests with chitosan in the control of *F. oxysporum* f.sp. *chrysanthemi*. However, similar results were found by Rocha (2015) when using chitosan to induce resistance against dumping off in vegetable crops. The increase of chitosan concentration resulted in a reduction of the initial mycelial growth but decreasing the efficiency during the trial.

Silva and Teixeira (2012) demonstrated that the fungus *F. solani* had greater mycelial growth in continuous light and photoperiod of 12 hours when compared with continuous darkness. Thus, the findings cited above and this research, reinforce the claim that chitosan has an inhibitory effect on the mycelium of *Fusarium* sp., even in the presence of light, revealing the promising potential of using the substance as biofungicide, although this effect may not be permanent.

The use of different concentrations of chitosan showed no inhibition on the mycelial growth of *Rhizoctonia solani* since there was no statistical difference in fungal growth with or without the use of chitosan. On the contrary, in some

concentrations, the mycelium growth matched or even exceeded that of the fungus on the chitosan-free plate (0%) (Table 1).

Some studies using lower amounts of chitosan compared to this research revealed that concentrations below 1% had a higher fungistatic effect on *Rhizoctonia* sp. but that this effect was reduced when increasing the concentration of that substance. Rocha (2015) observed that chitosan had a fungistatic effect on the initial growth of *R. solani*, at a concentration of 0.25%. However, this effect was not maintained, as the fungus continued growing. Similarly, Freddo (2012) observed that 0.5% of chitosan was the only one suitable for the reduction of *R. solani* growth and that this effect decreased at following concentrations.

Table 1.
Use of chitosan (0, 1, 2, 4, 8 and 16%) on the inhibition of the mycelial growth of *Rhizoctonia solani*, during a seven-day interval (D1 – D7).

Chitosan	D1	D2	D3	D4	D5	D6	D7
0%	0,60[a]	1,03[a]	1,35[a]	1,83[a]	2,18[a]	2,50[a]	2,80[a]
1%	0,55[a]	1,01[a]	1,29[a]	1,90[a]	2,20[a]	2,56[a]	2,78[a]
2%	0,60[a]	0,94[a]	1,36[a]	2,03[a]	2,44[a]	3,06[a]	3,71[a]
4%	0,63[a]	0,89[a]	1,40[a]	1,70[a]	2,06[a]	2,94[a]	3,48[a]
8%	0,53[a]	0,89[a]	1,31[a]	1,80[a]	2,09[a]	2,23[a]	2,34[a]
16%	0,65[a]	0,80[a]	1,38[a]	1,70[a]	2,13[a]	2,50[a]	2,81[a]

* Values with the same letter (a), in the same column, did not present significant differences (p> 0.05) by the Tukey test at 95% confidence.

The photoperiod did not influence *Trichoderma* spp. mycelial growth nor did the presence of light. On days one, two and three, all the concentrations (1, 2, 4, 8 and 16%) showed an inhibitory effect on the mycelial growth and, after that, no inhibition was observed because the fungus reached the edges of the Petri dishes (Figures 3 and 4).

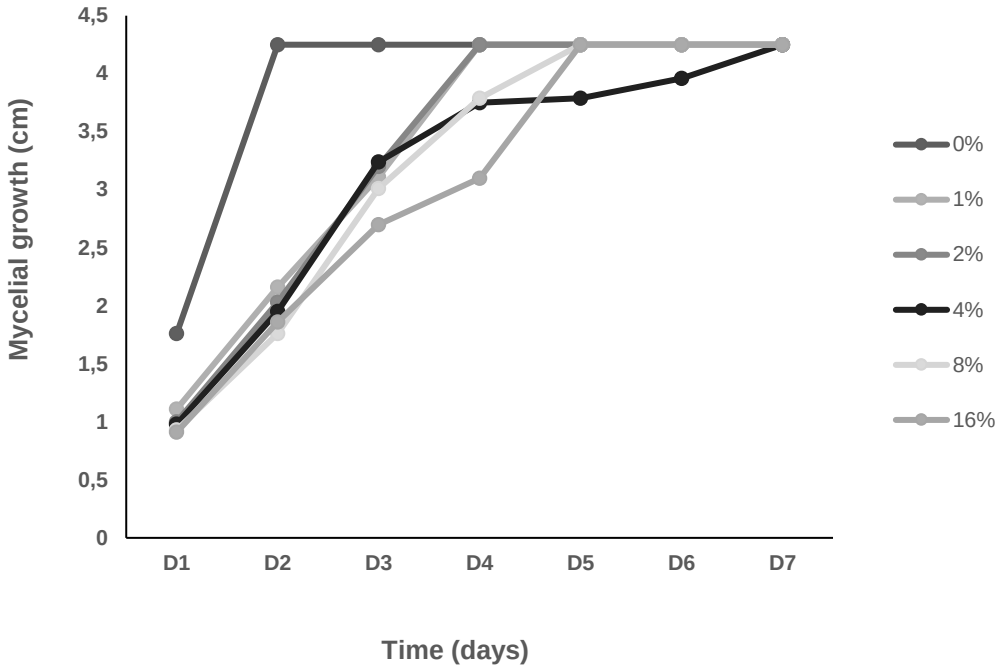

Figure 3. Effect of chitosan (0, 1, 2, 4, 8 and 16%), on the inhibition of the mycelial growth of *Trichoderma* spp., with 12-hour photoperiod, during a seven-day interval (D1 - D7).

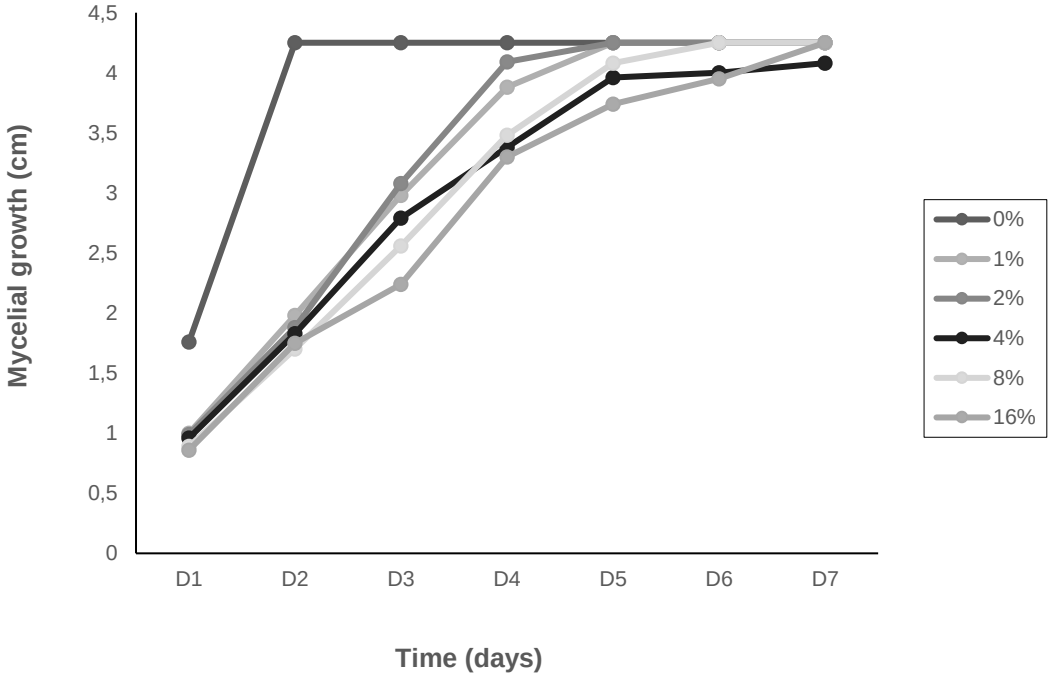

Figure 4. Effect of chitosan (0, 1, 2, 4, 8 and 16%), on the inhibition of the mycelial growth of *Trichoderma* spp., without 12-hour photoperiod, during a seven-day interval (D1 - D7).

Different studies have revealed the successful use of *Trichoderma* spp. for biocontrol of phytopathogens. Lobo Junior (2005) demonstrated a fungistatic effect of a commercial product based on *Trichoderma harzianum* 1306 on *Fusarium solani* and *Rhizoctonia solani* artificially inoculated on beans. Görgen et al. (2009) carried out an experiment to control white mold in soybean using *Brachiaria ruziziensis* and *Trichoderma harzianum* 1306. As a result, there was an increase in soybean yield and a decrease in the number of sclerotia, thus reducing the incidence of white mold. Later, Fipke et al. (2015) applied *Trichoderma* spp. for controlling white mold and observed that it inhibited the mycelial growth of *Sclerotinia sclerotiorum in vitro*.

In this study, it was possible to observe a decrease in the mycelial growth of *Trichoderma* spp. However, it must be emphasized that this fungus is used for controlling phytopathogens and promoting plant growth and that these attributes allowed this to be one of the most researched fungi in Brazil, both in vitro and in vivo conditions (Mota, 2015). So, according to this study, the association of *Trichoderma* spp. and chitosan for disease control in plants is not indicated, since chitosan inhibits the mycelial growth of *Trichoderma* spp. and may inhibit its activity as a biocontrol agent for phytopathogens.

Thus, it was concluded that chitosan has an inhibitory effect of the mycelial growth of *Fusarium oxysporum* and *Trichoderma* spp., but did not inhibit *Rhizoctonia solani* and that the use of chitosan in conjunction with *Trichoderma* spp. in the biocontrol of phytopathogens should be cautious.

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