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In vitro control of *Fusarium oxysporum* f. sp. *passiflorae* using chitosan

Controle in vitro de Fusarium oxysporum f. sp. passiflorae com a utilização de quitosana

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Abstract

Fusarium wilt, caused by the fungus Fusarium oxysporum f. sp. passiflorae (Fop), is one of the main diseases affecting passion fruit production. Given the lack of effective control measures, this study aimed to evaluate the inhibitory potential of chitosan in vitro for controlling Fop. The experiment was conducted at the Phytopathology Laboratory of IF Baiano, Guanambi Campus, using a completely randomized experimental design, with five concentrations of chitosan (0.1, 0.2, 0.3, 0.4, 0.5 g/L) in potato dextrose agar medium, and five replicates. The results showed that chitosan exhibited an inhibitory effect on the fungal mycelial growth and spore production at a concentration of 0.3 g/L. These results indicate that chitosan has the potential to be used as a control agent for Fusarium wilt in passion fruit, highlighting the need for further research, particularly on the activity of chitosan in vivo.

Keywords: Fugistatic action; Passion fruit; Fusarium wilt.

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Resumo

A murcha de fusarium, causada pelo fungo *Fusarium oxysporum* f. sp. *passiflorae* (Fop), é uma das principais doenças que comprometem a produção do maracujá. Diante da ausência de medidas de controle eficazes, objetivou-se com este trabalho avaliar o potencial inibidor da quitosana *in vitro* para controle do Fop. O experimento foi realizado no Laboratório de Fitopatologia do IF Baiano, *Campus* Guanambi, em delineamento experimental inteiramente casualizado, com cinco concentrações de quitosana (0,1, 0,2, 0,3, 0,4, 0,5 g/L) em meio de cultura batata-dextrose-ágar, e cinco repetições. Os resultados demonstraram que a quitosana apresentou efeito inibitório sobre o crescimento micelial do fungo e produção de esporos na concentração de 0,3 g/L. Esses resultados indicam que a quitosana tem potencial para ser utilizada como um agente de controle para a murcha de fusarium no maracujazeiro, sendo necessário a continuação das pesquisas, principalmente da atividade da quitosana *in vivo*.

Palavras-chaves: Ação fugistática; Maracujá; Murcha de fusarium.

INTRODUCTION

Passion fruit (*Passiflora* edulis) is cultivated in all Brazilian regions, presenting great socioeconomic importance, generating employment and income. Brazil is currently the world's largest producer, with a production of 697,859 tons in 2022, grown on an area of 45,602 hectares (IBGE, 2022). The Northeast region stands out as the main producing area, accounting for 69.8% of national production. The state of Bahia leads the Brazilian ranking, with 227,867 tons harvested on an area of 18,574 hectares (IBGE, 2022).

However, passion fruit production is severely affected by various diseases, with fusarium wilt or fusariosis, caused by the fungus *Fusarium oxysporum* f. sp. *passiflorae* (Fop), being one of the main ones. This disease causes serious economic losses due to plant death, the lack of effective control measures, and the ability of Fop to survive in the soil for long periods through chlamydospores (Fischer and Resende, 2016), potentially making cultivation unfeasible in areas with high disease incidence.

There is still no curative method proven to be effective for controlling Fusarium wilt. Control methods have relied on prevention, implementing proper irrigation management, eradicating crop residues, and using areas with no history of the disease, with attention to the tools and agricultural implements used. The use of resistant wild species has gained traction to obtain selected hybrids or rootstocks for yellow passion fruit (Junqueira et al., 2016; Lima et al., 2021; Nogueira, 2021). There are still no registered chemical products for controlling the disease (AGROFIT, 2024).

Among studies on plant disease control, chitosan, a natural heteropolymer obtained by deacetylation of chitin and most often extracted from the exoskeleton of crustaceans (Arnaud et al., 2016), has attracted the interest of researchers worldwide due to the various biological activities in which it can be used.

Due to its properties such as biocompatibility, biodegradability, non-toxicity, and high antimicrobial activity, chitosan has been used in various applications in areas such as medicine, agriculture, and the chemical and food industries (Arnaud et al., 2016; Campos et al., 2020; Ren et al., 2021).

In agriculture, chitosan is capable of inducing increased plant resistance to biotic and abiotic stresses, providing micronutrients, and coating structures such as seeds and fruits, as well as serving as the basis for various research aimed at creating fertilizers and hydrogels (Ahmed et al., 2021; Arnaud et al., 2016; Xing et al., 2018).

Additionally, chitosan can act directly on microorganisms as a fungistatic or fungicidal agent. Many studies indicate positive results against a wide range of pathogens (Huang, 2021; Meng, 2021;

Sun, 2021), showing activity against fungi of the genus *Fusarium oxysporum*, *Botrytis cinerea* and *Candida lambica* (Macedo et al., 2022).

Recent research has shown that chitosan is a potential inhibitor of potato dry rot, caused by *F. oxysporum* (Ren, 2021). Although such information is encouraging, up to the present moment, there are no studies that define the effects of chitosan on *Fusarium oxysporum* f. sp. *passiflorae*, form that acts more severely on the passion fruit plant.

In another study evaluating chitosan in mycelial growth of *F. oxysporum*, in the treatments without and with photoperiod, mycelial growth inhibition was observed from the 4th day of evaluation. The concentrations of 8% (without photoperiod) and 2% (with photoperiod) were considered the lowest inhibitory concentrations, with potential for use *in vitro* (Siqueira et al., 2020).

Considering the importance of the disease in passion fruit cultivation, the lack of studies on the use of chitosan in controlling Fop, the existence of few control methods, and the socioeconomic importance of the crop, this study aimed to evaluate the inhibitory potential of chitosan *in vitro* in control of *Fusarium oxysporum* f. sp. *passiflorae*.

MATERIALS AND METHODS

The study was carried out at the Phytopathology Laboratory of the Federal Institute of Education, Science, and Technology of Bahia (*Instituto Federal de Educação, Ciência e Tecnologia Baiano*) - Guanambi Campus, located in the district of Ceraíma, Guanambi - BA

The inhibitory potential of chitosan in vitro was evaluated based on the fungal mycelial growth *Fusarium oxysporum* f. sp. *passiflorae* (Fop) and in spore production. The Fop isolate used in all analyses of the experiment comes from a preservation bank at the State University of Southwest Bahia (*Universidade Estadual do Sudoeste da Bahia - UESB*). The chitosan used was obtained from a compounding pharmacy, in its pure form (free of adjuvants, excipients, and the like), where the degree of deacetylation was not reported, but the minimum value is estimated to be 60%.

The production of the chitosan solution occurred through its dissolution in glacial acetic acid to form the stock solution (SS) at 10 g/L. On a magnetic stirrer, the SS was kept for 8 hours at 720 rpm, and then the pH of this solution was adjusted to 5.5 by adding 1M NaOH, due to the solubility characteristics of chitosan.

To assess the effect of chitosan on the inhibition of Fop mycelial growth, Petri dishes containing potato dextrose agar (PDA) culture medium were used. After autoclaving and allowing the medium to reach room temperature, the proposed different concentrations of chitosan and streptomycin sulfate (antibiotic) were added. The poured plates were then inoculated with a 3.0 mm mycelial disc of Fop in the center of each dish and subsequently stored in a BOD (biochemical oxygen demand) growth chamber at 28°C, in complete darkness, for seven days.

Assessments were conducted daily starting from the 3rd day, where the diameters of the Fop colonies were measured with the aid of a digital caliper in two fixed transverse directions, obtaining the average of the colony's mycelial growth. To calculate the percentage of inhibition (% I) of Fop in the treatments relative to the control, the following formula was applied Edington et al. (1971):

$$\%I = \left(\frac{(C-T)}{C}\right)*100, \quad (1)$$

where C represents the mycelial growth diameter of Fop on the control plate, and T the mycelial growth diameter of Fop on the treated plate.

After analyzing mycelial growth, the effect of chitosan on Fop spore production was evaluated on each Petri dish. To this end, seven days after inoculation, a spore suspension was prepared on the plates containing the treatments. Five treatments with different chitosan dosages (0.1, 0.2, 0.3, 0.4, 0.5 g/L) and a control treatment were maintained, each with three repetitions.

The collection of spores was performed through gentle washes on the Petri dishes, using an aliquot of sterile distilled water, carrying out light scrapes with a sterile Drigalski loop to promote the release of the spores into the distilled water.

With the aid of an optical microscope and a Neubauer Chamber, spore counting was carried out for each treatment and its respective repetitions.

The evaluation of the determination of the average inoculum concentration ($\bar{x}Ci$), was carried out using the following formula:

$$\overline{x}\ Ci = \left(\frac{(\sum \overline{x}R)}{nR}\right) * 1{,}6 * 10^5\ , \qquad (2)$$

where $\overline{x}R$ represents the average of spores in one repetition of the treatment, and nR the number of repetitions in the treatment.

Data analysis

The experimental design was completely randomized (CRD), with five chitosan concentrations (0.1, 0.2, 0.3, 0.4, 0.5 g/L) and five repetitions, with the experimental unit consisting of a Petri dish. The control (Control) consisted of Petri dishes containing only BDA medium and a disk of Fop mycelium.

The data regarding the inhibitory activity of chitosan were subjected to the Shapiro-Wilk test for normality of residual distribution and the Bartlett test for homoscedasticity. Even with the Box-Cox transformation, there was no homogeneity of variances, so the nonparametric Kruskal-Wallis test was chosen, using the R statistical software (R CORE TEAM, 2024) and the Agricolae package (Mendiburu, 2015).

RESULTS AND DISCUSSION

The results obtained from the mycelial growth analysis demonstrate that chitosan has a significant inhibitory effect on the growth of Fop (Table 1; Figures 3 and 4). From the concentration of 0,3 g/L, a stabilization of the inhibition was observed, suggesting that the maximum effectiveness of the treatment was reached at this point.

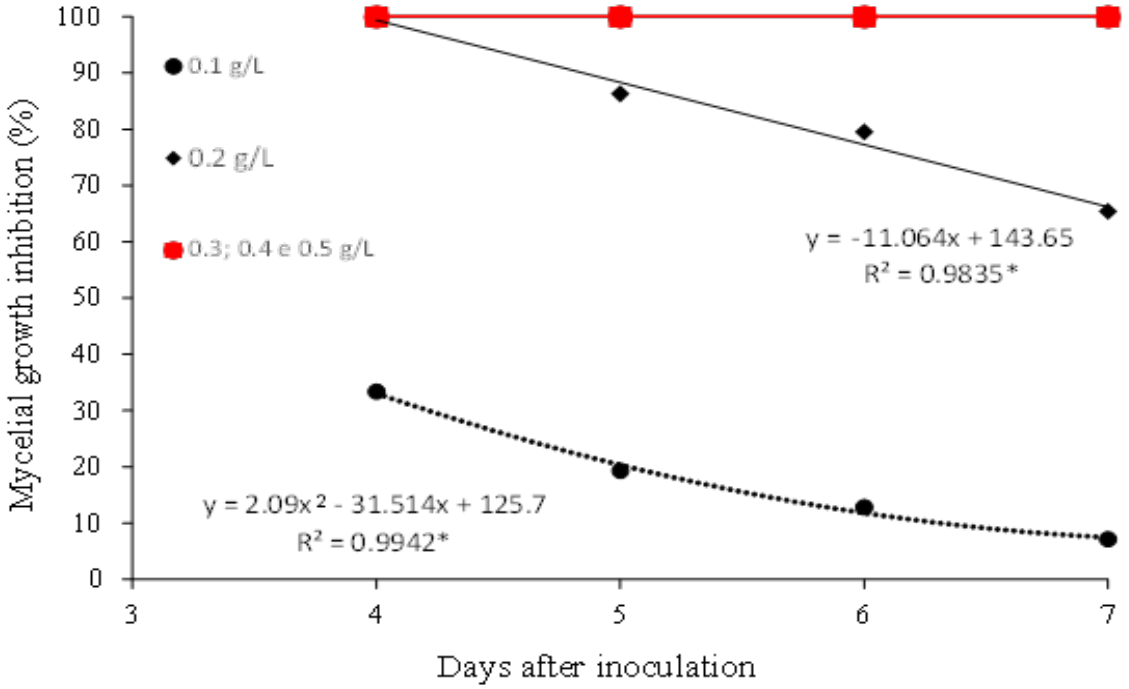
Table 1 - Mycelial growth rate of Fop in relation to chitosan concentrations. Guanambi - BA. 2024.

Treatment	Average mycelial growth rate (cm/day)
Control (BDA)	3,3 a
0,1g/L	2,8 b
0,2g/L	0,7 c
0,3g/L	0 d
0,4g/L	0 d
0,5g/L	0 d

Means followed by the same letter in the columns do not differ from each other according to Tukey's test at 5% significance.
Source: Authors (2024).

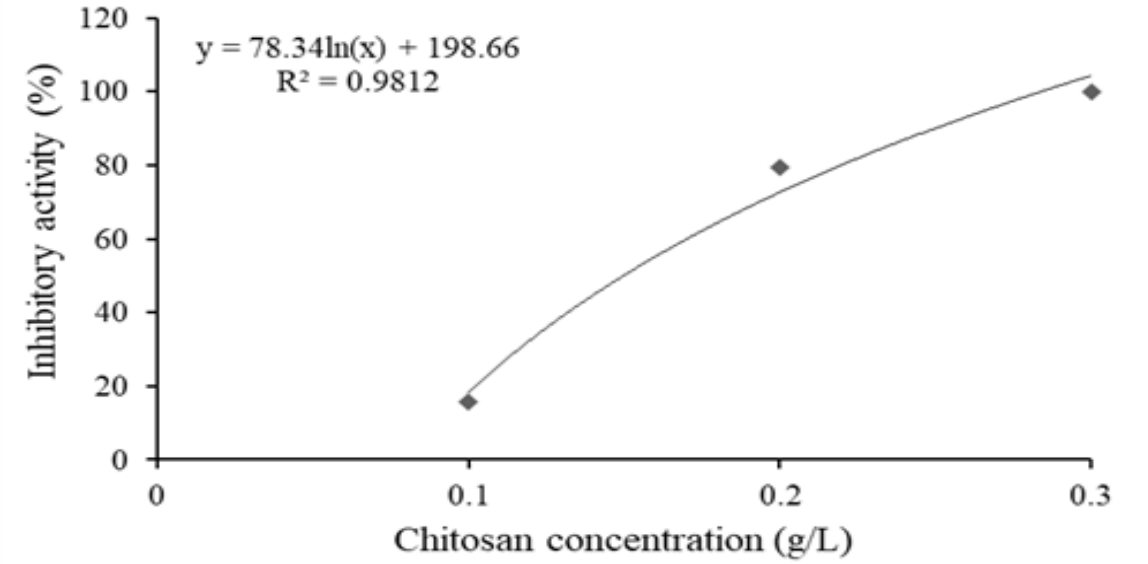
The percentage of inhibition decreases as the pathogen grows in the treatment, as observed at concentrations of 0.1 and 0.2 g/L. The low inhibitory rates of the 0.1 g/L treatment demonstrate the ineffectiveness of this concentration for the *in vitro* control of Fop. Although it was possible to delay the onset of mycelial growth by one day, the average reduction in inhibition was 77.4% between the 4th and 7th day. The 0.2 g/L concentration promoted complete inhibition of Fop until the 4th day, after which gradual reductions occurred, reaching a 34.6% reduction in inhibition during the evaluated period (Figures 1, 3, and 4), indicating that the pathogen may be able to continue its metabolic activities, growing as it resists the presence of chitosan in the medium at this concentration.

Figure 1 - Inhibition of mycelial growth of Fop (%), at different chitosan concentrations and analyzed days. Guanambi - BA. 2024. *The adjustment coefficients were significant.



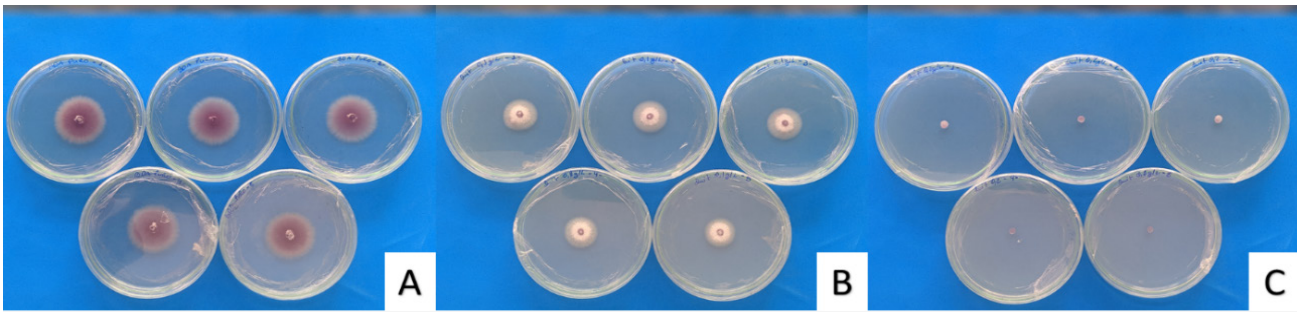
Source: Authors (2024).

Figure 2 - Calibration curve of the inhibitory activity on Fop mycelial growth.



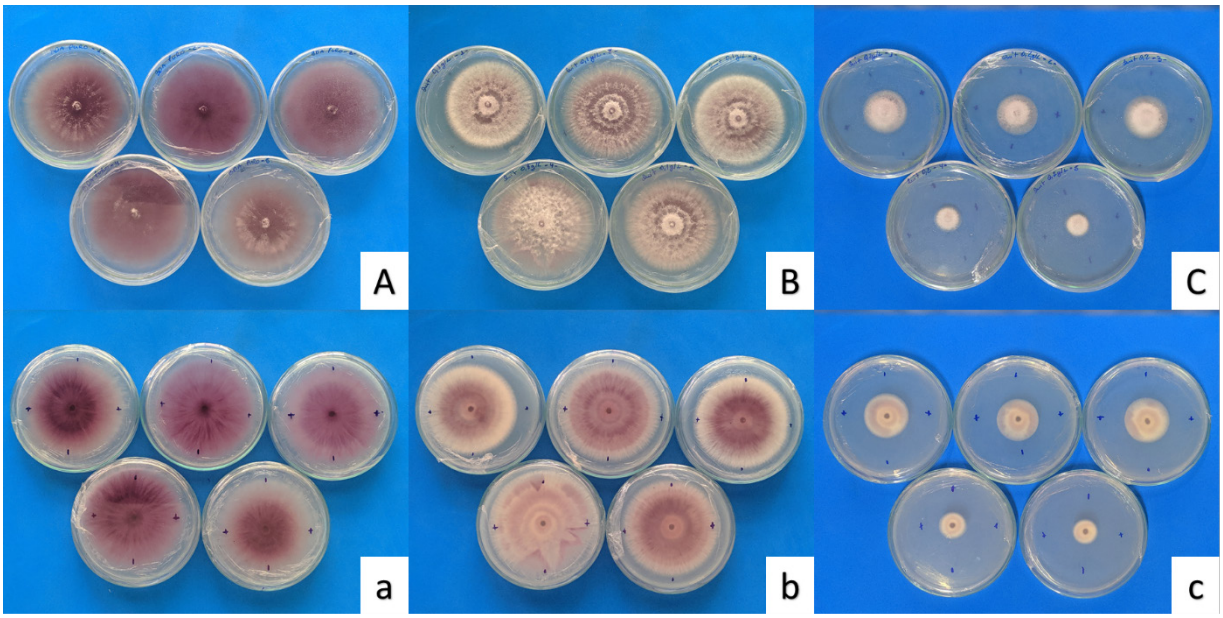
Source: Authors (2024).

Figure 3. Fourth day of Fop mycelial growth: Control (BDA) (A); Treatment 0.1 g/L (B); and Treatment 0.2 g/L (C).



Source: Authors (2024).

Figure 4. Seventh day of Fop mycelial growth: Control (BDA), (A); Treatment 0.1 g/L (B); and Treatment 0.2 g/L (C). Front of the Petri dish (A, B, and C) and back of the dish (a, b, and c).



Source: Authors (2024).

The treatments with concentrations of 0.3, 0.4, and 0.5 g/L stood out for showing 100% inhibitory activity in controlling Fop in the present experiment, not differing from each other, and with sustained inhibition throughout the evaluation period, concluding that a concentration of 0.3 g/L is sufficient for complete inhibition of Fop under the conditions established in this study (Figure 1).

After identifying the concentration range capable of exerting inhibitory activity, the inhibitory concentration of 50% (IC50) was determined by logarithmic fit, which is defined as the concentration of the agent capable of promoting 50% inhibition (Swinney, 2011). The correlation between the average inhibitory activity (%) and the different concentrations of chitosan (Figure 2) ($y = 78.34\ln(x) + 198.66$) with $R2 = 0.9812$, provided a IC50 of 0.1499 g/L, thus proving that the concentration of 0.3 g/L provides complete inhibition, making it unnecessary to increase the concentration of the solution in the present experiment.

In a study conducted using chitosan to control *F. oxysporum* in potato cultivation, a similarity with the data observed in this experiment is evident, where in their evaluations the

plateau of the inhibition effect was reached at concentrations higher than 0.4 g/L, with an IC50 of 0.215 g/L (Ren et al., 2021). The discrepancy between the observations is possibly related to the differences in Fusarium isolates, as well as the molecular weight and degree of deacetylation of the chitosan used.

In another study, they demonstrated that chitosan is capable of promoting the death of pathogens, due to its ability to penetrate the fungal cell membrane, destroy intracellular components, interrupt normal physiological processes, and even bind directly to genetic material and interfere with DNA replication (Xing et al., 2015).

The mechanism of action of chitosan is not yet fully understood and is the subject of study by many researchers. However, its benefits are undeniable, as it has both fungicidal and fungistatic activity. The prevailing view to explain its action involves the electrostatic interaction between chitosan and the anionic components of the cell wall, forming a polymeric membrane layer on the surface of the fungus, which can alter the selective permeability of the cells, thereby blocking nutrient entry or breaking the fungal cell wall membrane, allowing chitosan to enter the cell and leading to inhibition of protein synthesis (Verlee et al., 2017).

Regarding the alteration in the composition of the *F. oxysporum* cell wall, based on the analysis of the transcriptome sequencing results, it was observed that chitosan is capable of promoting the negative regulation of the gene encoding the plant cell wall-degrading enzyme (CWDE) secreted by *F. oxysporum*, leading to a reduction in its pathogenicity (Ren et al., 2021). In the same study, a correlation was found between CWDEs and Fusarium pathogenicity, since as CWDEs are activated, there is an increased ease of mycelial dissemination, as well as a high possibility of blockage of the plant's vessels (Ren et al., 2021).

Regarding the analysis of spore production inhibition, it was found that the concentration of 0.3 g/L is also more effective in inhibiting the sporulation of Fop. In the 0.1 g/L treatment, the highest sporulation rate was observed, almost tripling the number of spores (Table 2). This increase in sporulation may be related to the nutrition of Fop, as Elagamey (2022) suggests that chitosan at low concentration can be used by the pathogen as an alternative source of plant polysaccharides (since it uses CWDEs to obtain them to ensure its nutrition and energy), whereas chitosan at high concentration has antifungal activity, suppressing fungal growth and, consequently, sporulation. The 0.2 g/L treatment shows spore inhibition values close to the control treatment, although it should be noted that it had lower fungal growth (Tables 1 and 2; Figures 1, 3, and 4).

Table 2 - Average spore rate and inoculum concentration for the different chitosan concentrations. Guanambi - BA. 2024.

Treatment	Average spore rate	Inoculum concentration
Control (BDA)	39,6 b	6,3. 10^6 b
0,1g/L	100,6 a	1,6. 10^7 a
0,2g/L	35,0 b	5,6. 10^6 b
0,3g/L	0 c	0 c
0,4g/L	0 c	0 c
0,5g/L	0 c	0 c

Means followed by the same letter in the columns do not differ from each other according to the Tukey test at 5% probability.
Source: Authors (2024).

Fop spores can remain in the soil for several years, being a survival structure capable of creating challenges for researchers seeking to develop an effective control mechanism. Thus, studies related to sporulation are of great importance to help with control measures.

Chitosan is a very important bioactive compound with great potential for controlling Fusarium wilt in passion fruit cultivation. Therefore, it is hoped that this work will stimulate new research focusing on this crop, leading to the improvement of the Fop control mechanism in order to achieve sustainable, accessible, and effective control.

It is concluded that chitosan is a potential inhibitor *in vitro* of *Fusarium oxysporum* f. sp *passiflorae*. When concentrations of 0.3 g/L or higher are used, both mycelial growth and sporulation are inhibited.

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