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Antifungal effect of chitosan of different molecular weight against *Colletotrichum alatae* under in vitro conditions

Abstract

Colletotrichum alatae is a specific pathogen of *Dioscorea alata* responsible for the foliar anthracnosis in this Yam specie. The only product registered in Puerto Rico for the control of the anthracnose is based on the active ingredient Azosystrobin, which have shown to developed resistance on *Colletotricum* spp. Chitosan is a de-acetylated product of Chitin which have shown plant protection capabilities, particularly as a fungicide. To evaluate the efficacy of Chitosan on *Colletotrichum alatae* in vitro, the radial growth and a microtiter assay was performed. Three chitosan products and three concentrations were evaluated, a positive (Azosystrobin) and a negative control were also included. The results of the radial growth show that the best treatment in the control of the mycelial development is the Azosystrobin followed by the low molecular weight at 1000 ppm. In the other hand, the microtiter assay shows that the best treatment is the low molecular weight at 1000ppm. These results suggest that chitosan could be a potential product to control anthracnose which works better in the conidial stage of the fungi.

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Introduction

One of the main challenges for food production worldwide is the presence of pathogens that cause diseases, among which are phytopathogenic fungi. The genus *Colletotrichum* spp. includes a large number of phytopathogenic fungi species that have a wide range of hosts including many economically important crops. Yam (*Dioscorea* spp.) is one of the most consumed crops in the tropics for its high nutritional value and for being an excellent source of carbohydrates, vitamin C, minerals and proteins. *D. alata* is the specie with the higher yields, even in poor soils and is the most simple to propagate. However, a drastic decrease in its production has been observed in Puerto Rico due to foliar disease known as anthracnose. This disease can be caused by a complex of species of *Colletrotrichum* spp., being *C. alatae* specific of *D. alata*. Anthracnose is considered the most damaging disease of *D. alata* worldwide. In the island has been reported that can cause losses in yields of 50 to 90%, specifically in the variety Florido.

Colletotrichum spp. is considered one of the most important fungi worldwide, not only for its wide diversity of hosts, but also for its great ability of infection. *Colletotrichum* species that cause anthracnose show two main phases of nutrition in the process of colonization of the plant. The biotrophic phase is first, which ensures the establishment of the pathogen because (due) the fungus presents a poor enzymatic expression to degrade the tissue, causing that the host plant is inefficient recognizing it. The necrotrophic phase is associated with the appearance of anthracnose symptoms, which include brown circular lesions where the spores are produced. In order for the infection process to be completed, a series of morphological changes in the fungus must occur. Through splashes of water, the spores land on the surface of the leaves and in the presence of the appropriate humidity conditions they germinate and followed by germination is formed the apresorium. This structure is essential for successful infection as it is necessary for tissue penetration and obtaining nutrients. After initial penetration, a spherical vesicle is formed from which the hyphae grow out to colonize the host cells intercellularly.

Given the high capacity of this pathogen to cause disease in all stages of development of the crop, it is necessary to implement efficient control measures. Numerous studies have reported that agricultural production systems dependent on high amounts of chemicals have developed an increasingly serious problem of resistance to fungicides. In addition, this is attributed to environmental pollution problems. In Puerto Rico, the only registered product for the control of anthracnose is Quadris (Azosystrobin), which in other species of *Colletrotrichum* resistance development has been demonstrated. Given this, it is essential to find durable alternatives for the control of anthracnose and to conserve the environment.

Chitosan is a polycationic polymer obtained from the alkaline N-deacetylation of Chitin. It is used as an antimicrobial, chelating agent and in addition to shown to induce defense response in plants. Many in vitro researches have demonstrated its effectiveness against phytopathogenic fungi such as *Rhizopus stolonifer*, *Botrytis cinerea*, *Sclerotinia sclerotium*, *Penicillium digitatum*, *Alternaria alternata*, among others. Where it has been proven that chitosan is able to reduce mycelial growth and spore germination. On the other hand, considerable control has been observed in several species of the genus *Colletotrichum*, where a reduction in the diameter of the lesion occurs. A viable alternative for the control of anthracnose is the use of chitosan because it is a product of natural origin, biodegradable and non-toxic. Based on all its properties, it could be considered as an option to meet the needs of sustainable agriculture worldwide. However, the antifungal activity of chitosan may be affected by factors such as molecular weight, degree of polymerization, pH and temperature. Due to the lack of research in Puerto Rico to control the disease in yam crop, this study leads to test the in vitro antifungal effect of chitosan of different origin and molecular weights at various concentrations as an alternative control to *Colletotrichum alatae*.

Materials and methods

Fungal isolate

Colletotichum alatae was obtained from yam germplasm collection

(provided by Dra. Merari Feliciano, professor Epidemiology at University of Puerto Rico). The isolate was replicated in PDA culture medium (lactic acid), incubated for 7 days, then transferred by hypha-tip in green beans agar (454 g of green beans/L, mixed with 18 g of agar) under continuous fluorescent light at 28.5ºC for 7 days to induce sporulation.

In vitro evaluation of the antifungal activity of chitosan with different molecular weight and procedence

Three chitosan products with different molecular weight (Table 1) were evaluated for their antifungal activity against *Colletotrichum alatae* in vitro. Chitosan was dissolved in 1% acetic acid by heating the solution on hotplate stirrers for aproximately 1 hour continuosly. The pH of chitosan solutions was adjusted to 5.6 with 1N NaOH after dissolving in acetic acid. For all *in vitro* experiments, solutions were filtered with sterile syringe (0.45µm, VWR) in aliquoted. For three chitosans products tested, three concentrations were evaluated: 250, 500 and 1000µg/mL, reported by Rivero et al.,[1] and Seyfarth et al.[2] By using the volume concentration formula, the exact amount of chitosan to be used per treatment was determined. This compound was added when the culture medium had a temperature of 40°C, after sterilization.

Table 1 Chitosan-based products evaluated on *in vitro* experiments

Chitosan	Molecular weight	Deacetylation (%)	Concentration	Company
Shrimp Shells	190-375 KDa	75	100	Sigma Aldrich
LMW	50-190 KDa	75-85	100	Sigma Aldrich
MMW	–	75-85	90-100	Sigma Aldrich

LMW, low molecular weight; MMW, medium molecular weight

In vitro evaluation of the antifungal activity of chitosan in amended media

PDA amended with chitosan shrimp shells, low and medium molecular weight was used to test the antifungal activity of chitosan in vitro on the radial growth of *C. alatae*. PDA plates non amended and amended with acid acetic alone at 1%µg/mL (pH was also ajusted to 5.6) and Azoxystrobin (Quadris 22.9%) at 10µg/mL were used as negative and positive controls, respectively in all experiments. An agar disk (5 mm diameter) from a pure culture of *C. alatae* was placed in the center of the PDA plates and incubated under continuous light at 28.5ºC. The radial growth was measured each two days until the non-amended control reached the edge of the plate. The experiment was performed twice with three replicates per treatment.

Quantification of spores' suspension

Conidia purification

A conidia' solution was developed using *C. alatae* from a culture in Green bean agar during 7 days at 28°C. The solution was created by putting 10mL of distilled sterile water into the petri dishplate, scraping the conidia using a scalpel and filtrating the obtained suspension using sterile gauzes (Walgreens Brand) in a 50mL centrifuge tube. The volume was completed to 20mL with sterilized distilled water. For the conidia' wash, the solution was vortexed for 90 seconds and 10mL of the suspended material were collected to transfer them into a 15mL tube. The suspension was centrifuged at 7,000rpm for 10 minutes, discarding the supernatant and repeating this step to a total of three times. Finally, the remaining supernatant was discarded to obtain a pellet that was diluted in 20mL to obtain a concentration of 10^6 colony forming units (CFU) by mL.

Colorimetric essay

During this part, 12 tubes of 1.5mL were used for the evaluation of each Chitosan type and the positive and negative control. In these tubes, 400µL of PDB and 100µL of conidia (10^6 by mL) were added to group them in 3 categories, having the dose type of each Chitosan as the classification criteria (25, 50, and 100µg/mL). All these tubes were filled up with 1000µL of distilled sterile water (Table 2 & 3 for detailed recipes). These groups were placed randomly in a 96 wells ELISA plate. The same solution without any conidia added was used as internal control. All the plates were covered with paraffin-wax and incubated for 48 hours at 28°C. After incubation the plates were taken to the spectrophotometer (Multiskan FC, Fisher Scientifcic) to obtain the absorbance lecture.

Table 2 Colorimetric assay recipe

Treatment (µg/mL)		PDB (µL)	Conidia (µL)	Fungicide (µL)	Acetic Acid (µL)	Water (µL)	Final Volume (µL)
	250	400	0	25	0	575	1000
Shrimp	500	400	0	50	0	550	1000
	1000	400	0	100	0	500	1000
	250	400	0	25	0	575	1000
LMW	500	400	0	50	0	550	1000
	1000	400	0	100	0	500	1000
	250	400	0	25	0	575	1000
MMW	500	400	0	50	0	550	1000
	1000	400	0	100	0	500	1000
		400	0	5	0	595	1000
Quadris	10	400	0	0	0	600	1000
		400	0	0	10	590	1000

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Table 3 Colorimetric assay internal control recipe

Treatment (µg/mL)		PDB (µL)	Conidia (µL)	Fungicide (µL)	Acetic Acid (µL)	Water (µL)	Final Volume (µL)
	250	400	100	25	0	475	1000
Shrimp	500	400	100	50	0	450	1000
	1000	400	100	100	0	400	1000
	250	400	100	25	0	475	1000
LMW	500	400	100	50	0	450	1000
	1000	400	100	100	0	400	1000
	250	400	100	25	0	475	1000
MMW	500	400	100	50	0	450	1000
	1000	400	100	100	0	400	1000
		400	100	5	0	495	1000
Quadris	10	400	100	0	0	500	1000
		400	100	0	10	490	1000

Statistical analysis

All results were analyzed using INFOSTAT Statistical Software Version 2016 (InfoStat, FCA, Córdova Argentina). Analysis of variance (ANOVA) was performed at the significance level of P≤0.05. Fishers least significance test (LSD, P≤0.05) was performed to separate means.

Results

In vitro evaluation of the antifungal activity of chitosan-amended media

The inhibitory effect of chitosan on mycelium of *C. alatae* was determined by measuring the radial growth at several concentrations (Figure 1). Low molecular weight (LMW) and Chitosan shrimp shells at 1000µg/mL produced significant reductions (P<0.05) in the radial growth of *C. alatae* (14 and 9% respectively) compared with other treatments with chitosan (Figure 1). Azoxystrobin effect significant the radial growth compared with all treatments in the experiments. No effect on radial growth of mycelium was observed by chitosan Shrimp (250 and 500µg/mL), low (250 and 500µg/mL) and medium molecular weight (250, 500 and 1000µg/mL). Growth progression of *C. alatae* for 10 days in media modified with chitosan of different molecular weight was evaluated for each replicate of the experiment (Figure 2 & 3). Where growth had a linear trend from day to day 10. In replicate 1 and 2 treatments of shrimp and low molecular weight at 1000µg/mL were the ones that had the greatest effect in inhibiting the growth of the fungus compared to treatment not amended. There were no significant differences between the other treatments, because no treatment completely inhibited mycelial growth.

Chitosan effects on *C. alatae* using microtiter analysis.

The results of the microtiter analysis show a significant effect of the treatments (p<0.0001) in both repetitions. The treatment with low molecular weight (LMW) at 1000µg/mL show to have the highest mycelial growth inhibition (MGI) when compared to the untreated control (with 1% acetic acid pH 5.6). This treatment is significantly different than our positive control which is Azoxystrobin (AZ). The chitosan treatment that is from Shrimp at 1000µg/mL is not significantly different but is numerally different that the LMW 1000. All the other treatments are significantly different than the best treatment (Figure 4).

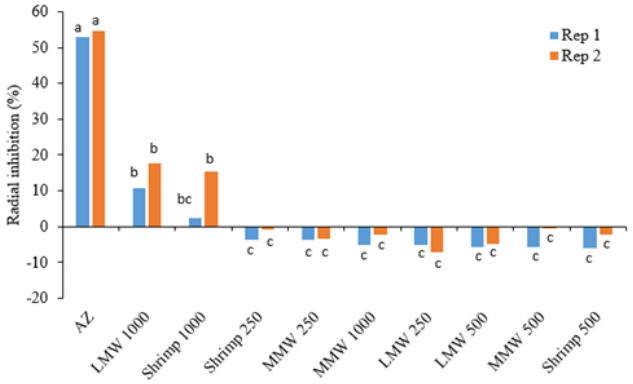

Figure 1 Radial inhibition of chitosan with different molecular weight at various concentrations on mycelial growth of *C. alatae* during 10d incubation period.

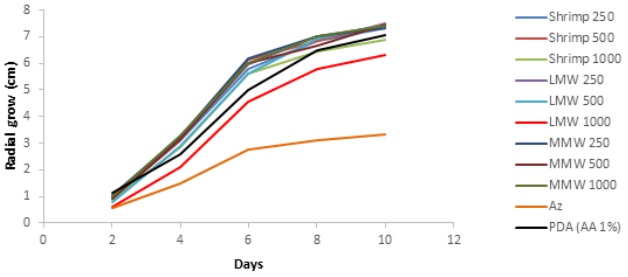

Figure 2 Effect of chitosan with different molecular weight at various concentrations on mycelial growth of *C. alatae* during 10d incubation period (Repetition 1).

After 10 days growing of *C. alatae* in modified media with different concentrations of chitosan, and positive control were visually evaluated (Figure 5). Both repetitions are presented. Similar growth was observed among PDA; LMW 250, and 500 ppm; MMW 250, 500, and 1000ppm, and shrimp 250, and 500 ppm. The treatment that shows less mycelial growth was the positive control azoxystrobin. Also we observe that LMW 1000 ppm, and Shrimp 1000 ppm treatments have an inhibitory effect. Sporulation was not observed among treatments. All treatments do not show control in relation of the formation of structures like sclerotia was observed with the exception of azoxystrobin.

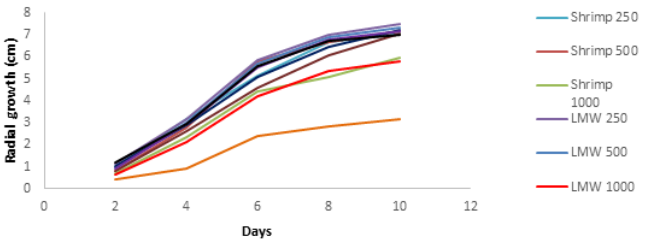

Figure 3 Effect of chitosan with different molecular weight at various concentrations on mycelial growth of *C. alatae* during 10d incubation period (Repetition 2).

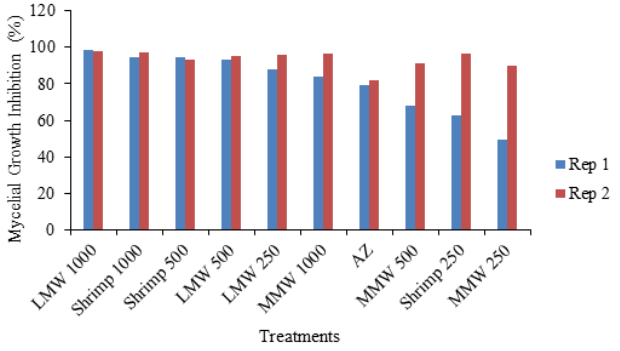

Figure 4 Mycelial growth inhibition of chitosan with different molecular weight at various concentrations of *C. alatae*.

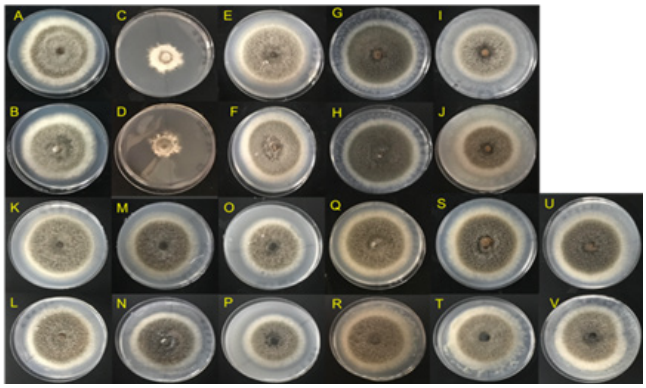

Figure 5 Colony phenotypes of *Colletotrichum alatae* on media amended with three different types, and concentrations of chitosan. A,B) non-amended media (PDA), C,D) Positive control (Quadris® Flowable) E,F) chitosan LMW 250 ppm, G,H) chitosan LMW 500 ppm, I,J) chitosan LMW 1000 ppm K,L) chitosan medium grade 250 M,N) chitosan medium grade 500 ppm, O,P) chitosan medium grade 1000 ppm, Q,R) chitosan shrimp 250 ppm, S,T) chitosan shrimp 500 ppm U,V) chitosan shrimp 1000 ppm.

Discussion

Since the development of the chitosan molecule, several in situ and in vitro experiments have been carried out to verify the antimicrobial activity,[1,3,4] defense of plants as elicitors[5,6] and preservation of food quality.[7] In this study, the treatments with the highest inhibition of *C. alatae* mycelial growth were "low" and "shrimp" with the highest dose (1000µg/ml) also Azoxystrobin (10µg/ml) (positive control). These results are similar with the study of Rivero et al.,[1] where the highest dose of chitosan showed inhibition of the mycelial growth of *Bipolaris oryzae.* The same tendency occurred with *Botrytis cinerea* and *Penicillium expansum* where not only inhibited the mycelial growth but also the germination of spores and elongation of the germinative tube with higher doses (500µg/ml).[8]

In the same way, Zhan et al.,[9] evidenced the inhibitory effect of chitosan on mycelial growth as the concentration increased in *Leotographium procerum*, *Sphaeropsis sapinea* and *Trichoderma harzianum*. Related results were reported by Ait Barka et al.,[10] Liu et al.,[11] and Ben Shalom et al.,[12] for *B. cinerea* where they showed a relation between the chitosan concentration and the inhibition caused. According with García et al.,[13] Kong et al., (2008) Torr et al.,[14] and Liu et al.,[15] the inhibitory effect has to do with chitosan adhering to components of the cell membrane (i.e., proteins, phospholipids) that are negatively charged. This could be the reason of the results of other researchers which suggest that Chitosan affects the fungal membrane inducing leakage of cellular content (Kong et al., 2008).[16,17] Once adhered it avoids that the different structures of the fungus continue to develop, causing greater or less inhibition depending on the species of the organism, because they block the transport of solutes into the cell.

Another factor to consider is the growth stage of the organism, for example, chitosan was more efficient in the exponential phase of *Staphylococcus aureus*.[18] The growth phase in which chitosan can be effective varies from one organism to another,[19] due to the components that make up each of these phases to a greater or lesser extent.

Moreover, the antifungal activity could be due to the presence of phenolic compounds that activate or increase when chitosan is added,[12] evidencing the elicitors of the plant.[5] Then, Ben-Shalom et al.,[12] demonstrated increased POD (peroxidase) in the presence of chitosan and inhibition of growth of *B. cinerea* in tomato (*Solanum lycopersicum*) and Liu et al.,[8] reported an increase in the levels of PPO (Polyphenoloxidases), POD and phenolic compounds in chitosan treatments. Other important aspect of the antimicrobial effect of chitosan, is that it is pH dependent.[15] This confirms the results of Leuba & Stossel,[16] where the authors suggest that Chitosan diluted in acetic acid at pH 5.8 induced a massive protein denaturation. The results of these researchers suggest that the antifungal activity is associated to the type of solvent used to dilute the chitosan.[20-22]

Conclusion

Chitosan can be considered a safe alternative to control foliar diseases such as anthracnose, because it is attributed important properties such as the anti-fungal effect on pathogens of economically important crops; it is a non-toxic product for humans and has a minimal impact on the environment. This and previous studies have observed that the anti-fungal effect affects both mycelium and conidia; being the conidia the structures most affected by the presence of chitosan. This happens because the chitosan acts preventively avoiding the germination of the conidia, which is the first inoculation structure. In our experiment, chitosan had a better effect on LMW and Shrimp treatments with a maximum concentration of 1000 ppm, this proves that the methodology established to meet our objectives was correct. Inhibition of mycelial growth and germination of conidia in *C. alatae* was observed. Our results did not show that the molecular weight influenced on the effectiveness of chitosan in inhibiting the development of *C. alatae*, but we do see an effect when the concentrations of each product are increased. It is important to evaluate the correct concentration of chitosan to control the pathogen, because some treatments show induction in mycelial growth. It is advisable to perform other procedures that reaffirm our results, such as the evaluation of the products on the development of aspersorium and the use of scanning and transmission electron microscopy. In addition, an in vivo assay should be performed to evaluate the effect

of chitosan at the field level, because chitosan can reduce the severity of the disease and induce defense response in the plant.

Acknowledgments

None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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