Antifungal Activity of Chitosan against *Fusarium oxysporum* f. sp. *cubense*

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ABSTRACT: The *in vitro* antifungal activity of chitosan against *Fusarium oxysporum* f. sp. *cubense* Race 4 (FocR4) the causal agent of banana wilt was investigated. Chitosan at all concentrations tested reduced the hyphal growth of FocR4 on potato dextrose agar media and recording maximum inhibition of 76.36% at 8 mg/mL. The inhibitory effect was found to increase as chitosan concentration increases. The 50% effective concentration value was estimated by probit analysis, and it was 1.4 mg/mL. Chitosan was more effective in potato dextrose broth where it completely inhibited the mycelial growth of FocR4 at all concentrations tested. Chitosan inhibited the sporulation of FocR4 by a maximum of 96.53% at 8 mg/mL chitosan, and 100% inhibition for spore germination was recorded at all concentrations tested. Chitosan at concentrations of more than 1.6 mg/mL was also found to induce morphological changes in FocR4 characterized by agglomeration of hyphae, abnormal shapes, vesicles, or empty cells devoid of cytoplasm in the mycelia. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 120: 2434–2439, 2011

Key words: chitosan; *Fusarium oxysporum*; antifungal; banana wilt

INTRODUCTION

Fusarium wilt affects important cultivars in regions in which bananas are cultivated world wide. *Fusarium oxysporum* f. sp. *cubense* (FocR4), a soil-borne fungal pathogen, is the causal agent of vascular wilt disease called Panama disease. It is capable of attacking cultivars, which were resistant to the disease in subtropical banana growing regions. No cultural or chemical control strategy has proven to be effective against this disease.[1] Recently, there are many encouraging attempts to control this pathogen using extracts of organic amendments, such as neem cake and groundnut cake,[2] endobacterium agents such as *Serratia marcescens*[3] and *Psudomonas* sp.,[4] fungal endophytes, which showed the ability to produce several types of volatile metabolites that inhibit the growth of FocR4[5] and compositions comprise a polyene fungicide such as natamycin and at least one phosphite-containing compound.[6]

Chitosan is a natural polycationic biopolymer derived by deacetylation of chitin. It can be obtained from crustacean shells (crabs, shrimp, and crayfishes) either by chemical or microbiological processes and also it can be produced by some fungi (*Aspergillus niger*, *Mucor rouxii*, *Penicillium notatum*, etc.).[7] Thus, chitosan has attracted tremendous attention as a potentially important resource for its biological properties including nontoxicity, biodegradability, and biocompatibility.[8] It has shown to be fungicidal against a wide range of phytopathogenic fungi whether *in vitro* or *in situ* studies.[9,10] Inhibitory effect of chitosan was also demonstrated with soil-borne phytopathogenic fungi including Fusarium wilt pathogens.[11–13]

Antifungal activity of chitosan was observed on various development stages such as mycelia growth, sporulation, spore viability and germination, and the production of virulence factors.[13] Microscopic observations of several fungi treated with chitosan showed mycelial swelling, abnormal shapes, excessive branching, and hyphal size reduction.[14,15] Chitosan also caused morphological changes such as cytoplasm aggregation, dissolution of protoplasm, large vesicles, or empty cells devoid of cytoplasm in the mycelium.[16,13] Area, length, and form of spores of *Colletotrichum gloeosporioides*, *Rhizopus stolonifer*, *Penicillium digitatum*, *Aspergillus niger*, and *F. oxysporum* were affected in chitosan solutions.[17–19] The main objectives of this work were to investigate the antifungal properties of chitosan on mycelial growth, sporulation, and conidial germination of FocR4 and to detect any morphological alterations to the fungus.

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MATERIALS AND METHODS

Preparation of chitosan

Locally prepared shrimp shell chitosan (88% deacetylated, viscosity 370 cps at 0.5% in 0.5% glacial acetic acid, moisture content 10–13%, and ash <0.3%) was obtained from the manufacturer, Chito-Chem, Malaysia.

Pathogen and culture

FocR4 isolate was obtained from the Department of Plant Protection, Universiti Putra Malaysia (UPM). The isolate was maintained on potato dextrose agar (PDA) in the dark at 25°C.

Effects of chitosan on mycelial radial growth

Fungicidal activity was determined by a radial hyphal growth bioassay.[12] Chitosan solutions were prepared by weighing 0.1, 0.2, 0.4, 0.8, and 1.6 g in amounts and dissolving in 100 mL of distilled water in 2 mL of glacial acetic acid and heated to 35°C, while constantly agitating for 24 h. The pH of the solution was adjusted to 5.6 by adding 2*N* sodium hydroxide. The chitosan solutions and PDA were autoclaved separately at 121°C for 20 min and were subsequently combined to obtain concentrations of 0.5, 1, 2, 4, and 8 mg/mL and poured onto sterile Petri dishes (9 cm). Control plates consisted of PDA only. A 5-mm diameter plug from the advancing margins of 3–5 days old colonies of FocR4 on PDA was seeded centrally onto eight plates of each chitosan concentration. All plates were incubated in the dark at room temperature in completely randomized design (CRD). The radial colony growth was measured daily until the leading edge of the fastest-growing colony had reached the edge of the plate. The percentage of inhibition was calculated based on percentage inhibition of radial growth (PIRG%) as follows:

$$\text{PIRG}\% = [(R1 - R2)/R1] \times 100\%$$

whereby, $R1$ = radial growth of FocR4 in control plate, and $R2$ = radial growth of FocR4 in treatment plate.

In another experiment, concentrations of 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg/mL chitosan were tested to determine the 50% effective concentration value and MIC (the minimum concentration showing over 90% inhibition of mycelial growth) by probit analysis.[20,13] Three experiments were performed with eight replicates per treatment per experiment. As the results of these experiments were similar, only the results from the first test were presented.

Effects of chitosan on sporulation

Sporulation was assessed using amended growth media as similarly described above. Mycelial growth was permitted to extend to the edge of the control plate, and after 10 days of incubation at room temperature, plates were flooded with sterile-distilled water, which was then placed on an orbital shaker running at $38 \times g$ for 20 min to liberate spores from the mycelia. The resulting spore suspension was filtered through two layers of cheesecloth, and the spore density of the suspension was determined using a haemacytometer. Spores were counted for each treatment and the spore population density was reported as a percent value (percentage inhibition of sporulation) using the formula described above for mycelial growth measurements. Three experiments were performed with four replicates per treatment per experiment and only the results from the first test were presented.

Effects of chitosan on spore germination

Germination was confirmed by spreading 300 μL of a diluted spore suspension (300 spores/mL) onto a PDA plate amended with different concentrations of chitosan (0, 0.5, 1, 2, 4, and 8 mg/mL). The plates were incubated at room temperature in the dark for 48 h to germinate the spores and the number of colonies counted. The percentage inhibition of spore germination (PIG %) was determined using the formula described above for mycelial growth measurements. In another experiment, involving concentrations of 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg/mL of chitosan, 250 μL of suspension (800 spores/mL) were spread onto a PDA plate amended with chitosan. Three experiments were performed with four replicates per treatment per experiment.

Effects of chitosan on mycelial growth in broth culture

Chitosan solutions and potato dextrose broth (PDB) were autoclaved separately at 121°C for 20 min and combined after autoclaving to obtain concentrations of 0.5, 1, 2, 4, and 8 mg/mL. Three mycelial discs (5 mm) from 7 days old culture were added to 100-mL sterilized PDB medium amended with chitosan in 250-mL Erlenmeyer flasks and were incubated at room temperature for 14 days. The mycelium was filtered through a preweighed filter (Whatman no. 1), washed five times with deionized water, dried in an oven at 100°C for 24 h, and then weighed. Four flasks were prepared for each treatment and the means were compared. The inhibition (%) of chitosan was calculated by weighing dried biomass of FocR4 using the formula:

$$[(\text{Biomass of control sample}) - (\text{Biomasss of treated sample})]/[\text{Biomass of control sample}]$$

TABLE I
Effect of Chitosan at Various Concentrations on Mycelial Radial Growth of FocR4 after 8 Days of Incubation

Experiment	Concentration (mg/mL)	Mycelial diameter (mm)	PIRG%
1	0	85.13 ± 0.30 a	
	0.5	50.00 ± 1.12 b	41.25 ± 1.40 a
	1	42.25 ± 1.01 c	50.36 ± 1.25 b
	2	38.25 ± 1.37 d	55.06 ± 1.63 c
	4	28.50 ± 0.89 e	66.52 ± 1.05 d
	8	20.13 ± 1.49 f	76.36 ± 1.79 e
2	0	82.29 ± 0.97 a	
	0.1	60.00 ± 0.87 b	27.05 ± 1.16 a
	0.2	58.29 ± 1.48 b	29.12 ± 1.93 a
	0.4	58.29 ± 2.17 b	29.14 ± 2.68 a
	0.8	50.57 ± 0.72 c	38.46 ± 1.35 b
	1.6	39.29 ± 1.84 d	52.18 ± 2.47 c
	3.2	29.29 ± 1.44 e	64.32 ± 2.01 d

(PIRG% = Percentage inhibition of radial growth). Means followed by the same letter within column in same experiment are not significant at $P < 0.05$ (Tukey test).

In another experiment, concentrations of 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mg/mL chitosan were tested. Three experiments were performed with four replicates per treatment per experiment.

Assessment by light microscopy

Mycelial samples, taken from the margin of 6 days old colonies grown on chitosan amended or chitosan free PDA, were observed under the light microscope to determine whether the observed inhibition correlated with morphological and (or) cytological changes. Images were obtained using a Nikon, Eclipse-E200 microscope with a charged coupled camera (Nikon, Coolpix 995).

Statistical analysis of experiments

All experimental treatments were arranged in a CRD. Means separation by Tukey test ($P = 0.05$) was carried out for comparison of all parameters.

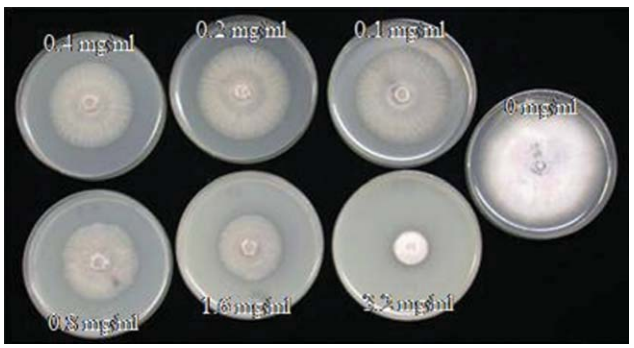

Figure 1 Effect of chitosan at various concentrations on mycelial radial growth of FocR4 after 8 days of incubation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

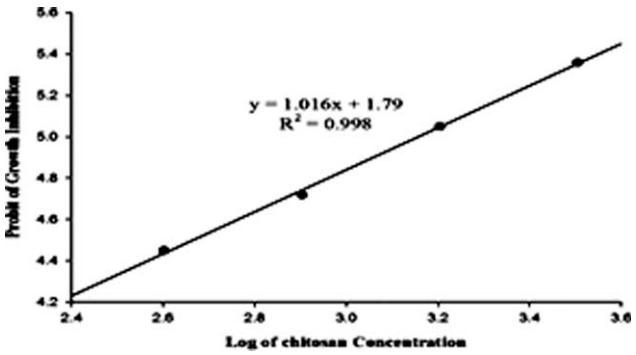

Figure 2 Relationship between inhibition probability and chitosan concentration logarithm for FocR4.

RESULTS

Effects of chitosan on mycelial radial growth

In all experiments, control cultures (without chitosan) of FocR4 reached the edge of culture plates after 8 days of incubation at room temperature, and measurements of colony diameters were therefore done daily until 8 days of incubation. The addition of chitosan to the PDA medium-inhibited mycelial growth of FocR4 significantly at all concentrations tested. This inhibition was chitosan concentration dependent, with a maximum growth inhibition of 76.36% recorded at 8 mg/mL (Table I and Fig. 1).

The correlation coefficients were obtained and then the EC_{50} % and MIC_{90} % of chitosan solution to FocR4 were calculated. The results showed EC_{50} % and MIC_{90} % of chitosan were 1.4 and 26 mg/mL, respectively (Fig. 2).

Effects of chitosan on sporulation

The results in Table II showed chitosan was partially inhibitory to sporulation at all concentrations. The number of spores produced by FocR4 at chitosan concentrations ranging from 0 to 8 mg/mL steadily decreased with an increase in concentration. The maximum inhibition was 96.53% at 8 mg/mL.

TABLE II
Effect of Chitosan at Various Concentrations on Sporulation of FocR4 after 10 Days of Incubation

Concentration (mg/mL)	No. of spores/mL	PIS%
0	36.98×10^6	
0.5	22.67×10^6	38.74 ± 5.31 a
1	18.67×10^6	49.38 ± 3.27 b
2	10.30×10^6	72.02 ± 4.85 c
4	4.41×10^6	88.04 ± 1.41 d
8	1.29×10^6	96.53 ± 1.37 e

(PIS% = Percentage inhibition ofsporulation). Means followed by the same letter within column are not significant at $P < 0.05$ (Tukey test).

TABLE III
Effect of Chitosan at Various Concentrations on Germination of FocR4 Spores after 2 Days of Incubation

Experiment	Concentration (mg/mL)	No. of colonies[a]	PIG%
1	0	77.25	86%
	0.5	0	100
	1	0	100
	2	0	100
	4	0	100
	8	0	100
2	0	153.25	77%
	0.1	0	100
	0.2	0	100
	0.4	0	100
	0.8	0	100
	1.6	0	100
	3.2	0	100

[a] Four replications. (PIG% = Percentage inhibition of germination).

Effects of chitosan on spore germination

The results obtained for spore germination were presented in Table III. After 48 h of incubation, spore germination in control plates were 86 and 77%, respectively, in two experiments. Chitosan at all concentrations tested completely inhibited spore germination of FocR4.

Effects of chitosan on mycelial growth in broth culture

When a liquid culture was used, chitosan after 14 days caused 100% inhibition of mycelial growth of FocR4 at all concentrations tested as shown in Figure 3.

Assessment by light microscopy

Evidence of effects by chitosan on mycelial growth of FocR4 on PDA was assessed by light microscopy (Fig. 4). It was revealed that chitosan at 1.6 mg/mL had markedly induced changes in FocR4 morphology characterized by hyphal agglomeration [Fig. 4(A)], abnormal shapes [Fig. 4(B,C)], and presence of large vesicles in the mycelia [Fig. 4(D,E)].

DISCUSSION

Fusarium wilt of banana is one of the most destructive diseases of banana world wide. Considerable attention has been made to develop methods of effective disease management especially with the discovery of FocR4 known for its ability to attack banana clones that were resistant to the disease. However, no effective control measure has been proven to date.

Various studies have shown that natural compounds such as chitosan could promote the plant to defend itself and also interfere with growth of the pathogen.[21] Chitosan was proven to inhibit the growth of several pathogenic fungi,[22] and therefore, this polycationic compound has potential for biotechnological applications. The results of this study provide new information regarding the inhibitory effects of various concentrations of chitosan on the growth of FocR4. Chitosan was demonstrated to be not only effective in reducing the growth but also causes morphological and ultrastructural changes to the pathogen.

Plates assay showed that FocR4 was highly sensitive to chitosan because radial growth was significantly affected at all concentrations including the lowest level of 0.1 mg/mL tested. However, complete inhibition was not achieved even at 8 mg/mL. Radial mycelial growth and sporulation were reduced as chitosan concentration increases. This was corroborated by light microscopy evidence of normal hyphal cells present in the vicinity of abnormal hyphae. Similar results for inhibition in mycelial growth were reported by Stossel and Leuba[23] for *Fusarium oxysporum* f. sp. *phaseoli*; Hirano and Nagao[22] for *Fusarium oxysporum* f. sp. *melonis* and *Fusarium oxysporum* f. sp. *lycopersici*; Benhamou[14] for *Fusarium oxysporum* f. sp. *radicis-lycopersici*; Bell et al.[24] for *Fusarium oxysporum* f. sp. *apii*; Laflamme et al.[25] for *Fusarium oxysporum* recovered from black spruce; Borgers et al.[26] for *Fusarium oxysporum* f. sp. *lycopersici*; El Hassni et al.[27] for *Fusarium oxysporum* f. sp. *albedinis*; Bautista et al.[17] for *Fusarium oxysporum* isolated from papaya; Tikhonov et al.[12] for *Fusarium oxysporum* f. sp. *radicis lycopersici*; Guo et al.[11] for *Fusarium oxysporum* f. sp. *vasinfectum*; Xu et al.[13] for *Fusarium oxysporum*.

Mycelial growth, sporulation, and spore germination were affected by chitosan demonstrating its effects on various stages in the growth and development of FocR4. The maximum inhibition to sporulation was 96.53% and increasing gradually with increase in chitosan concentration. Previous studies have shown similar effects in *Fusarium oxysporum*

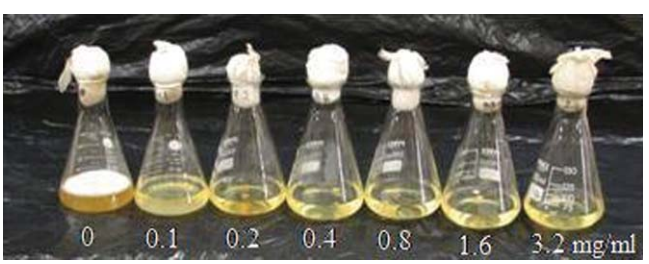

Figure 3 Effect of chitosan at various concentrations on mycelial growth of FocR4 in PDB after 2 weeks of incubation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

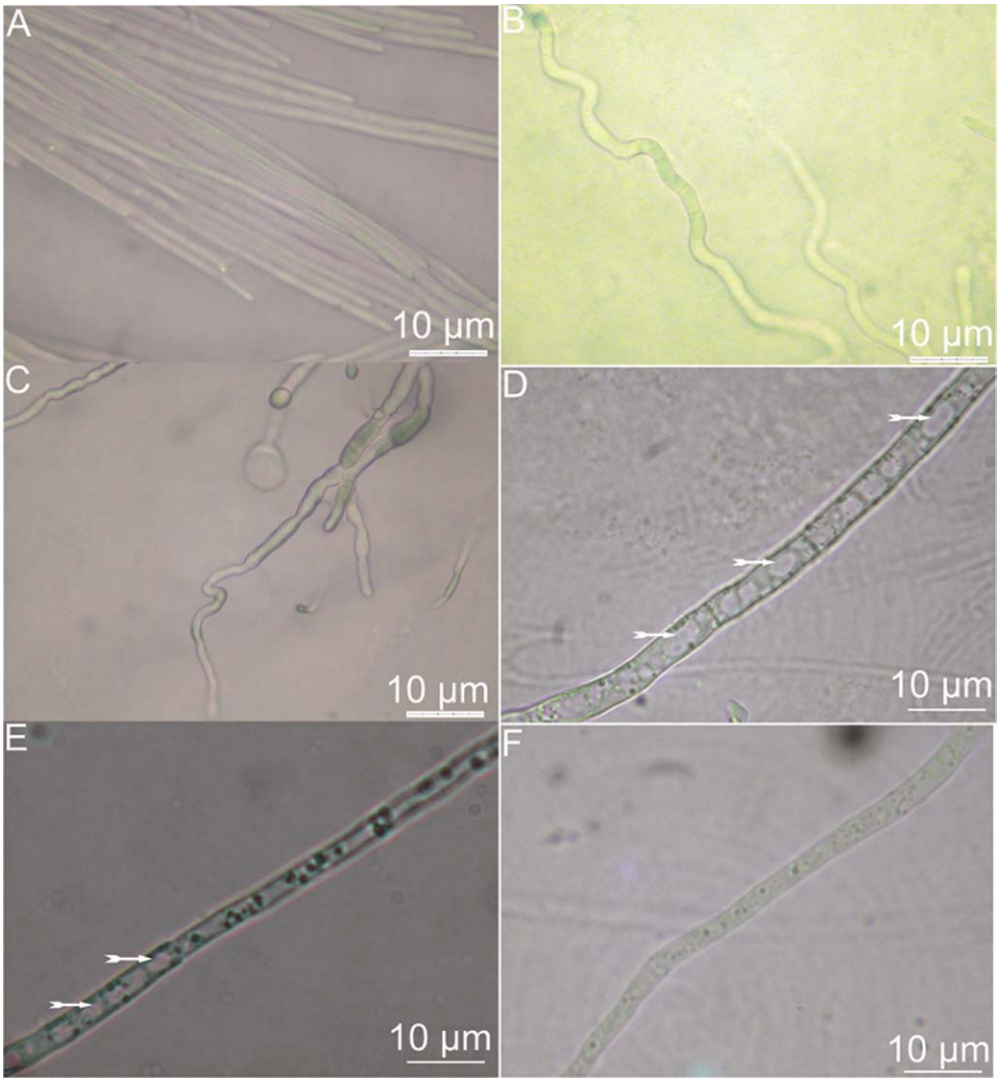

Figure 4 Light microscopy assessment of mycelia of FocR4 treated with chitosan. A: hyphal agglomeration. B and C: Abnormal shapes. D and E: Large vesicles in the mycelia (see arrows). F: Normal hyphal growth in control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

isolated from papaya,[17] *Fusarium solani*[28] and *Phytophthora capsici.*[13] Other studies have shown that chitosan stimulated sporulation of *Penicilium digitatum*,[17] *Alternaria alternate*,[29] and *Colletotrichum gloeosporioides.*[18] The studies indicated that high sporulation could be associated to a stress response caused by chitosan. Conidia of FocR4 were very sensitive to chitosan and no germination was observed upon plating on media amended at different concentrations. The complete inhibition of spore germination was also reported for *Botrytis cinerea*,[19] *Fusarium oxysporum* f. sp. *radicis lycopersici*,[12] *Colletotrichum gloeosporioides*,[17] and *Phytophthora capsici.*[13] The high sensitivity was shown in broth culture amended with chitosan where fungal growth was totally absent at all concentrations tested. Similar results on broth cultures were obtained by Prapagdee et al.[30] for *Fusarium solani* f. sp. *glycines*. Inhibition on broth culture also demonstrated in work involving *Fusarium oxysporum* f. sp. *albedinis*,[27] *Botrytis cinerea*,[16] *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Fusarium solani*.[28]

Light microscopy assessment revealed that growth inhibition of FocR4 in response to chitosan was accompanied by marked cellular changes. These changes included hyphal agglomeration, contortions, and increased vacuolations. Similar effects were reported for *Fusarium oxysporum* f. sp. *radicis lycopersici*,[14] *Botrytis cinerea* and *Rhizopus stolonifer*,[15] *Cylindrocarpon destructans*, *Fusarium acuminatum* and *Fusarium oxysporum*,[25] *Fusarium oxysporum* f.sp. *albedinis*,[27] *Botrytis cinerea*,[16] *Fusarium solani* f. sp. *glycines*,[30] and *Phytophthora capsici.*[13] The possible mechanism for the antifungal action of chitosan was discussed. At least three concepts were suggested to explain the mode of action of chitosan. First, its activity appeared to be the interaction between positively charged chitosan molecules due to ammonium groups and negatively charged residues on the fungal cell wall surface due

to phospholipids head groups, which led to increase in plasma membrane permeability.[31,32] El Ghaouth et al. (1992) reported leakage of proteins and amino acids from cells of *Rhizopus stolonifer* and *Botrytis cinerea* occurred upon treatment with chitosan. Second, it is thought that chitosan may enter the fungal cell, interact with DNA, and alter its conformation, thus inhibiting the synthesis of mRNA and proteins.[33] Third, chitosan exhibit chelating activity of metals, spore elements, and essential nutrients.[34] In this study, the observed vesicles supported the evidence of increased membrane permeability, whereas the results of germination and growth in broth culture could be related to the chelating activity of chitosan. The results of our studies strongly concur with these observations and confirm that chitosan is a potential compound for application in integrated disease management. To our knowledge, this study is the first report of the effect of chitosan on FocR4, an important pathogen of banana plantations in the tropics. These results show promise and potential use of chitosan for effective control of FocR4. Further investigations will be needed to determine whether chitosan is able to induce defense mechanism reaction in banana plants as previously demonstrated in tomato plants,[14,35] cucumber,[19,36] rice,[37] date palm,[27] pearl millet,[38] carrots,[39] grapevine,[40] soybean,[30] and tobacco.[10,41] In conclusion, this study has proven the effectiveness of chitosan in the growth suppression of FocR4. The radial growth and sporulation of FocR4 were reduced gradually by chitosan, whereas germination and submerged growth in broth cultures were completely inhibited even at low concentrations of chitosan.

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