Molecular Weight-Dependent Antifungal Activity and Action Mode of Chitosan Against *Fulvia fulva* (Cooke) Ciffrri

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ABSTRACT: Antifungal activities of chitosans (CTS) with different molecular weights (M_w) and different concentrations against *Fulvia fulva* (cooke) ciffrri (*F. fulva*) causing leaf mold in tomato plants were studied *in vitro* and *in vivo*; the action mode and its inhibition at different stages during the life cycle of *F. fulva* were observed. The results showed that: (1) *in vitro*, CTS exhibited strong antifungal activity against *F. fulva*, especially for the medium M_w (213 and 499 KDa) CTS. Almost complete inhibition of *F. fulva* conidia germination and mycelia colony radial growth was found when CTS was at concentration of 0.5 and 2 mg mL^{-1}, respectively; however, inhibitory effect on sporulation was not very obvious for all CTSs tested in this experiment. *In vivo*, CTS of 213 KDa CTS at 6 mg mL^{-1} concentration produced stronger antifungal effect than others. (2) The morphological study by scanning electron microscope (SEM) showed that CTS could induce the hyphal swelling, and the surface of hypha which was treated with low M_w (82 KDa) chitosan was smooth, but was rough treated with high M_w (1320 KDa) chitosan. The further study using a confocal laser scanning microscopy (CLSM) coupled with fluorescein isothiocyanate (FITC)-fluorescence detection system showed fluorescence of the FITC-labeled chitosans of which M_ws were below 500 KDa could enter into the inner of hypha; however, 1320 KDa chitosan was blocked off the outer of hypha. J Appl Polym Sci 119: 3127–3135, 2011

Key words: chitosan; molecular weight; antifungal activity; action mode; *Fulvia fulva* (Cooke) ciffrri

INTRODUCTION

Chitosan (CTS), a high molecular weight cationic polysaccharide, is a β-1,4-linked polymer of glucosamine (2-amino-deoxy-β-D-glucose), and is formed by the deacetylation of chitin (poly-*N*-acetylglucosamine), an abundant byproduct of the crab and shrimp processing industries. It is inexpensive and nontoxic, and possesses reactive amino groups. Therefore, it has been widely used in many different fields, for instances, as an antimicrobial compound in agriculture, as a potential elicitor of plant defense responses, as a flocculating agent in wastewater treatment, as an additive in the food industry, as a hydrating agent in cosmetics, and as a pharmaceutical agent in biomedicine. Recently, its use of bioactive substance has attracted much more attention due to its antimicrobial,[1–7] antitumor[8–10] activities, and its immune enhancing effects.[11]

Leaf mold, caused by the fungus *Fulvia fulva* (cooke) ciffrri, is one of the most common and destructive foliar diseases of tomato grown under humid conditions, especially in the greenhouse, which could kill large portions of the leaves and result in significant yield reduction (20–80%).[12] Cultivars carrying one or more resistance genes are available for use against the disease, but the fact that the fungus mutates easily leads to a limited application of these cultivars, so the use of chemical fungicides is still necessary.[13] Here brings another question, the applications of various fungicides could lead to resistance development and appearance of different cross-resistance groups. Hence, the frequent development of *F. fulva* isolates resistant to common fungicides and the desire to reduce pesticide use have led to efforts to develop alternatives.[14]

CTS and its derivative are proved to have strong antimicrobial activity against different groups of microorganisms,[1–7] and have attracted much attention as a potential environment safe means in controlling plant disease. Because of the positive charge on the C-2 of the glucosamine monomer below pH 6, CTS is more easily soluble and has stronger

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antimicrobial activity compared with chitin.[15] As a new, natural antimicrobial agent, CTS is biodegradable, nontoxic, biocompatible, and antimicrobial against a wide range of target organisms. Numerous studies on antifungal activity of CTS and its derivative against plant pathogens have been carried out,[16–20] and several fungi were studied[17,21–23] such as *Fusarium accuminatum, Cylindrocladium floridamum, Botrytis cinerea, Fusarium solani* f. sp. *Glycines*, etc. But little research focused on its antifungal activity against *F. fulva* and even less in its mode of action. Because the activity varies with the type of CTS and the target organism and the environment in which it is applied, it is necessary to study the ability of different M_w and concentration CTS to kill *F. fulva*.

Different mechanisms have been proposed although the exact mechanisms of the antimicrobial action of chitin, CTS, and their derivatives are still uncertain. For instance, CTS is adsorbed to bacterial walls leading to walls covering, membrane disruption and cell leakage,[24–27] whereas mainly focused on fungal cell wall[28–30] and cell membrane[31,32] and resulted in antifungal activity. Recently, penetrability of fluorescent-labeled CTS oligomers with molecular weight under 8 KDa into living cells of *Escherichia coli* (bacterium) was observed and oligochitosan was suggested to inhibit bacteria from inside of the cell.[33–35] However, little information about action mode of different M_w CTS on the fungal hypha has been made. It's necessary to further study the exact action mechanism of different M_w CTS on the fungal hypha because the action mode of CTS varies with the difference of CTS M_w and the structure of microbe (fungi or bacteria).

In this investigation, experiments were carried out to test the antifungal activity of different concentration CTS with different M_w against *F. fulva*, and its inhibition in different development stages such as spore viability, germination, mycelia growth, and sporulation. The possible mechanism of the antifungal activity of different M_w CTS was discussed to gain more accurate information on its mode of action.

EXPERIMENTAL

Materials

CTS (85% deacetylated, M_w: 1540, 1320, 499, 213, 144, 82, 38, 3 KDa) were acquired using acetic acid hydrolyzes in our laboratory.[36] All other chemicals and reagents used in this study were of analytical grade (A. R., Sigma Co., St. Louis, USA).

Isolation and culture of *F. fulva*

Fulvia fulva (cooke) ciffrri used in this study was isolated from infected tomato leaf (cultivated in greenhouse) with leaf mold disease. The diseased leaves were first disinfected with 75% (V/V) ethanol for 10 s, 0.1% (V/V) $HgCl_2$ for 5 min, and then rinsed the treated leaves with the sterile distilled water (SDW) for five times; small pieces (1–2 mm^2) of the boundary tissue between the healthy area and the region showing leaf mold symptoms were removed and placed on potato dextrose agar medium (PDA) consisting 200 g L^{-1} potato infusion, 20 g L^{-1} dextrose, and 18 g L^{-1} agar, cultured in the dark at 22.5°C. Single-spore cultures were maintained on PDA for the following use.

Antifungal assays

Antifungal assay of CTS (M_w: 3, 38, 82, 144, 213, 499, 1320, 1540 KDa) was conducted for the radial growth determination of *F. fulva* on PDA. The same quantity CTS solutions (dissolved in 1% (v/v) acetic acid) were added to the different quantity sterile molten PDA to obtain the desired CTS concentration. The pH value was adjusted to 5.6 with 1*M* NaOH, and then distributed to different plates (7.5 cm in diameter). Five-millimeter-diameter fungal plug taken from the margin of a freshly growing colony of *F. fulva* were placed on PDA medium supplemented with CTS of different concentrations (0, 0.125, 0.25, 0.5, 1, and 2 mg mL^{-1}) and M_ws, and incubated at 22.5°C in the dark. Two control treatments were set up for the experiments, one contained PDA only (abbreviated as CK_W) and the other contained acetic acid but not CTS (abbreviated as CK_H). The quantity of acetic acid in all treatments was equal except for CK_W to eliminate the influence of solvent. The growth of colony was monitored daily, and the diameter of colony was measured using crossing method when the mycelium of fungi reached the edges of the control plate (CK_W). Growth inhibition was expressed as the percentage of inhibition of radial growth relative to the control, and EC_{50} (the middle effective concentration) and EC_{90} (the 90% effective concentration) were calculated to evaluate the antifungal activity of different M_w CTS on *F. fulva.* All experiments were repeated thrice with triplicates.

$$\text{the percentage of inhibition } (\%) = (1 - D_a/D_b) \times 100$$

where D_a is the diameter of the growth zone in the test plates, D_b is the diameter of growth zone in the control plate (CK_W), and the data were average.

EC_{50} and EC_{90} values were calculated according to the toxic regression equation between the logarithm (x) of chitosan concentration and the probability (y) of the percentage of inhibition.

Effect of CTS on sporulation

The influence of CTS on control of sporulation was determined. Six mycelium plugs (5 mm in diameter)

were cut from the edge of the growing fungal colony on the medium containing 0, 0.063, 0.125, 0.25, 0.5, 1, and 2 mg mL^{-1} CTS of which M_w were 213, 499, and 1320 KDa, and immersed in 0.5 mL of SDW in 1.5 mL tube containing 0.05% Triton X-100, and rinsed for 0.5 min using a medical ultrasonic syringe (KQ-250E, KunShan ultrasonic apparatus, China). The number of spore was counted using haemacytometer under inverted microscope and calculated the magnitude of sporulation per squared millimeter and the inhibition percentage. Experiments were repeated thrice.

In vitro assay on activity of CTS against conidia germination of *F. fulva*

The same concentration of conidial suspensions (about 1×10^6 conidia mL^{-1}) supplemented with different concentration (0, 0.0313, 0.0625, 0.125, 0.25, and 0.5 mg mL^{-1}) and different M_w (38, 213, 499, and 1320 KDa) CTS were kept on six-well microscope concave slides, and were incubated at 22.5°C in the dark, in a wet chamber. The numbers of germinated conidia, which was defined as that the germ tube length of conidium was as long as the length of conidium, were counted in three different microscopic fields at a magnification of 400× under the light microscope (CX31RTSF, OLYMPUS, Tokyo, Japan) after 24 h. A total of 100 spores per replicate were observed. The germination inhibition percentages were calculated. Each experiment was performed twice with three replications.

$$\text{The germination inhibition percentage } (\%) = (1 - Q_2/Q_1) \times 100$$

where Q_1 is the quantity of germination of the control and Q_2 is the quantity of germination of the treatment containing CTS.

Effect of CTS on hypha growth and morpha

Effect of CTS on hypha growth was conducted for submerged growth determination. Mycelial plugs (5 mm in diameter) of *F. fulva* were cut from the margins of the colony cultured for 5 days on PDA medium, and aerobically cultivated in potato dextrose (PD), which is liquid at 25°C with continuous shaking at 121 rpm (rotary oscillating shaker, ZHWY-2102, Zhicheng analysis apparatus LTD., ShangHai, China). Every treatment was three replicates containing 100 mL PD supplemented with different concentration (0, 0.5, 1, and 2 mg mL^{-1}) CTS of which M_ws were 82, 499, and 1320 KDa. After 20 days, the colony growth was monitored by dry weight determination (after repeated washing with SDW). Growth inhibition was expressed as the percentage of inhibition (relative to the control).

Single-hypha cultured in the PD mentioned earlier was isolated and the morphology of hypha was observed under light microscope and scanning electron microscope (SEM) (KYKY2800B, KYKY Technology Development, Beijing, China) to determine the putative mechanism of the action of CTS.

Preparation of FITC-labeled CTS and observation of their penetrability using confocal laser scanning microscopy

CTS was flocculated when the pH value of different M_w CTS solution (10 mg mL^{-1}, dissolved in 0.1*M* CH_3COOH) was adjusted to 6.8 using 0.2*M* NaOH, and the FITC-labeled CTS was synthesized by adding 10 mL of FITC solution dissolved in dehydrated methanol (2.0 mg mL^{-1}) to 20 mL of CTS solution, stirring in the dark at ambient temperature. After 15 h, the labeled flocculation was centrifuged at 12,000 $\times g$ (10 min) and washed with methanol/water solution (v/v: 70 : 30). The washing and flocculation were repeated until no fluorescence was detected in the supernatant when analyzed by fluorescence spectrophotometers (Hitachi F-4500, Tokyo, Japan; $\lambda_{ex} = 490$ nm, $\lambda_{em} = 520$ nm). The FITC-labeled chitosan was redissolved in 10 mL of 0.1% CH_3COOH and dialyzed in the dark against 5 L of 70% methanol, the methanol being replaced with fresh 70% methanol every 6 h until no fluorescence in the methanol. Finally, the FITC-labeled CTS was freeze-dried and weighed.[33]

FITC-labeled CTS at 0.5 mg mL^{-1} (dissolved in sterile 1% acetic acid) was incubated with actively growing fungal bead in PD. The fungal bead was picked out after 24 h, and washed by SDW repeatedly until no fluorescence in the water was detected. Hypha was isolated from mycelial fungal pellet, and observed under CLSM (LSM5PASCAL, ZEISS, German) to judge whether the FITC-labeled CTS penetrated into the hypha. Conidia were observed simultaneously according to the method described earlier to determine the putative mechanism of inhibition of CTS to conidium.

In vivo field trials to evaluate the inhibitory effect of chitosan on leaf mold

The use of chitosan as a natural antifungal agent against leaf mold in tomato was investigated in greenhouse. Tomato (*Lycopersicon esculentum* Mill.) cv. "Qianxi" was used as the target plant in this experiment and cultivated to the stage of the fourth true leaf. The plants were sprayed thrice with different concentrations (based on preliminary dose response studies) and different M_w (82, 144, 213,

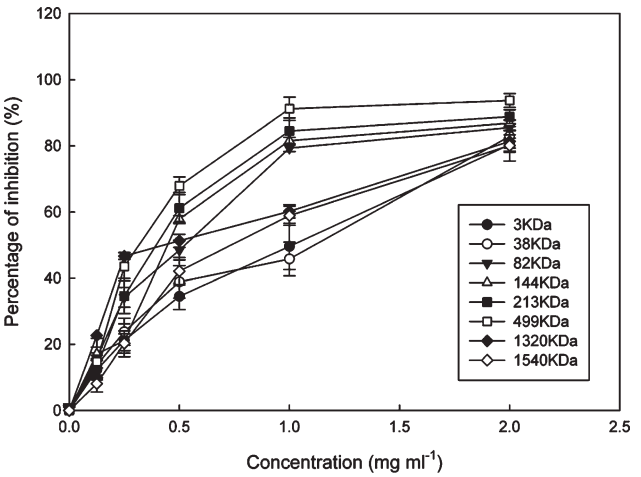

Figure 1 Effect of different M_w and concentrations of chitosan on antifungal activity (the inhibition percentage of the mycelial radical growth) of *F. fulva* at pH 5.6. (mean ± SD, $n = 9$).

1320, 1540 KDa) CTS at an interval of 10 days, and the control plants were sprayed with water only. Conidia were harvested from the PDA plates by adding 5 mL SDW containing 0.05% (v/v) Triton X-100 to each plate on which the colony was cultured 20 days and gently rubbing the sporulation mycelial mat with a bent glass rod. The conidial suspension concentration was adjusted to 1×10^6 conidia mL^{-1} with the aid of a haemocytometer.

After 24 h, plants sprayed with CTS and water thrice were artificially inoculated with conidial suspension of *F. fulva*, and incubated for another 48 h at 22.5°C and 90% relative humidity in greenhouse. Two weeks later, the disease was investigated every 5 days, a total of three times. Leaf mold was evaluated on a scale of 0–7 with: 0 = no necrosis, leaf area is completely healthy; 1 = 25% of the leaf area is with symptoms; 3 = 50% of the leaf area is with symptoms; 5 = 75% of the leaf area is with symptoms; 7 = 100% of the leaf area is covered with symptoms. A disease index was calculated as the sum of the area of the five leaves. Nontreated plants served as the control. Treatments were arranged in a randomized complete block design with three replications, and each treatment was consist of 30 plants.

Statistical analysis

Results were analyzed with the software package Sigmaplot 8.0 and SPSS13.0 (SPSS, Chicago, IL). Mean values and standard deviations from the different replicates per treatment were calculated, and the significance between the treated group and the control was analyzed by two tailed paired *t*-test with significance set at $P < 0.01$.

RESULTS AND DISCUSSION

Antifungal activity of chitosan as a function of its concentration and M_w *in vitro*

Antifungal activities of CTS with different M_w and concentration against *F. fulva* were tested. The colony of *F. fulva* reached the edges of the control plate (CK_W) after 4 weeks cultivation. The inhibition percentage of different concentration and M_w CTS was shown in Figure 1, their EC_{50} and EC_{90} were calculated (Table I). All CTS samples tested in this experiment had antifungal activity, the difference of antifungal activity with the difference of concentration and M_w was obvious, and the antifungal activity improved with the increase of concentration, and the middle M_w CTS of 499 KDa had strongest inhibition, and its EC_{50} and EC_{90} were 0.318 and 1.204 mg mL^{-1}, respectively (Table I). CTS had strong antifungal activity (the inhibition percentage was bigger than 80%) at concentration of 2 mg mL^{-1} for all tested CTS samples, and at 1 mg mL^{-1} for 82, 144, 213, and 499 KDa CTS, respectively (Fig. 1).

There are several factors, both intrinsic and extrinsic, that affect the antimicrobial activity of CTS, and the concentration and M_w are more important factors among these. In this experiment, it demonstrated that CTS with different M_w could inhibit the radial growth of *F. fulva* with an optimal effect at concentrations ranging from 0.25 to 2 mg mL^{-1}, and the middle M_w CTS were more effective than others used in this study. It is in accordance with the study of Qin et al.,[37] although the CTS and pathogen used in the experiment are different. Although in other studies, CTS with M_w below 300 kDa, the antimicrobial effect on *Staphylococcus aureus* was strengthened as the M_w increased. In contrast, the effect on *E. coli* was weakened.[38] CTS samples with M_w from 55 to 155 KDa have antimicrobial activities at the concentration higher than 0.2 mg ml^{-1}. The antimicrobial activity of low M_w CTS is higher than that of the high M_w samples.[6] These diverging results may originate from the differences of CTS and pathogen,

TABLE I
Effect of Different Concentration and M_w CTS on EC_{50} and EC_{90} Value of Hyphal Radical Growth Inhibition (mean ± SD, $n = 9$, mg mL^{-1})

Chitosan (M_w: KDa)	EC_{50} value	EC_{90} value
3	0.777 ± 0.008	4.838 ± 0.003
38	0.718 ± 0.007	4.802 ± 0.009
82	0.442 ± 0.008	2.241 ± 0.007
144	0.445 ± 0.005	2.009 ± 0.006
213	0.396 ± 0.009	1.677 ± 0.008
499	0.318 ± 0.003	1.204 ± 0.004
1320	0.438 ± 0.006	5.260 ± 0.007
1540	0.704 ± 0.009	3.466 ± 0.007

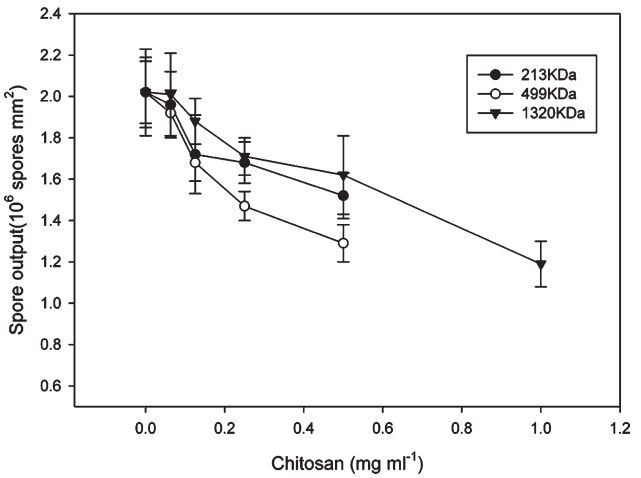

Figure 2 Effect of different M_w and concentration chitosan on the sporulation of *F. fulva*. (mean ± SD, $n = 3$).

because the sensibility is different for the different microorganisms (mold and/or bacteria) for CTS and it is related to the M_w of the CTS.

Efficiency of CTS on the quantity of sporulation

The mycelial plugs on the medium containing 0–2 mg mL^{-1} CTS were cultured in the dark at 25°C. After about 20 days, there was not sufficient radial growth for the mycelial plugs growing on mediums containing 1 and 2 mg mL^{-1} for 213 and 499 KDa CTS, respectively; and 2 mg mL^{-1} for 1320 KDa CTS, so no data were obtained for these treatments. Inhibitory effects of these tested CTS to sporulation were not notable compared with the control (Fig. 2). At the concentration of 0.5 mg mL^{-1}, the biggest inhibitory percentage was 24.8% and 34.9% for 213 and 499 KDa M_w CTS, respectively. At the concentration of 1 mg mL^{-1}, the biggest inhibitory percentage was 41.1% for the 1320 KDa CTS.

In this experiment, the method assaying inhibitory effect on sporulation is calculate the quantity of sporulation for every 5 mm diameter of mycelial plug growing on the medium containing different M_w and concentration CTS, the inhibitory effect on sporulation was not obvious by this way. However, the total of sporulation on whole colony growing on the medium containing different CTS is still very different, this can be explained by its antifungal activity as described earlier.

In vitro activity against conidia germination

In vitro the effect of CTS on spore germination was studied. The result showed that all CTSs with different M_ws could inhibit germination of *F. fulva* conidia (Table II). A low concentration of 499 KDa CTS

TABLE II
Effect of Different Concentration and M_w CTS on the Value of EC_{50} and EC_{90} of Conidia Germination Inhibition (mean ± SD, $n = 6$, mg mL^{-1})

Chitosan (M_w: KDa)	EC_{50} value	EC_{90} value
38	0.083 ± 0.003	0.211 ± 0.003
213	0.070 ± 0.002	0.160 ± 0.003
499	0.034 ± 0.001	0.146 ± 0.001
1320	0.088 ± 0.003	0.195 ± 0.002

EC_{50}, median effect concentration; EC_{90}, 90% effect concentration.

(0.034 mg mL^{-1}) caused 50% inhibition of germination, and almost complete inhibition was observed at 0.25 mg mL^{-1} of all tested CTSs (Fig. 3). The percentage of spore germination in CK_H was 99.1% after 24 h incubation. Table II and Figure 3 showed that the antifungal activity of 499 KDa CTS on *F. fulva* was better than other M_ws.

Figure 4 showed various shapes of conidia after 24 h incubation, when compared with the shape of normal spore [Fig. 4(A)] and germinated spores [Fig. 4(B)] which were not treated with CTS. Conidia which treated with CTS did not germinate and appeared distorted or plasmolyzability [Fig. 4(D)] observed under light microscope, and its appearance was covered with a layer of FITC-chitosan further observed with 400× CLSM [Fig. 4(C)]. Incubation for another 24 h, these results did not change. Inhibition of CTS to spores germination may be related with its solution osmotic potential and adhesion of CTS to spore which led to conidial distortion, affected spore viability.

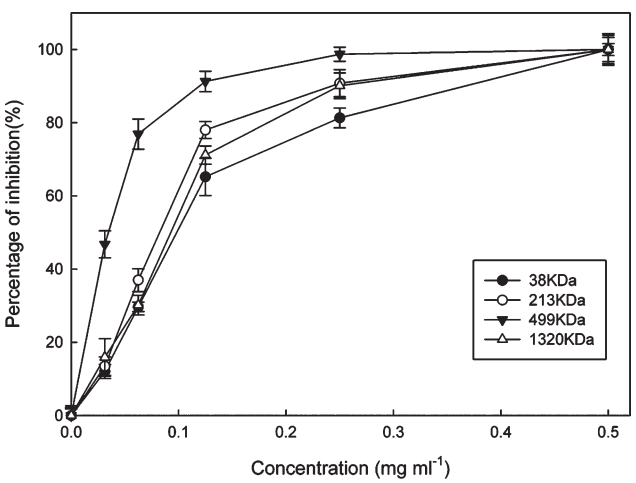

Figure 3 Effect of various M_w and concentration of chitosan on the inhibition percentage of conidia germination of *F. fulva*. (mean ± SD, $n = 6$).

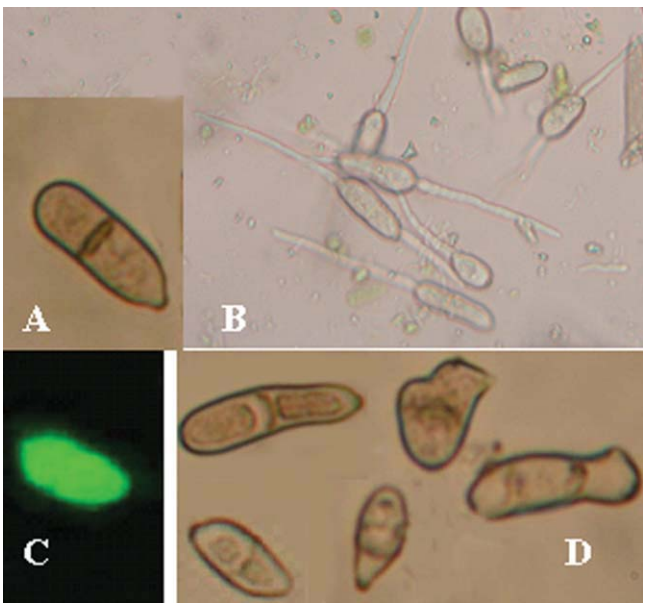

Figure 4 The shapes of normal spore (A) and normal germination spores (B) which were not treated with chitosan; the shapes of spore treated with chitosan (D); A, B, and D are the images observed under light microscope. The shape of spore treated with FITC-chitosan observed under a confocal laser scanning microscope (C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Effect of CTS on hyphal growth and morpha

Effect of CTS on hyphal growth was determined. After 20 days, mycelial disks cultured in PD, and the inhibitory effects of all CTSs on hyphal growth at 1–2 mg mL^{-1} concentration were obvious (Table III). The mycelial plugs formed fungal ball in PD containing 0 and 0.5 mg mL^{-1} CTS, but they came into being fragments in the PD supplemented with 1 and 2 mg mL^{-1} CTS. It was because CTS might destroy the whole mycelia which led to form fragments. The dry weight of *F. fulva* mycelia treated with 1320 KDa CTS was the most weight, and the 499 KDa CTS was the lightest among the three CTSs (Table III), this result is correlated with antifungal activity which 499 KDa CTS has strongest inhibitor to *F. fulva* among the tested CTS (Fig. 1), especially at the concentration of 1–2 mg mL^{-1}.

Hypha cultured in PD supplemented with 0.5 mg mL^{-1}, 82 and 1320 KDa CTS was isolated and examined by light microscope and SEM. The morpha of *F. fulva* hypha growing in the absence of CTS showed typical outline feature of the genus [Fig. 5(A,A′)], but the hypha cultured in PD supplemented CTS showed hypha swelling, distortion, excessive branching, and shorting of mycelial segments observed under light microscope [Fig. 5(B,C)]. The further observation by SEM showed that the swelling hypha which was cultured in PD containing 0.5 mg mL^{-1} 82 KDa CTS was smooth in appearance [Fig. 5(B′)], whereas rough in PD containing 0.5 mg mL^{-1} 1320 KDa CTS [Fig. 5(C′)].

SEM observation showed the possible action of CTS and its derivatives on different fungi.[30,31,39–42] CTS and its derivatives induced obvious structural alterations including cell wall loosening, vacuolation, and protoplasm degradation. Moreover, a histochemical assay on *Rhizopus stolonifer* with chitin specified wheat germ agglutinin/ovomucoid–gold complex has suggested that cell wall loosening was the result of upset balance between biosynthesis and turnover of chitin at the hyphal apex.[30] In this study, the results showed that the antifungal action mode of different M_w CTS may be different; although all CTS samples could induce the hypha swelling, the surface of mycelia treated with CTS of lower M_w was smooth, and it was rough with higher M_w CTS.

Effect of different M_w of FITC-labeled CTS on permeability through hypha

The action mode of different M_w CTS was investigated through the research of the permeability of FITC-labeled CTS by laser fault images using CLSM in different depth of mycelium. The stacking of 1320 KDa CTS to the hyphal surface of *F. fulva* was confirmed, and there was no fluorescence at the inner of hypha (Fig. 6). It demonstrated that the CTS of 1320 KDa could not permeate into the inner of hypha. The fluorescence of the FITC-labeled 499 KDa M_w CTS at the inside of the *F. fulva* cell was observed (Fig. 7), and the brightness of green fluorescence increased with the increase of depth [Fig. 7(A–G)], but weakened from the inner to the surface [Fig. 7(G–L)]. It demonstrated that the CTS of which M_w below 499 KDa could permeate into the inner of hypha.

It was further confirmed that the antifungal mechanism of different M_w CTS was different. The permeation of lower CTS into hypha was also observed (Fig. 7) by CLSM. The growth of *F. fulva* may be inhibited by CTS from the inside of cell, and the antifungal mechanism of CTS with low M_w may be caused mainly by the inhibition of the transcription from DNA.[43] Although the higher M_w CTS, acted

TABLE III
Dry Weight (g) of *F. fulva* Mycelia Cultured in LPD Containing Different CTS after 20 Days

Concentration of CTS (mg mL^{-1})	82 KDa CTS	499 KDa CTS	1320 KDa CTS
0 (CK_W)	1.608 ± 0.15	1.608 ± 0.15	1.608 ± 0.15
0 (CK_H)	1.504 ± 0.10	1.504 ± 0.10	1.504 ± 0.10
0.5	0.422 ± 0.03[a]	0.382 ± 0.03[a]	0.653 ± 0.05[a]
1	0.142 ± 0.01[a]	0.101 ± 0.01[a]	0.309 ± 0.03[a]
2	0.092 ± 0.02[a]	0.053 ± 0.02[a]	0.118 ± 0.28[a]

[a] Compared with CK_W $P < 0.01$.

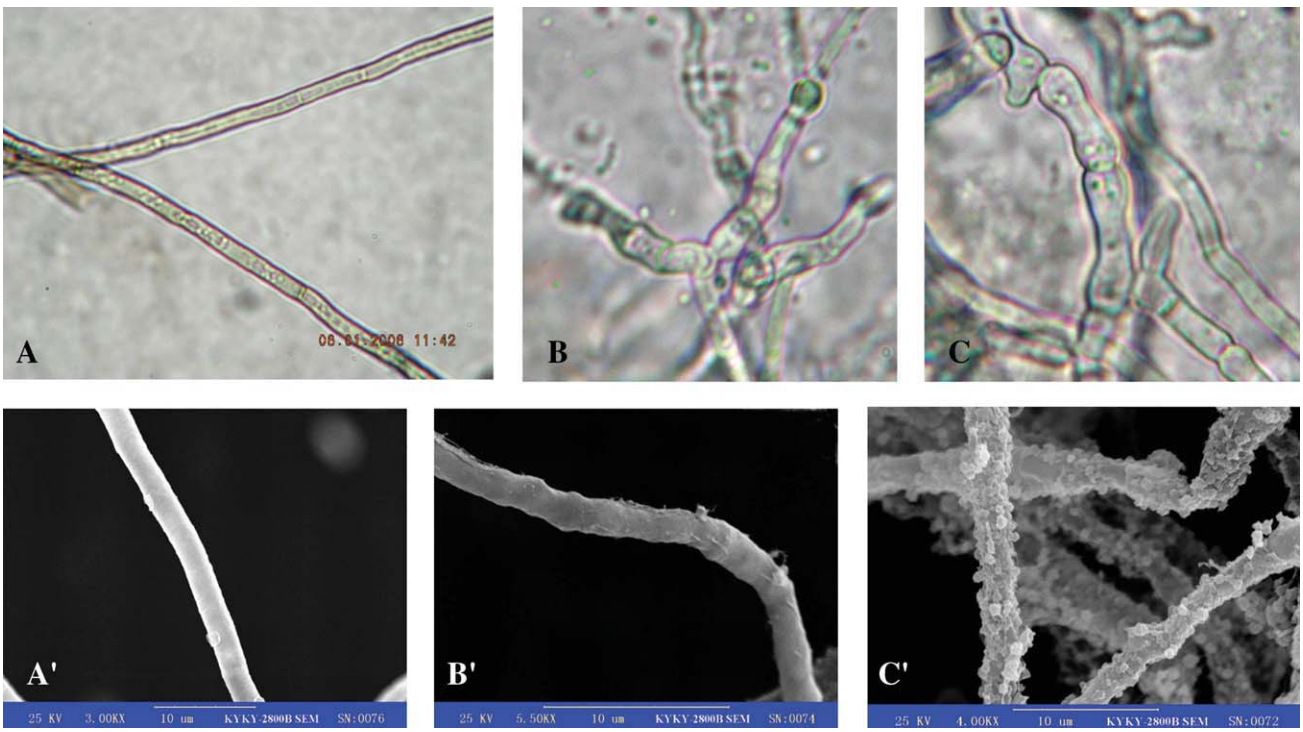

Figure 5 Effect of chitosan on the hyphal morpha under both the light microscope and SEM. (A) is the typical micrographs of the genus of *F. fulva* not treated with CTS, (B and C) is the micrographs of *F. fulva* treated with 82 and 1320 KDa chitosan, respectively, which were observed under the light microscope. (A′) is the typical micrographs of the genus of *F. fulva* not treated with CTS, (B′ and C′) is the micrographs of *F. fulva* treated with 82 and 1320 KDa chitosan, respectively, which were observed under the scanning electronic micrographs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary..com.]

mainly on the outer surface of the mycelia, formed an impervious layer around hypha [Fig. 5(C′)], and directly stunted the development of mycelia. On the other hand, the effective growth inhibition of hypha was assumed to be the prevention of nutrition delivery through cell wall.

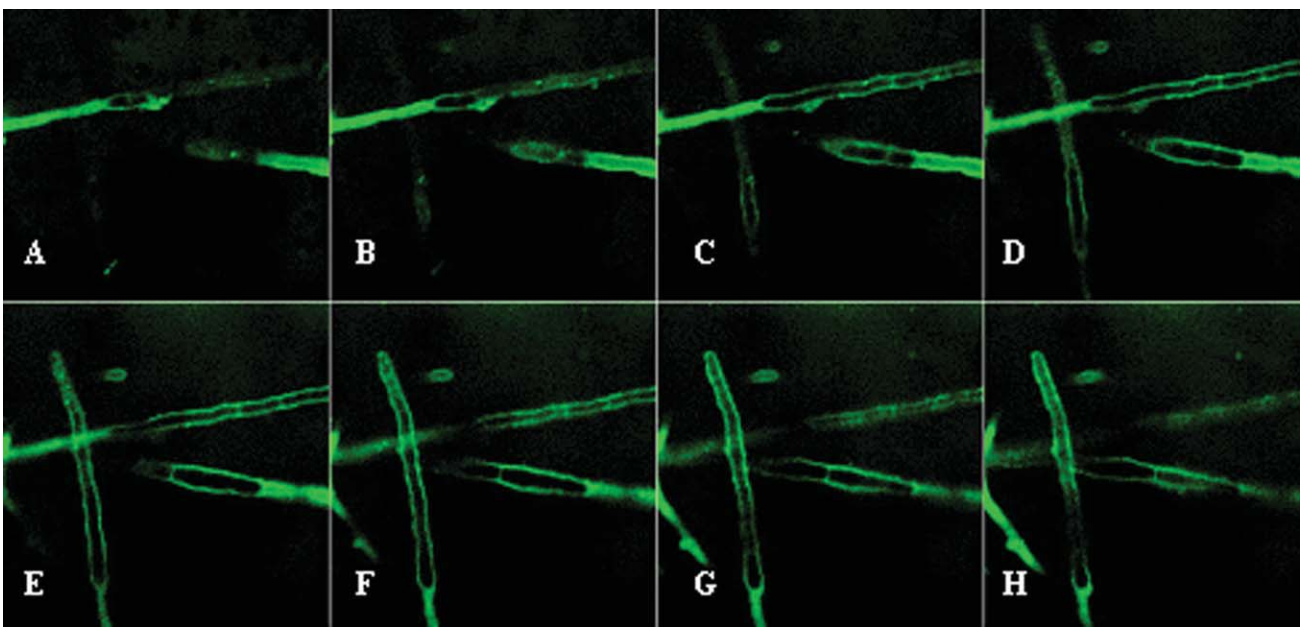

Figure 6 Confocal laser scanning micrographs (×1000) of hypha treated with 1320 KDa chitosan. (A–H) is the different layers of hypha, which is from one surface to another surface of the hypha. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

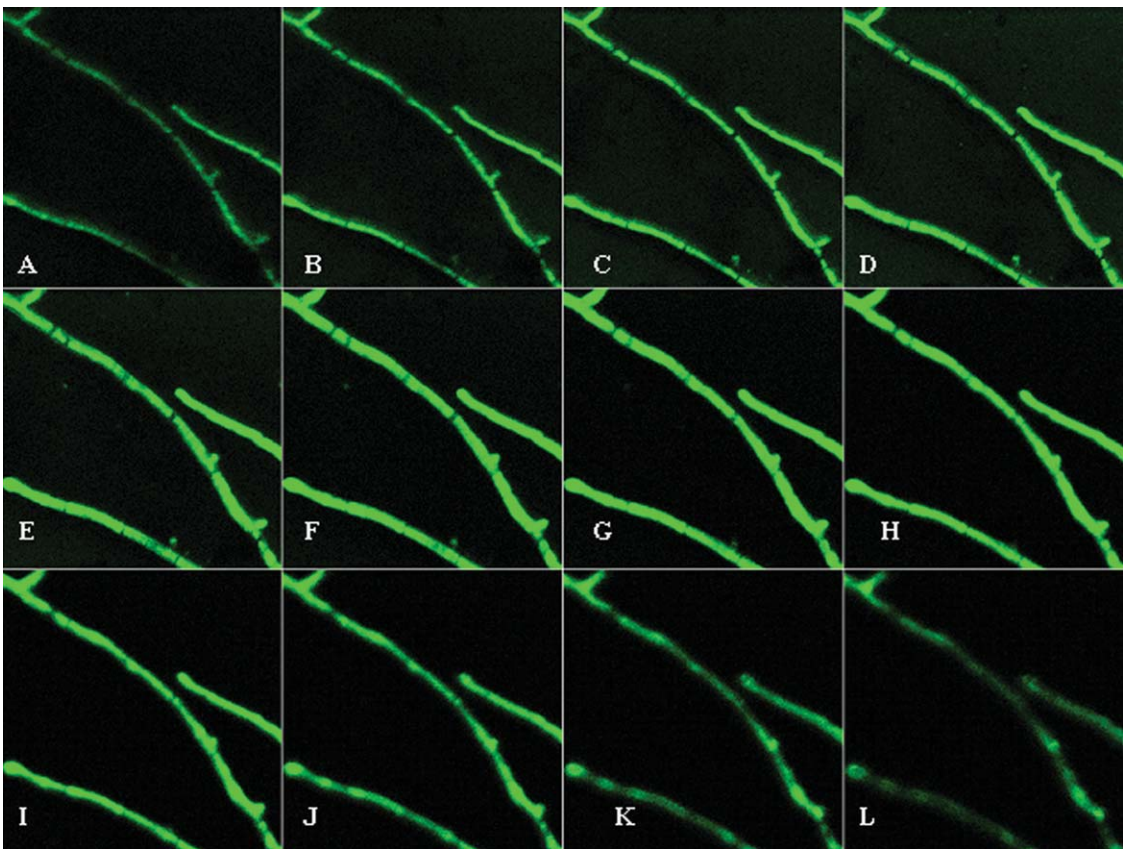

Figure 7 Confocal laser scanning micrographs (×1000) of hypha treated with 499 KDa chitosan. From (A–F) is from the surface to the inner, and (G–L) is from the inner to against surface of hypha. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Penetrability of FITC-labeled CTS oligomer with molecular weight of 9300 Da stacking to the cell wall of *Escherichia coli* was confirmed.[35] This is obviously different from that CTS with 499 KDa can be diffused into inner of mycelia. The main reason may be that M_w of CTS and target pathogen were different in this experiment.

In vivo the effect of chitosan on leaf mold in the field

The adverse effects on growth of tomato plants causing by CTS were not found in this experiment. Spraying CTS on tomato plants thrice before inoculation of conidia caused a decrease in the diseased leaf rates and the disease index, which were shown in Table IV. Thrice foliar application of the 213 KDa CTS at 1–10 mg mL^{-1} concentration and different M_w CTS at 5 mg mL^{-1} significantly reduced disease index as compared with the control, and the 213 KDa CTS at 6 mg mL^{-1} concentration reduced disease index from 29.2 in the control to 11.1; however, no very obvious difference in the disease severity among the different M_w CTS but at the same concentration of 5 mg mL^{-1} was observed, 213 KDa CTS was little better than others.

In this study, the reason that the effect of different M_w CTS on diseased index was not obvious may be the concentration of 6 mg mL^{-1} CTS was appropriate for control tomato leaf mold. It's not exactly accordance with the antifungal activity tested *in vitro*, this may be correlated with the method of

TABLE IV
The Rates of Diseased Leaf and Diseased Index of Tomato Cultivars after Leaves Sprayed with Chitosan

Chitosan (M_w: KDa)	Treatment (mg mL^{-1})	Investigation leaves	Diseased leaf rate (%)	Diseased index
CK	0	295	70.5 ± 3.4	29.2 ± 1.3
213	0.1	352	67.9 ± 3.6	23.2 ± 0.9
	1	363	41.7 ± 1.9[a]	17.1 ± 1.1[a]
	3	379	44.1 ± 1.9[a]	16.8 ± 1.1[a]
	6	413	33.0 ± 3.7[a]	11.1 ± 0.8[a]
	10	301	47.3 ± 1.9[a]	14.6 ± 1.2[a]
82	5	387	45.8 ± 2.3[a]	15.5 ± 1.4[a]
144	5	369	47.3 ± 2.0[a]	16.2 ± 0.9[a]
213	5	422	35.4 ± 5.2[a]	13.5 ± 2.4[a]
1320	5	365	49.2 ± 2.8[a]	18.5 ± 1.0[a]
1540	5	329	55.5 ± 1.7[a]	14.4 ± 1.2[a]

The data in the table are the average of three times investigation data.

[a] Compared with CK $P < 0.01$.

experiment that tomato plants were sprayed thrice CTS before inoculated with conidia of *F. fulva*. The reason that CTS could decrease the diseased index was not only the antifungal activity of CTS but also the inducing resistance caused by CTS.

CONCLUSIONS

In vitro assay showed that CTS exhibited strong antifungal activity against *F. fulva* at optimal concentration, and the middle M_w CTS had stronger antifungal activity than larger or smaller M_w CTS tested in this study, and the inhibition rate enhanced with the increase of CTS concentration. Among the three stages of life cycle of *F. fulva*, CTS showed the strongest inhibition on spores germination, and the weakest inhibitory effect on the sporulation. CTS could significantly reduced the occurrence of tomato leaf mold in the greenhouse, especially for the 213 KDa CTS, at 6 mg mL^{-1} concentration had better antifungal effect than that of other CTS tested in this experiment.

CTS could induce the mycelia distorted. The stacks of 1320 KDa CTS to the hyphal surface was found but not of 82 KDa. The further research using CLSM demonstrated that the CTS samples of which M_w were lower than 499 KDa could enter into the inner of mycelia, and 1320 KDa CTS was block off outer of the membrane of mycelia. The action mode of different M_w CTS is different. An investigation with CLSM is under carrying out in our laboratory to find the exact action site of different M_w CTS.

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