Antifungal activity of chitosan against soil-borne plant pathogens in cucumber and a molecular docking study

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In this study, we investigated the antifungal activity of four different chitosan against Sclerotinia sclerotiorum and Fusarium oxysporum f. sp. cucumerinum (FOC). In addition, molecular docking studies of the chitosan compound against plant pathogens have been evaluated. Concentrations of 0.2, 0.3, 0.5, 1 and 2 mg/mL of each chitosan were used. The activity rate increased as the amount of concentration increased. Strong antifungal effects were observed at concentrations of 1 and 2 mg/mL of chitosan used against test fungi. For the first time with this study, the activity of chitosan against F. oxysporum f. sp. cucumerinum and molecular docking studies against both test pathogens were determined.

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1. Introduction

Soil-borne Sclerotinia sclerotiorum Lib. De Bary and Fusarium oxysporum (Schlechtend.;Fr) f. sp. cucumerinum (Owen) Snyder & Hansen are two pathogenic groups that cause disease in cucumber cultivated areas throughout the world. White mould disease is caused by S. sclerotiorum [1]. This pathogen affects plants belonging to 64 families, 225 genus and 361 species with broad host circles [2]. Fusarium wilt in the cucumber is a pathogen that causes serious economic losses by F. oxysporum f. sp. cucumerinum in many parts of the world [3]. The pathogen is host specific to cucumber [4]. This is one of the main factors limiting the production of cucumber, since there is no plant species resistant to pathogens and effective control method. Currently, there has been no effective method of struggle against soil-borne plant pathogenic fungi. Pesticides used in control lose importance because of the difficulties encountered in struggling with soil-borne pathogens, limited and short-term effects in practice, and the emergence of pathogenic resistant forms. For these reasons, it is necessary to develop new alternative control methods.

Chitosan is the second most common polysaccharide in nature, obtained by deacetylation of chitin, a natural non-toxic carbohydrate polymer. Chitosan reduces [5] disease development in plants and strengthens the defense system against pathogens [6]. The use of chitosan to control plant pathogens has been ongoing for many years. Chitosan is an important polymer with antibacterial, antifungal, antiviral and antioxidant properties. Six chitosan with different molecular weights showed more antibacterial activity than gram-negative bacteria in gram-positive bacteria [7]. In a different study, they demonstrated that chitosan showed antifungal activity against Alternaria solani at 1 mg/mL concentration and reduced the disease severity on tomato leaves [8]. When chitosan is sprayed onto the leaves of various plant species, it causes local and systemic resistance to infections caused by Alfalfa mosaic virus and Tobacco mosaic virus [9]. Antioxidant activities of chitosan have been examined [10,11]. The activity of chitosan against fungus [12], viruses, viroids [13] and bacteria [14] varies according to the molecular weight. Chitosan is often used as an elicitor to control plant diseases. The activity depends on properties such as concentration, molecular weight, acetylation grade, solvent, pH and viscosity [15,16]. Chitosan acts as a barrier for the penetration of fungi and reduces disease development [17].

In this paper, antifungal activities of four different chitosan polymers were determined against two pathogens of cucumber S. sclerotiorum and F. oxysporum f. sp. cucumerinum under in vitro and in vivo conditions, and molecular docking studies were carried out.
2. Experimental

2.1. Fungi culture

Fungus cultures, white mould (S. sclerotiorum) and fusarium wilt (F. oxysporum f.sp. cucumerinum), were used for antifungal activity studies. Fungus cultures were inoculated in cucumber cultivation areas in Antalya (in Turkey). These are located in Gaziosmanpaşa University, Faculty of Agriculture, as stock culture in phytopathology laboratories. The plant pathogens were grown on potato dextrose agar (PDA) medium. The fungus cultures were then incubated for 7 days at 22 ± 2°C for use in activity assays.

2.2. Chitosan production

Shrimp waste sourced from Marmara Sea (Turkey) was used for chitin and chitosan production. The shrimp waste is washed with tap water, dehydrated at room temperature and shred into pieces smaller than 2 mm using a blender (Fakir Harmony 400, People’s Republic of China). Biotransformation of chitin to chitosan by enzymatic deacetylation can be achieved by chitin deacetylases (EC).3.5.1.41, ChDa. Most importantly, these enzymatic reactions have many advantages over conventional chemical processing, producing chitosan with higher molecular weight and desired degree of deacetylation [18]. The activity of chitosan depends on the properties of pH. Optimum extraction conditions were carried out by using the response surface methodology (RSM). The chitin was experimented using 0.73-mol/L hydrochloric acid for 132.61 min at room temperature for demineralization and 0.95-mol/L sodium hydroxide for 75.65 min at 60.49°C for deproteinization. The obtained chitin was used to optimize chitosan production and two chitosans were defined as chitosan-1 (C1) and chitosan-2 (C2). C1 was obtained by deacetylation with 40% sodium hydroxide at 120°C for 300 min and C2 was carried out by deacetylation with 50% sodium hydroxide at 100°C for 720 min. Demirdöven and Tokatli [18] provided detailed information about optimization. C1 has deacetylation degree of 78.2%, molecular weight of 182 kDa, viscosity of 120 cPs and C2 has deacetylation degree of 84.95%, molecular weight of 127 kDa, viscosity of 40 cPs [19]. Chitosan-3 (C3) [Low molecular weight (50–190 kDa)]; Sigma Aldrich-448869] and chitosan-4 (C4) [Medium molecular weight (190-310 kDa)]; Sigma Aldrich-448877] were obtained from Sigma-Aldrich (* Based on viscosity). Figure 1.

2.3. In vitro antifungal activity

Antifungal activities of chitosans (C1, C2, C3 and C4) were determined using the agar plate method [20]. To determine the final concentration of each chitosan, 10 mL (prepared in 1% (v/v) acetic acid) solutions containing 200, 300, 500, 1000 and 2000 mg of chitosan were added to 90 mL PDA (cooled to 40°C). PDA with final concentrations of 0.2, 0.3, 0.5, 1 and 2 mg/mL for each chitosan were poured (∼ 10 mL/plate) in petri plates (60 mm in diameter). Seven-day-old agar discs (5 mm in diameter) bearing the desired fungus mycelium growth were transferred to the Petri plates. These fungus cultures were incubated at 22 ± 2°C for 10 days. Fungus mycelial growth was recorded daily [21]. Commercial fungicide Thiram 80% (w/v) was used as a positive control. Acetic acid 1% (v/v) was used as a negative control. Each chitosan was adjusted for pH = 6.0 by NaOH. The experiment was set up with 4 replication and repeated 2 times [22].

The percentage of mycelial growth inhibition (MGI) was calculated accordingly to the formula mentioned by [23].

\[
MGI = 100 \times \frac{dc - dt}{dc}
\]

where dc is the mycelial growth in control, dt is the mycelial growth in treatment.

2.4. In vivo antifungal activity

During the in vitro experiment, 2 mg/mL chitosan concentration showing the highest impact against test fungi was used in in vivo assays. Sclerotinia sclerotiorum and F. oxysporum f.sp. cucumerinum were grown on PDA medium for 7 days at 22 ± 2°C. Cucumber fruits are surface sterilized by dipping in 2% NaOCl for 5 min and washed by sterile water. Then, they were left to dry on blotting paper in laminar flow cabinet. Afterward pathogen inoculations were performed, respectively. Cucumber fruits were inoculated with S. sclerotiorum and F. oxysporum f.sp. cucumerinum. According to Ji et al. [24], the application method was modified and implemented. In this method, each fruit was opened with 8 mm wound twice by cork borer. The 100µL−1 concentration (2 mg/mL) of chitosans was applied to wounds. Then mycelial discs (8 mm diameter) were inserted into the opened wounds. All inoculated plant materials were incubated in a test chamber (Sanyo Group) under 16:8 hours (light/dark) photoperiod at
22 ± 2°C and 75–90% humidity. The assay was conducted with 4 replications and repeated twice. The recommended concentration (2 mg/mL) of fungicide (80% Thiram; w/v) was adjusted to be 100 μL\(^{-1}\) in the assays as a positive control. A volume of 1% of acetic acid (v/v) was added in 100μL\(^{-1}\) in the assays as a negative control. Test fungi were examined on fruit surface after 15 days. The observed pathogen symptoms which appeared on the fruit surface were measured and recorded. Pathogen inoculation was performed 12 h after the application of chitosan.

### 2.5. Molecular docking studies

Trial docking studies with these enzymes suggested that the crystal structures 2X2T of *Sclerotinia sclerotiorum* and 3U7B of *Fusarium oxysporum* as most appropriate target of the chitosan compound. Molecular docking studies were performed by using Autodock Vina [25] and Discover studio Visualizer 4.5 [26] programs. Before the docking process, the stable structure of the chitosan molecule was obtained using the quantum chemical calculation 6-311G++(d,p), with the base set [27] DFT (B3LYP) [28] theory and the Gaussian 09 program [29]. The Gaussian 5 program [30] was also used to graphically examine the Gaussian outputs.

### 2.6. Statistical analysis

All statistical data were performed by SPSS 16.0 software (SPSS, Chicago, IL). Means were compared by using Duncan’s multiple range test and differences were considered significant when \(P < 0.05\). Lethal doses (LD) estimates for LD\(_{10}\), LD\(_{50}\), and LD\(_{90}\) were determined with the software Polo Plus (LeOra software).

### 3. Results

#### 3.1. In vitro antifungal activity

The antifungal effects of each chitosan at different concentrations are shown in Figure 2 and Table 1.

![Figure 2](image)

**Figure 2.** Antifungal activity of chitosans against test fungi on PDA medium mycelial growth. Photographs were taken 10 days after inoculation.

| Test fungi                  | Concentration (mg/mL) | C1 | C2 | C3 | C4 |
|-----------------------------|-----------------------|----|----|----|----|
| *S. sclerotiorum*           |                       |    |    |    |    |
| C-                          |                       |    |    |    |    |
| 0.2                         | 15                    | 23 | 18 | 16 |
| 0.3                         | 20                    | 32 | 37 | 49 |
| 0.5                         | 28                    | 48 | 47 | 83 |
| 1                           | 70                    | 78 | 72 | 96 |
| 2                           | 100                   | 93 | 96 | 100|
| C+                          | 100                   | 100| 100| 100|
| *F. oxysporum f. sp. cucumerinium* |                     |    |    |    |    |
| C-                          |                       |    |    |    |    |
| 0.2                         | 27                    | 28 | 26 | 37 |
| 0.3                         | 31                    | 44 | 50 | 54 |
| 0.5                         | 37                    | 51 | 60 | 67 |
| 1                           | 75                    | 83 | 77 | 79 |
| 2                           | 98                    | 100| 90 | 96 |
| C+                          | 100                   | 100| 100| 100|

(-), No activity.

When all chitosan treatments were compared with negative control, they significantly reduced the development of *S. sclerotiorum* and *F. oxysporum f. sp. cucumerinium* (\(P < 0.05\)) (Figure 2). For every chitosan polymer, there is a noticeable antifungal activity in all concentrations, although this varies according to the test fungi. As the amount of concentration increased, the activity increased. The most effective concentrations in both pathogenic groups were 1 and 2 mg/mL. For each chitosan, 70–96% (for 1 mg/mL) and 93–100% (for 2 mg/mL) were determined in *S. sclerotiorum*, respectively. For FOM, these rates were 75–83% (for 1 mg/mL) and 90–100% (for 2 mg/mL) (Table 1). Within both pathogens, 100% mycelial growth inhibition occurred in the positive control. Similarly, 100% inhibition was observed against *S. sclerotiorum* at 2 mg/mL concentration of C1 and C4, and against *Fusarium oxysporum f. sp. melonis* (FOM) 100% inhibition of C2 was observed at 2 mg/mL concentration (Table 1). As shown in Figure 2, when compared to the negative control as a result of the effects of the chitosan applied against the test fungi, aerial mycelium growth, morphological deterioration in the mycelial structure was observed. For *S.*
**Table 2. Lethal doses of in vitro test fungi against chitosan (mg/mL).**

| Test fungi | C1 (mg/mL) | C2 (mg/mL) | C3 (mg/mL) | C4 (mg/mL) |
|------------|------------|------------|------------|------------|
| Ss LD10    | 0.23       | 0.18       | 0.13       | 0.10       | 0.14       | 0.11       | 0.16       | 0.15       | 0.13       | 0.17       |
| LD50       | 0.61       | 0.53       | 0.70       | 0.47       | 0.43       | 0.52       | 0.49       | 0.44       | 0.53       | 0.32       | 0.30       | 0.34       |
| LD90       | 1.63       | 1.30       | 2.21       | 1.78       | 1.49       | 2.18       | 1.74       | 1.47       | 2.13       | 0.65       | 0.59       | 0.73       |
| LDP9       | 13.1       | 7.65       | 28.8       | 29.0       | 18.2       | 52.8       | 25.9       | 16.5       | 45.9       | 2.98       | 2.31       | 4.14       |
| Slope      | 2.92 ± 0.16| 2.24 ± 0.15| 2.32 ± 0.15| 4.12 ± 0.27|
| Het.       | 0.98       | 0.31       | 0.95       | 0.89       |
| X2         | 12.74      | 4.04       | 12.31      | 11.59      |
| Foc LD10   | 0.14       | 0.10       | 0.18       | 0.12       | 0.09       | 0.15       | 0.07       | 0.05       | 0.10       | 0.06       | 0.04       | 0.08       |
| LD50       | 0.49       | 0.42       | 0.57       | 0.38       | 0.34       | 0.43       | 0.38       | 0.33       | 0.43       | 0.29       | 0.25       | 0.34       |
| LD90       | 1.74       | 1.35       | 2.50       | 1.24       | 1.03       | 1.60       | 1.93       | 1.55       | 2.59       | 1.50       | 1.22       | 1.99       |
| LDP9       | 25.3       | 12.8       | 70.9       | 15.1       | 8.85       | 31.9       | 62.2       | 31.5       | 159.4      | 48.6       | 24.4       | 128.1      |
| Slope      | 2.34 ± 0.16| 2.51 ± 0.18| 1.80 ± 0.15| 1.80 ± 0.16|
| Het.       | 1.93       | 1.28       | 0.42       | 0.35       |
| X2         | 25.12      | 16.70      | 5.51       | 4.54       |

**Ss,** Sclerotinia sclerotiorum; **Foc,** Fusarium oxysporum f.sp. cucumerinum; **LD,** Effective dose (Lethal Dose); **Low,** Lower bound (95% limits); **Upr.** Upper bound (95% limits); **Het.** Heterogeneity; X2, Chi-square.

sclerotiorum the formation of sclerot was inhibited at all concentrations.

Depending on the increase in the concentration of C4, the activity was faster than the other chitosan. 16% at 0.2 mg/mL and 83% at 0.5 mg/mL in S. sclerotiorum. These rapid effects continue in the form of C2, C3 and C1, respectively. According to these results, antifungal activity is different according to chitosan inoculation, molecular weight and pathogen type.

The lethal doses of each chitosan against the test fungi are given in Table 2. In S. sclerotiorum, it was found that the most effective C2 (0.13 mg/mL) for LD10, C4 (0.32 mg/mL) for LD50 and C4 (0.65 mg/mL) for LD90. Likewise, the most effective lethal doses were found in FOC, C4 (0.06 mg/mL) for LD10, C2 and 3 (0.38 mg/mL) for LD50 and C2 (1.24 mg/mL) for LD90 (Table 2).

The amount of standardized residuals of each chitosan is given in Figure 3. The test fungi showed varying amounts of residue depending on the concentration of chitosan, the reaction of the pathogen to each concentration, and the concentration increase.

### 3.2. In vivo antifungal activity

In this section, the effectiveness of plant pathogens was evaluated on cucumber fruits against chitosan. In the study, 2 mg/mL concentration was used which showed the highest activity in in vitro experiments. In the absence of chitosan, 48.53 mm (for S. sclerotiorum) and 37.50 mm (for FOC) lesions were observed 15 days after inoculation of the pathogen on the fruit. After the application of chitosan, lesion development was completely (100%) inhibited in all other chitosan types against S. sclerotiorum (except C2 (59%)) and FOC. However, it was determined that the lesion length differs according to the chitosan type. According to these results, in vivo experiments also support in vitro results (Figure 4, Table 3).

### 3.3. Molecular docking studies

The crystal structure of Sclerotinia sclerotiorum (pdb code: 2X2T) and Fusarium oxysporum (pdb code: 3U7B) were obtained for docking studies from the Protein Data Bank (http://www.rcsb.org/pdb).

Before the docking process, hydrogen atoms and Gasteiger charges were added after the water molecules were removed from all protein structures. After these processes, as a result of the docking process the states of chitosan ligand embedded in the binding sites of both receptors are shown in Figure 5(a,b) and the three and two dimensional interactions between the ligand molecule and the target protein structures are shown in Figures 6(a,b). The estimated binding energy and RMSD

![Figure 3. Standardized residual values: Sclerotiorum sclerotiorum (a) and F. oxysporum f.sp. cucumerinum (b).](image-url)
Figure 4. Antifungal activities of chitosans against *S. sclerotiorum* (a) and *F. oxysporum* f.sp. *cucumerinum* (b) on cucumber fruit under in vivo conditions. Photographs were taken 15 days after inoculation.

Table 3. Antifungal activity values (MG and MGI) of chitosans under in vivo conditions.

| Test fungi | Doses | C1 | C2 | C3 | C4 |
|------------|-------|----|----|----|----|
|            |       | MG (mm) | MGI (%) | MG (mm) | MGI (%) | MG (mm) | MGI (%) | MG (mm) | MGI (%) |
| *S. sclerotiorum* |       | 48.53 | - | 48.53 | - | 48.53 | - | 48.53 | - |
| 2 mg/mL | C- | 0.00 | 100 | 19.98 | 59.0 | 0.00 | 100 | 0.00 | 100 |
| C+ | 0.00 | 100 | 0.00 | 100 | 0.00 | 100 | 0.00 | 100 |
| *F. oxysporum* f.sp. *cucumerinum* | 37.50 | 40.00 | 0.00 | 100 | 0.00 | 100 | 0.00 | 100 |
| 2 mg/mL | C- | 0.00 | 100 | 0.00 | 100 | 0.00 | 100 | 0.00 | 100 |
| C+ | 0.00 | 100 | 0.00 | 100 | 0.00 | 100 | 0.00 | 100 |

*Ss, Sclerotinia sclerotiorum; Fol, Fusarium oxysporum f.sp. cucumerinum; MG, mycelial growth (mm); MGI, mycelial growth inhibition (%); (–), no inhibition.

Values obtained from the molecular docking results and the numerical results are shown comparatively in Table 4.

It has been indicated that the protein structure and ligand are selected correctly when the obtained docking results of RMSD value are smaller than 2 Å [31]. The criterion to be considered after RMSD values is binding energy. The energy of the binding of the ligand to the active site of the protein is checked. The binding energy of the ligand to the active site of the protein is checked.

Chitosan ligand was found to bind with weak covalent interactions with the substrate-binding sites of receptors, more specifically with hydrogen bond interactions. As shown in Figure 6(a), hydrogen bonds were formed between VAL124 and ASN145 with the O atoms of the ligand at 5.49 and 5.00 Å respectively, while hydrogen bonds at the lengths of 3.80 and 2.85-4.03 Å between the N atoms of the ligand and LYS141 and SER139, respectively. These interactions between the ligand molecule and the receptor are shown in detail in Table 5.

Figure 5. Pictorial representation of the ligand chitosan embedded in the active site of (a) 2X2 T and (b) 3U7B receptors.
Figure 6. Representation of the result of docking studies of the chitosan molecule with (a) 2X2T and (b) 3U7B protein structures. H-bond, Van der Waals and other interactions are shown in green, light green and red lines, respectively.

Table 4. Affinity and RMSD values of the different conformations that the chitosan molecule has made with protein structures.

| Sclerotinia sclerotiorum | Fusarium oxysporum |
|-------------------------|--------------------|
| Mode | Binding Affinity (kcal/mol) | RMSD | Mode | Binding Affinity (kcal/mol) | RMSD |
| 1   | -5.0 | 0.0 | 1   | -5.8 | 0.0 |
| 2   | -4.5 | 23.638 | 2 | -5.8 | 38.062 |
| 3   | -4.4 | 2.543 | 3 | -5.8 | 52.509 |
| 4   | -4.3 | 2.429 | 4 | -5.8 | 53.252 |
| 5   | -4.3 | 23.03 | 5 | -5.6 | 38.71 |
| 6   | -4.3 | 1.827 | 6 | -5.6 | 1.791 |
| 7   | -4.3 | 1.206 | 7 | -5.5 | 33.928 |
| 8   | -4.2 | 23.622 | 8 | -5.5 | 53.077 |
| 9   | -4.1 | 23.96 | 9 | -5.5 | 37.649 |

In the same manner, as shown in Figure 6(b), hydrogen bonds were formed between HIS82, GLU245, TRP285 and GLU45 with the O atoms of the ligands at 5.47, 4.92, 5.21 and 4.84 Å, respectively, while hydrogen bonds at the lengths of 5.63–5.75 and 5.56 Å between the N atoms of the ligand with GLU131 and GLU245, respectively. These interactions between the ligand molecule and the receptor are shown in detail in Table 6.

It can be said that according to the calculated binding affinity of ligand, it may be determined as the molecule which may be the potential inhibitor compound for the target protein structures.
Table 5. Interactions between chitosan ligand and 2X2T receptor.

| Bond name     | Bond length (Å) | Bond category | Bond type       | Donor atom | Acceptor atom | Bond angle (DHA) (°) |
|---------------|-----------------|---------------|-----------------|------------|---------------|---------------------|
| LYS141:H23-ligand:N | 3.01649         | Hydrogen Bond | Conventional Hydrogen Bond | LYS141:H23 | ligand:N       | 126.964             |
| ASN145:HD21-ligand:O | 2.10533         | Hydrogen Bond | Conventional Hydrogen Bond | ASN145:HD21 | ligand:O       | 173.637             |
| ligand:N SER139:O | 2.4517          | Hydrogen Bond | Conventional Hydrogen Bond | ligand:N   | SER139:O       | 98.514              |
| ligand:N SER139:O | 2.21947         | Hydrogen Bond | Conventional Hydrogen Bond | ligand:N   | SER139:O       | 113.586             |
| ligand:N SER139:O | 2.6316          | Hydrogen Bond | Conventional Hydrogen Bond | ligand:N   | SER139:O       | 114.349             |
| ligand:O VAL124:O          | 2.58722         | Hydrogen Bond | Conventional Hydrogen Bond | ligand:O   | VAL124:O       | 120.03              |

Table 6. Interactions between chitosan ligand and 3U7B receptor.

| Bond name     | Bond length (Å) | Bond category | Bond type       | Donor atom | Acceptor atom | Bond angle (DHA) (°) |
|---------------|-----------------|---------------|-----------------|------------|---------------|---------------------|
| HIS82:HE2-ligand:O | 1.97459         | Hydrogen Bond | Conventional Hydrogen Bond | ASP126:HN  | ligand:O       | 163.843             |
| TRP285:HE1-ligand:O | 1.79721         | Hydrogen Bond | Conventional Hydrogen Bond | TRP285:HE1 | ligand:O       | 167.297             |
| ligand:O GLU245:OE2 | 2.7678          | Hydrogen Bond | Conventional Hydrogen Bond | ligand:O   | GLU245:OE1     | 103.673             |
| ligand:O GLU245:OE2 | 2.82597         | Hydrogen Bond | Conventional Hydrogen Bond | ligand:O   | GLU131:OE2     | 110.113             |
| ligand:O GLU245:OE2 | 2.90323         | Hydrogen Bond | Conventional Hydrogen Bond | ligand:O   | GLU245:OE1     | 128.711             |
| ligand:O GLU245:OE2 | 2.38815         | Hydrogen Bond | Conventional Hydrogen Bond | ligand:O   | GLU45:OE2      | 129.197             |

4. Discussion

The use of natural compounds against plant pathogens will significantly reduce the use of fungicides. In this way, the negative effects of fungicides on the environment, human and natural balance will also be reduced.

In the present study, we showed that the use of chitosan on PDA medium and cucumber fruit in four different types and molecular weights inhibited white mould diseases caused by S. sclerotiorum, and Fusarium wilt which is caused by Fusarium oxysporum f. sp. lycopersici (FOL). Among the natural compounds, chitosan offers a biodegradable potential with antifungal [8], antibacterial [7], antiviral [13] and antioxidant [11] activities. In our study, strong activity results were obtained in vitro and in vivo conditions against chitosan plant pathogenic fungi. These results are similar to the previous studies showing that chitosan can control various plant diseases. Some of these studies in vitro conditions have reported by Cheah and Page [32] that chitosan significantly reduced the growth of S. sclerotiorum at concentrations of 1%, 2% and 4% in vitro conditions. Microscope studies revealed that fungal mycelium exposed to chitosan appeared to be deformed and dead, whereas untreated mycelium was normal in appearance.

It was reported that enzymatically hydrolysed chitosan did not affect the radial growth of S. sclerotiorum colonies on potato dextrose agar plates [33]. S. sclerotiorum isolated from canola plant was not effective in mycelial growth of oligochitosan used in 50 µg/L dose in vitro conditions [34].

In a different study, chitosan strongly inhibited mycelial growth against S. sclerotiorum. Using fluorescent microscopy, the plasma membrane of chitosan-treated S. sclerotiorum mycelia was observed to be markedly damaged [35]. According to the previous studies, there has been no study on the activity of chitosan against Fusarium oxysporum f. sp. cucumerinum. There are activity studies of chitosan with the disease-causing species in different hosts in the same genus.

In studies of the effects of low molecular weight and high molecular weight chitosan against certain pathogens including Fusarium oxysporum f. sp. radicis-lycopersici (FORL) and Fusarium oxysporum f. sp. melonis (FOM), inhibition of mycelial growth of FORL by 69% to 51% and FOM by 86% to 56%, respectively [36]. Benhamou [37] reported that chitosan against FORL inhibited radial growth at 3–6 mg/mL concentration. They also observed swelling and distortion in fungus hyphal in light microscope. It was reported that chitosan inhibited both mycelial growth and sporulation in vitro (1.5% in concentration) against Fusarium oxysporum. They also reported that F. oxysporum was the most sensitive from Test fungi [38]. In a study of the effect of chitosan on cucumber plants, El-Ghaouth et al. [39] reported that chitosan does not show any phytotoxic effect on cucumber plants. The coating of chitosan solution (2% and 4%) of carrot roots in vivo conditions significantly (from 88% to 28%) inhibited the effect of S. sclerotiorum in the roots. At the same time, they noted that the growth of the distal lesion was 26–12 mm [31]. In a similar study, the application of enzymatically hydrolysed chitosan 3 days before inoculation of the carrot plant reduced the height and severity of decay compared to control [32]. In studies investigating the effect of oligochitosan against S. sclerotiorum in canola plants, 25.8%, 41.4%, 57.1%, 68.7%, and 48.8% of the inhibition were obtained at 0, 24, 48, 72, and 96 h before pathogen inoculation, respectively [33]. It was reported that, when chitosan was compared to control against S. sclerotiorum rot on a carrot plant, it provided effective control by inducing enzymes related to the defense system [34]. Chitosan applied as root dipping to Tolerance Celery reduced the development of F. oxysporum FOL.
in the soil population [40]. In another study, the application of pea oligochitosan inhibited the macroconidi germination of *Fusarium solani* to 8 μg/mL concentration [41]. Whether chitosan was applied by leaf spraying and root coating method, chitosan was found to importantly reduce the amount of root lesions caused by the fungi and to severely increase the formation of physical barriers in infected root tissues. The activity of chitosan on the induction of host cell reactions was observed at concentrations between from 0.5 to 2 mg/mL [42]. Lafontaine and Benhamou [17] reported that 12.5 and 37.5 mg/mL in the concentration of chitosan applied to FORL in vivo in tomato significantly reduced root rot symptoms and yield loss.

Chitosan did not adversely affect plant growth and fruit yield in the absence of FORL. According to our information, the activity of chitosan against the FOC has been determined for the first time. In addition, the effect of chitosan against *S. sclerotium* and *F. oxysporum* was demonstrated for the first time by molecular docking studies. According to the data in Table 6, it was observed that the citosan molecule (C2 for 2 mg dose) was more effective on *F. oxysporum* than *S. sclerotium* in the in-vivo study. The same situation was observed in the molecular docking study, and the Binding Affinity value was higher in *F. oxysporum* (−5.0 kcal/mol) compared to *S. sclerotium* (−5.8 kcal/mol). According to this result, it can be said that in-vitro and molecular docking studies are compatible with each other.

5. Conclusion

The results obtained in this study clearly demonstrate that four types of chitosan significantly reduce the development on PDA medium and cucumber fruit of plant pathogens. This is supported by molecular docking studies.

**Abbreviations**

- **FOC** *Fusarium oxysporum* f.sp. *cucumerinum*
- **PDA** potato dextrose agar
- **RSM** response surface methodology
- **C1** Chitosan-1
- **C2** Chitosan-2
- **C3** Chitosan-3
- **C4** Chitosan-4
- **MGI** mycelial growth inhibition
- **LD** lethal dose
- **MG** Mycelial growth (mm)
- **Low** Lower bound (95% limits)
- **Upr** Upper bound (95% limits)
- **Het** Heterogeneity
- **X2** Chi-square
- **FOM** *Fusarium oxysporum* f.sp. *melonis*
- **FOL** *Fusarium oxysporum* f. sp. *lycopersici*
- **FORL** *Fusarium oxysporum* f.sp. *radicis-lycopersici.*

**Authors’ contributions**

AO and YB contributed to antifungal studies, evaluation and analysis of data and preparation of article draft. KT and MB contributed to the production of chitosan, activity studies of chitosan and critical writing of the article. TK carried out molecular docking studies. YY supervised the laboratory work and contributed to the critical writing of the article. All the authors revised the final manuscript and approved the submission.

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