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Antifungal efficacy of chitosan and its thiourea derivatives upon the growth of some sugar-beet pathogens

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Abstract

Chitosan (CS) was modified by reaction with benzoyl thiocyanate to give a thiourea derivative (TUCS). The antifungal behavior of chitosan and its thiourea derivative was investigated in vitro on the mycelial growth, sporulation and germination of conidia or sclerotia of the following sugar-beet: *Beta vulgaris* pathogens isolated in Egypt, *Rhizoctonia solani* Kühn ($AG_{2\text{-}2}$) *Sclerotium rolfsii* Sacc. and *Fusarium solani* (Mart.) Sacc. All the prepared thiourea derivatives had a significant inhibiting effect on the different stages of development on the germination of conidia or sclerotia of all the investigated fungi in the polymer concentration range of 5–1000 μg ml^{-1}. In the absence of chitosan and its derivative, *R. solani* exhibited the fastest growth of the fungi studied. However, growth tolerance of the modified chitosan was highest for *F. solani* and lowest for *R. solani*. The most sensitive to the modified chitosan stress with regard to their germination and number produced were the sclerotia of *S. rolfsii*. It has been found that the TUCS is a much better fungicidal agent (about 60 times more) than the pure CS against most of the fungal strains tested. The molecular weight and the degree of deacetylation were found to have an important effect on the growth activities of the pathogens.

Keywords: Chitosan; Modified chitosan; Growth activity; Sugar-beet; *Rhizoctonia solani*; *Sclerotium rolfsii*; *Fusarium solani*

1. Introduction

Chitin is one of the most abundant natural polymers after cellulose and is obtained from the exoskeleton of crustacean such as shrimps, crabs, squids and from some fungi cell walls. Chitosan (CS) is derived from chitin by strong alkaline hydrolysis it is actually a copolymer of 2-acetamido-2-deoxy-glucopyranose (D-Acetyl-glucosamine) units linked by β-(1–4) linkage and 2-amino-2-deoxy-glucopyranose (D-glucosamine) units. Chitosan is soluble in water at pH >6 and being polycationic, non-toxic biodegradable finds numerous applications especially in the agriculture, food and pharmaceutical industries, such as food preservation [1], fruit juice clarification [3,8] water treatment especially removal of heavy metals ions [9–11]; sorption of dyes and flocculating agent. Chitosan and its derivatives can be also used as biological adhesive for its hydrogel-forming ability [12] wound healing accelerator [13] and also in cosmetic industries. Chitosan also has immunological and anti-tumoral properties. Hirano and Nagao [14] and Sekiguchi et al. [15] have suggested that low molecular weight chitosan in an agar system inhibited a range of phytopathogenic fungi more effectively than high molecular weight chitosan inhibited the organisms. The depolymerized products of chitosan were found to be potent inhibitors of tumor-induced angiogenesis [16].

Chitosan is known to prevent the development of phage infections in cultures of various microorganisms [17], it also exhibits antimicrobial activity against some strains of filamentous fungi [18], yeast [19] and bacteria [2,4,5–7,20]. Many chitosan derivatives, for example, *N*,*O*-acyl and *N*-alkyl and *N*-aryl chitosan derivatives were found to have insecticidal and fungicidal activity [21,22]. Thiourea chitosan was prepared by the reaction of chitosan with ammonium thiocyanate in ethanol [23,24]. The antimicrobial activity of the Ag^{+} complexes of this thiourea compound was evaluated against six species of bacteria and molds. The complex showed a wide spectrum of antimicrobial activities, whose minimum inhibitory concentration (MIC) values against bacteria were 20 times lower than those of chitosan, 100 times lower than those of sodium diacetate and 200 times lower than those of sodium benzoate, respectively; the complex has a better antibacterial activity than antifungal activity [23].

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Chitosan was also modified by grafting thiourea through glutaraldehyde linkage [25]. This graft copolymer showed enhanced sorption properties for platinum ions from solution.

Sugar-beet (*Beta vulgaris* L., Chenopodiaceae) is one of the most important crops grown mainly in the areas of temperate climatic conditions for sugar production. It has great economic importance for Egypt [26] since it is the second crop plant for the sugar production after sugar cane. Sugar-beet is attacked by several root-rot diseases the most serious of which are those caused by *Rhizoctonia solani* and *Sclerotium rolfsii* [27] and also a wilt disease caused by *Fusarium* species [28,29]. Taking this economic importance into consideration the present work was designed to investigate the in vitro effect of chitosan and a new thiourea derivative (TUCS) on the growth activities of the sugar-beet pathogens: *R. solani, S. rolfsii* and *Fusarium solani*.

2. Experimental

2.1. Materials

Chitosan was isolated from red shrimp that was harvested from the red sea.

The exoskeleton was extracted with HCl to remove the carbonates (45%) then boiled with 1–2 M NaOH to remove the protein and finally refluxed for 8 h in 40% by weight NaOH solution. The slurry was filtered washed thoroughly with distilled water till free from the alkali and finally boiled in acetone for 6 h and dried. The degree of deacetylation DDA of all chitosan samples was determined by acid-base potentiometric titration [30] and by elemental analysis [31]. The high molecular weight sample was kindly donated by professor Furuhata of Tokyo Institute of Technology.

3. Preparation of the TUCS

The thiourea derivatives prepared in this work has not been prepared before according to our knowledge. It was prepared by two procedures: in the first one the chitosan was suspended in acetonitrile and in the second method the chitosan was dissolved in 5% acetic acid and the reaction was conducted in a homogeneous condition.

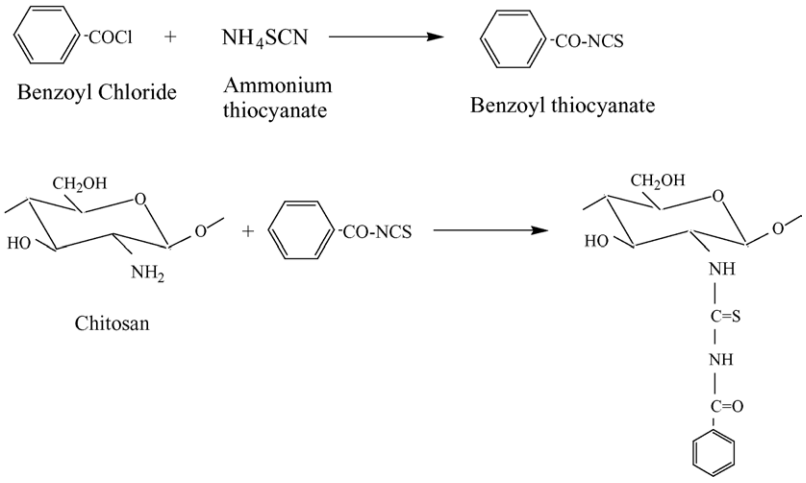
Procedure 1: Dry ammonium thiocyanate (NH_4SCN) (4.0 g) was added to dry acetonitrile at 0 °C and an equivalent amount of benzoyl chloride (6.1 g) in acetonitrile was added drop wise. The reaction medium was stirred at 0 °C for 1 h, filtered, and the filtrate was added while cold to a chitosan suspension in acetonitrile with stirring. The yellowish white product was filtered washed with acetonitrile and ether and dried at 60 °C. The obtained material was analyzed by elemental analysis and FTIR spectroscopy. The elemental analysis was found to be C = 43.03%, H = 5.00%, N = 8.71%, S = 8.25%. The used chitosan had a degree of deacetylation of about 83% this means that the benzoyl thiocyanate reacted not only with the more reactive NH_2 group but also with some of the OH groups in chitosan.

Procedure 2: The benzoyl thiocynate (PhCONCS) (in excess) was added at room temperature to a chitosan solution in acetic acid (5%). After stirring for 2–3 h the homogenous solution was neutralized with Na_2CO_3 to give a white precipitate, which was filtered and washed with distilled water and alcohol and dried. This sample was found to be soluble in dilute acids. The found elemental analysis was C = 45.22%, H = 5.10%, N = 10.94% and S = 9.44%. An increase in the N, and S content confirms that OH group has reacted and that it reacted to a greater extent in the homogeneous method. The preparation of TUCS can be illustrated by the following Scheme 1.

The molecular weight of the used chitosan was assessed by measuring the intrinsic viscosity in acetic acid solution (5%) containing 0.20 M KCl using the conventional Ubbelohde viscometer. Since the literature contains several equations relating the intrinsic viscosity to molecular weight so we used simply the viscosity as an indicator of the molecular weight of the used samples.

3.1. Sources and culture of fungi

The fungi used in this work were isolated from diseased sugar-beet roots [27]. These fungi were maintained in culture



Scheme 1. Scheme for the synthesis of TUCS.

Table 1
Effect of chitosan concentrations on the percent germination (G), average length of hyphal extension (L_h), dry mass yield (D_m), production of sclerotia of *Rhizoctonia solani, Sclerotium rolfsii* and macroconidia of *Fusarium solani* at 27 °C

Purified chitosan concentration (μg ml^{-1})	G (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of sclerotia/plate
R.solani						
Control	56.1	843.3	1020.1	5.8	7.9	63
100	51.6	830.2	983.2	5.8	7.4	60
200	48.3	713.7	962.1	5.8	7.1	57
300	40.2	651.5	830.7	5.8	7.2	51
400	32.7	410.2	614.2	5.7	7.2	44
500	29.6	390.1	403.4	5.7	7.0	39
600	21.3	250.2	337.2	5.7	7.0	30
700	18.4	190.4	217.1	5.7	6.5	19
800	12.3	90.2	170.2	5.6	6.5	12
900	4.5	52.2	93.4	5.6	6.4	8
1000	0	0	0	5.6	6.1	0
L.S.D.						
1%	6.3	13.7	16.9	–	–	6.8
5%	4.5	8.4	8.7	–	–	3.1
S. rolfsii						
Control	53.1	662.4	612.1	5.8	3.3	623
100	51.2	640.3	591.2	5.8	3.7	612
200	46.3	612.2	570.3	5.8	3.7	601
300	40.2	581.2	534.7	5.8	3.7	592
400	32.7	500.3	491.2	5.7	3.7	571
500	29.6	403.7	400.3	5.7	3.8	513
600	20.7	361.2	313.2	5.7	3.8	474
700	13.2	301.2	219.	5.7	3.8	310
800	10.1	196.2	107.2	5.6	4.0	81
900	6.2	66.3	84.3	5.6	4.1	56
1000	0	0	0	5.6	4.2	0
L.S.D.						
1%	5.9	12.8	16.5	–	–	17.2
5%	3.7	8.0	8.5	–	–	10.9

	G (%)	GT (μm)	D_m (mg)	Initial pH	Final pH	Number of macrconidia × 10^4 ml^{-1}
F.solani						
Control	63.2	12.4	357.2	5.8	4.3	314.7
100	61.5	12.1	310.1	5.8	4.3	301.2
200	59.2	11.3	297.2	5.8	4.3	296.3
300	55.3	11.0	283.7	5.8	4.3	289.4
400	43.1	9.6	279.1	5.7	4.2	274.1
500	37.4	8.5	253.4	5.7	4.2	251.7
600	31.2	7.1	211.1	5.7	4.2	203.6
700	29.1	6.2	193.2	5.7	4.0	187.1
800	21.2	5.1	151.5	5.6	4.0	171.2
900	17.1	3.1	101.2	5.6	4.0	113.2
1000	0	0	0	5.6	4.0	0
L.S.D.						
1%	7.8	2.4	11.9	–	–	18.6
5%	5.4	1.5	5.8	–	–	7.4

Note: Percent germination (G) and average length of hyphal extension (L_h) of sclerotia were assessed after 12 h for *R. solani* and 30 h for *S. rolfsii*. Percentage germination (G) of macroconidia of *F. solani* and average length of germ-tubes (GT) were assessed after 9 h. Dry mass yield (Dm) and production of sclerotia by *R. solani* and *S. rolfsii* were assessed after 9 days.

on modified Czapek-Dox agar [32] which consisted of (g/l): sucrose, 20; KNO_3, 4.0; Na_2HPO_4, 2.0; $MgSO_4{\cdot}7H_2O$, 1.0; KCl, 0.50; yeast extract, 0.02; microelement mixture, 1 ml; agar, 15 and distilled water, 1l. Chitosan was dissolved in 0.04 M HCl and the pH was adjusted to 5.6 with 2.0 N KOH. The polymer was added to the nutrient solution to obtain chitosan concentration of 100–1000 μg ml^{-1}.

3.2. Germination of macrcoconidia

Microscope slides were covered, each, with 1 ml of the microconidal suspension of *F. solani* in aqueous solution of the desired chitosan concentration in Petri dishes and then incubated at 27 °C for 9 h in complete darkness. The percentage of germination and the average length of the germ-tubes (GT) were assessed

Table 2
Effect of TUCS1 concentrations on the percent germination (G), average length of hyphal extension (L_h), dry mass yield (D_m), production of sclerotia of *Rhizoctonia solani*, *Sclerotium rolfsii* and macroconidia of *Fusarium solani* at 27 °C

Purified TUCS (μg ml^{-1})	G (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of sclerotia/plate
R.solani						
Control	56.1	843.3	1020.1	5.8	7.9	63
100	33.2	300.0	347.2	5.8	7.4	42
200	17.6	107.2	118.1	5.8	7.1	24
300	6.5	68.2	80.3	5.7	6.5	13
400	0	0	0	5.7	6.1	0
L.S.D.						
1%	5.3	12.7	15.9	–	–	5.7
5%	3.4	7.3	8.7	–	–	2.1
S. rolfsii						
Control	53.1	662.4	612.1	5.8	3.3	623
100	15.6	114.7	207.3	5.8	3.7	171
200	7.9	36.2	100.1	5.8	4.1	114
300	1.3	14.1	44.1	5.7	4.1	31
400	0	0	0	5.7	5.7	0
L.S.D.						
1%	4.7	11.8	15.6	–	–	18.3
5%	2.6	8.3	8.4	–	–	11.2

	G (%)	GT (μm)	D_m (mg)	Initial pH	Final pH	Number of macroconidia × 10^4 ml^{-1}
F.solani						
Control	63.2	12.4	357.2	5.8	4.3	314.7
100	55.1	7.9	293.2	5.8	4.3	292.1
200	41.2	5.9	227.1	5.8	4.2	213.0
300	23.1	3.8	158.3	5.7	4.0	154.1
400	9.5	2.4	91.3	5.7	4.0	39.2
500	0	0	0	5.7	4.0	0
L.S.D.						
1%	7.5	2.1	11.6	–	–	19.3
5%	5.3	1.4	5.7	–	–	6.8

[29]. Five plates were prepared for each treatment and the means were compared.

3.3. Germination of sclerotia

Sclerotia of *R. solani* and *S. rolfsii*, produced on potato dextrose agar (PDA) and Czapek-Dox agar, respectively, were surface disinfected by soaking them for 5 min in 1:400 (w/v) bromine:water to kill hyphal extensions, washed thoroughly with distilled water, and dried. Ten sclerotia per Petri dish for either pathogen were plated on the surface of tap water agar (1.5% w/v) supplemented with the relevant amounts of purified chitosan to produce concentrations in the range of 25–1000 μg ml^{-1} in the medium. The dishes were incubated at 27 °C for 12 h for *R. solani* and 30 h for *S. rolfsii*, and the percentage of germinated sclerotia and average length of hyphal extensions were determined. Five plates were prepared for each treatment and the means were compared.

3.4. Dry mass

Purified chitosan or chitosan derivative was mixed aseptically with Czapek-Dox to produce medium concentrations of 100–1000 μg ml^{-1} and dispensed in 50 ml aliquots into 250 ml Erlenmeyer flasks. A 6 mm agar disk bearing hyphae of either *R. solani* or *S. rolfsii* from 7-days old colonies were incubated at 27 °C for 9 days. The mycelium was harvested, dried to constant weight at 80 °C and the dry mass yield and final pH value were recorded. Five flasks were prepared for each treatment and the means were compared.

3.5. Production of sclerotia

PDA was used for *R. solani* and Czapek-Dox agar for *S. rolfsii*. Dried chitosan powder was mixed with the medium to produce the required concentration and poured into Petri dishes. The fungi were transferred to the dishes and incubated at 27 °C for 9 days. For *R. solani*, 1 ml of the hyphal suspension was added to each dish. This was prepared by transferring two 6 mm diameter PDA disks bearing hyphae into potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks, each containing 50 ml of the medium. The flasks were incubated at 27 °C for 3 days and filtered; the mycelial mats were washed with sterile distilled water. This mycelium was homogenized with 100 ml sterile water in a sterile micro blender for 3 min to form a heavy suspension [33]. For *S. rolfsii*, one 6 mm diameter agar disk bearing hyphae of

Table 3
Effect of TUCS2 concentrations on the percent germination (G), average length of hyphal extension (L_h), dry mass yield (D_m), production of sclerotia of *Rhizoctonia solani, Sclerotium rolfsii* and macroconidia of *Fusarium solani* at 27 °C

Purified TUCS concentration (μg ml^{-1})	G (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of sclerotia/plate
R.solani						
Control	56.1	843.3	1020.1	5.8	7.9	63
50	34.1	341.3	368.2	5.8	7.4	44
100	21.1	127.2	139.1	5.8	7.1	11
150	13.5	77.1	88.2	5.7	6.5	7.0
200	0	0	0	5.7	6.1	0
L.S.D.						
1%	5.1	12.5	15.8	–	–	5.6
5%	3.2	7.1	8.6	–	–	2.0
S. rolfsii						
Control	53.1	662.4	612.1	5.8	3.3	623
50	16.5	209.3	303.0	5.8	3.7	179
100	8.8	49.2	109.2	5.8	4.1	124
150	2.4	17.1	56.1	5.7	4.1	34
200	0	0	0	5.7	5.7	0
L.S.D.						
1%	4.6	11.9	13.7	–	–	18.0
5%	2.5	8.5	9.5	–	–	12.0

	G (%)	GT (μm)	D_m (mg)	Initial pH	Final pH	Number of macroconidia × 10^4 ml^{-1}
F.solani						
Control	63.2	12.4	357.2	5.8	4.3	314.7
50	56.1	8.1	299.2	5.8	4.3	278.1
100	47.3	6.0	239.1	5.8	4.2	188.1
150	21.1	3.9	163.2	5.7	4.0	134.2
200	2.1	2.3	68.2	5.7	4.0	23.4
L.S.D.						
1%	7.3	2.2	11.8	–	–	19.1
5%	5.1	1.4	7.7	–	–	6.8

the fungus was transferred to each dish. The number of sclerotia produced per plate in each treatment was visually counted. Five plates were prepared for each treatment and the means were compared.

3.6. Production of macroconidia

Modified Czapek-Dox agar was mixed aseptically with purified chitosan powdered extracted in amounts calculated to produce the required concentration and poured into Petri dishes. The dishes were incubated with a 6 mm disk of mycelium of *F. solani,* incubated for 9 days at 27 °C and the number of spores produced was calculated by a haemocytometer [29].

3.7. Statistics

The experiments were conducted in three to five replicates and the results obtained were treated statistically with an analysis of variance and the significance was expressed at L.S.D. 5% and 1%.

4. Results and discussion

The fungicidal activity of chitosan towards three soil-borne sugar-beets pathogens was investigated in vitro and the results are depicted in Table 1. Chitosan thiourea derivatives having two functional groups (C=S, and C=O) have been prepared with different molecular weights and degrees of deacetylation and were also investigated against the growth activities of the same pathogens. Their fungicidal activity data are depicted in Tables 2–5.

From Table 1 (pure chitosan with no modification) one can see that the percent germination of sclerotia of *R. solani* and *S. rolfsii* decreased with increasing the chitosan concentration reaching nil at 1000 μg ml^{-1} for both species. The average length of hyphal extension and dry mass yield was affected similarly, decreasing proportionally to the chitosan concentration. The pH of the growth medium shifted toward alkalinity for the *R. solani*. The pH increase in the culture medium during fungal growth may have been caused by differential uptake of cations and anions. Transport of anions such as phosphates may act as the hydroxide exchange system with the medium becoming more basic [34]. The rapid decline in the initial pH of the culture was probably

Table 4
Effect of TUCS3 concentrations on the percent germination (G), average length of hyphal extension (L_h), dry mass yield (D_m), production of sclerotia of *Rhizoctonia solani, Sclerotium rolfsii* and macroconidia of *Fusarium solani* at 27 °C

Purified TUCS concentration ($\mu g\,ml^{-1}$)	G (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of sclerotia/plate
R.solani						
Control	56.1	843.3	1020.1	5.8	7.9	63
25	30.2	209.1	267.7	5.8	7.4	33
50	19.1	103.3	103.2	5.8	7.1	12
75	7.3	36.2	66.4	5.7	6.5	9.0
100	0	0	0	5.7	6.5	0
L.S.D.						
1%	5.3	12.7	14.3	–	–	5.6
5%	3.4	7.3	8.9	–	–	2.3
S. rolfsii						
Control	53.1	662.4	612.1	5.8	3.3	623
25	13.3	188.3	209.7	5.8	3.7	109
50	7.5	44.2	119.1	5.8	4.1	39.1
75	1.3	13.3	44.3	5.8	4.1	19.3
100	0	0	0	5.7	5.7	0
L.S.D.						
1%	4.7	12.0	13.8	–	–	18.3
5%	2.6	8.7	9.6	–	–	12.3

	G (%)	GT (μm)	D_m (mg)	Initial pH	Final pH	Number of macroconidia $\times 10^4$ ml^{-1}
F.solani						
Control	63.2	12.4	357.2	5.8	4.3	314.7
25	12.3	6.2	109.2	5.8	4.3	108.2
50	9.4	3.2	88.6	5.8	4.2	66.3
75	2.6	1.6	48.3	5.7	4.0	23.1
100	0	0	0	5.7	4.0	10.4
L.S.D.						
1%	7.5	2.4	12.0	–	–	19.3
5%	5.7	1.7	8.0	–	–	6.8

due to the production of organic acids (oxalic acid) through the oxidation of carbon source [35].

The number of sclerotia produced by *R. solani* and *S. rolfsii* at chitosan concentration ranging from 100 to 1000 $\mu g\,ml^{-1}$ were reduced proportionally to the chitosan concentration. No sclerotia were produced by either species at concentration of 1000 $\mu g\,ml^{-1}$. Macroconidia of *F. solani* germinated in a wide range of chitosan concentration 100–1000 $\mu g\,ml^{-1}$, although the percentage germination steadily decreased with an increase in the chitosan concentration. Maximum inhibition was recorded at a concentration of 1000 $\mu g\,ml^{-1}$ with a decrease in the germination rate from 63.2% in the absence of chitosan to 17.1% in its presence. Similar results were obtained when measuring the length of the germ-tube after 9 h incubation. Maximum reduction in the germ-tube length was obtained at chitosan concentration of 900 $\mu g\,ml^{-1}$ (from 12.4 to 3.1 μm).

Dry weight estimations showed that mycelial tolerance to chitosan concentration was highest for *F. solani* and lowest for *R. solani* and *S. rolfsii*. For *F. solani*, the results recorded at chitosan concentrations 100–900 $\mu g\,ml^{-1}$ were significantly different from the control.

From Table 1 it can easily be seen that chitosan has a profound effect on the growth activities of sugar-beet pathogens. A similar report by Benhamou et al. [36] indicated that chitosan derived from crab-shell at concentration of 0.5 and 1 mg/ml was effective in reducing disease incidence caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Chitosan at pH 5.8 (when most of the amino groups are protonated) was found to induce massive leakage of uv absorbing materials in *Pythium paroecandrum* [37]. At the same time El-Ghaouth et al. [38] revealed that chitosan was effective in inhibiting mycelial growth of *Pythium aphanidermatum* completely at a concentration of 400 $\mu g\,ml^{-1}$. While at a concentration of 100 $\mu g\,ml^{-1}$ it causes a 75% reduction of the mycelial dry weight. In addition, chitosan appeared to affect the development of *P. aphanidermatum* in submerged culture. At a concentration of 400 $\mu g\,ml^{-1}$, *P. aphanidermatum* grew in the form of cell clusters, indicating that chitosan may have affected the process of hyphal extension [38].

Tables 2–4 contain data of TUCS prepared by the heterogeneous method, which means that the chitosan derivatives are insoluble in the medium. From the data in the three tables one can deduce that the DDA of the starting chitosan and its molecular weight have a strong effect on the growth activities of the pathogens higher than that of the original unmodified chitosan. Table 6 contains a summary of the characteristics of the investigated materials.

Table 5
Effect of soluble TUCS4 (prepared by the homogeneous method) concentrations on the percent germination (G), average length of hyphal extension (L_h), dry mass yield (D_m), production of sclerotia of *Rhizoctonia solani, Sclerotium rolfsii* and macroconidia of *Fusarium solani* at 27 °C

Purified TUCS concentration (μg ml^{-1})	G (%)	L_h (μm)	D_m (mg)	Initial pH	Final pH	Number of sclerotia/plate
R.solani						
Control	56.1	843.3	1020.1	5.8	7.9	63
5	32.7	450.1	503.2	5.7	7.2	39
10	21.9	200.4	313.7	5.7	7.0	31
15	4.1	54.2	70.3	5.6	6.4	8.0
20	0	0	0	5.6	6.1	0
L.S.D.						
1%	6.8	12.9	13.4	–	–	6.5
5%	5.1	6.8	8.2	–	–	3.4
S. rolfsii						
Control	53.1	662.4	612.1	5.8	3.3	623
5	29.7	380.1	373.2	5.7	3.8	415
10	14.1	282.1	201.3	5.7	3.8	309
15	7.2	69.3	85.4	5.6	4.2	44.0
20	0	0	0	5.6	4.2	0
L.S.D.						
1%	6.0	12.6	15.7	–	–	16.3
5%	3.6	8.2	8.6	–	–	10.5

	G (%)	GT (μm)	D_m (mg)	Initial pH	Final pH	Number of macroconidia × 10^4 ml^{-1}
F.solani						
Control	63.2	12.4	357.2	5.8	4.3	314.7
5	60.4	11.9	307.4	5.8	4.3	290.3
10	28.3	6.1	190.1	5.7	4.0	180.2
15	13.4	3.5	100.1	5.6	4.0	87.3
20	0	0	0	5.6	4.0	0.0
L.S.D.						
1%	7.6	2.7	11.6	–	–	17.3
5%	5.5	1.6	5.8	–	–	8.6

The higher the DDA the stronger the effect on the growth activities, at the same time, the lower the molecular weight the higher the activities (samples 2 and 3, respectively). The entire TUCS derivatives have a much higher fungicidal activity than the original chitosan in Table 1. An even higher fungicidal activity was found for the soluble TUCS derivative as shown in Table 5.

All other investigated parameters (length of hyphal extension, dry mass and number of sclerotia per plate for *R. solani* and *S. rolfsii* or macroconidia for *F. solani*) show the same trend. The solubility of the TUCS enhances dramatically the diffusion of the active ingredient inside the pathogens, which may lead to a disturbance of the enzyme activities responsible for the growth criteria, instead of the adsorption of the compounds on the fungal hyphae surface as in the case of the insoluble TUCS.

All these observations and findings bring further evidence that chitosan and its thiourea derivative are active inducer of plant defense action and thus have the potential of becoming powerful and safe alternative means of disease control instead of the harmful pesticides.

Table 6
Characteristics of the used materials

Material	N%	S%	DDA%	[η]* dl/g
Chitosan	7.6	0	83	33.50
TUCS1	8.71	8.25	83	33.50
TUCS2	8.80	5.40	79	19.69
TUCS3	9.69	8.12	88	5.76
TUCS4	10.94	9.44	83	33.50

* Viscosity of the original chitosan before reaction with benzoyl isothiocyanate.

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