

## The Mutagenic Activity of Chitosan and its Effect on the Growth of *Trichoderma harzianum* and *Fusarium oxysporum* F. Sp. *Sesami*

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**Abstract:** Five concentrations of chitosan; 0.38, 0.75, 1.50, 3.00 and 4.50 mg/ml were used to study its effect on the growth of *Fusarium oxysporum* f. sp. *Sesami* and *Trichoderma harzianum*. Chitosan inhibited the growth of *Fusarium* increasingly with the doses; 0.38, 0.75 and 1.50 mg/ml, while no growth was obtained when the two concentrations; 3.00 and 4.50 mg/ml were used. In case of *Trichoderma*, in addition to growth inhibition with chitosan treatment, two doses; 3.00 and 4.50 mg/ml resulted in changing the growth color from green to yellow. It was found that chitosan treatment of *Trichoderma* with the two doses; 3.00 and 4.50 mg/ml has affected the fungus genetically. The induced mutants were isolated and characterized. Chitosan Addition to the media enhanced the antagonistic properties of *Trichoderma* against *Fusarium*. Protein fingerprinting and Statistical analysis of the obtained data for the chitosan concentrations and its control were done.

**Key words:** *Fusarium oxysporum* f. sp. *sesami*, *Trichoderma harzianum*, Chitosan, Biocontrol agent, Genetic effect and SDS-PAGE

### INTRODUCTION

Control of sesame wilt disease depends on fungicides as seed or soil treatment<sup>[17]</sup>. Fungicide application is expensive and can cause environmental pollution. Moreover, it may induce pathogen resistance<sup>[15]</sup>. Recently a potential approach in biological control involves the use of the natural bioactive substance chitosan, which inhibits fungal growth and also activates the biological efficiency of the antagonistic microorganisms<sup>[10,27]</sup>. Chitosan has a fungistatic activity demonstrated against some plant diseases, i.e., virus infection<sup>[3]</sup> and pathogenic fungi<sup>[28,21]</sup>. Chitosan also stimulates the plant to defend itself through the activation of resistant gene(s) for induced multiple biological reactions<sup>[12,5]</sup>. Efficiency of *Trichoderma* spp. as a biocontrol for plant pathogenic fungi may be increased by combining different organisms and/or simple chemical substances. Chitosan could promote the plant to defend itself besides; it interferes with the growth of the Pathogen<sup>[18]</sup>. Chitosan (Poly- -1, 4 glucosamine) is prepared commercially by alkaline decelylation of chitin obtained from the exoskeletons of marine crustaceans.<sup>[21]</sup> The induction of mutation and the auxotrophic mutant were studied also by Kanemaru and Migamoto<sup>[16]</sup> and Daminko<sup>[6]</sup> as a result of chitosan treatment. The present study deals with the mutagenic activity of chitosan and its effect on the growth of *Fusarium oxysporum* f. sp. *sesame*,

the causal agent of sesame wilt and the antagonistic effect of *Trichoderma harzianum* isolates as a biocontrol agent.

### MATERIALS AND METHODS

**Fungal Strains:** Two fungal strains were used in this work; *Fusarium oxysporum* f. sp. *sesami* known to be highly virulent on sesame plant and the antagonistic isolate of *Trichoderma harzianum* which obtained from plant pathology Dept. NRC of Egypt.

**Chitosan Treatments:** Purified Chitosan (obtained from Sigma chemical steinham, Germany) was prepared by the method which described by El-Ghaouth *et al.*<sup>[8]</sup>. To study the effect of different doses of chitosan on the linear growth (mm) of *Fusarium oxysporum* f. sp. *sesami* and *Trichoderma harzianum*, five concentrations of chitosan were used. A disc of (5 mm-dm) of pure cultures of the tested fungi was placed on the center of PDA plate containing; 0.00, 0.38, 0.75, 1.50, 3.00 and 4.50 mg/ml of chitosan. Plates were incubated at 27±2°C for seven days and the diameter of the fungal growth was recorded twice daily. Four replicates were used for each particular treatment.

**Mutagenicity Test:** Chitosan was applied as mentioned before to PDA medium in concentrations of; 0.38, 0.75, 1.5, 3.00 and 4.5 mg/ml based on active

ingredient. The surviving colonies and the morphologically different colonies (Yellow) were isolated and tested on both minimal and complete media. The colonies which showed growth on complete medium but not on minimal medium were considered as auxotrophs (mutants). Survival and mutation percentages were determined for each treatment according to Mckennes and Meton<sup>[20]</sup> and Sahab *et al.*<sup>[24]</sup>.

**Isolation of Auxotrophs and Their Nutritional Requirements:** Nutritional requirements of each mutant obtained from different mutagenic treatment were identified by replica plating method on the minimal medium supplemented with one or more of the amino acid (250 mg/l) or vitamins (2.5 mg/l) or nitrogen bases (25 mg/l) as described by Abou Sereih *et al.*<sup>[11]</sup>.

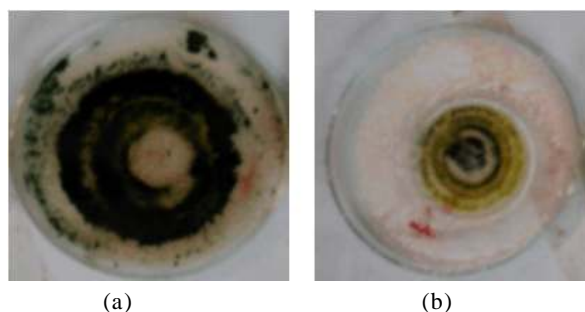
**Antagonistic Screening Test:** Screening procedure for detecting the antifungal activity of different *Trichoderma harziesum* against *F. oxysporium* f. sp. *sesami* was adapted by Ziedan and Elewa<sup>[29]</sup>. Five ml of the pathogenic fungal suspension containing 10<sup>6</sup> CFU/ml was added to 200 ml of melted PDA medium (50°C) before pouring into the Petri dishes (9 cm diameter). The Petri dishes were inoculated with disks (5 mm diameter) loaded with *Trichoderma* culture of 7 days old and incubated at 25±2°C for 5 days. Zones of inhibition were estimated in mm and the degree of antagonism observed using a scale ranges from zero (no inhibition) to 3 + (excellent inhibition), Ziedan<sup>[28]</sup>.

**Total Protein Extraction:** Total protein extraction and banding patterns SDS-polyacrylamide gel electrophoresis was performed according to Sheri *et al.*<sup>[26]</sup>.

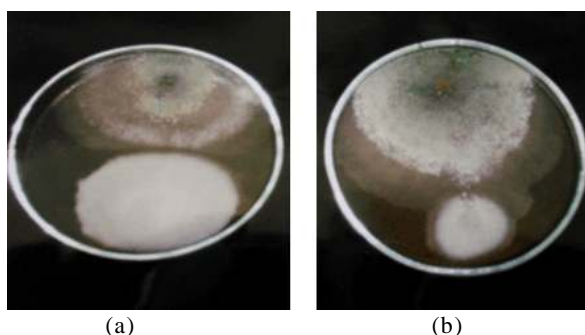
**Gel Documentation System:** The gel documentation system image analysis Gel works 1D advanced software was used for more accurate analysis and comparison between the treatments via biochemical genetic analysis. This method is recommended to determine the relationship within and between the treatments<sup>[11]</sup>.

## RESULTS AND DISCUSSIONS

**Effect of Chitosan on Fungal Growth:** In this work, five doses of chitosan; 0.38, 0.75, 1.50, 3.00, 4.50 mg/ml were used to study the effect of this material on linear growth (mm) of *Fusarium oxysporum* f. sp. *sesami* and *Trichoderma harzianum*. Data in Table (1) showed that chitosan treatments decreased the linear growth of both *Fusarium* and *Trichoderma*



**Fig. 1 (a,b):** The effect of chitosan in concentration 3.00 mg/ml on *Trichoderma harzianum*; Fig. (a) showed the green color of *Trichoderma* before treatment and Fig. (b) showed the yellow color of *Trichoderma* after treatment with chitosan.



**Fig. 2 (a,b):** The effect of *Trichoderma harzianum* on the growth of *Fusarium oxysporum* f. sp. *sesami* (a) without chitosan and (b) combined with chitosan.

in positive reaction to chitosan concentration of 3 mg/ml concentrations. The two higher doses of chitosan 3.00 and 4.50 mg/ml were lethal with *Fusarium* while in case of *Trichoderma* they resulted in the change in growth color from green to yellow. These results were in agreement with Ryan<sup>[22]</sup> who reported that chitosan induced multiple biological reactions including induction of phytoalexin synthesis of B-1-3 gluconase and chitinase. On the other hand, Chung and Choi<sup>[5]</sup> reported that the *Trichoderma harzianum* is considered as a biocontrol agent caused damage on *Fusarium oxysporum* f. sp. *sesami*. Concomitant use of *Trichoderma harzianum* and chitosan improved the antagonistic effect of *Trichoderma* against *Fusarium*. This result was in agreement with Ziedan and Elewa<sup>[29]</sup> (Fig. 2. a & b).

**Genetic Effect of Chitosan:** *Trichoderma* treatments were tested on Minimal Media (MM) and Complete Media (CM) at two chitosan concentrations; 3.00 and 4.50 mg/ml of each medium. On contrary of complete medium, no growth was found on Minimal media.

**Table 1:** Effect of different chitosan concentrations on the linear growth (mm) for *Fusarium oxysporum* f. sp. *sesami* and *Trichoderma harzianum*.

Chitosan concentrations (mg/ml)	Fungi	
	<i>Fusarium oxysporum</i> f. sp. <i>sesami</i>	<i>Trichoderma harzianum</i> .
0.00	90.00	93.75
0.38	71.23	80.50
0.75	62.25	69.00
1.50	50.50	60.25
*3.00	00.00	23.00
4.50	00.00	16.75

\* Lethal dose

**Table 2:** Characterization of chitosan induced mutant produced from *Trichoderma harzianum*

	Chitosan concentration		
	1.5	2.00	4.50
Colonies tested	500	500	500
Total No. of colonies	500	500	500
Total No. of mutant	41	43	44
Mutant %	8.2	8.6	8.8
Stable mutant %	0.0	6.97	35.5
Unstable mutant %	100	93.03	64.5
Morphological mutants	0	2	4
Biochemical mutant			
Nitrogen requiring mutants	0	0	0
Vitamins requiring mutants	0	0	0
Amino acid requiring mutants	1* 1***	2* 1**	1* 3***

\* Arginine - requiring mutants.

\*\* Adenine - requiring mutants.

\*\*\* Methionine - requiring mutants.

For *Trichoderma*, it was able to grow on both M.M. and C.M. at the rest of chitosan concentrations. These results indicated that the two chitosan concentrations 3.00 and 4.50 mg/ml have a genetic effect and induced mutation. The *Trichoderma* colonies which grown on C.M. and not on M.M. at these concentrations are considered as mutants.

Table (1) shows that the increase in chitosan concentration was associated with an increase in mutation and decrease in survival. The lethal dose (3 mg/ml) affected *Fusarium oxysporum* f. sp. *sesami*; these indicate that chitosan has an antimicrobial activity in *Trichoderma* but its effect is on a gene responsible for color and make a mutation. These results are like those reported by [21].

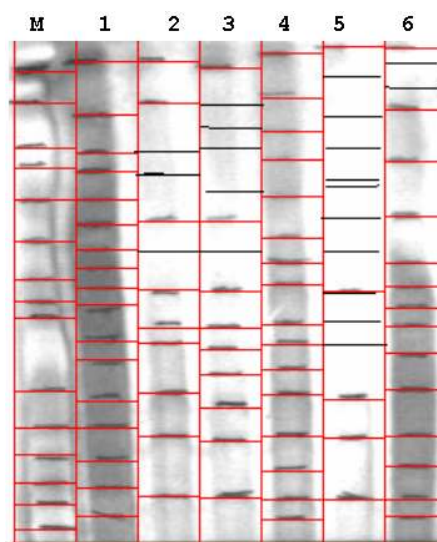
Data in Table (2) shows that chitosan can induce stable and unstable mutants. Generally, the stable mutants were much less than those of unstable mutants in both isolates. In this respect, Beremand [21] isolated, identified and chemically characterized three stable mutants.

The effect of chitosan on the cell wall of the pathogenic fungi has been cleared by Ryerson and Heath [23], they found no effect on a non-pathogen. The wall fragments were derived from differentiated or

**Table 3:** In-vitro test of the antagonistic activity of different mutant strains of *Trichoderma harzianum* against *Fusarium oxysporum* f. sp. *sesami*.

Chitosan Concentrations mg/ml	No. of tested colonies(*)	Average zone inhibition (mm)
1.5	2	20.1
	4	22.0
3.00	5	22.2
	7	25.5
	2	26.1
4.50	4	16.5
	5	27.1
	7	26.6
	5	27.5
Wild type	1	14.5

(\*) Number of tested colonies (mutant strains) of *Trichoderma harzianum* have nearly a same size of zone inhibition (mm).

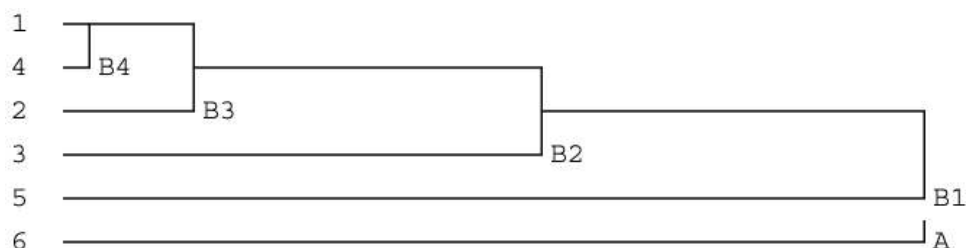


**Fig. 3:** The SDS-PAGE total protein pattern for the effect of five concentrations (mg/ml) of chitosan on *Trichoderma harzianum*; 0.38 (lane 1), 0.75 (lane 2), 1.50 (lane 3), control (lane 4), 3.00 (lane 5), 4.50 (lane 6) and (lane M) the marker with molecular weight (12 to 97 KDs).

undifferentiated fungal structures. On the other hand, El-Ghaouth *et al.* [19] suggested that the induction of morphological and cytological alterations in *Rhizopus stolonifer*.

Data in Table (3) reveal that the tested isolated of *T. harzianum* mutants were significantly changed in their antagonistic action. The lower value of zone of inhibition (20-22 mm) in the comparison with the wild type isolate (14.5 mm). On the other hand, isolates of (3.00, 4.50 mg/ml) chitosan were considered a high antagonistic activity against *Fusarium oxysporum* f. sp. *sesami*.

Different doses of chitosan which caused increase or decrease the antagonistic activity by *T. harzianum*



**Fig. 4:** Dendrogram tree for the effect of five concentrations (mg/ml) of chitosan on *Trichoderma harzianum*; 0.38 (1), 0.75 (2), 1.50 (3), wt (4), 3.00 (5), 4.50 (6).

may be due to the changes of the genetic structure or the gene regulation system responsible for antagonistic activity<sup>[25]</sup>, or it may be effect on the responsible gene for endochitinase which degraded the cell wall of the pathogen<sup>[13]</sup> and some times antifungal compounds enhances inhibition of spore germination<sup>[19]</sup>.

**3-Protein Fingerprinting:** Total protein extraction and banding patterns SDS-polyacrylamid gel electrophoresis for five treatments resulting from using five concentrations of chitosan on *Trichoderma harzianum* and its control are illustrated in Fig. (3). There are observable differences in the protein banding pattern for all five treatments and the control. Some minor differences in banding patterns between the control and the treatments which resulting from the chitosan concentrations; 0.38 (lane 1), 0.75 (lane 2), 1.50 (lane 3), control (lane 4) were observed, but variation to the same extent was also present between the two treatments; 3.00 (lane 5), 4.50 (lane 6). Data from Fig. (3) also reveal that the total bands number for all the five treatments and their control ranged from 12 bands (0.75, lane 2), to 17 bands (0.38, lane 1). The higher total number of bands for the treatments of chitosan concentrations (mg/ml); 0.38(lane 1), control (lane 4) and 4.50 (lane 6) were 17,16 and 16, respectively. The molecular weight ranged from 14, 4 to 94 KDs. There are common bands found in all strains. The results also indicate that the two treatments of chitosan concentrations (mg/ml); 3.00 (lane 5) and 4.50 (lane 6) have one band at molecular weigh 73.05 KDs and two different specific (monomorphic) bands at molecular weigh 46.6 and 44.88 KDs, respectively, while the rest of the treatments of chitosan concentrations have not any monomorphic band. These results could be used to distinguish between these treatments for example, each of the treatments of chitosan concentrations (mg/ml) on *Trichoderma harzianum* 0.38 (lane 1), 0.75 (lane 2), 1.50 (lane 3) and control (lane 4),

have not any unique or specific band, while only 3.00 (lane 5) and 4.50 (lane 6) have one specific band. This specific band of the two chitosan treatments may be referring to the occurred change of growth color and appearance the yellow color of the *Trichoderma harzianum*.

**Statistical Analysis for SDS-PAGE data:** Package SPSS system. Significant different were determine at  $p < 0.05$ . Data from SDS-PAGE were pooled and transferred into 1 and 0, they were interred into the input of the program as shown in the dendrograms below (Fig 4). The statistical analysis data were carried out with the statistical software according to the method which described by Iruela<sup>[14]</sup>. The dendrogram generated by (Gel works 1D) analysis confirmed the above pattern of diversity using SDS-PAGE found genetic difference the two chitosan concentration treatments, 3.00 & 4.50 mg/ml and the rest of treatments and in additions of the control The total five chitosan concentration treatments and in additions of the control were classified for the dendrograme into five pool clusters. The first and the second clusters (A & B1) include two chitosan concentration treatments, 3.00 & 4.50 mg/ml, these two clusters are so far from other cluster (about 100% dissimilarity) they have not genetic similarity of approximately 0%. This result was inagreement with obtained data at Fig (3). In the contrary, the first chitosan concentration treatment, 0.38mg/ml (No.1) and the control (No. 4) have a one group ( cluster B4) with very close distance between them (about 98% similarity). The second chitosan concentration treatment, 0.75 mg/ml (No.2) have one cluster (B3) near the last cluster but it is little distance from them (88% similarity) and near the last cluster but it is a little distance from them and have a common ancestor the clusters B 2. The third chitosan concentration treatment, 1.50mg/ml (No.3) also have one cluster about 40 % similarity with cluster (B3) and 48% dissimilarity with the two clusters A & B1.

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