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Research Article **Microbiology**

Effect of chitosan on *Fusarium* spp. isolated from the soil of the Nile Delta region in Egypt

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KEY WORDS

Fusarium isolates, chitosan, antifungal activity and ultrastructure changes

ABSTRACT

Continuous and over use of fungicides to reduce phytopathogens has harmful effect on health and induces natural resistance in plant pathogens. Present study aimed to use natural compound such as chitosan to inhibit growth of Fusarium spp. isolated from soil of Egyptian governments (Kafr El-sheikh, El-Gharbia, and El-Monofia), *Fusarium* isolates identified based on morphological characteristics as *Fusarium oxysporum*, *F. solani*, *F. verticolliodes*, and *F. chlamydosporum*. Potato dextrose agar medium (PDA) containing 1.6 g/L of chitosan acetic acid distilled water solution recorded remarkable mycelial growth inhibition (77, 76.7 , 71.4 and 67%) for *Fusarium verticilloides*, *F. oxysporum*, *F. chlamydosprum* and *F. solani* respectively. Ultrastructural changes in conidia of *F. chlamydosprum* treated with 1.2g/L chitosan acetic acid distilled water solution were also investigated by means of transmission electron microscopy (TEM) which indicated alterations of cytoplasm and cell wall thickness, compared with control.

Introduction

Fusarium species are common phytopathogen present in the environment, including soil and water that associated with various plant diseases such as root rot disease, vascular wilt of more than hundred species of plants and this is lead to large losses on crops yields of world agriculture every year (Wu, 2007). *Fusarium* spp. is one of the main cereals pathogen and mycotoxin contamination of wheat, oats, barley, and maize (Thomas, et al., 2019). Many species of *Fusarium* such as *F. graminearum, F. proliferatum*, *F. culmorum*, *F. sporotrichioides*, *F. verticilliodes* and *F. subglutinans* produce a range of phytotoxic compounds, such as fusaric acid, fumonisins, beauvericin, enniatin, moniliformin and trichothecenes (Nivalenol, Deoxynivalenol and T-2 toxin) (Ismaiel & Papenbrock, 2015, Ferrigo et al., 2016, Tima et al., 2016 and Tola & Kebede, 2016). These toxins possess a variety of biological activities and cause morphological, physiological and metabolic effects on plant including necrosis, chlorosis, growth inhibition, wilting, and inhibition of seed germination (Rocha et al., 2005). In addition to, infection by *Fusarium* species causes reduction in crops yield; these toxins accumulated in grains to become

carcinogenic to humans and cause a number of animal diseases (Danielsen et al., 1998 and Desjardins, 2006).

Fusarium has ability to remain in soil for several years through their resting structures in the form of chlamydospores (Wharton et al., 2007). The continuous use of fungicides for long-term has side effects on human health and the environment (Song et al., 2004; Binod & Bhupendra, 2009), including immune suppression, hormonal disruption, reproductive abnormalities, cancer and the induction of plant resistance (Gupta, 2004). With increasing awareness of the harmful caused by chemical control, scientists do their best efforts to find alternative methods for reducing crop damage with minimum ecological hazards (Khan et al., 2011).
Biological control is a safe method for the suppression of plant pathogens using natural compounds (Daami-Remadi, et al., 2006; Mejdoub-Trabelsi, 2017). Among the most widely used natural reduction compounds for phytopathogens, chitosan which display antibiotic activity against microorganisms, either bacteria (Liu et al., 2004; Tikhonov et al., 2006) or fungi (Feliziani et al., 2013).

Chitosan is a straight-chain copolymer composed of D-glucosamine and N-acetyl-D-glucosamine, non-toxic compound derived from chitin and biodegradable it applied in solution after stirring in acids or as powder (Kurita, 2006, Rinaudo, 2006 and Romanazzi et al., 2013). Chitosan is a safe to plants and used as soil modifier, to improve seed and flower quality, as well as increasing crop yields (Ren et al., 2001). Chitosan has antifungal activity against several fungal pathogens, such as *Aspergillus parasiticus* (Cota-Arriola et al., 2011), *Geotricum candidum* (El-Mougy et al., 2012), *Alternaria alternata* (Reddy et al., 2000), *Fusarium solani* and *Sclerotium rolfsii* (Eweis et al., 2006).
The aim of this research was to study the effect of Chitosan on the growth of different *Fusarium* species isolated from the soil of the Nile Delta region and to investigate the morphological ultrastructure changes occurred for their conidia.

Materials and methods

Fungal isolation, purification and identification

45 soil samples were collected from different locations of three Egyptian governments (Kafr El-sheikh, EL-Gharbia and El-monofia). Each soil sample were air dried, ground well and sieved, 10 g of each sample was transferred to 100ml of sterile dist.H_2O in a 250 ml flask. After flasks were shaken on a rotary shaker for 30 min 10-fold dilutions were made serially from the original suspension. 1ml from each dilution was spread on Potato Dextrose Agar medium (PDA) [20g dextrose, 20g agar and the broth from 250g white potatoes made up to 1L with dist. H_2O], in Petri dish (three replicates for each dilution). All plates were incubated at 25 ±1^oC for 3-7days and examined daily for the occurrence of fungal growth. The growing fungi were examined microscopically, purified by cutting the emerging colonies, and transferred individually onto new PDA plates.
For morphological identification three types of media were used, Potato Dextrose Agar medium (PDA), Synthetic Nutrient Agar (SNA) [1.0g KH_2PO_4, 1.0g KNO_3, 0.5g $MgSO_4$.$7H_2O$, 0.5g KCl, 0.2g glucose, 0.2g sucrose and 20g agar/L] and Carnation leaf Agar medium (CLA)[prepared by placing fresh, sterile carnation leaf pieces into Petri dish and adding sterile 2% Water agar]. These media support the formation microconidia, macroconidia and chlamydospores (Leslie and Summerell, 2006).

Chitosan preparation

Chitosan (Mw 150 kDa, 75% –85% deacetylated) acquired from Sigma-Aldrich. It was prepared as a stock solution. 2g of chitosan was dissolved in 100 ml of 1% (v/v) acetic acid-distilled water solution (pH 5.6) (Benhamou et al., 1998). The obtained solution was autoclaved for 20 min., and then adjusted to get different concentrations of chitosan (0.5, 1.0, 1.2, 1.6 and 2.0 g/L in acetic acid-distilled water solution).

Effect of chitosan on fungal growth

Effect of chitosan on fungal growth was carried out on PDA plates amended with different chitosan concentrations (0.5, 1.0, 1.2, 1.6 and 2 g/L acetic acid distilled water solution).

Unamended PDA plates served as controls. Three plates for each chitosan concentration were inoculated in the center with a plug (5 mm diameter) from the edge of 7-day-old-colony of each *Fusarium* spp. to be tested. Two colony radii were measured for each plate, when controls reached the edge of the plate. The inhibition percentage of mycelial growth was calculated according to the following formula outlined by Pandey et al., 1982.

Percentage of mycelial growth inhibition:

$$(I\%) = [(dc - dt/dc)] \times 100$$

Where dc = Average diameter of fungal colony in control. dt = Average diameter of fungal colony in treatment.

Transmission electron microscopy (TEM)

Since *Fusarium chlamydosporum* produce large number of macroconidia than other isolates it was more suitable to use in making transmission electron microscopy.

Both untreated (control) and treated mycelia of *F. chlamydosporum* that grown on liquid Czapek's media containing 1.2 mg/ml chitosan for 7 days at 25 ±1°C, were fixed overnight by immersion in 2.5% (V/V) glutaraldehyde phosphate buffer (pH 7.4) at 4°C for 2 h. samples were then post fixed with 0.1% osmic acid for 30 min at room temperature. Samples were dehydrated in a graded ethanol series for 30min. then infiltrated with acetone for 1 hour.

After dehydration, samples were embedded in Araldite 502 resin. The plastic molds were cut in the LEICA Ultracut UCT ultra-microtome, stained with 1% toleudine blue. After examination of semi-thin sections ultra-thin sections were cut, stained with uranyl acetate. Then counter stained with lead citrate and examined and photographed using JEOL-JEM-100SX electron microscope, Japan, Faculty of Medicine, Electron Microscope Unit, Tanta University.

Results

All *Fusarium* isolates were identified according to their morphological characters such as color of mycelium and colony pigmentation, colony diameters on PDA medium, microscopic examination for presence or absence of microconidia, macrocondia and chlamydospores and type of phialid after grown on different types of media potato dextrose agar medium (PDA) for 7 days, Synthetic Nutrient Agar (SNA) and Carnation leaf Agar medium (CLA) for two weeks. The isolates were identified as *Fusarium oxysporum*, *F. solani*, *F. verticilliodes* and *F. chlamydosporum* (Table 1 and Figure 1).

Table 1: Identification of *Fusarium* spp. according to their morphological and microscopic characters

Fusarium spp.	Morphological characters on PDA medium	Microscopic characters on SNA medium		
		Microconidia	Macroconidia	Chlamydospore
F. oxysporum	Abundant white mycelium with pale violet pigment	Abundant, oval to reniform	Short to medium in length, almost straight, with 2-3 septa	Produced singly or intercalary chain
F. solani	Dense, white to pale violet mycelium	Oval with one septa formed in false head	Slightly curved, 3-5 septa	Produced single or in pairs
F. verticilliodes	White to violet mycelia with dark blue pigment	Abundant, oval	Straight, oval,1-2 septa	Not present
F. chlamydosporum	Off white, floccose, fast growing mycelium with pigment ranging from yellow to pale brown	Abundant, oval with various size formed in heads	straight, long, slender falcate, with rounded apex with 3-5 septa	Abundant, rough, formed rapidly, in single, chain or clusters

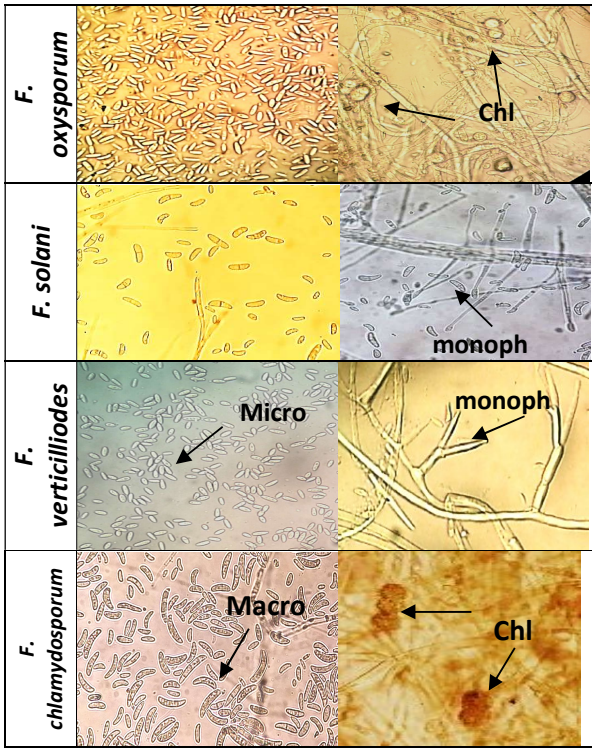

Fig. 1: Micro, macro-conidia and chlamydosporesof different *Fusarium* spp., (Chl): chlamydospore, (monoph): monophyalide pairing conidia, (Micro): microconidia, (Macro): macroconidia

Table 2 and Figure 2 showed that inhibition of mycelial growth of all tested *Fusarium* spp. increased with increasing chitosan concentration. 1.6 g/L chitosan acetic acid distilled water solution recorded high inhibition percentage for growth of *F. verticilliodes, F. oxysporum*, *F. chlamydosporum* and *F. solani* was reduced by 77, 76.7, 71.4 and 67% respectively, compared with their respective untreated controls. At concentration 2.0 g/L of chitosan acetic acid distilled water solution complete inhibition occurred for all *Fusarium* isolates.

Table 2: The mean of colony diameters of *Fusarium* isolates after grown on PDA medium containing different concentrations of chitosan and their standard error (SD) compared with control

Chitosan conc. (g/L)	Mean of colony diameters of *Fusarium* isolates on PDA medium (mm± SD)			
	F. verticilliodes	***F. solani***	***F. chlamydosporum***	***F. oxysporum***
Control	90.0 ± 0.00	90.0 ± 0.00	90.0 ± 0.00	90.0 ± 0.00
0.5	69.7 ± 0.07	73.7 ± 0.08	71.0 ± 0.05	65.7 ± 0.08
1.0	52.3 ± 0.07	61.0 ± 0.09	56.7 ± 0.07	45.0 ±0.09
1.2	34.7 ± 0.09	45.0 ± 0.09	33.0 ± 0.09	31.0 ± 0.07
1.6	20.7 ± 0.03	29.7 ± 0.07	25.7 ± 0.03	21.0 ±0.09
2.0	7.0 ± 0.03	12.3 ± 0.03	8.3 ± 0.03	5.0 ±0.00

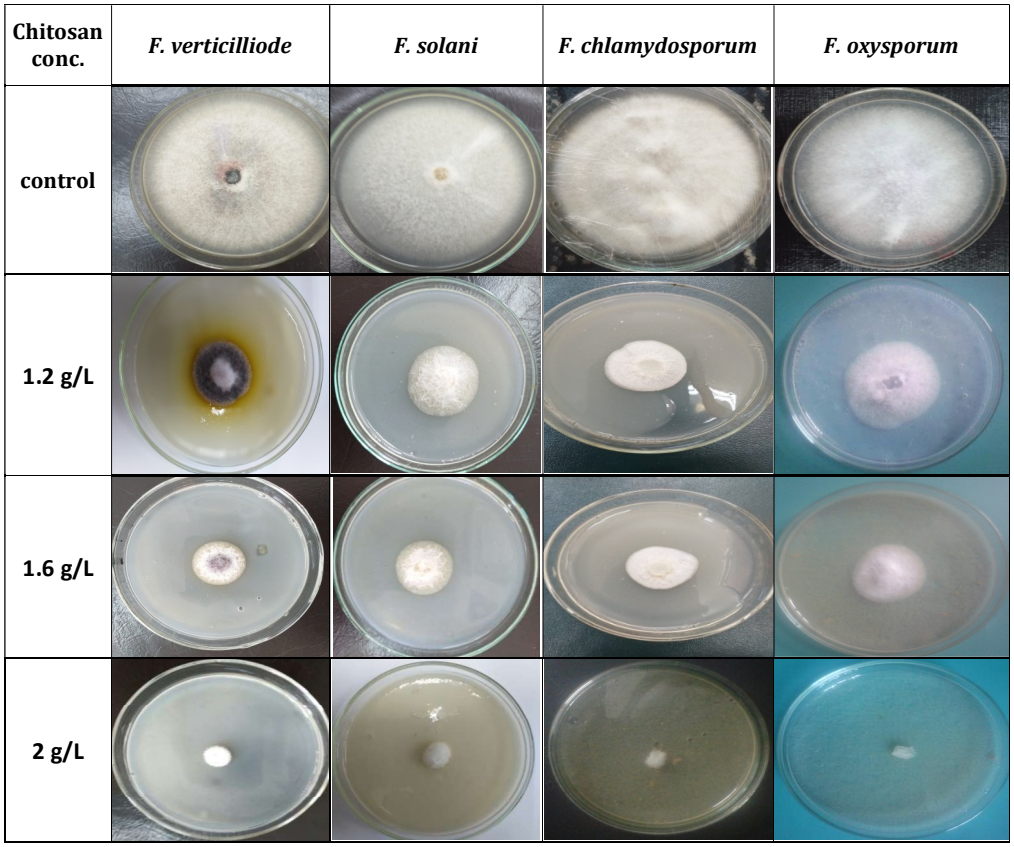

Fig.2: Increase the mycelial growth inhibition with increasing chitosan concentration

At ultrastructural level, conidia of *F. chlamydosporum* untreated with chitosan appear to be covered with an extracellular sheath and have the organelles usually present in the fungal cells, like nucleus, vacuoles, mitochondria, lipid bodies, etc. Plasma membrane of control pressed to the cell wall and the structured cytoplasm (Figure 3A). while, that treated with 1.2 g/L chitosan acetic acid distilled water, appear to be damaged at cellular level: the organelles are partly and/or entirely destroyed, the cytoplasm is degenerated, the external sheath was slightly modified and the cell wall had irregular shape on the outside in addition to the accumulation of lipid bodies and the thickening of the cell wall (Figure 3B).

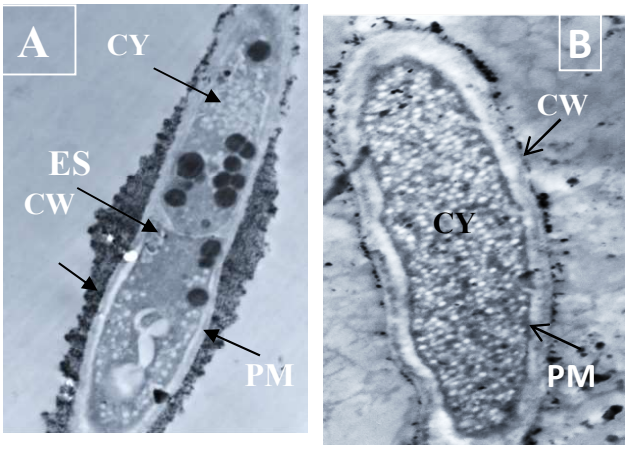

Fig. 3: Transmission electron microscopy (TEM) of *Fusarium chlamydosporum*. A) Untreated conidia (control) (6000X). B) Treated conidia with 1.2 g/L chitosan (6000X). (ES): external sheath, (CY): cytoplasm, (CW): cell wall and (PM): plasma membrane.

Discussion

Identification of *Fusarium* isolates based on morphological, microscopic characters is the same with Gherbawy et al., 2019. They isolated and identified different *Fusarium* spp. collected from four Governorates in Upper Egypt (Aswan, Luxor, Qena and Sohag) depending on morphological characteristics as *F. sambucinum*, *F. oxysporum*, *F. incarnatum* and *F. verticillioides*. In addition, Taha et al., 2016 collected 43 *Fusarium* isolates from roots of sugar beet plants from different sugar beet grown fields of lower and middle Egypt. They demonstrated that *F. oxysporum*, *F. solani*, *F. proliferatum*, *F. equiseti* and *F. veticillioides* were the most abundant species and sugar beet pathogens.

It is clear from the present study that chitosan can be used as an inhibitory compound for Fusarium isolates growth. Our results are in agreement with many researchers, Benhamou et al., 1998 demonstrated that chitosan inhibits the growth of *Fusarium oxysporum* f. sp. *radices*. EL Hassni, 2004 suggested that chitosan at 1 mg/ml reduced the growth of *Fusarium oxysporum* f. sp. *albedinis* on PDA medium by an average of 75%. Ashour et al., 2017 studied the effect of different concentrations of chitosan against growth of *Fusarium culmorum* that is the most virulent causative agent for dry rot of potato collected from Egyptian governorates. They proved that chitosan recorded inhibition for fungal growth increases with increasing the chitosan concentration; this is lead to disease reduction between 13-87%.

Chitosan used as an alternative agent to chemical fungicides for the control of *F. sambucinum*, *F. graminearum* and *F. oxysporum* that cause tuber dry rot and plant wilting (Mejdoub-Trabelsi et al., 2019).

The antimicrobial activity of the chitosan on pathogenic microorganisms depends on different factors like the strain, molecular weight, concentration, degree of deacetylation, and type of chitosan (Rinaudo, 2006).

The interaction of chitosan with the microorganism results in different changes: (a) changes on cell permeability, due to the polycationic nature of the chitosan amino group and the electronegative charges in the outer surface of the fungal membrane (Palma-Guerrero et al., 2010); (b) affectation on homeostasis (K+, Ca2+), leading to the efflux of small molecules affecting fungal respiration (Peña et al., 2013 and Hosseinnejad & Jafari, 2016). (c) microbial starvation, when chitosan acts as chelating agent of metals and essential nutrients affecting microbial development (Wang et al., 2005); and (d) inhibition on synthesis of mRNA and proteins, related to their ability to pass through the cell membrane of a microorganism and subsequently bind to DNA (Hosseinnejad & Jafari, 2016).

Conclusion

Chitosan has a great antifungal activity for *Fusarium* spp. and it considers a promising alternative natural compound for chemical fungicides.

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"تأثير الكيتوزان على عزلات فطرة *الفيوزاريوم* المعزولة من أراضى منطقة الدلتا بمصر"

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قسم النبات و الميكروبيولوجى- كلية العلوم – جامعة طنطا- طنطا - مصر

يعتبر جنس الفيوزاريوم واحدا من اهم الاجناس الفطرية التى تنتشر فى التربة ولها اهمية اقتصادية حيث انه يضم العديد من الأنواع الممرضة والتى تسبب مدى واسعا من الأمراض النباتية.

تم عزل 45 عزلة من الجنس فيوزاريوم من عينات تربة مجمعة من اماكن مختلفة من ثلاث محافظات (محافظة كفر الشيخ ومحافظة الغربية ومحافظة المنوفية) من دلتا جمهورية مصر العربية وتم تعريف هذه العزلات بناء على الصفات المورفولوجية لنمو الفطروالأصباغ التى ينتجها عند تنميته على وسط غذائى صلب والصفات المورفولوجية بعد الفحص المجهرى للجراثيم من حيث الشكل والحجم وتقسيمها الى مجموعات منها فيوزاريوم اوكسيسبورم *F. oxysporum* وفيوزاريوم سولانى *F. solani* وفيوزاريوم فيرتيسليدس *F. verticilliodes* وفيوزاريوم كلاميدوسبورم *F. chlamydosporum*.

تم عمل مقاومة بيولوجية للعزلات باستخدام مادة طبيعية يتم استخلاصها من قشور الكائنات البحرية (الكيتوزان) وليس لها اى تأثيرضار على كلا من التربة اوالنبات وذلك عن طريق تنمية العزلات المختلفة من الفطر على وسط غذائى صلب يحتوى على تركيزات مختلفة من مادة الكيتوزان (1، 1,2، 1,6، 2جم/لترمن الكيتوزان المذاب فى 1% من حمض الاسيتك)، حيث اظهرت النتائج ان تركيز 2جم/لترمن الكيتوزان استطاع ان يحدث تثبيط كلى لنمو العزلات المختلفة .

بدراسة التغيرات الحادثة لجراثيم فطر الكلاميدوسبورم باستخدام الميكروسكوب اللألكترونى نتيجة لاستخدام مادة الكيتوزان حدث زيادة فى سمك الجدار الخلوى، اصبح شكل الجدار الخلوى والغشاء البلازمى غير منتظم مقارنة بجراثيم الفطرالاصلى بالأضافة الى تغيرات فى السيتوبلازم.