1. 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), Geotrichum 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.

2. Materials and methods
2.1. 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. H2O 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. H2O], in Petri dish (three replicates for each dilution). All plates were incubated at 25 ±1°C for 3-7 days 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 KH2PO4, 1.0g KNO3, 0.5g MgSO4.7H2O, 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 Sommerell, 2006).

2.2. 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).

2.3. 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.

\[
(\%) = \left[ \frac{(dc - dt)}{dc} \right] \times 100
\]

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

### 2.4. 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.

### 3. Results

All Fusarium isolates were identified according to their morphological and microscopic characters such as color of mycelium and colony pigmentation, colony diameters on PDA medium, microscopic examination for presence or absence of microconidia, macroconidia 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

<table>
<thead>
<tr>
<th>Fusarium spp.</th>
<th>Morphological characters on PDA medium</th>
<th>Microscopic characters on SNA medium</th>
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<tbody>
<tr>
<td></td>
<td>Abundant white mycelium with pale violet pigment</td>
<td>Abundant, oval to reniform</td>
</tr>
<tr>
<td>F. oxysporum</td>
<td>Abundant white mycelium with pale violet pigment</td>
<td>Short to medium in length, almost straight, with 2-3 septa</td>
</tr>
<tr>
<td>F. solani</td>
<td>Dense, white to pale violet mycelium</td>
<td>Oval with one septa formed in false head</td>
</tr>
<tr>
<td>F. verticilliodes</td>
<td>White to violet mycelia with dark blue pigment</td>
<td>Slightly curved, 3-5 septa</td>
</tr>
<tr>
<td>F. chlamydosporum</td>
<td>Off white, floccose, fast growing mycelium with pigment ranging from yellow to pale brown</td>
<td>Abundant, oval</td>
</tr>
<tr>
<td></td>
<td>Abundant, oval, various size formed in heads</td>
<td>Abundant, rough, formed rapidly, in single, chain or clusters</td>
</tr>
</tbody>
</table>

![Figure 1](image_url): Micro, macro-conidia and chlamydospores of 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

<table>
<thead>
<tr>
<th>Chitosan conc.</th>
<th><em>F. verticilliodes</em></th>
<th><em>F. solani</em></th>
<th><em>F. chlamydosporum</em></th>
<th><em>F. oxysporum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.0 ± 0.00</td>
<td>90.0 ± 0.00</td>
<td>90.0 ± 0.00</td>
<td>90.0 ± 0.00</td>
</tr>
<tr>
<td>0.5 g/L</td>
<td>69.7 ± 0.07</td>
<td>73.7 ± 0.08</td>
<td>71.0 ± 0.05</td>
<td>65.7 ± 0.08</td>
</tr>
<tr>
<td>1.0 g/L</td>
<td>52.3 ± 0.07</td>
<td>61.0 ± 0.09</td>
<td>56.7 ± 0.07</td>
<td>45.0 ± 0.09</td>
</tr>
<tr>
<td>1.2 g/L</td>
<td>34.7 ± 0.09</td>
<td>45.0 ± 0.09</td>
<td>33.0 ± 0.09</td>
<td>31.0 ± 0.07</td>
</tr>
<tr>
<td>1.6 g/L</td>
<td>20.7 ± 0.03</td>
<td>29.7 ± 0.07</td>
<td>25.7 ± 0.03</td>
<td>21.0 ± 0.09</td>
</tr>
<tr>
<td>2.0 g/L</td>
<td>7.0 ± 0.03</td>
<td>12.3 ± 0.03</td>
<td>8.3 ± 0.03</td>
<td>5.0 ± 0.00</td>
</tr>
</tbody>
</table>

**Figure 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).

**Figure 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.

4. 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*, and *F. equiseti* and *F. verticillioides* 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).
5. Conclusion

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

References


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


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


"تأثير الكيتوزان على عزلات فطرة *فيوزاريوم* المعزولة من أراضي منطقة الدلتا بمصر" 
أ/ علاء مصطفى ابوزيد, أ/ سعيدة محمد عامر, أ/ محمد ياسر بديوى, د/ محمد عبد الصمد الهيتى
قسم النبات وال mikrobiyoloji, كلية العلوم – جامعة طنطا - طنطا - مصر

يعتبر جنس الفيوزاريوم واحدا من اهم الاجناس الفطرية التي تنتشر في التربة ولها أهمية اقتصادية حيث انه يضم العديد من الأنواع الممرضة والتي 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 وتتم تعرف هذه العزلات بناء على الصفات المورفولوجية لمрон الفطر واصابات التي ينتجها عند تنموته على وسط غذائي صلب والصفات المورفولوجية بعد الفحص المجهرى للجراثيم من حيث الشكل والحجم وتسميتها إلى مجموعات منها فيوزاريوم اوکسپوروم *F. oxysporum* وفيوزاريوم سولاني *F. solani* وفيوزاريوم فيرتيلايديس *F. verticilliodes* وفيوزاريوم كلاميدوسورم *F. chlamydosporum*.

تم عمل مقاومة بيولوجية للعزلات باستخدام مادة طبيعية يتم استخلاصها من شور الكائنات البحرية (الكتوزان) وليس لها اي تأثير ضار على كلا من التربة أو النباتات وذلك عن طريق تنمية العزلات المختلفة من الفطر على وسط غذائي صلب يحتوي على تركيزات مختلفة من مادَّة الكيتوزان (1, 1,2, 1,6, 2)جم/لتر من الكيتوزان المذاب في 1% من حمض الاسيت، حيث اظهرت النتائج ان تركيز 2جم/لتر من الكيتوزان استطاع ان يحدث تأثير كبير لنمو العزلات المختلفة.

بدراسة التغييرات الحادة لجراثيم قطر الكلاميدوسورم باستخدام الميكروسكوب الاكثونائي نتيجة استخدام مادة الكيتوزان حدد زيادة في سمك الخلايا، أصبح شكل الخلايا البلانزيمي غير منتظم مقارنة بجراثيم الفطر الأصلي بالإضافة إلى تغيرات في السيتيولازم.