sciendo

Agriculture (Poľnohospodárstvo), 68, 2022 (2): 75− 86

Original article

DOI: 10.2478/agri-2022-0007

CHITOSAN AND *TRICHODERMA HARZIANUM* A NEW CHALLENGE FOR MANAGING FUSARIUM CROWN AND ROOT ROT IN ASPARAGUS PLANT

KOWSAR SHIRAZI[1], NARJES KHATON RAMESH[2], MOHAMMAD REZA ASEF[3]*

[1]Zabol University Zabol, Iran
[2]Islamic Azad University, Tehran, Iran
[3]Education and Extension Organization (AREEO), Tehran, Iran

Shirazi, K., Ramesh, N.K., and Asef, M.R. (2022). Chitosan and *Trichoderma harzianum* a new challenge for managing Fusarium crown and root rot in asparagus plant. *Agriculture (Poľnohospodárstvo), 68*(2), 75–86.

Fusarium oxysporum f. sp. *asparagi* is one of the main problems associated with the roots of asparagus plants and inflicting serious losses worldwide. A novel and eco-friendly strategy to reduce pathogen resistance and management of the Fusarium crown and root rot is using chitosan and *Trichoderma harzianum* as a sustainable treatment approach to improve both disease resistance and yields, while also effectively managing the asparagus product. In this study, the effect of chitosan and *Trichoderma harzianum* on growth promotion and control of Fusarium crown and root rot in asparagus plants was investigated. Diseased plants from the main production areas of Iran were surveyed. The *in vitro* antifungal properties of chitosan solutions (concentrations of 5, 10, 15 and 20 mg/mL) and *T. harzianum* strain were evaluated by the dual culture method. *T. harzianum and* chitosan solution (20 mg/mL) gave the largest inhibition zone against *F. oxysporum* and were selected for further studies in the greenhouse. Chitosan (20 mg/mL) and *T. harzianum* were applied as seed treatments and they significantly reduced disease incidence by 92% and 73% respectively, compared to the control.

Key words: *Asparagus officinalis* L., chitosan, *Trichoderma harzianum*, Fusarium crown and root rot, antifungal activity, biological control

Asparagus (*Asparagus officinalis* L.) is one of the most important vegetable and medicine products worldwide, belongs to the lily family grown as a perennial crop (De la Lastra *et al*. 2021). In recent decades, the crop has the greatest increase due to the demand of consumers for healthy foods with high nutritional value such as dietary fibre and high content of natural antioxidants (Lee *et al*. 2014). Soil-borne pathogens can have considerable detrimental effects on asparagus growth and production. *Fusarium oxysporum* f. sp. *asparagi* is one of the most important and economic diseases (Brizuela *et al*. 2020) which can cause Fusarium crown and root rot of asparagus in Iran and most producing areas and was first described more than a century ago by Cohen and Heald (1941).

Efforts to manage the Fusarium crown and root rot are challenging and difficult because of asparagus wide and deep-spread root system (Brizuela *et al*. 2020), the persistence of the *Fusarium* sp. in plant debris and soil over many years and its systemic propagation of asparagus plants (Elmer 2015). The chemical fungicidal application is inefficient and affects human health, wildlife, and the environ-

Kowsar Shirazi, Department of Plant Protection, Faculty of Agriculture, Zabol University, Zabol, Iran.
Narjes Khaton Ramesh, Department of Plant Protection, College of Agricultural Sciences and Food Industries, Science and Research Branch, Islamic Azad University, Tehran, Iran.
Mohammad Reza Asef (*Corresponding author), Department of Botany, Research Institute of Plant Protection, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran. E-mail: asefmr@yahoo.com

© 2022 Authors. This is an open access article licensed under the Creative Commons Attribution-NonComercial-NoDerivs License (http://creativecommons.org/licenses/by-nc-nd/4.0/).

ment negatively. Currently, to avoid the use of fungicides, alternative methods are emerging to control plant pathogens (Kappel *et al*. 2022).

Biological control has emerged as one of the most sustainable control strategies in the world that is highly recommended and encouraged (Lahlali *et al*. 2022). The potential of biological control agents to suppress the disease caused by the Fusarium crown and root rot of asparagus has been evaluated, but so far, the success of these efforts has been moderate (Farahani-Kofoet *et al*. 2020). The stimulation of root health by colonizing roots with arbuscular mycorrhizae (Haque & Matsubara 2018), endophytic fungi (Surono & Narisawa 2018) and rhizospheric fungi (Ruangsanka 2014) has been used for achieving tolerance to *Fusarium* attack.

The fungus *Trichoderma harzianum* is known to be a promising tool in biological control, which is commonly existing in soil, root, and foliar ecosystem and can be effective against many soil-borne pathogenic fungi such as *Fusarium* strains (Xu *et al*. 2022). *T. harzianum* can confer biocontrol either directly by interacting with pathogens via mycoparasitism, competition for nutrients, colonization of root surfaces, and penetrating the epidermal cells to enhance root growth indirectly and induce the host resistance (Hermosa *et al*. 2012). Accordingly, *T. harzianum* has become one of the most extensively used beneficial fungi for the biocontrol of soil-borne fungal diseases and agricultural crop improvement (Pedrero-Méndez *et al*. 2021; Mukherjee *et al*. 2022).

Chitosan is also known worldwide for its potential as a strong antimicrobial agent and plant resistance enhancer considered against a variety of plant pathogens (Orzali *et al*. 2017) that can control pathogenic microorganisms by preventing growth, sporulation, spore viability, germination and cell disrupting and triggering the defence responses in the host plant by inducing and or inhibiting different biochemical activities during the plant pathogen interaction, but their widespread use in agriculture is still pending (Hassan & Chang 2017). To control plant pathogens, chitosan has been applied to seeds or roots and is utilized in the form of seed soaking, spraying, soil remediation, and foliar spraying, thereby promoting pathogen resistance and increasing growth simultaneously (Badawy & Rabea 2011).

The objective of this work was to evaluate the ability of biological control agents, including chitosan and *T. harzianum*, to control the disease caused by *F. oxysporum* f. sp. *asparagi* under both *in vitro* and greenhouse conditions. Furthermore, it was determined that these biocontrol agents could increase the growth of asparagus plants.

MATERIAL AND METHODS

Isolation of the fungal pathogen

Fusarium sp. was isolated from root samples of symptomatic asparagus plants collected from the fields in Iran. The infected roots were washed under tap water, chopped into 2 cm small pieces, and surface sterilized in 0.5% NaOCl for two minutes then rinsed twice with triple distilled water, placed on WA (water agar) and kept in an incubator at 25°C dark conditions. After five days of incubation, small colonies of fungus appeared and were transferred to the PDA (potato dextrose agar- PDA: Merck, Germany) media under the dark condition to isolate the fungus pathogen. All the procedure was carried out in a laminar hood under sterile condition (Corpas-Hervias *et al*. 2006).

Morphological characterization

For morphological identification, single spore isolates were grown for 10 – 15 days on PDA media. A total of twenty isolates of *Fusarium* spp. were identified based on colony morphology, characteristics of macro and microconidia, conidiophores and chlamydospores measurements by following the standard mycological manual of Leslie and Summerell (2006).The fungal isolates were stained using lactophenol cotton blue (Hardy Diagnostics, California, USA). The microscopic slides of each isolate were observed under a light fluorescence microscope (Olympus Bx51, Tokyo, Japan).

Molecular identification and phylogenetic analyses

The DNA of all twenty *Fusarium* isolates was extracted using the method given by Zhong and Steffenson (2001). The molecular identification of *Fusarium* isolates was carried out based on conserving ribosomal internal transcribed spacer (ITS) region. The universal primers of ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTC-

CGCTTATTGATATGC-3') were used for amplification of the ITS1-5.8S-ITS2 region of rDNA (ITS-rDNA) (Singha *et al*. 2016) for the identification of *Fusarium oxysporum*. PCR reactions were carried out in a 25µl reaction volume containing 2.5 µl of PCR buffer (10 mM Tris-HCl, 2.5 mM MgCl2 and 50 mM KCl, pH 8.8), 2 mM each dNTP, and 1 µM of each primer ITS-1 and ITS-4, 8 µl of Taq DNA Polymerase, 4 ng genomic DNA and 6.5 µl deionized water. DNA amplification was performed on an Eppendorf Thermal Cycler consisting of an initial denaturation temperature of 95°C for 2 min, followed by 35 cycles of template denaturation for the 30s at 95°C, primer annealing for 1 min at 56°C and chain elongation for 2 min at 72°C, with a final extension of 8 min at 72°C. Amplification conditions were similar to those described by Mishra *et al*. (2003). The amplified PCR products were verified using 1.5% agarose gel electrophoresis at 65 V for 150 min and directly sequenced in one direction by Bioneer Company (Seoul, South Korea). The ITS nucleotide sequence was compared with sequences available in the public domain databases NCBI (National Center for Biotechnology Information; www.ncbi.nih.gov) using the basic local alignment search tool (BLAST). The DNA sequence result was deposited in GenBank, and the accession number is provided. The original sequence was edited using MEGA version 7.0 software and aligned using the Clustal W version 1.8 program. The Phylogenetic tree was created using the neighbor-joining (NJ) method with bootstrap values calculated from 1,000 replicate runs (Tamura *et al*. 2013) and *Aspergillus oryzae* was used as an outgroup in the analysis.

Preparation and stored of Trichoderma harzianum strain.

The strain of *T. harzianum* (5233 PTCC No.) was obtained from the Persian Type Culture Collection (PTCC), Tehran, Iran. The strain was cultured in PDA and was stored at 25ºC in an incubator with a 12 h/12 h light cycle.

Preparation of chitosan

A commercial preparation of chitosan (degree of N-deacetylation 75%; Sigma-Aldrich, USA) of crustacean origin was purchased from Sigma-Aldrich. The solution was adjusted to pH 5.6 by dispersing it in 0.25% acetic acid solution (Merck, Germany) and for the complete dissolution of its particles kept overnight under a magnetic stirring. Then concentrations of 5, 10, 15, and 20 mg/mL chitosan solution were prepared from this stock and the best concentration was used (Qing *et al*. 2015).

Antifungal activity of Chitosan in vitro

Fusarium oxysporum f. sp. *asparagi* was grown on PDA and prepared suspension 10^6 CFU/mL concentration (an optical density of OD_{600}=1.00) by using a spectrophotometer, 1 mL of this suspension was added to 15 mL of PDA (45°C) and poured into a Petri dishes. The disk diffusion method was done. The filter paper disks (5 mm diameter) were autoclaved and impregnated with different chitosan concentrations (5, 10, 15, and 20 mg/mL), then allowed to dry and eventually, placed on PDA plates. Negative controls were done with sterile distilled water in the same way. All the plates were incubated for a week at 25ºC. The extent of growth inhibition zones around each of the disks were quantified (Berkow *et al*. 2020).

Antifungal activity of Trichoderma harzianum in vitro

To evaluate the antifungal activity of *T. harzianum* strain against *F. oxysporum* f. sp. *asparagi,* dual culture technique, was performed. The 5-day-old culture mycelial plugs (5 mm in diameter) were taken from the edge of an actively growing colony of antagonistic strain and fungal pathogen. Then inoculated on two opposite sides of 90 mm PDA plates with a distance of 75 mm, while only mycelial plugs of pathogen isolate inoculated PDA plates were used as controls. To record the distance of the mycelial inhibition zone, all plates were incubated at 25°C for 7 days in the darkness (Xu *et al*. 2022). The percentage mycelial inhibition (PMI) rate was calculated as follows:

$$(PMI)\% = (Rc - Ri) / Rc \times 100$$

where Rc refers to the mycelial growth of fungal pathogens in control plates (mm) and Ri refers to the mycelial growth of fungal pathogens in dual culture plates [mm] (Chang *et al*. 2020).

Fungicide chemical activity of propiconazole in vitro

Propiconazole (1,000 ppm) as a commercial

chemical fungicide was considered against *F. oxysporum*. A fungicide was added to PDA Petri dish, then centrally inoculated with a 5 mm plug of *F. oxysporum* and incubated for a week at 25ºC. The inhibition effects were assessed by measuring the diameters of the inhibition zones (Mondani *et al*. 2021).

Greenhouse experiment

Greenhouse experiments were performed to evaluate the control efficacy of chitosan (20 mg/mL showed the highest percentage of fungal growth inhibition), *T. harzianum* strain and propiconazole (1,000 ppm) against *F. oxysporum*. For this purpose, asparagus seeds were sterilized by immersion with 10% sodium hypochlorite for 10 min, then rinsed three times with sterile water and dried on a sterile paper towel at 28°C in the dark. Seeds were immersed separately in 1 mL chitosan solution (20 mg/mL), 1 mL of fresh suspension of *T. harzianum* on 10^6 CFU/mL and 1 mL propiconazole (1,000 ppm) respectively and incubated at room temperature for 1 h (Reid *et al*. 2002). Treated seeds were germinated on WA (water agar 3.8%) plates for 2 weeks at 25°C in the dark. Once seedlings reached an appropriate size (1 – 1.5 cm), they were inoculated by immersion of the roots in a suspension of 10^6 CFU/mL of *F. oxysporum* for 4 h. Control plants were root-dipped in distilled water. Then clean, uniform seedlings were transferred to plastic pots (15 × 15 cm) filled with 500 gr sterilized sandy loam soil. Plants were watered twice weekly. These experiments were carried out three times for each biological agent with three replications. Plants were grown for 8 weeks in a completely randomized design and evaluated weekly for disease severity based on a 1 – 5 scale, where 1 = 1 – 20%, 2 = 21 – 40%, 3 = 41 – 60%, 4 = 61 – 80% and 5 = 81 – 100% of Fusarium crown and root rot. Finally, the roots were removed from the pots and rinsed with tap water (Brizuela *et al*. 2020), then the disease incidence and biocontrol efficacy were assessed by the following formula:

Crown and root rot incidence [%] = {(scale ×number of plants infected)/(highest scale × total number of plants)} ×100

Biocontrol efficacy [%] = {(Disease incidence of control – Disease incidence of antagonist) / Disease incidence of control} × 100

Asparagus growth promotion

The effectiveness of the treatments was evaluated by counting the number of dead plants weekly and measuring the shoot and root length, fresh and dry weight. The shoots of all the asparagus plants were placed in an oven and dried at the 70°C for 24 h. Dry shoots were weighted, and the growth rate was calculated using the formula subcriteria:

GPE [%] = {(Plant parameter of treated plants – Plant parameter of control) / Plant parameter of control} ×100 (Alelign 2021).

Statistical analysis

All *in vitro* experiments with four repetitions and the greenhouse experiments with three repetitions were statistically evaluated using SAS 9.1 software. Most data obtained were subjected to Duncan's multiple range test, when one-way ANOVA revealed significant differences ($P < 0.05$).

RESULTS

Morphological characterization

In this experiment, twenty *Fusarium oxysporum* subspecies were derived from the roots of infected asparagus plants. The morphological characteristics of this serious and common disease are as follows: *Fusarium oxysporum* f. sp. *asparagi* Schltdl., *Fl. berol.* (Berlin) 2: 139 (1824), (also see Species *Fusarium asparagi* Briard 1890); *Nectriaceae.*

Colonies on PDA reached 75 mm in diameter after 10 days at 25°C under dark conditions and became white cottony with pigmentations from pale violet to peach-violet. Aerial hyphae were white to white-violet. Spores were oval to ellipsoid and kidney-shaped. The conidiophores were branched monophialides, short and wide. Sporodochiums were orange to brown and relatively common. Macroconidia were straight and relatively slender to develop singly, formed in false heads on short monophialides, with 3 septate macroconidia and 35 – 48 (41.5) × 3 – 6.5 (4.75) µm and 4 – 12 (8) × 4 – 7 (5.5) µm. Chlamydospores with a smooth or rough wall appearance formed singly or in pairs (Figure 1). The morphological features of the investigated isolate were similar to the description provided by Leslie and Summerell (2006).

Molecular identification

Molecular identification of *F. oxysporum* isolate was done by amplifying the internal transcribed spacer (ITS) region of the conserved ribosomal DNA using primers ITS1 and ITS4. The total size of the ITS1-5.8S-ITS2 region of rDNA isolates studied varied from 380 to 620 bp (Figure 2).

The sequence obtained from the amplification of conserved ribosomal ITS region was compared with sequences from National Center for Biotechnology Information (NCBI) database using BLAST. This sequence was identified and deposited in NCBI GenBank and the accession number which was provided is ON350777. ITS sequence of *Fusarium oxysporum* f. sp. *asparagi* isolates KSH86 was aligned with the consensus region using the Clustal W program. The Phylogenetic tree based on the neighbor-joining method with 1,000 bootstrap replicates were performed and *Aspergillus oryzae* was used as an outgroup in the analysis (Figure 3).

Antifungal effect of chitosan in vitro

Based on our observation, all concentrations of chitosan (5, 10, 15, and 20 mg/mL) had an antifungal effect against *F. oxysporum* in the filter paper disk method (Figure 4). Data has shown there was a significant difference among the concentration of chitosan in the percentage reduction of inhibition zones [cm] about 19.65 mm ($P \leq 0.05$) (Table 1). Concentrations of 20 mg/mL had the greatest effect compared to the other ones and showed 77.46% mycelial growth inhibition. An increase in the inhibition zone was also clearly observed with increasing chitosan concentration. Therefore, it was chosen for the greenhouse experiment.

Antifungal effect of Trichoderma harzianum in vitro

Dual culturing results showed that *T. harzianum* strain limited the growth of *F. oxysporum* and had a significant inhibition zone of about 32.92 mm against the growth of this pathogen as shown in

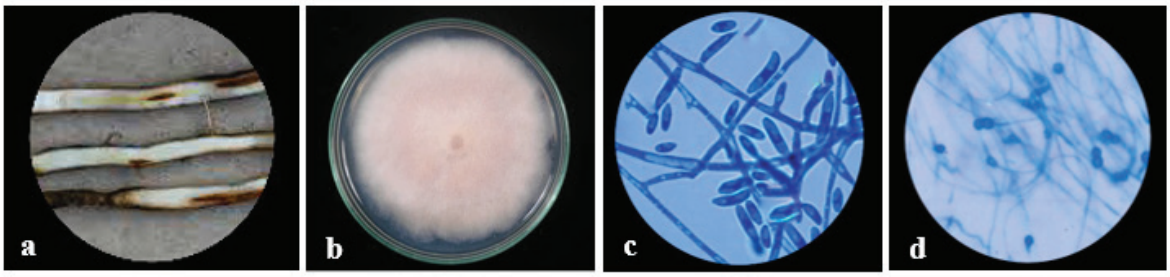


Figure 1. Morphological characteristics of *Fusarium oxysporum*. a: Fusarium crown and root rot of asparagus, b: white cottony colony and pigmentation, c: microconidia and macroconidia and d: chlamydospores.

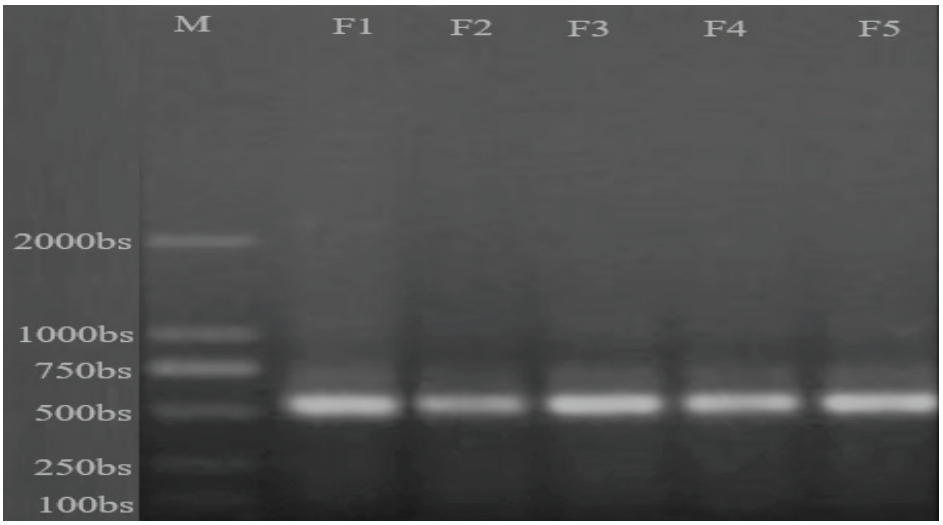


Figure 2. Amplification of conserved ribosomal ITS regions of *Fusarium oxysporum* f. sp. *asparagi* using the primers ITS-1 and ITS-4.

Figure 5. Furthermore, *T. harzianum* suppressed the mycelial growth of *F. oxysporum* at a relative percentage of 62.24%, which was significantly higher ($P \leq 0.05$) than the control (Table 1), so that was also selected for the greenhouse experiment.

Fungicide chemical activity of propiconazole in vitro

The fungal pathogen isolates included in the study, behaved differently on the fungicide applied. Propiconazole had the weakest impact on *F. oxysporum* (Figure 6), around 15% while both biocontrol

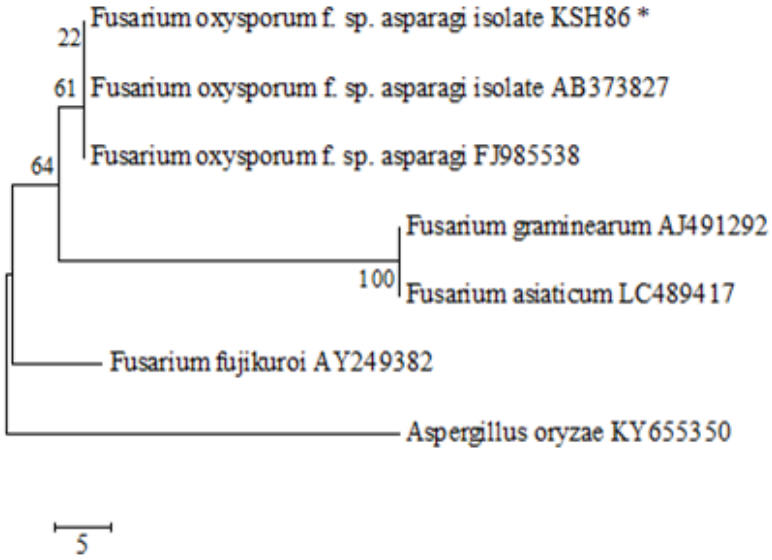


Figure 3. Phylogenetic tree based on ITS rDNA gene sequences of the *Fusarium oxysporum* isolated from asparagus along with the sequences from NCBI. The analysis was conducted with MEGA (version 7.0) using the neighbor-joining method with 1,000 bootstrap replications. *Aspergillus oryzae* (KY655350) was used as an outgroup in the analysis.

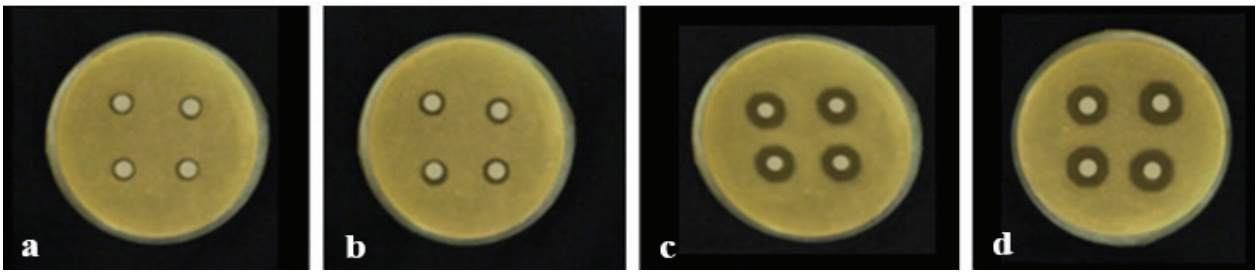


Figure 4. Antagonistic activity of chitosan against growth of *F. oxysporum*. a: concentration of 5 mg/ml, b: concentration of 10 mg/mL, c: concentration of 15 mg/mL and d: concentration of 20 mg/mL.

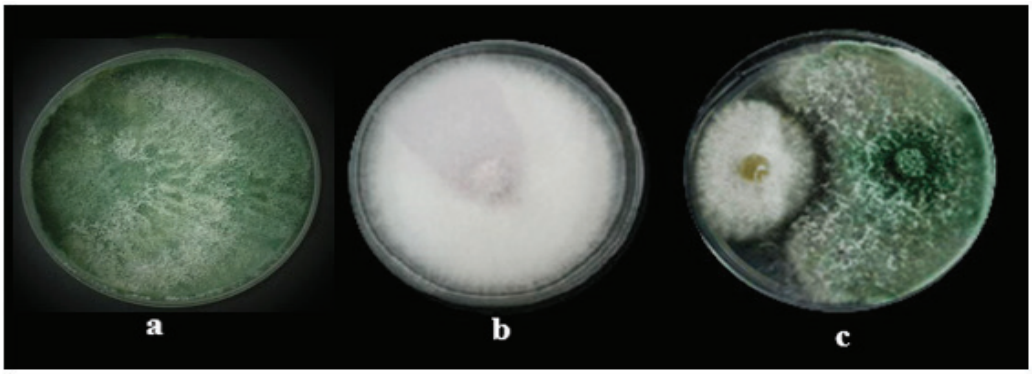


Figure 5. Antifungal activity of *T. harzianum* against growth of *F. oxysporum*. a: *T. harzianum*, b: *F. oxysporum* and c: inhibitory zone of *T. harzianum* against *F. oxysporum*.

agents were more effective on *F. oxysporum*. The interaction between propiconazole and the tested fungal pathogen was significant and higher ($P \leq 0.05$) than in the control (Table 1).

Biocontrol of Fusarium crown and root rot in greenhouse

The influence of chitosan (20 mg/mL) and *T. harzianum* significantly reduced Fusarium crown and root rot intensity, compared with control plants. The results recorded the the highest percentage reduction of disease incidence and efficacy, respectively. Both these biological control agents applied as a seed treatment significantly reduced Fusarium crown and root rot, 92.19% and 72.99% respectively. Furthermore, propiconazole showed growth inhibition of 45.60% and had less effect when compared to other biological control agents. In this study, the chitosan applied was the best treatment for reducing the severity of asparagus crown and root rot (Table 2).

Asparagus growth promotion

In this study, both chitosan (20 mg/mL) and *T. harzianum* strain stimulated the growth of inoculated asparagus plant and increased growth promotion. Chitosan (20 mg/mL) and *T. harzianum* strain in the presence and absence of *F. oxysporum* significantly

T a b l e 1

Inhibitory effect of chitosan, propiconazole and *T. harzianum* against *F. oxysporum in vitro*

Treatments	Mean diameter of mycelial growth of pathogen [mm]	Percentage of mycelial growth inhibition [%]
F. oxysporum + Chitosan 5 mg/mL	66.12^{c}	24.17^{c}
F. oxysporum + Chitosan 10 mg/mL	58.72^{d}	32.66^{d}
F. oxysporum + Chitosan 15 mg/mL	40.18^{e}	53.92^{c}
F. oxysporum + Chitosan 20 mg/mL	19.65^{g}	77.46^{a}
F. oxysporum + *T. harzianum*	32.92^{f}	62.24^{b}
F. oxysporum + Propiconazole	73.45^{b}	15.76^{f}
F. oxysporum + Distilled water (control)	87.20^{a}	0.00^{g}

Different letters in the same column indicated that the values were significantly different ($P < 0.05$) according to Duncan's multiple range test.

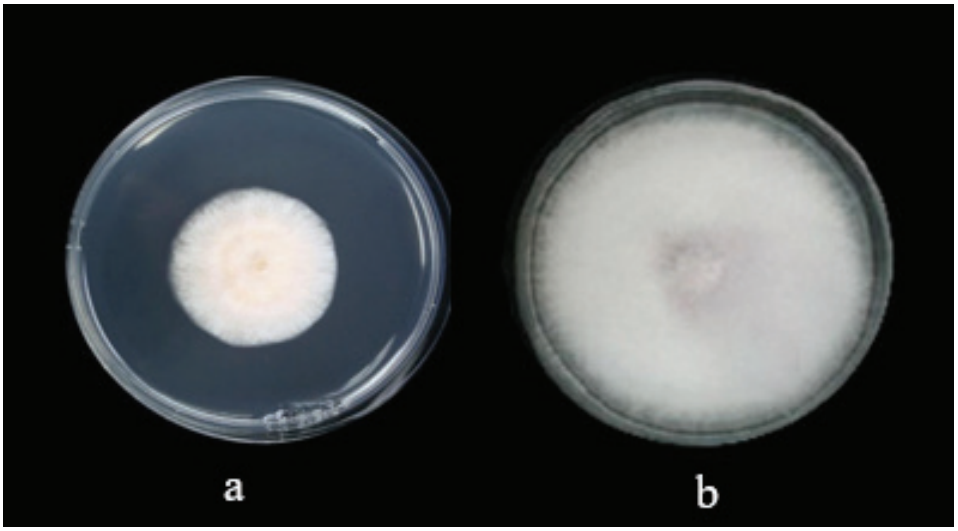


Figure 6. Fungicide impact against growth of pathogen. a: *F. oxysporum* on a Petri dishes with propiconazole and b: Control.

increased shoot and root length and their fresh and dry weight, compared with control plants. Although, the most significant increase was observed in infected plants which were treated with chitosan (20 mg/mL) (Table 3 and Figure 7).

Chitosan (20 mg/mL) and *T. harzianum* strain also increased the growth of asparagus plants which were non-infected by *F. oxysporum* in comparison with the control, 32.65 cm and 30.66 cm respectively. Although, chitosan (20 mg/mL) had the most stimulatory effect on asparagus plants' growth. However, there were no significant differences between the effect of chitosan (20 mg/mL) and *T. harzianum* strain on the shoot and root length, of non-infected plants with *F. oxysporum*. But, both fresh and dry shoot and root weights of inoculated plants with *T. harzianum* strain without any isolate of *F. oxysporum* were significantly differences in comparison with the control.

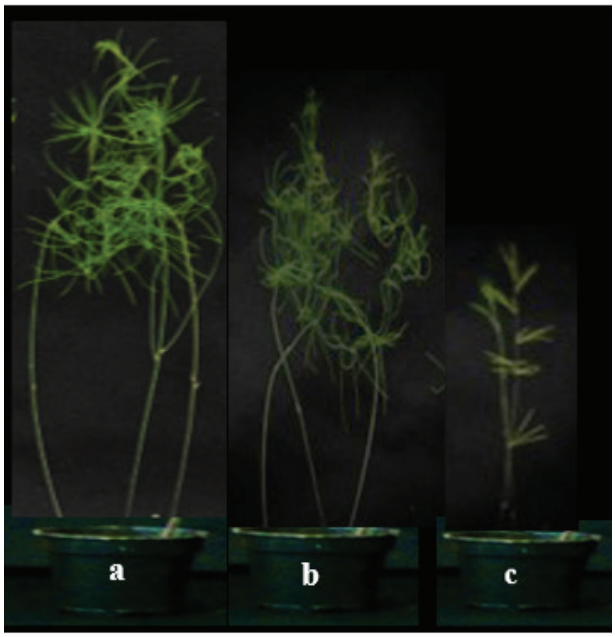


Figure 7. Effect of chitosan and *T. harzianum* on asparagus growth promotion, a: chitosan, b: *T. harzianum*, c: control.

T a b l e 2

Effect of chitosan and *T. harzianum* on Fusarium crown and root rot in asparagus plant in the greenhouse

Treatments incidence [%]	Crown and root rot incidence [%]	Biocontrol efficacy [%]
F. oxysporum + Chitosan (20 mg/mL)	6.50^{c}	92.19^{a}
F. oxysporum + *T. harzianum*	22.50^{b}	72.99^{b}
F. oxysporum + Propiconazole	45.33	45.60
F. oxysporum + Distilled water (control)	83.33^{a}	0.00^{c}

Values followed by the same letter are not statistically significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

T a b l e 3

Effect of chitosan and *T. harzianum* on plant growth promotion of asparagus, under greenhouse conditions

Treatments	Shoot height [cm]	Root length [cm]	Shoot fresh weight [g]	Root fresh weight [g]	Shoot dry weight [g]	Root dry weight [g]
Chitosan (20 mg/mL) + *F. oxysporum*	23.45^{b}	7.50^{b}	8.33^{c}	12.44^{c}	1.05^{b}	1.33^{c}
T. harzianum + *F. oxysporum*	19.33^{c}	4.10^{bc}	5.25^{d}	9.25^{d}	0.20^{c}	0.85^{d}
Chitosan (20 mg/mL)	32.65^{a}	16.95^{a}	18.25^{a}	22.85^{a}	2.50^{a}	3.40^{a}
T. harzianum	30.66^{a}	15.00^{a}	12.10^{b}	16.75^{b}	1.50^{b}	2.25^{b}
Distilled water (control)	9.53^{d}	2.50^{c}	1.30^{c}	2.10^{c}	0.09^{c}	0.20^{d}

Values followed by the same letter are not statistically significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

DISCUSSION

Soil-borne fungal pathogens cause irreparable economic losses to agriculture worldwide. Nowadays, alternative control methods without using fungicides, mainly on chemical-synthetic origin, that can be harmful to human health and the environment are considered (Du *et al*. 2022). Numerous studies have been published about the ability of eco-friendly agents to control soil-borne fungal pathogens in different crops (Carlucci *et al*. 2022; Gao *et al*. 2022; Viejobueno *et al*. 2021), however there are few studies based on biological agents to control Fusarium crown and root rot in asparagus plant, as far as we know (Elmer 2015; Farahani-Kofoet *et al*. 2020).

In this study, we have evaluated two of these promising alternatives, named the natural product chitosan (5, 10, 15 and 20 mg/mL) and *T. harzianum* strain, because of their demonstrated biological control activity against different crop diseases tested earlier (Zohara *et al*. 2019; Stasinska-Jakubas & Hawrylak-Nowak 2022; Tyskiewicz *et al*. 2022). This is the first report on the potential use of chitosan and *T. harzianum* to control Fusarium crown and root rot and asparagus plant growth promotion, which is consistent with the result of Kappel *et al.* (2022), who reported that chitosan had strong *in vitro* antifungal activities against *F. oxysporum* on sugar beet plants.

In vitro tests, through dual culture and filter paper disk experiments, clearly demonstrate that the *F. oxysporum* showed inhibition by *T. harzianum* and all concentrations of chitosan (5, 10, 15 and 20 mg/mL). The comparison of the different chitosan concentrations enabled us to select the most effective biocontrol agent against *F. oxysporum*. The concentration of 5 mg/mL of chitosan showed the least efficacy, but the concentration of 20 mg/mL turned out to be the most promising agent with high fungicidal effects on *F. oxysporum* and inhibition of nearly 80% was observed. This is in line with other published findings on fungal pathogens (Stamford *et al*. 2010; Onaran *et al*. 2021) and other *Fusarium* spp. (Li *et al*. 2009; Deshaies *et al*. 2022; Filyushin *et al*. 2022).

Greenhouse studies with asparagus seedlings in the pots using chitosan (20 mg/mL) and *T. harzianum* indicated that both increased shoot height, root length, root and shoot weights and decreased root disease compared with the infested control when *F. oxysporum* f. sp. *asparagi* was used. In other research, chitosan solution at different concentrations showed effective antifungal activity against the strain *Candida albicans* compared to the control (Shih *et al*. 2019). Also, in the current study, chitosan reduced the Fusarium crown and root rot in asparagus with a seed treatment. Reducing disease with chitosan has been demonstrated in previous studies, for example, the ability of chitosan has been shown to inhibit the development of leaf spot disease caused by *Lasiodiplodia theobromae* in Kiwifruit trees (Wang *et al*. 2022). Other studies have shown that chitosan reduces wilt incidence, mainly caused by *Harpophora maydis* in maize cultivations (Hassan *et al.* 2022). Also, chitosan solution had shown the potential for control of early blight and stem rot of *Solanum tuberosum* (Kumar *et al*. 2022). Chitosan can alter cell permeability by forming an impermeable layer and preventing nutrients from entering the cell. As a result, the growth of the hyphae may stop due to inadequate nutrient intake (Ke *et al*. 2022).

Furthermore, in this research, *T. harzianum* had an inhibitory effect against *F. oxysporum* and reduced Fusarium crown and root rot in the asparagus plant compared to the control conditions, which is in agreement with the result of Hewedy *et al.* (2019) that showed *T. harzianum* reduced *F. oxysporum* infection of pepper. Similarly, AL-surhanee (2022) found that the application of *T. harzianum* reduced disease caused by *F. oxysporum* in tomato plants. Also, our findings were consistent with the results of Bochicchio *et al.* (2022), who found *T. harzianum* increased the shoot, root and dry weight of wheat plants. *Trichoderma* species in biocontrol programs are mainly used as seed coatings. After some research, similar to our data with *T. harzianum* coating, it has been shown that the germination of plant species improves their vital index and defence mechanism (Vitti *et al*. 2015; Harman *et al*. 2021). Some species of *Trichoderma* could penetrate the epidermis and cause long-term colonization at the root surfaces, which in turn leads to changes in metabolism and plant expression characteristics (Subramaniam *et al*. 2022). Our data suggest that *T.*

harzianum is a candidate to replace common fungicides by promoting seedling germination, growth, and plant health, as well as inducing multiple systemic resistance.

Our trials with both active ingredients had a positive effect on the development, growth promotion, and induction of systemic resistance in asparagus. It is thus conceivable that using chitosan and *Trichoderma* spp., may be used as a powerful tool to activate crop control that can reduce pathogen resistance but stimulate plant defence responses. Combined or intermittent applications of these biological control agents would be a new and fascinating tool, but there are still many studies to be done in this field.

CONCLUSIONS

The current work clearly showed the biocontrol efficiency of chitosan (20 mg/mL) and *T. harzianum* against Fusarium crown and root rot in asparagus plants caused by *F. oxysporum*, reduced pathogen growth and increased plant growth. Overall, eco-friendly agents, such as those studied in this research, have demonstrated multifaceted beneficial effects related to increasing plant growth and health. However, more studies on the precise mode of action and the mechanism of these biocontrol agents may well help in the timely and appropriate use of these agents, which could improve the growth and health of plants. The potential increased use of these beneficial agents may further help in reducing problems associated with the use of synthetic chemicals in agriculture.

Conflict of Interests: The authors declare that they have no conflict of interest.

REFERENCES

Alelign, S. (2021). Evaluation of the efficacy of *Trichoderma* and *Pseudomonas* species against bacterial wilt *Ralstonia* isolates of tomato (*Lycopersicum* species). *African Journal of Microbiology Research*, *15*(5), 262 – 271. DOI:10.5897/AJMR2021.9523.

AL-surhanee, A.A. (2022). Protective role of antifusarial eco-friendly agents (*Trichoderma* and salicylic acid) to improve resistance performance of tomato plants. *Saudi Journal of Biological Sciences*, *29*(4), 2933 – 2941. DOI:10.1016/j.sjbs.2022.01.020.

Badawy, M.E.I. and Rabea, E.I. (2011). Biopolymer chitosan and its derivatives as promising antimicrobial agents against plant pathogens and their applications in crop protection. *International Journal of Carbohydrate Chemistry*, *2011*(29), 1 – 29. DOI:10.1155/2011/460381.

Berkow, E.L., Lockhart, S.R., and Ostrosky-Zeichner, L. (2020). Antifungal susceptibility testing: current approaches. *Clinical Microbiology Reviews*, *33*(3), 1 – 30. DOI:10.1128/CMR.00069-19.

Bochicchio, R., Labella, R., Vitti, A., Nuzzaci, M., Logozzo, G., and Amato, M. (2022). Root morphology, allometric relations and rhizosheath of ancient and modern tetraploid wheats (*Triticum durum* Desf.) in response to inoculation with *Trichoderma harzianum* T-22. *Plants*, *11*(2), 1 – 14. DOI:10.3390/plants11020159.

Brizuela, A.M., De la Lastra, E., Marín-Guirao, J.I., Gálvez, L., de Cara-García, M., Capote, N., and Palmero, D. (2020). *Fusarium* consortium populations associated with asparagus crop in Spain and their role on field decline syndrome. *Journal of Fungi, 6*(4), 1 – 23. DOI:10.3390/jof6040336.

Carlucci, A., Raimondo, M.L., Colucci, D., and Lops, F. (2022). *Streptomyces albidoflavus* strain CARA17 as a biocontrol agent against fungal soil-borne pathogens of fennel plants. *Plants*, *11*(11), 1 – 12. DOI:10.3390/plants11111420.

Chang, X., Yan, L., Naeem, M., Khaskheli, M.I., Zhang, H., Gong, G., Zhang, M., Song, C., Yang, W., Liu, T., and Chen, W. (2020). Maize/soybean relay strip intercropping reduces the occurrence of *Fusarium* root rot and changes the diversity of the pathogenic *Fusarium* species. *Pathogens*, *9*(3), 1 – 16. DOI:10.3390/pathogens9030211.

Cohen, S.I. and Heald, F.D. (1941). Wilt and root rot of asparagus is caused by *Fusarium oxysporum* (Schlecht.). *Plant Disease Reporter*, *25*(1), 503 – 509.

Corpas-Hervias, C., Melero-Vara, J.M., Molinero-Ruiz, M.L., Zurera-Muñoz, C., and Basallote-Ureba, M.J. (2006). Characterization of isolates of *Fusarium* spp. obtained from asparagus in Spain. *Plant Disease*, *90*(11), 1441 – 1451. DOI:10.1094/PD-90-1441.

De la Lastra, E., Marín-Guirao, J.I., López-Moreno, F.J., Soriano, T., de Cara-García, M., and Capote, N. (2021). Potential inoculum sources of *Fusarium* species involved in asparagus decline syndrome and evaluation of soil disinfestation methods by qPCR protocols. *Pest Management Science*, *77*(10), 4749 – 4757. DOI:10.1002/ps.6519.

Deshaies, M., Lamari, N., Ng, C.K.Y., Ward, P., and Doohan, F.M. (2022). The impact of chitosan on the early metabolomic response of wheat to infection by *Fusarium graminearum*. *BMC Plant Biology*, *73*(22), 1–17. DOI:10.1186/s12870-022-03451-w.

Du, S., Trivedi, P., Wei, Z., Feng, J., Hu, H., Bi, L., Huang, Q., and Liu, Y. (2022). The proportion of soil-borne fungal pathogens increases with elevated organic carbon in agricultural soils. *mSystems*, *7*(2), 1 – 12. DOI:10.1128/msystems.01337-21.

Elmer, W.H. (2015). Management of *Fusarium* crown and root rot of asparagus. *Crop Protection*, *73*(1), 2 – 6. DOI:10.1016/j.cropro.2014.12.005.

Farahani-Kofoet, R.D., Witzel, K., Graefe, J., Grosch, R., and Zrenner, R. (2020). Species-specific impact of *Fusarium* infection on the root and shoot characteristics of asparagus. *Pathogens*, *9*(6), 1 – 20. DOI:10.3390/pathogens9060509.

Filyushin, M.A., Shagdarova, B.T., Shchennikova, A.V., Il'ina, A.V., Kochieva, E.Z., and Varlamov, V.P. (2022). Pretreatment with chitosan prevents *Fusarium* infection and in-

duces the expression of chitinases and β-1,3-glucanases in garlic (*Allium sativum* L.). *Horticulturae*, *8*(5), 1 – 18. DOI:10.3390/horticulturae8050383.

Gao, Y., Zhang, Y., Cheng, X., Zheng, Z., Wu, X., Dong, X., Hu, Y., and Wang, X. (2022). Agricultural jiaosu: An eco-friendly and cost-effective control strategy for suppressing *Fusarium* Root Rot disease in *Astragalus membranaceus*. *Frontiers in Microbiology*, *13*(1), 1 – 15. DOI:10.3389/fmicb.2022.823704.

Haque, S.I. and Matsubara, Y. (2018). Arbuscular-mycorrhiza-induced salt tolerance and resistance to *Fusarium* root rot in asparagus plants. *Acta Horticulturae*, *1227*(1), 365 – 372. DOI:10.17660/ActaHortic.2018.1227.45.

Harman, G., Khadka, R., Doni, F., and Uphoff, N. (2021). Benefits to plant health and productivity from enhancing plant microbial symbionts. *Frontiers in Plant Science*, *11*(1), 1–21. DOI:10.3389/fpls.2020.610065.

Hassan, E.O., Shoala, T., Attia, A.M.F., Badr, O.A.M., Mahmoud, S.Y.M., Farrag, E.S.H., and EL-Fiki, I.A.I. (2022). Chitosan and nano-chitosan for management of *Harpophora maydis*: Approaches for investigating antifungal activity, pathogenicity, maize-resistant lines, and molecular diagnosis of plant infection. *Journal of Fungi*, *8*(5), 1 – 16. DOI:10.3390/jof8050509.

Hassan, O. and Chang, T. (2017). Chitosan for eco-friendly control of plant disease. *Asian Journal of Plant Pathology*, *11*(2), 53 – 70. DOI:10.3923/ajppaj.2017.53.70.

Hermosa, R., Viterbo, A., Chet, I., and Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. *Microbiology*, *158*(1), 17 – 25. DOI:10.1099/mic.0.052274-0.

Hewedy, O.A., Abdel-Lateif, K.S., and Bakar, R.A. (2019). Genetic diversity and biocontrol efficacy of indigenous *Trichoderma* isolates against Fusarium wilt of pepper. *Journal of Basic Microbiology*, *60*(2), 126 – 135. DOI:10.1002/jobm.201900493.

Kappel, L., Kosa, N., and Gruber, S. (2022). The multilateral efficacy of chitosan and *Trichoderma* on Sugar Beet. *Journal of Fungi*, *8*(2), 1 – 23. DOI:10.3390/jof8020137.

Ke, Y., Ding, B., Zhang, M., Dong, T., Fu, Y., Lv, Q., Ding, W., and Wang, X. (2022). Study on inhibitory activity and mechanism of chitosan oligosaccharides on *Aspergillus flavus* and *Aspergillus fumigatus*. *Carbohydrate Polymers*, *275*(1), 118673. DOI:10.1016/j.carbpol.2021.118673.

Kumar, R., Duhan, J.S., Manuja, A., Kaur, P., Kumar, B., and Sadh, P.K. (2022). Toxicity assessment and control of early blight and stem rot of *Solanum tuberosum* L. by mancozeb-loaded chitosan-gum acacia nanocomposites. *Journal of Xenobiotics*, *12*(2), 74 – 90. DOI:10.3390/jox12020008.

Lahlali, R., Ezrari, S., Radouane, N., Kenfaoui, J., Esmaeel, Q., El Hamss, H., Belabess, Z., and Barka, E.A. (2022). Biological control of plant pathogens: A global perspective. *Microorganisms*, *10*(3), 1–33. DOI:10.3390/microorganisms10030596.

Lee, J.W., Lee, J.H., Yu, I.H., Gorinstein, S., Bae, J.H., and Ku, Y.G. (2014). Bioactive compounds, antioxidant and binding activities and spear yield of *Asparagus officinalis* L. *Plant Foods for Human Nutrition*, *69*(2), 175 – 181. DOI:10.1007/s11130-014-0418-9.

Leslie, J.F. and Summerell, A.B. (2006). *The Fusarium Laboratory Manual*. UK: BlackWell Publishing Oxford, 388.

Li, Y., Sun, X., Bi, Y., Ge, Y., and Wang, Y. (2009). Antifungal activity of chitosan on *Fusarium sulphureum* in relation to dry rot of potato tuber. *Agricultural Sciences in China*, *8*(5), 597 – 604. DOI:10.1016/S1671-2927(08)60251-5.

Mishra, P.K., Fox, R.T.V., and Culham, A. (2003). Development of a PCR-based assay for rapid and reliable identification of pathogenic Fusaria. *FEMS Microbiology Letters*, *218*(2), 329 – 332. DOI:10.1111/j.1574-6968.2003.tb11537.x.

Mondani, L., Chiusa, G., and Battilani, P. (2021). Fungi associated with garlic during the cropping season, with focus on *Fusarium proliferatum* and *F. oxysporum*. *Plant Health Progress*, *22*(1), 37 – 46. DOI:10.1094/PHP-06-20-0054-RS.

Mukherjee, P.K., Mendoza-Mendoza, A., Zeilinger, S., and Horwitz, B.A. (2022). Mycoparasitism as a mechanism of *Trichoderma*-mediated suppression of plant diseases. *Fungal Biology Reviews*, *39*(1), 15 – 33. DOI:10.1016/j.fbr.2021.11.004.

Onaran, A., Bayar, Y., Karakurt, T., Tokatli, K., Bayram, M., and Yanar, Y. (2021). Antifungal activity of chitosan against soil-borne plant pathogens in cucumber and a molecular docking study. *Journal of Taibah University for Science*, *15*(1), 852 – 860. DOI:10.1080/16583655.2021.2006434.

Orzali, L., Corsi, B., Forni, C., and Riccioni, L. (2017). Chitosan in agriculture: A new challenge for managing plant disease. In Shalaby, E.A. (Ed.). *Biological activities and application of marine polysaccharides*. 1st ed., Croatia: InTech, Rijeka, 328p.

Pedrero-Méndez, A., Insuasti, H.C., Neagu, T., Illescas, M., Rubio, M.B., Monte, E., and Hermosa, R. (2021). Why is the correct selection of *Trichoderma* strains important? The case of wheat endophytic strains of *T. harzianum* and *T. simmonsii*. *Journal of Fungi*, *7*(12), 1 – 21. DOI:10.3390/jof7121087.

Qing, W., Jin-hua, Z., Qian, W., Yang, N., and Li-pu, G. (2015). Inhibitory effect of chitosan on growth of the fungal phytopathogen, *Sclerotinia sclerotiorum*, and *sclerotinia* rot of carrot. *Journal of Integrative Agriculture*, *14*(4), 691 – 697. DOI:10.1016/S2095-3119(14)60800-5.

Reid, T.C., Hausbeck, M.K., and Kizilkaya, K. (2002). Use of fungicides and biological controls in the suppression of *Fusarium* crown and root rot of asparagus under greenhouse and growth chamber conditions. *Plant Disease*, *86*(5), 493 – 498. DOI:10.1094/PDIS.2002.86.5.493.

Ruangsanka, S. (2014). Identification of phosphate-solubilizing fungi from the asparagus rhizosphere as antagonists of the root and crown rot pathogen *Fusarium oxysporum*. *Science Asia*, *40*(1), 1 – 16. DOI:10.2306/scienceasia1513-1874.2014.40.016.

Shih, P., Liao, Y., Tseng, Y., Deng, F., and Lin, C. (2019). A potential antifungal effect of chitosan against *Candida albicans* is mediated via the inhibition of SAGA complex component expression and the subsequent alteration of cell surface integrity. *Frontiers in Microbiology*, *10*(1), 1 – 14. DOI:10.3389/fmicb.2019.00602.

Singha, I.M., Kakoty, Y., Unni, B.G., Das, J., and Kalita, M.C. (2016). Identification and characterization of *Fusarium* sp. using ITS and RAPD causing *fusarium* wilt of tomato isolated from Assam, North East India. *Journal of Genetic Engineering and Biotechnology*, *14*(1), 99 – 105. DOI:10.1016/j.jgeb.2016.07.001.

Stamford, T.C.M., Alcântara, S.R.C., Berger, L.R.R., Stamford, N.P., Silva, M.C.F., Borges, T.K.S., Laranjeiras, D., and Campos-Takaki, G.M. (2010). Antimicrobial activity of chitosan against *Fusarium oxysporum* f. sp. *tracheiphilum*. *The 3t international conference on microorganisms in industry and environment*, Lisbon: Scientific and industrial research to consumer product, pp. 12 – 15.

Stasinska-Jakubas, M. and Hawrylak-Nowak, B. (2022). Protective, biostimulating, and eliciting effects of chitosan and its derivatives on crop plants. *Molecules*, *27*(9), 1 – 17. DOI:10.3390/molecules27092801.

Subramaniam, S., Zainudin, N.A.I.M., Aris, A., and Hasan, Z.A.E. (2022). Role of *Trichoderma* in plant growth pro-

motion. In Gupta, V.K. and Tuohy, M.G. (Eds.). *Fungal biology*. 1st ed. USA: Springer, pp. 257 – 280.

Surono, and Narisawa, K. (2018). The inhibitory role of dark septate endophytic fungus *Phialocephala fortinii* against *Fusarium* disease on the *Asparagus officinalis* growth in organic source conditions. *Biological Control*, *121*(1), 159 – 167. DOI:10.1016/j.biocontrol.2018.02.017.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725 – 2729. DOI:10.1093/molbev/mst197.

Tyskiewicz, R., Nowak, A., Ozimek, E., and Jaroszuk-Sciseł, J. (2022). *Trichoderma*: The current status of its application in agriculture for the biocontrol of fungal phytopathogens and stimulation of plant growth. *International Journal of Molecular Sciences*, *23*(4), 1 – 28. DOI:10.3390/ijms23042329.

Viejobueno, J., Albornoz, P.L., Camacho, M., de los Santos, B., Martínez-Zamora, M.G., and Salazar, S.M. (2021). Protection of strawberry plants against charcoal rot disease (*Macrophomina phaseolina*) induced by *Azospirillum brasilense*. *Agronomy*, *11*(2), 1–12. DOI:10.3390/agronomy11020195.

Vitti, A., Bevilacqua, V., Logozzo, G., Bochicchio, R., Amato, M., and Nuzzaci, M. (2022). Seed coating with *Trichoderma harzianum* T-22 of Italian durum wheat increases protection against *Fusarium culmorum*-induced crown rot. *Agriculture*, *12*(5), 1 – 12. DOI:10.3390/agriculture12050714.

Wang, Q., Li, H., Lei, Y., Su, Y., and Long, Y. (2022). Chitosan as an adjuvant to improve isopyrazam azoxystrobin against leaf spot disease of kiwifruit and enhance its photosynthesis, quality, and amino acids. *Agriculture*, *12*(3), 1 – 13. DOI:10.3390/agriculture12030373.

Xu, H., Yan, L., Zhang, M., Chang, X., Zhu, D., Wei, D., Naeem, M., Song, C., Wu, X., Liu, T., Chen, W., and Yang, W. (2022). Changes in the density and composition of rhizosphere pathogenic *Fusarium* and beneficial *Trichoderma* contributing to reduced root rot of intercropped soybean. *Pathogens*, *11*(4), 1 – 16. DOI:10.3390/pathogens11040478.

Zhong, S. and Steffenson, B.J. (2001). Virulence and molecular diversity in *Cochliobolus sativus*. *Phytopathology*, *91*(5), 469 – 476. DOI:10.1094/PHYTO.2001.91.5.469.

Zohara, F., Surovy, M.Z., Khatun, A., Prince, M.F.R.K., Akanda, A.M., Rahman, M., and Islam, T. (2019). Chitosan biostimulant controls infection of cucumber by *Phytophthora capsici* through suppression of asexual reproduction of the pathogen. *Acta Agrobotanica*, *72*(1), 1–8. DOI:10.5586/aa.1763.

Received: August 31, 2022
Accepted: November 11, 2022