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Production of *Aspergillus quadrilineatus* MT083999 Chitinase, β-1,3-Glucanase and Nano-silver Important for Biocontrol of *Fusarium* spp. Infecting Crops

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In our search for an active biocontrol agent against *Fusarium solani* and *F. oxysporum*, the major species causing wilt, root rot and damping-off in plants affecting crops. The fungal isolate genetically identified as *Aspergillus quadrilineatus* MT083999 displayed high antagonistic activities against the tested root-rot fungi, and presented valuable chitinase and β-glucanase activities of 235.484 and 508.953 U/gds, respectively. Solid state fermentation conditions were optimized by applying 20 trails of the central composite design (Three-factor-five-level) and two responses of chitinase R1 and β-glucanase R2 activities, respectively. The optimum activities of chitinase (386.70 U/gds) and β-glucanase (1094.70 U/gds) were obtained in trial 4 and 2 respectively, with about 1.64-fold increase in chitinase activity and 2.15-fold increase in β-glucanase activity when compared with initial fermentation. Biosynthesis of nano-silver from *A. quadrilineatus* was evaluated in 10 experiments of another design of 2-factors-5-levels. The antifungal effects of each biosynthesized nano-silver against *F. solani* (R1) and *F. oxysporum* (R2) were evaluated as Inhibition Zone Diameter (IZD).The high fungicide nano-silver against both tested phytopathogens was characterized by UV-visible spectrophotometry, Transmission Electron Microscopy (TEM), Zeta Dynamic Light Scatter (DLS) and Fourier Transform Infrared Spectroscopy (FTIR). The results indicated that *A. quadrilineatus* MT083999 chitinolytic enzymes and nano-silver can be applied as a novel candidate in biocontrol of Fusarium wilt infecting crops worldwide.

Keywords: Fungi, Inhibition zone diameter, Phytopathogens, Statistical designs

Introduction

Fusarium plant wilt is a major fungal disease devastating many crops and leading to huge loses in the commercially domesticated plants and crops around the world. The fatal soil-borne fungi *Fusarium solani* and *F. oxysporum* are spread in the most agricultural fields affecting many plants by producing micro and macro conidia which infect the plant roots feeding, also infecting the vascular tissues leading to wilting and gradual death of the plants.[1] Several ways have been formulated to control the spread of such devastating fungi, but have many difficulties due to its ability to remain in a dormant state in field for a long period and have wide range of hosts.[2] Out of the possible ways that have been applied to control the fungal pests, the scientists have found that, some resistant microorganisms that have been used as biocontrol agents, have high potentiality against many phytopathogens resistance without affecting the other non-target beneficial organisms and the environment.[3] Soil fungi able to produce a huge amount of effective bioactive metabolites, which can be applied as biological tools against pathogenic fungi, bacteria, pests and also applied as antitumor pharmaceutical agents.[4] *Aspergillus* is one of the most valuable fungal genera and can be found ubiquitous in nature and have high biotechnological potential depending on their economic relevance in microbial fermentations, as well as on their genomic diversity leading to the production of several bioactive metabolites.[5] Chitinase (E.C. 3.2.1.14) and β-1,3-glucanase (EC 3.2.1.6) are considered to be important hydrolytic enzymes in the lysis of fungal cell wall and play an important role in bio control of phytopathogens.[6,7] Chitinolytic enzymes have been known to be produced by several fungi of the genus Aspergillus as *Aspergillus griseoaurantiacus*, *A. parasiticus*, *A. niger* LOCK 62 and *A. nidulans* AKB-25 under solid-state fermentation techniques.[8–10] On the other hand, Nano materials have been considered as another solution to control phytopathogenic organisms and they are required urgently as a novel mechanism to avoid and control pathogens' resistance development.[11] Nano-silver is one of the main nano-fungicides used to enhance the resistance of plants' diseases at minimum

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concentrations, with low toxicity to animals, humans and the environment when compared with many other different chemical agents.[12,13]

Central composite designs (CCD) are useful statistical tools for more advanced results with less development time and less overall costs.[14,15] In this design a few numbers of experiments with large number of various variables, can be studied to optimize chitinolytic enzymes production.[16] In addition, the shape and size of biosynthesized nano-silver can be controlled recently by applying a statistical design of (CCD) with the analysis of variance (ANOVA), that is highly effective on the nano silver antifungal and antibacterial properties.[17] The aim of the present study was to find a novel active fungal isolate that has high antagonistic potential activities against phytopathogens. Application of statistical experimental designs for maximum optimization of chitinolytic enzymes; Chitinase and β-1, 3-Glucanase as bio control agents against Fusarium wilt fungi. And biogenesis of nano-silver from the locally isolated fungus with statistically controlled size and shape effective against tested root rot fungi.

Materials and Methods

Chemicals

Chitin and laminarine were purchased from (Sigma-Aldrich-MO-USA). Silver nitrate was purchased from Sigma–Aldrich (Poole, UK). Other used chemicals were of analytical grade.

Isolation, Morphological Identification and Maintenance of Rhizospheric Fungi

Roots of wild plants, collected from Hafr Al-Batin City, in the Eastern Province, Saudi Arabian have been cut into pieces (~0.5–1.0 cm), and placed on potato dextrose agar medium (PDA) containing streptomycin to control the bacterial growth. Plates were incubated at 28°C and the observed fungal mycelium tips were transferred into a PDA slant. The previous step was repeated for further purification of the fungal strains.[18] The fungal genera and species of the obtained pure cultures were first identified on the basis of their macro and microscopic characteristics of colony and hyphae shape, using the standard guidelines of taxonomy.[19] The pure cultures were numerated and preserved at 4°C on (PDA) slants.

Antagonism Assay of Isolated Fungi Against *Fusarium* spp.

Three different rhizospheric fungi were tested as bio control agents *in-vitro* against root-rot pathogens of *F. solani* NRC15 and *F. oxysporum* NRC 25. The pathogens stored in the culture unite of National Research Centre, Egypt. For inoculum preparation, a10 mm-diameter-disc of all tested isolates were suspended separately in 5.0 ml sterilized distilled water. One ml of each prepared inoculum was inoculated in the medium of PDA and left to solidify. After that, 10 mm-diameter-disc of the pathogen was placed in the plates at the center position. Controls were prepared using pathogens alone. All plates were incubated for 7 days at 28°C. Three replicates were made for each treatment. After incubation period, the pathogen inhibition percentage was evaluated using the following equation:

$$\text{Inhibition \%} = (R1 - R2)/R1 \times 100.$$

R1 (Controln) and R2 (Tested antagonist); Refers to the pathogen growth diameter.[20]

Evaluation of Hydrolytic Enzymes Production

Three isolated fungal strains were screened for the production of both chitinase and β-glucanase enzymes using solid-state-fermentation (SSF) of the dry collected wild plants without any nutritional additives. According to Mostafa & Abd El Aty [21] the SSF was applied by addition of 2.0 g dried cut plant (~ 0.5–1.0 cm) moisture with 15 mL of distilled water into Erlenmeyer flasks (250 mL). Flasks were autoclaved for 20 min at 121°C. Then the flasks cooled, and one ml (5×10^8 spores/mL) of 6 days old culture was inoculated to each flask. SSF were then incubated under static conditions at 28–30°C for 7 days. For enzyme extraction, 50 ml of distilled water was added to each flask and placed in a rotary shaker at150 rpm for 60 min. Chitinase and β-glucanase activities were presented in units of activity/gram solid substrate (U/gds).

Assay of Chitinase Enzyme

The colorimetric method for the estimation of N-acetyl amino sugar with the modified dinitros-alicyclic acid (DNS) method was performed for chitinase assay.[22]

Assay of 1, 3- β–Glucanase Enzyme

Glucanase activity was estimated by a colorimetric method according to Burner.[23]

Molecular Identification of Selected Fungal Isolate

The fungal strain showed the best antagonism against *Fusarium* spp. and high enzymes production was identified genetically according to Shaheen &

Abd El Aty.[24] 18S-rRNA sequence of the fungal strain was conducted using primers, 5′-TCCGTAGGT GAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGA TA TGC-3′, respectively. The retrieved sequences were deposited in GenBank with a specific accession numbers. Molecular phylogeny and sequence alignments were performed using Bio-Edit-software. TREE VIEW program is used for displaying the phylogenetic tree.

Antagonistic Potential of Aspergillus quadrilineatus and Microscopic Examination

The interaction between the fungal isolate and pathogens was evaluated according to Zohair *et al.*[25] a plate containing 15 ml of PDA medium, 10 mm-diameter-disc of the tested strain was placed oppositely at a distance of at least 3 cm from the fungal pathogen. Plates incubated in the dark for 3–5 days at 28°C. After incubation period, the hyphae from the contact zone between the phytopathogens and the fungal isolate were harvested for testing the *A. quadrilineatus* myco-parasitism. The examination was done using a light microscope 150 (Olympus CX40 RF100), which have a canon A620 digital camera.

Three-Factor-Five-Level Central Composite Design (CCD) for Enzymes Optimization

SSF of *A. quadrilineatus* was optimized for maximum chitinase and β-glucanase production using response surface methodology of 3-factor-5-level central composite design (CCD). Different weights of the dry plant (A), wheat bran (B) and incubation period (C) were evaluated in twenty experiments to determine the optimal values for high enzymes production. All variables were tested at zero level (0), high level (+1), and low level (−1), with α = (1.682) as shown in Table 1.

Statistical Biosynthesis of Aspergillus quadrilineatus Nano-Silver Production of Fungal Biomass

The broth medium, yeast extract-peptone-dextrose-medium (YPD), containing in (%): 1 yeast extract, 2 Peptone and 2 Dextrose used for biomass production. Erlenmeyer flask (250 ml) containing 50 ml (YPD) broth media was inoculated with two 10mm-diameter-discs. Flasks were placed in an incubator shaker at (150 rpm), for 6 days at 28–30°C. After incubation period, the fungal mycelium was filtered and separated by using sterile Whatmann filter paper No.1 (Oakland, California, USA), then the mycelium washed to remove all the medium traces using the sterile bi-distilled water. 5.0 g of wet biomass were placed in 50 ml-flask containing sterilized distilled water and incubation is taken place for 3 days.[26]

Two-Factors-5-Levels CCD

The best pH-value and $AgNO_3$ concentration, important for biosynthesis of bioactive nano-fungicide was determined depending on CCD application. According to Abd El Aty *et al.*[27], 10 experimental trails of CCD, were carried out to evaluate the optimum pH-value (A) and $AgNO_3$ concentration (B). Two variables were evaluated with α = (1.414).The actual and coded values of tested variables along with responses (R1) (R2) of the inhibition zone diameter (IZD) against *F. solani* and *F. oxysporum* are shown in Table 2.

In-vitro Antifungal Activity of Biosynthesized Nano-Silver

Ten biosynthesized nano-silver of central composite design (CCD) were tested against *F. solani* and *F. oxysporum* on PDA using agar well diffusion method.[28] About 300 µl of AgNPs were added to 10 mm-diameter-wells made in the solidified inoculated (1×10^6 spores/ml) plates. The plates were incubated at 28°C for 72 h. The anti-fungal effect was evaluated by measuring the zones diameter of inhibition that's formed around the wells in (mm). The average values were reported using MS Excel as Mean ± SD.

Characterization of Biosynthesized Nano-Silver

The metal ions were characterized in the range of (200–800 nm) using UV-Visible-spectrophotometer. The sharp peak given at the absorption range 400-450 nm indicated the formation of AgNPs. In addition, biosynthesized AgNps has been monitored by TEM (JEOL-2100) to determine out the size and shape of the most active nanoparticles. The distribution of particle size and Zeta potential, were measured by using "Dynamic Light Scattering" in the range of 0.1–1000 µm (Particle Sizing Systems, California, USA). The functional groups of the AgNPs solution were detected by Fourier Transform Infrared Spectroscopy (FTIR) (JASCO, FT/IR- 6100).

Table 1 — CCD design for optimization of *A. quadrilineatus* MT083999 chitinase and β-glucanase activity

Variable code	Variable	Levels				
		−α	−1	0	+1	+α
A	Dry plant (g/ flask)	−0.36	1	3	5	6.36
B	Wheat bran (g/flask)	0.32	1	2	3	3.68
C	Incubation period (Days)	0.95	3	6	9	11.05

A = 1.682

Table 2 — Factors and their levels which were studied by CCD for biosynthesis of antifungal nano-silver

Trial number	Coded levels		Actual levels		*R1: IZD of *F. solani* (mm)		*R2: IZD of *F. oxysporum* (mm)	
	Factor1 A: pH value	Factor 2 B: $AgNO_3$ (mM)	Factor1 A: pH value	Factor 2 B: $AgNO_3$ (mM)	Experimental	Predicted	Experimental	Predicted
1	0	**−α**	10.50	0.17	18 ± 0.17	19.05	13 ± 0.00	12.51
2	0	0	10.50	3.00	21 ± 1.02	21	20 ± 0.91	20
3	−1	−1	9.00	1.00	16 ± 0.19	14.52	14 ± 0.17	14.53
4	−1	+1	9.00	5.00	19 ± 0.00	18.13	19 ± 2.01	18.13
5	+1	−1	12.00	1.00	18 ± 1.51	17.63	14 ± 1.04	13.87
6	**−α**	0	8.38	3.00	12 ± 1.10	13.41	18 ± 0.18	18.03
7	0	**+α**	10.50	5.83	19 ± 0.91	19.19	14 ± 0.57	15.49
8	0	0	10.50	3.00	21 ± 1.03	21	20 ± 1.14	20
9	+1	+1	12.00	5.00	14 ± 0.17	14.23	16 ± 1.21	14.47
10	**+α**	0	12.62	3.00	13 ± 0.53	12.85	14 ± 0.27	14.97

*The anti-fungal effect of AgNPs (1–10) was determined by measuring the diameter of (IZ) formed around the well in mm; The average values were evaluated by using MS Excel as Mean ± SD

Results and Discussion

Evaluation of Isolated Fungi Against the Phytopathogens and Enzymes Production

Three isolated fungi of the genera: Aspergillus, Geotrichum and Alternaria were isolated from the rhizosphere of wild plants collected from Hafr Al-Batin City, in the Eastern Province, Saudi Arabia. *In-vitro* antagonistic potential of isolated fungi was evaluated against pathogenic *F. solani* and *F. oxysporum.* The fungal isolate *Aspergillus sp.* showed the best percentage of inhibition (%) against the phytopathogens *F. solani* (65.51%) and *F. oxysporum* (84.21%), respectively (Fig. 1 A). Solid state fermentation (SSF) of the dry plant without any additives using three fungal isolates indicated that, all isolates were able to produce chitinase and β-glucanase enzymes with different degree, after 7 days of incubation. Results in Fig. 1 (B) demonstrated that the optimum chitinase activity of 235.484 U/gds was obtained using *Aspergillus sp.,* where *Geotrichum sp.* and *Alternaria sp.* showed low activity of 52.201 and 10.935 U/gds, respectively. The results also emphasized that the highest extracellular β-glucanase activity (508.953 U/gds) was found in *Aspergillus sp.* culture fermentation, followed by *Alternaria sp.* (476.813 U/gds) and *Geotrichum sp.* (441.095 U/gds).

Screening study demonstrated that *Aspergillus sp.* was the most efficient tested fungal strain for the production of hydrolytic enzymes to control reproduction and growth of the phytopathogenic *Fusarium sp.* in this context, Geiser *et al.*[29] indicated that the members of the genus Aspergillus are intensely studied with respect to production of

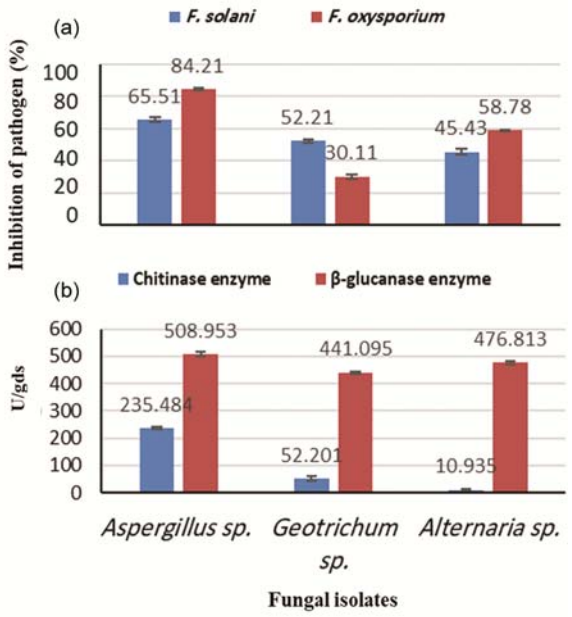

Fig. 1 — Screening of isolated fungi depending on (A) Antagonism assay against phytopathogens *F. solani* and *F. oxysporum* (B) Production of chitinase and β-glucanase hydrolytic enzymes

secondary metabolites and characterized by their ability to live as saprophytes in soils. Other workers Brzezinska and Jankiewicz[9] showed the ability of *A. niger* LOCK 62 to inhibit the growth of: *F. culmorum*, *F. solani* and *Rhizoctonia solani* depending on chitinase production after 6 days incubation, but not affecting the growth of *F. oxysporum.*

Application of Molecular Tools (18S-rDNA) for Fungal Isolate Identification

The fungal isolate representing the highest enzymes and antagonistic activities was selected and

identified according to 18S-rDNA. Sequence analysis and ITS phylogenetic tree showed high similarity (98%) to *A. quadrilineatus* isolate EN-8 (Gene Bank accession no. MN909163) and *A. quadrilineatus* isolate RSCF6 (MN871860) and others as shown in Fig. 2. According to the morphological characters, the microscopic examination and genetic analysis, the fungal isolated identified as *A. quadrilineatus*, and take a specific Accession No. MT083999 in GenBank database.

Myco-parasitism of Aspergillus quadrilineatus against Phytopathogenic Fusarium spp.

The interaction effect between *A. quadrilineatus* and phytopathogens was first observed as the difference in the growth rate of the fungal isolate and *Fusarium sp*. placed on the same plate after incubation for 4 days. Results in Fig. 3 (A & D) showed that *A. quadrilineatus* was able to grow faster than *F. solani* and strongly controlled the growth of *F. oxysporum.* The obtained results demonstrated the significant ability of the fungal isolate to secrete enzymes able to control the spread of pathogens, and causes an inhibition for the pathogen growth, that was in agreement with Pates *et al*.[30] Also, the microscopic examination of hyphae taken from the contact zone between the *A. quadrilineatus* and the phytopathogens demonstrated the hyphal interactions which could explain the parasitic effect of *A. Quadrilineatus* on *F. solani* and *F. oxysporum*. *Aspergillus quadrilineatus* hyphae were able to grow surrounding the mycelia of the phytopathogens and wrapping them tightly Fig. 3 (B & F)**.** The microscopic examination emphasized that, there were cytoplasmic disorganization in the mycelia of *F. solani,* moreover deformation was observed in the spores Fig. 3(C).

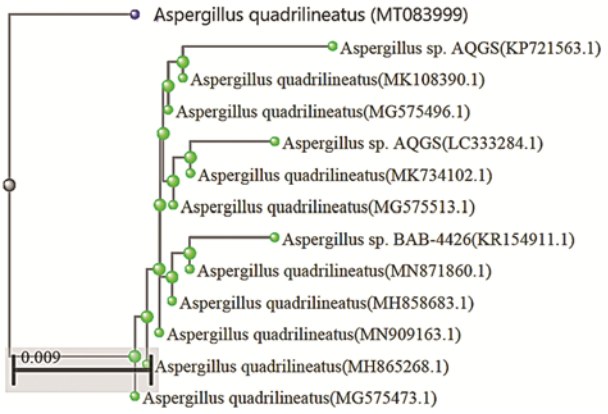

Fig. 2 — Phylogenetic analysis of *Aspergillus quadrilineatus* (MT083999)

Where the hyphae of *A. quadrilineatus* grew normally there were rupture take places in the *F. oxysporum* cell membrane which may lead to lyses of the cells as shown in Fig. 3(E). Changes occurred in the morphology and structure of the pathogenic strains caused by *A. quadrilineatus* were identical to those demonstrated by Cuervo-parra *et al.*[31] during the parasitism by *Trichoderma harzianum*.

Experimental Design and Optimization of A. quadrilineatus Chitinase and β-Glucanase Activity by RSM

Twenty experiments of Three-factor-five-level (CCD) were prepared to examine the combined effect of three tested factors in relation with chitinase (R1) and β-glucanase (R2) activities. The results obtained in Table 3 indicted that each enzyme has specific optimum fermentation condition. High chitinase activity of 386.70 U/gds was obtained with trial 4, that contains 3 g dry plant, 0.32 g wheat bran and incubated for 6 days,also good β-glucanase activity

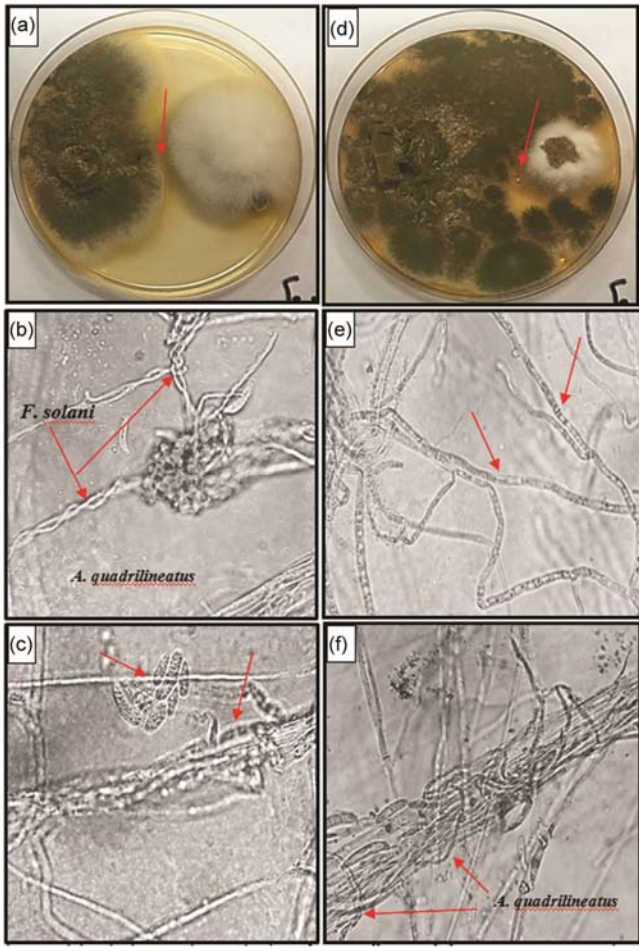

Fig. 3 — Antagonistic potential and microscopic examination of *Aspergillus quadrilineatus*MT083999 against *F. solani* (A, B, C) and *F. oxysporum* (D, E, F)

Table 3 — Central composite design of predicted and experimental values for *A. quadrilineatus* MT083999chitinase and β-glucanase activity

Trials	Dry plant (A)	Wheat bran (B)	Incubation period (C)	Chitinase activity (U/gds)		β-glucanase (U/gds)	
				experimental	predicted	experimental	predicted
1	−1	+1	−1	233.85	165.12	372.34	306.49
2	−1	−1	+1	196.81	204.18	1094.70	1054.64
3	0	0	0	256.77	256.91	286.44	281.34
4	0	**−α**	0	386.70	316.262	882.14	945.15
5	0	0	**-α**	42.89	62.95	85.72	179.40
6	0	**+α**	0	116.79	182.303	48.12	163.59
7	+1	+1	−1	88.18	84.30	281.26	195.11
8	0	0	0	256.77	256.91	286.44	281.34
9	0	0	0	256.77	256.91	286.44	281.34
10	−1	+1	+1	72.55	64.16	207.15	121.60
11	+1	−1	−1	91.70	103.58	232.15	191.50
12	0	0	**+α**	34.61	33.10	60.72	145.52
13	0	0	0	256.77	256.91	286.44	281.34
14	0	0	0	256.77	256.91	286.44	281.34
15	**+α**	0	0	63.45	62.95	34.61	120.12
16	+1	+1	+1	27.35	1.01	115.18	56.20
17	0	0	0	256.77	256.91	286.44	281.34
18	+1	−1	+1	68.90	141.12	396.45	336.10
19	**-α**	0	0	128.66	154.09	725.03	818.00
20	−1	−1	−1	154.49	184.31	1023.26	956.03

882.14 U/gds was obtained under the same conditions. Abdel Wahab *et al.*[32] observed an increase in exochitinase production on wheat bran as the substrate by *Trichoderma longibrachiatum*. On the other hand, the optimum β-glucanase activity 1094.70 U/gds was obtained with trial 2, that contains 1:1g(w : w) of dry plant and wheat bran and flasks incubated for 9 days. Other studies ofJabasingh & Nachiyar[33] reported maximum endoglucanase activity (28.84 IU/gds) with *A. nidulans* MTCC344 using SSF of sugarcane bagasse. Where the chitinase activity strongly decreased at high weights of dry plant and wheat bran ratio of 5:3 (trials 16), and minimum β-glucanase activity (34.61 U/gds) obtained at high ratio of 6.36:2 dry plant and wheat bran, respectively.

In accordance with Gao *et al.*[34] finding, the enzyme production is dependent on the nature of carbon source, degree of degradability, physico-chemical characteristics of substrates, and availability of some nutrients. Results indicated that the SSF of dry wild plant without any additives showed good enzymes activity and the addition of wheat bran rich in hemicellulose, protein, zinc, manganese, iron and copper improved *A. quadrilineatus* chitinase and β-glucanase production. The results obtained were in agreement with Mostafa & Abd El Aty[21] and Dhillon *et al.*[35] who found that the highest enzymes production of *A. niger* and *Alternaria alternata*, respectively was under SSF using wheat bran as the substrate.

The incubation period considered one of the main factors affecting both enzymes activity as shown in trials 5 and 12 with (-α of about one day) and (+α of about 11 days). The present findings showed the maximum chitinase and β-glucanase were after 6 and 9 days, where Vitcosque *et al.*[36] & Brzezinska and Jankiewicz,[9] reported that the optimum endoglucanase and chitinase activities were after 4 and 6 days incubation, respectively using different species of *A. niger*.

Equation of second-order regression demonstrated the levels of chitinase and β-glucanase activities as a function of dry plant, and wheat bran weights and incubation period in terms of coded factors were: Chitinase activity (U/gds) = + 256.91 – 35.97 × A – 39.83 × B – 15.86 × C – 0.021 × A × B + 4.42 × A × C – 30.21 × B × C – 57.74 × A2 – 2.70 × B2 – 78.00 × C2 β-glucanase activity (U/gds) = + 281.34 – 207.48 × A – 232.36 × B – 10.07 × C + 163.29 × A × B + 11.50 × A × C – 70.88 × B × C + 66.37 × A2 + 96.53 × B2 – 42.03 × C2

Analysis of variance (ANOVA) for the response surface quadratic model of chitinase and β-glucanase enzymes indicated that this regression was statistically significant with R-Squared 0.8813 and 0.9560 respectively.

The validation was applied at the achieved maximum fermentation conditions for each enzyme and the obtained experimental values were closer to the predicted values, with about 1.64-fold increase in chitinase activity and 2.15-fold increase in β-glucanase activity in comparison with initial fermentation.

Application of 2-Factors-5-Levels (CCD) for Nano-Silver Biosynthesis with Antifungal Effects

Biosynthesis of AgNPs from *A. quadrilineatus* was evaluated in 10 experiments of a statistical design to determine all possible combinations of the metal concentration and pH value which directly control and affect the antifungal activity of AgNPs within 3-D Space region.

Inhibition zone diameter (IZD) formed by AgNPs at each trial against *F. solani* (R1) and *F. oxysporum* (R2) are shown in Table 2. Results indicated that all biosynthesized AgNPs have antifungal activities against *Fusarium sp.* with different degree. The highest responses 21 and 20 mm-IZD of *F. solani* (R1) and *F. oxysporum* (R2), respectively were found at the center point (runs 2and 8) where the metal concentration and pH-value were 3.00 mM, and 10.50, respectively. On the other hand, results discussed by Abd El Aty & Ammar[26] indicated the biosynthesis of antifungal AgNps at optimum conditions of 4 mM $AgNO_3$ solution, with IZD = 16 and 17 mm against *F. solani* and *F. oxysporum* respectively.

The lowest response (R1) of 12 & 13 mm-IZD against *F. solani* was found at lower (8.38) and higher (12.62) pH values, runs 6 and 10 respectively. Also we noticed that very low metal concentration of 0.17 and 1.0 decrease the antifungal activity of AgNPs against *F. oxysporum* to 13&14 mm-IZD in runs 1, 3 and 5. Results also indicated that the reaction conditions of run 10 of high pH value (12.62) not appropriate with the metal concentration (3.00 mM) and the AgNPs showed low IZD of 13 and 14 mm with *F. solani* (R1) and *F. oxysporum* (R2) respectively. Results obtained by Abd El Aty *et al.*[17] partly agree with obtained results, who showed significant antifungal activity of AgNPs synthesized at pH 12.00 and 1 mM $AgNO_3$ concentration, against *F. solani* (13 mm) and *A. niger* (11 mm).

Analysis of variance (ANOVA) of both responses, showed a significant quadratic model. *Fusarium solani* (R1) and *F. oxysporum* (R2) showed "R-Squared" of 0.9323 and 0.9026 respectively, which advocates for high significance of the model.

The relation between variables and each response in terms of Actual Factors:

IZD of *F. solani* (R1) = $-191.11198 + 38.36785 \times$ pH value $+ 7.55714\ AgNO_3 - 0.58333 \times$ H value $\times\ AgNO_3 - 1.75000 \times$ pH value$^2 - 0.23437 \times AgNO_3^2$

IZD of *F. oxysporum* (R2) = $-74.37792 + 16.36193 \times$ pH value $+ 7.65089 \times AgNO_3 - 0.25000 \times$ pH value $\times\ AgNO_3 - 0.77778 \times$ pH value$^2 - 0.75000 \times AgNO_3^2$

Obtained results indicated that the design of 2-factors-5-levels was very successful in biosynthesis of high fungicide AgNPs, effective against both tested phytopathogens, therefore run 2 or 8 was selected and characterized.

Characterization Studies

The biosynthesis of AgNPs was first indicated by transforming the reaction mixture from pale yellow to dark brownish color. UV-visible absorbance spectra of AgNPs in range from 300–700 nm presented an optimum peak at 415 nm wavelength as seen in Fig. 4. The sharp peak given by UV visible spectrum at the absorption range 400–450 nm emphasized the biosynthesis of AgNPs according to Shaheen & Abd El Aty.[24]

TEM measurement of *A. quadrilineatus* AgNPs revealed that the particles were well dispersed without aggregation and has spherical shape, with size ranging from 5.44 to 12.97 nm diameter. HR-TEM has been performed to show the crystalline nature of the nanoparticles (Fig. 5 A)**.**These results were investigated by DLS analysis that showed characteristic peak in zeta sizer Fig. 5 (B) with the particle size means of 107.7 nm. Zeta potential was found to be −22.3, which indicated the good stability of nanoparticles. The high negative value of surface

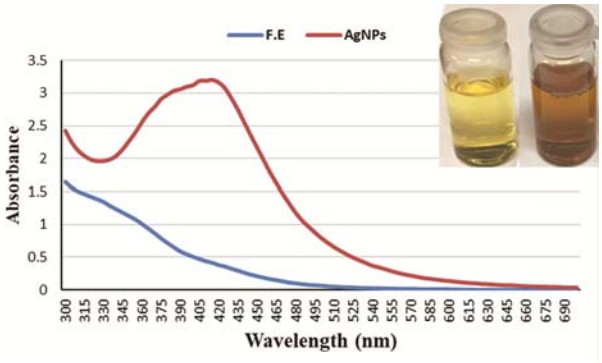

Fig. 4 — Changing the color of *A. quadrilineatus* MT083999 mixture, UV-Vis spectra analysis of biosynthesized AgNPs in comparison with the fungal extract (FE)

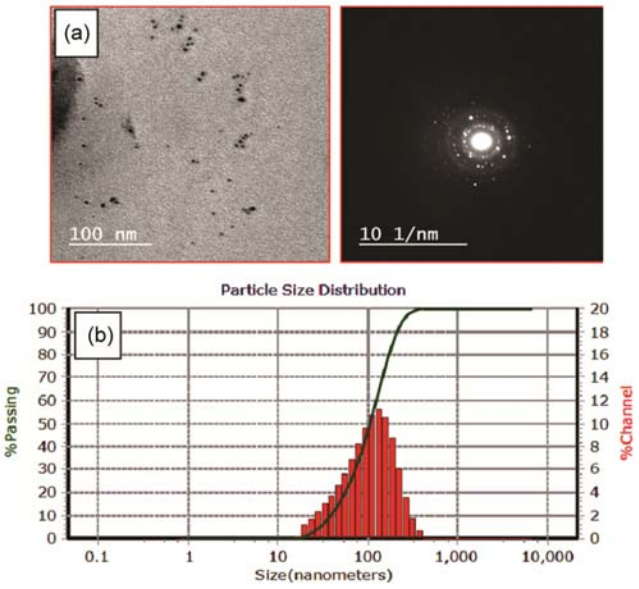

Fig. 5 — Nanosilver characterization (A) TEM image of AgNPs formed by the fungal strain *A. quadrilineatus* MT083999, (B) Dynamic light-scattering analysis showing particle size distribution of *A. quadrilineatus* AgNPs

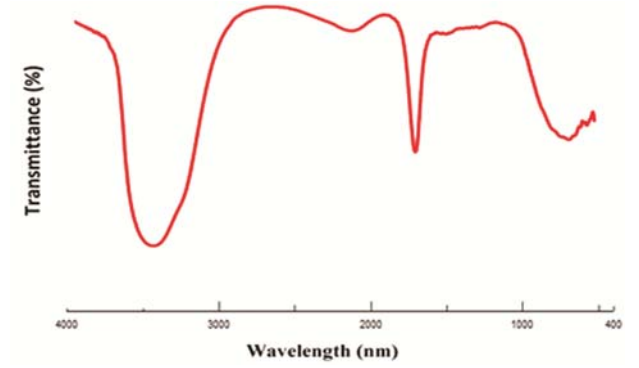

Fig. 6 — *A. quadrilineatus* MT083999AgNPs -FTIR-spectrum

charge showed the repulsion between the nano-particles which in turn indicated AgNP's stability.[37]

Fourier Transform Infra-red (FTIR) spectroscopy was applied to show the main groups that involved in AgNPs synthesis and stabilization. In Fig. 6 distinctive bands at 1640.2, 1495.55, 1460.77, 1430.15, 3519.67, cm^{-1} are shown which correspond to the binding vibrations of amide I and amide II of protein, with N–H stretching's. While the bands obtained at 1245.18, 1315.25, and 1193 cm^{-1} related to C–N stretching vibrations of aromatic and aliphatic amines, where 2081.84 and 1870.41 cm^{-1} represents C–H stretching vibration. Out of obtained results of FTIR-analysis, we concluded the availability of reducing and capping agents.[38]

Conclusions

This research has provided information about a novel biological control agent against the aggressive pathogens *Fusarium* spp. Affecting crops. *Aspergillus quadrilineatus* MT083999 isolated from the rhizosphere of wild plants collected from Hafr Al-Batin City, in the Eastern Province, Saudi Arabian displayed in-vitro high antagonistic activities against pathogenic *F. solani* and *F. oxysporum*. SSF of the agricultural wastes showed their ability to produce the valuable chitinolytic enzymes chitinase and β-glucanase important for degrading the pathogen cell wall and penetrate the fungal mycelium. Application of central composite design (Three-factor-five-level) increased the chitinase and β-glucanase activities with about 1.64 and 2.15 fold, respectively. In addition, another design of 2-factors-5-level was applied for the biosynthesis of antifungal nano-silver using *A. quadrilineatus* MT083999. All biosynthesised AgNPs were tested against the fungal pathogen and the runs 2 & 8 showed the highest responses 21 and 20 mm-IZD respectively against *F. solani* (R1) and *F. oxysporum* (R2) were characterized. Finally, we can conclude that *A. quadrilineatus* MT083999 can defend plants against fungal pathogens either in association with chitinases and β-glucanase or biosynthesized AgNPs.

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