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The Efficacy of Photosensitizers on Mycelium Growth, Mycotoxin and Enzyme Activity of *Alternaria* spp.

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Abstract

Vegetables are subjected to heavy yield losses in quality and quantity as a result of various diseases caused by dematiaceous fungi. However, the use of fungicides is hazardous to humans, animals, and the environment. In this study, emphasis is given to other methods of disease control through the employment of photodynamic treatments such as photosensitizers which are considered safer, more economical, and eco-friendly. The objective of the current study was to evaluate the possibility of using photodynamic inactivation against *Alternaria alternata*, the causal agent of tomato blight. Samples of tomato, squash, pepper and cucumber were collected from different fields, and the isolated fungi were identified morphologically and molecularly. The inhibitory doses of the photosensitizer regarding the growth of *A. alternata* were studied. The results showed that 100 µg/mL of toluidine blue-O (TBO) was effective inhibiting *Alternaria* spp. with a significance variation among different species of *Alternaria*. The *A. alternata* isolated from tomato was more resistant to TBO and less sensitive to light compared to other isolates. The effect of the photosensitizer on cellulase and pectinase as well as the production of alternariol mycotoxin were studied. The results showed that the photosensitizer was inhibitory for enzyme activity and the alternariol production especially in the presence of light. In conclusion, photosensitizers can be used for treating plant pathogenic fungi such as *Alternaria* spp.

KeyWords: *Alternaria* spp., Photosensitizer, Cellulase, Pectinase, Mycotoxin.

1. Introduction

Vegetables constitute the most important and inexpensive component of a balanced diet due to their high nutritional values indispensable for the body. Cucurbitaceae, Brassicaceae and Solanaceae families are considered important vegetables due to their nutritional and economical values, however, various diseases caused by dematiaceous fungi may lead to heavy yield losses in the quantity and quality. Dematiaceous fungi are characterized by a dark cellular pigment, resulting from a melanization process. Jacobson (2000) and Nosanchuck and Casadevall (2003) linked melanin with virulence, resistance, and susceptibility in plants.

Different *Alternaria* spp. were reported to be associated with different angiospermic families, but *Alternaria alternata* -a dematacious fungus- cause early blight diseases to Cucurbitaceae, Brassicaceae and Solanaceae families (Neeraj and Verma, 2010). Furthermore, different species of *Alternaria* had family-pathogenic infection specificity, for example, *A. tenuissima* and *A. cucumerina* on cucurbitaceous; *A. brassicae*, *A. brassicicola* and *A. raphani* on brassicaceous and *A. solani*, *A. longipes* and *A. crassa* on solanaceous plants (Neeraj and Verma, 2010).

There are several studies (Abada *et al*., 2008; Agamy *et al*., 2013; Bhatti *et al*., 2002; Metz, 2017) which investigated the controlling procedures for the early blight of these vegetables. One of the most commonly applied methods is the use of fungicides, but these compounds cause serious health hazards to human beings and cause environmental pollution. Hence, nowadays more emphasis is given to other methods of disease control. One of these methods is the employment of photodynamic treatments by using photosensitizers which are considered safer, more economical, and eco-friendly.

Several compounds when irradiated (photoactive compounds or photosensitizers) can cause toxic reactions in living cells (Matos and Ricardo, 2003). The importance of these compounds may increase, if they can react with different microbes that affect human or animal health, and crops. Polyetilenes, thiophenes, coumarins, furano-cocumarins, furanocromones, β-carbolines and other alkaloids and complex quinines are the main classes of plant photosensitizers (Arnason *et al*., 1992). By absorbing light, photosensitizers can trigger chemical modifications

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of a substrate or target. Photosensitizers are considered among the new environmentally safe and harmless pesticides to non-target microorganisms for crop protection.

The first utilization of light effects was for the control of insects (Robinson, 1983). At present, photosensitizers are promising compounds used to control insects, nematodes, weeds, algae, viruses, bacteria, yeast and fungi as well as tumor cells (Berenbaum, 1987; Wainwright, 1998; Hamblin and Hasan, 2004). A large number of synthetic and natural compounds for antimicrobial photodynamic therapy has been developed (Jori, 2006). Acridine hydrochloride used for the photoinactivation of microorganisms was the first to be described (Raab, 1900). The phenothiazine dyes as methylene blue and toluidine blue showed promising results against bacteria and fungi (Wainwright, 1998). The photosensitizers with highly conjugated molecules such as porphyrins and phthalocyanines possess improved optical properties (Sternberg and Dolphin, 1998). Phthalocyanines, which are characterized by far red wavelength absorption (>670 nm), long triplet life time (ms), and high quantum yields of singlet oxygen generation (>0.2), have been studied as drugs in microbial photodynamic inactivation (Jori and Tonlorenzi, 1999).

The photocatalytic technology is based on the interaction of light with semi-conductor particles to produce highly Reactive Oxygen Species (ROS) which destroy microbial cells (Biel, 2010). Thus, photochemically-active compounds in catalytic amounts (Aromatic Photosensitizers or APS) may be considered (Hamblin and Hasan, 2004). The elimination of microorganisms using APS is known as antimicrobial Photo-Dynamic Inactivation (PDI) (Maisch, 2007), and it uses visible light to activate the photosensitizers. The absorption of a photon by a photosensitizer leads to the production of ROS according to two pathways: (i) radicals formation, issued from electron transfer from the photosensitizer to molecules in its direct environment (Type I photo-oxidation reaction). In the presence of air, the most often produced ROS is thus superoxide radical-anion, $O_2^{\bullet -}$, (ii) energy transfer from the triplet excited state of photosensitizers to the ground state of oxygen, generating singlet oxygen, $^1O_2$, superoxide and radicals (Type II photo-oxidation reaction). These reactive species can oxidize the surrounding bioorganic molecules, such as proteins, nucleic acids, lipids, leading to cell death.

Toluidine blue (TBO) as antimicrobial photodynamic or photosensitizing agent is a promising method that can be used to control the phytopathogens. This method has been tried successfully under in vitro conditions against *Metarhizium anisopliae* and *Aspergillus nidulans* under different incubations of light conditions (Gonzales *et al*., 2010).

The objectives of the current study were: (i) to evaluate the impact of the photosensitizer in the presence of light on controlling the phytopathogenic *Alternaria* spp., and (ii) to determine the efficacy of photosensitizer on reducing secreted toxins, and (iii) to determine the efficacy of photosensitizer on inhibition of the hydrolytic degrading enzymes of isolated fungi in order to evaluate the possibility of using photozensitizer in plant protection against phytopathogenic fungi, and to reduce the use of hazardous chemical pesticides.

2. Materials and Methods

2.1. Collection, Isolation, Purification, and Preservation of Fungal Isolates

The samples for this study were collected in 2012 from naturally-infected vegetables including cucumbers (*Cucumis sativus* L.), squash (*Cucurbita pepo* L.), tomatoes (*Solanum lycopersicum* L.*)*, and pepper (*Capsicum annuum* L.) grown in different fields in Almadinah Almunawwarah region. The pathogens were isolated from different parts of the plant, such as the fruits, leaves, and stems which show symptoms of infection (Table 1).

For isolation, a small piece was removed from the lesion and healthy tissue of diseased plants and the surface was sterilized by 1 % sodium hypochlorite solution, then washed with sterile distilled water for one minute. After that, the specimen was air-dried under the laminar flow on sterilized filter paper, transferred to Potato Dextrose Agar plates (PDA) (formula/Liter; potato extract 4.0g, dextrose 20.0g, agar 15.0g; 4.0g of potato extract equivalent to 200g of infusion from potatoes (Oxoid LDT., Basingstoke, Hampshire, England), and incubated at 28º C±2 for one week.

A pure culture of each fungus was obtained by hyphal tipping, and the isolates were incubated onto PDA plates. For preservation, about ten agar plugs containing young and actively growing mycelia were taken from the margins of the colony using sterile 4-mm diameter cork-borer and were transferred into glass vials filled with 70 ml sterile distilled water and 30 ml glycerol and kept at -20° C.

Table 1. Information concerning host, locations and identity of the isolates collected from different fields at Almadinah Almunawwarah..

Isolate number	Isolate ID	Host	Field number	Location (GPS coordinate)		Organism	Gene bank accession number
				Coordinate 1	Coordinate 2		
2	AjKSA12-01	Tomato	F1	N24.620	E36.92	*A. japonica*	KF944473
5	PodKSA12-01	Tomato	F1	N24.620	E36.92	*Podospora* sp.	KF944474
6	AaKSA12-01	Tomato	F2	N24.709	E40.13	*A. alternata*	KF944475
7	ChaetKSA12-	Tomato	F1	N24.620	E36.92	*Chaetomium*	KF944476
8	AaKSA12-02	Tomato	F1	N24.620	E36.92	*A. alternata*	KF944477
9	TharKSA12-01	Tomato	F1	N24.620	E36.92	*Thielavia*	KF944478
10	PtKSA12-01	Tomato	F1	N24.620	E36.92	*Phoma tropica*	KF944479
11	AaKSA12-03	Tomato	F2	N24.709	E40.13	*A. alternata*	KF944480
12	AaKSA12-04	Tomato	F1	N24.620	E36.92	*A. alternata*	KF944481
13	AaKSA12-05	Tomato	F2	N24.709	E40.13	*A. alternata*	KF944482
14	AaKSA12-06	Tomato	F2	N24.709	E40.13	*A. alternata*	KF944483
16	AaKSA120-07	Squash	F3	N24.375	E39.54	*A. alternata*	KF944484
17	AaKSA12-08	Squash	F3	N24.375	E39.54	*A. alternata*	KF944485
18	AaKSA12-09	Squash	F3	N24.375	E39.54	*A. alternata*	KF944486
20	AaKSA12-10	Squash	F3	N24.375	E39.54	*A. alternata*	KF944487
21	AaKSA12-11	Squash	F3	N24.375	E39.54	*A. alternata*	KF944488
22	AaKSA12-12	Squash	F3	N24.375	E39.54	*A. alternata*	KF944489
23	AcKSA12-01	Pepper	F4	N24.515	E39.32	*A. compacta*	KF944490
24	AaKSA12-13	Pepper	F4	N24.515	E39.32	*A. alternata*	KF944491
25	ApKSA12-01	Pepper	F4	N24.515	E39.32	*A. porri*	KF944492
26	AaKSA12-14	Pepper	F4	N24.515	E39.32	*A. alternata*	KF944493

2.2. Identification of Fungal Isolates

2.2.1. Morphological Identification

Pure cultures were grown for seven days and were examined microscopically (Light Microscopic - LEICA DME) to view the morphological characters of the different fungi. Slide cultures were prepared for each isolate. They were photographed, and identified via identification key for the imperfect fungi of Barnett (1960), Barnett and Hunter (2003) and Ellis (1976).

2.2.2. Molecular Confirmation

A total of thirty isolates were sequenced in this study. The morphologically identified isolates were sub-cultured on PDA, and were sent for molecular identification to Fragment Analysis & DNA Sequencing Services (FADSS), Okanagan, British Columbia, Canada. Internal transcribed spacer region ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAG TAAAAGTCGTAACAAGG) were used for sequencing (White *et al*., 1990). DNA extraction, PCR's and sequencing using the ABI sequencer system were conducted on each isolate. Sequences were received as text files, and were edited via BioEdit version 5.0.6 (Hall, 2007) and BLASTn at the Gen Bank nucleotide database (http://www.ncbi.nlm.nih.gov/). Both, sequences of the thirty isolates and the reference isolates from the GenBank were aligned and a tree was executed using the software MEGA 5.1 (Tamura *et al*., 2011). The Maximum Likelihood method based on the Tamura-Nei model was used (Tamura and Nei, 1993). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985).

2.2.3. Fungal Isolates Used

Alternaria spp. were selected for studying the effect of the photosensitizer. Four species were chosen: *A. alternata* (AaKSA12-05 and AaKSA12-12), *A. compacta (*AcKSA 12-01), *A. porri (*ApKSA12-01), and *A. japonica* (AjKSA 12-01). *A. alternata* isolates were chosen because they were genetically different when they were molecularly confirmed.

2.3. Radiation and Photosensitization

2.3.1. White light lamp

The lamp (Lanzini Illuminazione, Italy) with a fluence rate of 400W/m^2 was used.

2.3.2. Photosensitizer

Toluidine blue O (TBO) (Sigma, UK) photosensitizer was used in this study. A 1000 µg/mL solution of TBO was used as stock solution in saline, and was kept at 2-4 C° in the dark. Different concentrations ranging from 0-100 µg/mL (0, 12.5, 25, 50, 100 µg/mL) were used for further experiments.

2.3.3. Linear Growth

Toluidine blue O (TBO) was prepared in different concentrations (125, 250, 500, and 1000 µg/mL). The medium was prepared in 500 mL flasks containing 270 mL sterilized Czapek Dox Agar (CZ) medium (formula/Liter; sucrose 30.0 g, $NaNO_3$ 2.0 g, K_2HPO_4 1.0 g, Mg_2SO_4 0.5

g, KCl 0.5 g, $FeSO_4$ 0.01 g, and agar 15.0 g) and 30 mL of TBO from each concentration. The control contained sterile distilled water instead of TBO. *Alternaria* spp. were grown on PDA from five to seven days depending on the species. A 10 mm diameter agar plug was transferred from PDA containing the fungal culture for each of the *Alternaria* spp. to a CZ plate that was previously treated with TBO. The culture of each species was divided into two groups: group 1 (no light) where the cultures were kept in the dark for sixty minutes, and group 2 (with light) where the cultures were exposed to fluent rate of 400 W/m^2 white light delivered for sixty minutes. After pre-incubated, both groups were incubated at 28°C in the dark. Colony diameters (in mm) were measured every forty-eight hours, until the mycelium filled the agar plate.

2.4. Production and Assay of Mycotoxin

In a preliminary experiment it was found that *A. alternata* was the only species that produces mycotoxins in a considerable amount. Therefore, the isolates AaKSA12-05 and AaKSA12-12 were selected for this purpose. For the mycotoxin production, Czapek Dox medium modified after Gatenbeck and Hermodsson (1965) was used (formula/Liter: 0.06 g, NH_4Cl, 0.25 g, $NaNO_3$, 1 g, KH_2PO_4, 0.5 g, $MgSO_4$ x 7 H_2O, 0.25 g, NaCl, 0.25 g, KCl, 0.01 g, $FeSO_4$ x 7 H_2O, 0.01 g, $ZnSO_4$ x 7 H_2O, 1 g, yeast extract, 30 g glucose (glucose is added separately after autoclaving). The pH was adjusted to 5.5. The inoculums were prepared as stated before in item 5.2. The alternariol was extracted from the medium with a mixture of acetonitrile and 4 % KCl in water (9:1 v/v, 40 mL) for thirty minutes, followed by the addition of 1 N HCl (8 mL). Alternariol was measured quantitatively by the mycotoxin analyzer (ROS-M Reader, Charm) using quantitative test kit (Burkin and Kononenko, 2011). The kit involves a quantitative lateral flow immunoassay with a range of sensitivity of 0 to 150 ppb and a limit of detection of 1 ppb.

2.5. Production of Pectinolytic and Cellulolytic Enzymes

Spore suspension of isolated AaKSA12-05 and AaKSA12-12 were prepared in 60 mL sterile distilled water flask. Then, 5 mL of the suspension were added to 5 ml of the different concentration of TBO (25, 50, 100 and 200 µg /mL). The control contained sterile distilled water instead of TBO. One group was kept in the dark for 60 minutes, and the other group was exposed to a fluent rate of 400W/m^2 white light delivered for sixty minutes. Ten milliliters of treated inoculums were grown on Czapek Dox broth (CDB) where the carbon source was substituted with one percent carboxy methyl cellulose (as substrate for cellulolytic enzymes), or one percent pectin (as substrate for pectinolytic enzymes). Carboxy methyl cellulose (CMC) medium consisted of (g/L): 7.5 g CMC, 7.5 g sucrose, 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g KCL, 0.01 g $FeSO_4$ g. Pectin medium has the same composition, but pectin replaced CMC. They were placed in incubator at 28°C in the dark for ten days. Then the treated inoculums were filtered for enzymes assay.

The cellulase and pectinase (polygalacturnase) activity were measured using the method of Bindo *et al*. (2005). The reaction mixture was prepared as follows ; 0.5 mL of 1 % substrate in phosphate citrate buffer (0.05 M, pH 5.2), 0.5 ml enzyme solution and 1 mL distilled water. They were placed in a shaking water bath at 50 °C for 10 minutes. Then three ml dinitrosalicylic acid reagent was added to stop the reaction. The tubes were heated in a boiling water bath for 5 minutes. After cooling, the tubes were centrifuged, and the absorbance was measured spectrophotometerically (APLE spectrophotometer PD-303UV) at 575 nm. One unit of enzyme activity (U) was defined as 1 µmol of reducing sugar released per minute.

2.6. Statistical Analysis

Analysis of variance (ANOVA) was performed using the PROC ANOVA in Statistical Analysis System (SAS 9.3; SAS Institute Inc., Cary, NC). Differences in means were compared using Fisher's least significant difference (LSD) test ($\alpha = 0.05$). Non parametric analysis was performed using MINITAB16 software (Minitab, State College, PA).

3. Results

3.1. Identification of Fungal Isolates

3.1.1. Morphological and Microscopical Characteristics

A total of thirty fungal isolates were collected from tomato, squash and pepper. Based on morphological characters, the isolates were related to six genera, namely: *Alternaria*, *Phoma*, *Chaetomium*, *Thielavia*, *Podospora* and *Trichoderma*. The most dominant genus was *Alternaria* which was represented by *A. alternat*a (n=23), *A. compacta* (n=1), *A. porri* (n=1) and *A. japonica* (n=1). As for the other genera, each was represented by one species namely: *Phoma tropica*, *Chaetomium* sp., *Thielavia arenaria*, *Podospora* sp. and *Trichoderma* sp.

3.1.2. Molecular Confirmation

The thirty isolates were sent for molecular identification and conformation. The ITS4/ITS5 gene region was amplified in the thirty isolates, but due to bad sequencing only twenty-one isolate were included in the dendogram (Figure 1). In the dendogram, eight species of the samples were shown clearly and included *Alternaria alternata*, *A. compacta*, *A. porri*, *A. japonica*, *Phoma tropica*, *Thielavia arenaria*, *Podospora* sp., and *Chaetomium* sp. (Figure 1). As shown in the dendogram, the *A. alternata* isolates recovered from the different hosts are distributed in different clusters indicating a genetic variation among the isolates. For example, *A. alternata* isolates number seventeen and twenty-two are found in cluster A, while isolates six and twenty-four are found in cluster B. Moreover, the *A. alternata* isolates within cluster A are found in different sub-clusters (Figure 1).

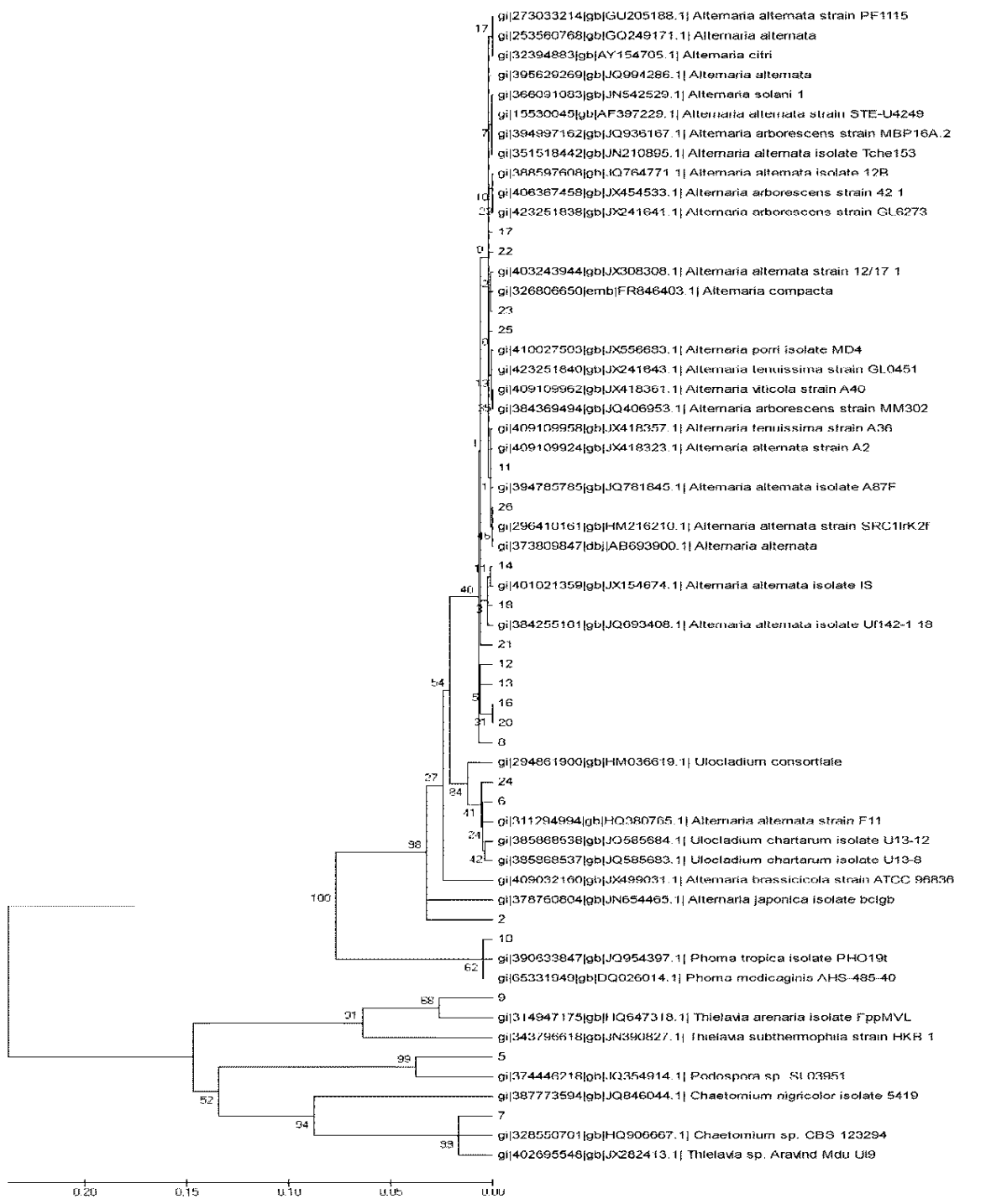

Figure 1. Dendogram of the 21 fungal isolates amplified using the ITS4/5 gene region. The dendogram was executed by MEGA 5 (Tamura *et al*., 2011) with 1000 bootstraps.

3.1.3. Linear Growth

Due to the dominance of *Alternaria* species in the isolated samples, the present work focused on the four *Alternaria* species, of which *A. alternata* was represented by two isolates. Among the five isolates of *Alternaria* spp. investigated in this study, *A. alternata* isolated from tomato had the highest mycelium growth regardless of the presence or absence of light compared to the other isolates (Table 2, Figure 2).

There were significant differences among the isolates (Table 3). Generally, the linear growth rate decreased as the concentration of the toluidine blue-O (TBO) increased with significant difference for the light, isolate, and concentration factors (Table 2, 3). Moreover, isolate-TBO concentrations' interaction showed highly significant differences (Table 2, 3, Figure 2).

Table 2. Linear growth (mm) of *Alternaria* spp. isolated from different hosts, treated with Toluidene Blue-O, and subjected to dark and light for 60 minutes [ab].

Isolate	Host	TBO (µg/ml) (Mean±se)									
		Dark					Light				
		0	12.5	25	50	100	0	12.5	25	50	100
Linear growth (mm)											
A.alternata	Tomato	66.84±3.45	62.00±2.18	55.17±1.54	53.38±1.28	49.78±0.65	57.33±1.70	54.26±1.84	49.25±1.50	47.57±1.14	42.15±0.46
A. alternata	Squash	61.03±2.56	55.73±2.78	46.08±3.11	43.89±2.59	40.26±1.60	53.90±1.32	47.42±1.27	39.58±2.21	34.18±1.07	30.96±1.18
A.compacta	Pepper	66.47±3.58	61.15±2.04	54.38±1.42	52.76±1.15	48.13±1.02	56.50±1.54	53.03±1.63	47.78±1.10	45.16±0.89	42.55±1.20
A. porri	Pepper	51.74±2.82	51.49±2.87	50.52±2.62	49.63±2.42	48.34±2.17	48.32±1.81	45.87±1.27	45.33±1.21	44.48±1.08	43.09±0.82
A. japonica	Tomato	45.27±4.14	44.73±4.01	42.67±3.60	41.34±3.50	40.16±3.27	41.03±3.07	40.55±2.69	39.16±2.43	35.55±2.48	33.72±2.13

[a] Each value is a mean of three replicates. [b] Isolates were incubated at 28 °C ± 2 for six days.

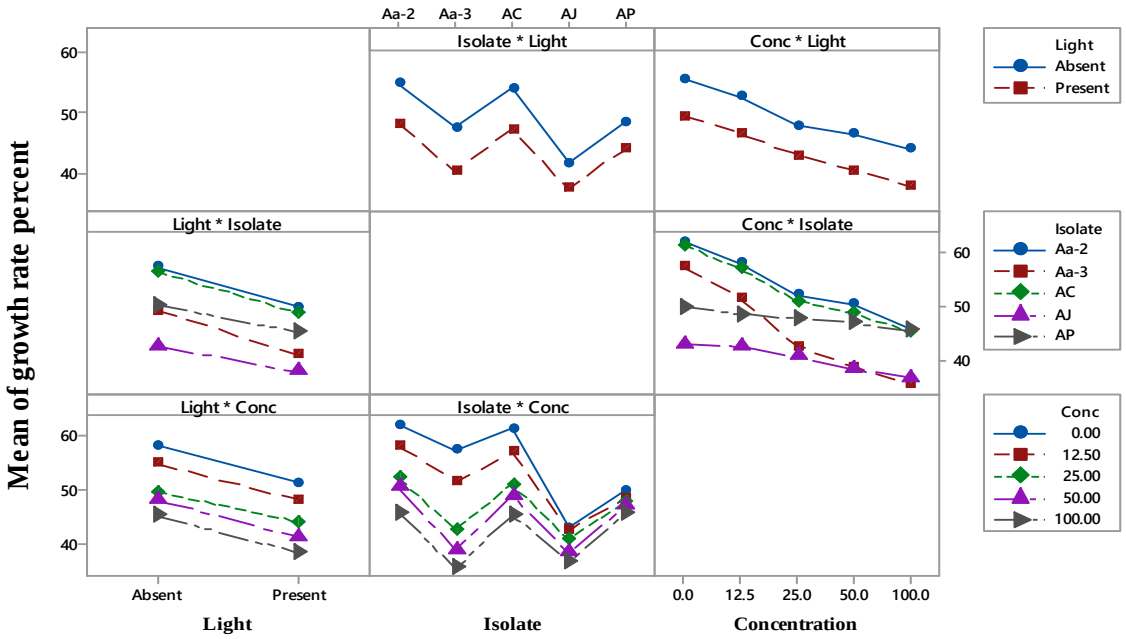

Figure 2. The linear growth under different isolates of *Alternaria* interactions between the main factors considered in this study.***Aa-2:** *A. alternata* isolated from tomato. **Aa-3:** *A. alternata* isolated from squash. **AC:** *A. compacta* isolated from pepper. **AJ:** *A. japonica* isolated from tomato. **AP:** *A. porri* isolated from pepper.

Table 3. Analysis of variance for linear growth using adjusted sum of squares for tests.

Source of variation		linear growth	
	df	F	*P*-value
Light [a]	1	108.33	0.000
Isolate [b]	4	60.78	0.000
TBO concentration [c]	4	54.39	0.000
Replicate [d]	2	0.01	0.990
Light*Isolate	4	1.25	0.291
Light*TBO concentration	4	0.16	0.956
Isolate*TBO concentration	16	3.87	0.000
Light*Isolate*TBO concentration	16	0.19	1.000
Error	398		
Total	449		

[a] Light source was either present or absent ,[b] *Alternaria compacata* isolated from pepper, *A. japonica* isolated from tomato, *A. porri* isolated from pepper, *A. alternata* isolated from tomato, and *A. alternata* isolated from squash. [c] Five concentrations were used: 0, 12.5, 25, 50, and 100 µg/µL of the TBO photosensitizer. [d] Three replicates were used for each treatment.

3.2. Production and assay of mycotoxin

In a preliminary test, the different isolates of *Alternaria* were investigated for the production of mycotoxin. *A. alternata* showed a considerable production of the mycotoxin alternariol, so the two isolates of this species were employed in this experiment. Alternariol was produced more and was highly significant in the *A. alternata* isolated from tomato with a mean of 26.45 compared to *A. alternata* isolated from squash with a mean of 14.46 µg/mL (Tables 4 and 5). The presence of light caused a significant decrease in the production of alternariol with a mean of 24.60 and 16.31 µg/mL in the case of absence and presence of light, respectively. Concerning toluidine blue O (TBO) concentration, the alternariol amount further decreased as the concentration increased, particularly in the presence of light. There was a significant difference among the concentrations (Table 5). Moreover, all the interactions among the different factors included in the analysis model were high and significantly different (Table 5).

Table 4. Effect of different concentrations of toluidine blue O (TBO) on the production of the mycotoxin, alternariol by *Alternaria allternata* isolated from squash or tomato.

	TBO concentration (µg/mL) (Mean±sd)									
Host	0		12.5		25		50		100	
	D *	L	D	L	D	L	D	L	D	L
A. alternata-squash	18.7±0.00	21.9±3.09	18.0±0.00	14.2±0.00	19.8±0.00	11.4±1.53	16.9±0.00	5.7±0.00	15.8±1.58	2.2±0.00
A. alternata-tomato	32.3±0.01	34.5±0.00	33.5±0.00	37.1±0.00	35.0±0.02	18.6±0.00	28.8±0.01	11.7±1.54	27.2±0.00	5.8±0.00

*D=TBO- treated inoculum of *A. alternata* was incubated in dark for one hour; L= TBO- treated inoculum of *A. alternata* was incubated in light for one hour.

Table 5. Analysis of variance for alternariol mycotoxin under different interactions.

Source	DF	Mean Square	F Value	Pr > F
Model	21	303.48	5.07E15	<.0001
Host [a]	1	2156.40	3.6E16	<.0001
Light [b]	1	1030.86	1.72E16	<.0001
Concentration [c]	4	450.69	7.53E15	<.0001
Replicate [d]	2	0.00	2.61	0.0865
Host*Presence or absence of light	1	35.11	5.87E14	<.0001
Host*concentration	4	62.43	1.04E15	<.0001
Light* Concentration	4	242.93	4.06E15	<.0001
Host*light*Concentration	4	31.61	5.28E14	<.0001
Error	38	0.001		
Corrected Total	59			

[a] Two hosts were studied: tomato and squash. [b] Treatments were divided into two groups: light-treated and dark-treated inoculums.

[c] The toluidine blue O (TBO) concentrations were used: 0, 12.5, 25, 50, and 100 µg/mL. [d] Three replicates were used for each treatment.

3.3. Production of Pectinolytic and Cellulolytic Enzymes

Pectinase and cellulase activities of both isolates of *Alternaria alternata* were significantly reduced when incubated in light as compared to dark (Table 6).

As the concentration of toluidine blue O (TBO) increased, the enzymes significantly decreased reaching 3.44 and 3.27 U/g substrate for pectinase and 1.66 and 1.47 U/g for cellulase at 100 µg/mL TBO incubated in the dark in the case of *A. alternata* isolated from tomato and squash, respectively (Table 6). The activity of both enzymes was steadily reduced when 100 µg/mL TBO-treated samples were exposed in light for sixty minutes reaching 1.03 and 0.73 U/g substrate for pectinase and 0.48 and 0.26 U/g substrate for cellulase in the case of *A. alternata* isolated from tomato and squash, respectively (Table 6). Generally, enzymes' activities were high in *A. alternata* isolated from tomato compared to *A. alternata* isolated from squash. Moreover, all the interactions among the different factors included in the regression analysis were high in significance (Table 7).

Table 6. Pectinase and cellulase activity (U/g substrate)* of *Alternaria alternata* isolates pre-incubated for 60 minutes with different concentrations of Toluidine Blue O (TBO) in dark (D) or in light (L).

alternata isolated from (host)	TBO concentration (µg/ml)									
	0		25		50		75		100	
	D	L	D	L	D	L	D	L	D	L
Pectinase										
Tomato	4.31±0.005	1.80±0.017	4.04±0.005	1.67±0.008	3.87±0.008	1.56±0.005	3.67±0.013	1.42±0.005	3.44±0.008	1.03±0.013
Squash	4.05±0.005	1.68±0.005	3.96±0.013	1.58±0.008	3.76±0.005	1.32±0.005	3.54±0.022	0.96±0.02	3.27±0.013	0.73±0.013
Cellulase										
Tomato	3.95±0.020	1.59±0.009	3.32±0.020	1.46±0.005	2.75±0.008	1.33±0.013	2.06±0.012	1.08±0.017	1.66±0.008	0.48±0.013
Squash	3.70±0.005	1.33±0.013	3.06±0.013	1.08±0.023	2.18±0.013	0.80±0.017	1.76±0.012	0.56±0.005	1.47±0.005	0.26±0.008

* One unit of enzyme activity is defined as the amount of enzyme required to release 1µmol reducing sugars per ml under assay condition.

Table 7. Analysis of Variance for enzyme activity, using adjusted sum of squares for tests.

Source	DF	Mean Square	F-Value	*P*-Value
Enzyme [a]	1	18.715	40197.62	0.000
Light [b]	1	120.683	259206.2	0.000
Isolate [c]	1	2.218	4764.36	0.000
Concentration [d]	4	6.017	12924.16	0.000
Replicate [e]	2	0.001	1.38	0.257
Enzyme*Light	1	5.059	10865.37	0.000
Enzyme*Isolate	1	0.165	354.37	0.000
Enzyme*Concentration	4	0.684	1468.54	0.000
Light*Isolate	1	0.053	113.66	0.000
Light*Concentration	4	0.421	905.12	0.000
Isolate*Concentration	4	0.036	78.15	0.000
Enzyme*Light*Isolate	1	0.002	4.04	0.048
Enzyme*Light*Concentration	4	0.449	964.19	0.000
Enzyme*Isolate*Concentration	4	0.035	75.65	0.000
Light*Isolate*Concentration	4	0.023	48.96	0.000
Enzyme*Light*Isolate*Concentration	4	0.007	15.48	0.000
Error	78	0.000		
Total	119	177.624		

[a] Two enzymes were studied: cellulase and pectinase. [b] Light source was either present or absent. [c] Tow isolates of *Alternaria* were used: *A. alternata* isolated from tomato, and *A. alternata* isolated from squash. [d] Five concentrations were used: 0, 12.5, 25, 50, and 100 µg/µl of the TBO photosensitizer. [e] Three replicates were used for each treatment.

4. Discussion

Many dematiaceous hyphomycetes cause economically critical diseases in all types of vascular plants, especially agricultural crops. The class hyphomycetes are primary pathogens of plants and weeds, causing, root, stem and leaf necrosis, dieback, cankers, wilts, and blight (Ellis and Ellis, 1987).

In the present study, thirty fungal isolates were recovered from tomato, squash and pepper samples including fruits, leaves and stems cultivated in different regions at Almadinah Almunawwarah. The identification was based on the morphological characteristics of the isolates, and was confirmed by DNA sequencing and BLAST analysis. The isolates were related to six species assigned to five genera. *Alternaria japonica*, *A. porri*, and *A. compacta*, are new records for Saudi Arabia. About 50 % of the isolated species were related to *A. alternata*. This specie is a quite common pathogenic fungus associated with vegetables. *A. alternata* is considered as an opportunistic pathogen on numerous hosts causing leaf spots, rots, and blights on many plant parts (Silva and Melo, 1999). Other isolated members of *Alternaria*, though recovered in low occurrence, included *A. compacta*, *A. porri* and *A. japonica*. Several authors indicated that the release of many dry-dispersed spores, including *A. porri*, shows diurnal periodicity, with the majority of spores being collected during midday when temperature, wind speed and the level of turbulence near the ground are highest, and relative humidity lowest (Humpherson-Jones and Phelps, 1989). This occurrence conditions of *Alternaria* spp. match the environmental conditions dominant at Almadinah region.

A. japonica and *A. compacta* are rarely encountered as phytopathogens. The former species were identified from

the leaf lesions of turnip and cabbage plants in Spain (Bassimba *et al*., 2013), and from the seeds of cruciferous vegetable crops in Japan (Tohyama and Tsuda, 1995), and from the Chinese cabbage seedlings in China (Ren and Zhang, 2012), while the later fungal species were recovered from climbing hydrangea (*Hydrangea anomala* subsp. petiolaris) (Garibaldi *et al.*, 2008). *Phoma tropica* has been identified as thermotolerant species, which is mainly found in greenhouses on a wide range of hosts, but probably has a tropical origin (Schneider and Boerema, 1975). *Phoma* species are common soil inhabitants. Overhead irrigation or rain splash may result in an excessive spread of the species.

Thielavia arenaria, isolated in the present research, may be interrelated to some other species especially those producing dark pigmented mycelium and brownish-black, hairy ascomata like *T. gigaspora* Mouchacca, *T. hyrcaniae Nicot*, *T. microspora* Mouchacca and *T. subthermophila* Mouchacca. (Mouchacca, 1973). The genotype of *T. subthermophila* is very close to *T. arenaria*, revealed in dendogram of the fungal isolates recovered in this study, and amplified using the ITS4/5 gene region (Figure 5). Thermotolerance tests of *T. arenaria* (unpublished data) showed that the isolate grew rapidly at both 35°C and at higher temperatures of 42°C, 45°C, and 50°C. This ecological manner is harmonizing with the environmental conditions predominant in Almadinah region during summer.

Each of *Podospora* and *Chaetomium*, was isolated once in the current study. These two genera are considered as soil mycobiota of date palm plantations in Elche, SE Spain (Abdullah *et al.*, 2010), a situation similar to that found in Saudi Arabia where vast areas are abundant with date palm trees. The investigations on soil fungi of several date palm plantations in Iraq have revealed several novel and interesting fungal taxa (Abdullah and Zora, 1993).

The present work is focused on the possibility of inactivating the phytopathogenic fungi by exploiting photosensitization. Toluidine Blue O (TBO) in different concentrations up to 100µg/mL was employed as photosensitizer. The application of higher TBO concentration (>100µg/mL) was significantly ineffective in exerting more reduction in growth when compared with that recorded at 100µg/mL (unpublished data). The decrease in the efficacy of the photosensitizer at the higher concentration can be explained by the decreased penetration of light through the suspension with increasing the TBO concentration rather than the decrease in the uptake. This would reduce the amount of light reaching the sensitized fungi, and would reduce the overall efficiency of the system (Manpreet *et al*., 1998).

Five of the isolated fungi were the target of the experiments, namely: *Alternaria alternata* (two molecularly different isolates, one isolated from tomato and one from squash), *A. compacta*, *A. porri* and *A. japonica*. The linear growth and spore germination of the investigated isolates were gradually decreased as the TBO concentration elevated up to100µg/mL. The reduction in growth and in spore germination was more obvious during the pretreatment of the samples with TBO in the presence of light for sixty minutes. *A. japonica* and *A. alternata* isolated from squash were more susceptible than the other isolates. Minnock *et al.* (1996) reported that gram-negative and gram positive bacteria can be killed after sensitization with phthalocyanine using 400 w metal halide spectral lambs. Ladan *et al.* (1993) demonstrated that Staphylococcus aureus can be killed by using a white light source and hematoporphyrin as a photosensitizer.

A. alternata is a common species of critical significance because it produces a number of mycotoxins. Natural occurrence of alternariol, alternariol methyl ester and other toxins has been frequently detected in sunflower seeds, apples, mandarins, olives, spices, tomatoes, soybean, cereals, melons, peppers, apples, pecans, wheat flour, oilseed rape meal, cucumbers, oranges, lemons as well as processed fruits and vegetables (Scott and Kanhere, 2001 and Asam *et al.*, 2011).

A steady reduction in the alternariol production for both isolates was noticed in case of the treatment of the inoculums with TBO; they were exposed to light for 60 minutes reaching 2.2 and 5.8 µg/mL for *A. alternata* isolated from squash and tomato, respectively as compared to 21.9 and 34.5 µg/mL regarding control in the absence of light. The results indicate that the reduction in alternariol may be attributed to the presence of the photosenistizer. This result is somehow consistent with that published by Söderhäll *et al*. (1978) who found that the production of alternariol and alternariol monomethyl ether by *A. alternata* (Fr.) Keissler, grown in drop culture, produced alternariol and alternariol monomethyl ether were almost completely inhibited when the fungal cultures were exposed to white light (180 W/m2), although mycelial dry weight was not significantly affected. However, the divergence in the results of Söderhäll *et al.* (1978) and those cited in the present work might be related to the fungus long exposure to light; its photosensitization may be attributed to the presence of melanin pigment. Häggblom and Unestam (1979) showed that light inhibits the production of the mycotoxins alternariol and alternariol monomethyl ether, both polyketids produced by *A. alternata*. The authors also indicated that the effect seems to be general because seven of the isolates of *A. alternata* with different alternariol- and alternariol monomethyl ether-producing abilities, all respond to continuous light with reduced levels of alternariol and alternariol monomethyl ether. DiCosmo and Straus (1985) indicated that the mycotoxin alternariol is phototoxic to *Escherichia coli* in the presence of near UV light (320-400 nm). The phototoxicity bioassays with a DNA repair-deficient mutant of *E. coli* suggested that DNA may be the molecular target for photo-induced toxicity of alternariol. Interactions between alternariol and double-stranded, supercoiled DNA suggest that alternariol interacts with DNA by intercalation. These results suggest that alternariol is a phototoxic, DNA-intercalating agent and is a DNA cross-linking mycotoxin in near UV light.

Phytopathogenic fungi that attempt to colonize higher plants must compete with physical barriers of the host as surface waxes and cell wall (Dyakov *et al.*, 2007). Phytopathogenic fungi produce several pectolytic enzymes, which are capable to degrade the plant cell wall components during plant pathogenesis. It was proved that hydrolytic enzymes have a significant role in pathogenesis due to their impact on the degradation of infected tissues and the expansion of disease (Bateman and Miller, 1966). However, some investigators have not proved that these enzymes are specific factors determining the degree of phytopathogenic virulence (Keen and Erwin, 1971). On

the other hand, some authors indicated a correlation between virulence of isolates and their ability to produce pectolytic enzymes (El-Ktatny, 1984). In a number of systems, a strong correlation has been found between the presence of pectinolytic enzymes and the disease symptoms and disease virulence (Durrands and Cooper, 1988).

On the whole, the mean activity of pectinase exhibited higher activity than cellulase for both *A. alternata* isolates. It is well-known that the relative quantitative production of the enzyme depends on the availability of the substrate in the cell wall of the host plant that provides all the necessary nutrients for the pathogen. The xylanase, pectinase and cellulase yields by *Sporotrichum thermophile* were 400-, 200-, and 20-fold higher in solid state fermentation as compared to those in submerged fermentation and similar increase in yields of enzymes in solid-state fermentation over submerged fermentation (Babu and Satyanarayana, 1995).

Steady inhibition of both investigated enzymes was recorded for the mycelium developed from photosensitized inoculums in the presence of light at 100 µg/ml TBO concentration. The activity of pectinase reached 1.01 and 0.72 while dropped down to 0.43 and 0.29 U/g substrate, in the case of *A. aternata* isolates recovered from tomato and squash, respectively. Paardekooper *et al*. (1995) showed that the photodynamic treatment of yeast with TBO causes a rapid uptake of a small amount of the dye, resulting in damage to intracellular enzymes (alcohol dehydrogenase, cytochrome-c oxidase, glyceraldehydes-3-phosphate dehydrogenase and hexokinase). These enzymes control the glycolysis and oxidative phosphorylation. It was found that when the *Kluyveromyces marxianus* is pretreated with 1 µM of chloroaluminium phthalocyanine and then illuminated with 150 W tungsten-halogen slide projector lamp, this did not affect the hexokinase activity, but the activity of the mitochondrial enzyme decreases. Ouf *et al.* (2003) evaluated the antifungal activity of solar simulator in presence of haematoporphyrin derivative, methylene blue and toluidine blue O as photosensitizers against seven dermatophytes. They indicated drastic inhibition of keratinase, phosphatases, amylase and lipase when the fungi were irradiated after their treatment with the photosensitizers. *Trichophyton verrucosum* and *T. mentagrophytes* were the most sensitive to photo-sensitization.

The relatively lower efficiency of TBO in light against *A. alternata* at 100 µg/mL compared to the corresponding lower concentrations or to that exposed to TBO in light may be due to the high density of the TBO particles which decrease light penetration of the photosensitizer. Moreover, the high concentration of TBO may increase the particles aggregation and consequently lowers fungitoxic effect. This phenomenon is known as self-shielding, in which saturated concentrations of the substance absorb a major portion of the incident light in the surrounding medium and superficial layers. A study by Rizvi *et al* (2013) stated that high concentrations of the photosensitizer may not induce the expected effective result. These observations point to the importance of "right" amount of photosensitizer and "right" light irradiance to achieve effective treatment.

5. Conclusions

It was possible using photosensitizers, such as the toluidine blue O (TBO), for the protection of plants against phytopathogenic fungi, above all, *Alternaria* spp. The photosensitizer (TBO) is useful as an alternative method in plant disease management and disease control in Saudi Arabia, a country characterized by sunshine throughout the year. When compared to the classical and traditional methods such as the usage of fungicides which cause environmental pollution and more significantly serious health hazards to human beings, the photosensitizers, such as (TBO), are safer, more economical, and eco-friendly.

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