



## Biological and Chemical Control of Some Tomato Fungal Diseases

Salwa M. Abdel Rahman<sup>(1)#</sup>, Hoda Yusef<sup>(1)</sup>, Jana Halawi<sup>(2)</sup>

<sup>(1)</sup>Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt; <sup>(2)</sup>Department of Biological Sciences, Faculty of Science, Beirut Arab University, Beirut, Lebanon.



**I**N RESPONSE to environmental and health concerns about the extended use of pesticides, it exists a considerable interest in finding alternative control approaches. The current study compared the antifungal activities of some biocontrol agents. Three fungal strains were isolated from infected tomato. Two belonged to genus *Aspergillus* and the third was *Fusarium* sp. Ten morphologically different bacterial strains were isolated from intact tomato roots and rhizospheric soils, they were screened for their antagonistic effects against the fungal isolates. *Achromobacter xylosoxidans* produced the most potent antifungal activity as indicated by the percentage of fungal weight loss recorded (equivalent to 73, 77, and 99% for *Aspergillus* sp. 1, *Fusarium* sp., and *Aspergillus* sp. 2, respectively). Four tested plants: Inula (*Dittrichia viscosa*), Onion (*Allium cepa*), Basilic (*Ocimum basilicum*), and Quinoa (*Chenopodium quinoa*) were tested for their antifungal activities against the isolated fungal pathogens. Inula extract showed the highest inhibition of fungal growth among the tested plants. The MIC values were evaluated for the tested biocontrol agents and compared with the standard antifungal chemicals: Between and Logico. In *vivo* study was performed using seedlings of Tomato (*Solanum lycopersicum*). The seedlings infected with the fungal pathogens using spraying technique showed severe symptoms of fungal disease, while the tomato plants infected with fungal pathogens and treated with the biocontrol agents (cell-free supernatant of *A. xylosoxidans* or Inula extract) exhibited observed reduction in the percentage of disease severity.

**Keywords:** *Achromobacter xylosoxidans*, Biocontrol agent, *Dittrichia viscosa*, Tomato.

### Introduction

In recent years, the biological control of plant pathogens has been considered as an alternative strategy because chemical control results in the accumulation of harmful chemical residues, which may lead to serious ecological problems (Madbouly, 2018). At present, the effective management of plant diseases and microbial contamination in several agricultural crops is generally achieved by synthetic pesticides. However, their continuous and indiscriminate application represents a health hazard for both animals and humans due to the residual toxicity associated with these chemicals (Heydari & Pessarakli, 2010; Singh et al., 2018). Tomato (*Solanum lycopersicum* L.) is one of the most widely grown vegetables in the world (Kumar

& Prabha, 2018). It is also an important source of potassium, phosphorus, magnesium, and iron which are essential to maintain the normal activity of nerves and muscles vitamins (e.g., A, B, and C) (Bhowmik et al., 2012). It is the third source of vitamin C in our diet, and the fourth of vitamin A, due to its content of beta-carotene or pro vitamin A; phytosterols, which help maintaining cholesterol under control; folic acid, which helps to eliminate homocysteine, an amino acid whose metabolism on the metabolism of B-complex vitamins (especially on that of folic acid) (Martí et al., 2016). Moreover, tomato contains lycopene, which is a powerful antioxidant that, unlike nutrients in most fresh fruits and vegetables, has an even greater bioavailability after cooking and processing. This fruit is also responsible for other protective mechanisms, such as the antithrombotic

#Corresponding author email: salwam218@gmail.com

Tel: 00201271862554

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and anti-inflammatory functions. In addition, studies have found a relationship between eating tomatoes and a lower risk of certain cancers, as well as other conditions, including cardiovascular disease, osteoporosis, ultraviolet light-induced skin damage, and cognitive dysfunction (Bhowmik et al., 2012). In recent years, Lebanese tomato has lost its original taste because of the abundant use of chemical fertilizers and pesticide residues in cultivations. According to the Lebanese Ministry of Agriculture, the most common tomato fungal diseases and pathogens found in Lebanon are: late blight (*Phytophthora infestans*), early blight (*Alternaria solani*), *Fusarium* wilt (*Fusarium oxysporum* f.sp. *lycopersici*), southern blight (*Sclerotinia sclerotiorum*), damping-off (*Pythium* spp.), *Verticillium* wilt (*Verticillium* spp.), powdery mildew (*Leveillulataurica* spp.), gray mold disease (*Botrytis cinerea*), and fruit rot (*Aspergillus* spp.). The widespread use of fungicides to control tomato diseases has led to an increase in health hazards due to their phytotoxic residual and pollution effects. Therefore, using alternative means of disease control to replace agrochemical compounds is strongly encouraged. The metabolites produced by rhizospheric bacteria and some plants include potent antifungal agents, and can be used as biocontrol agents. The aim of the present study was therefore to evaluate the effectiveness of biopesticides in the control of the tomato fungal diseases, and compare the performance of chemical and biological control strategies.

## **Materials and Methods**

### *Sample collection*

Different samples of infected tomatoes (leaves and fruits), tomato roots, and rhizospheric soils were collected from several tomato cultivation areas located in Saida, Lebanon. Also, fresh leaves of Inula, onion, basil, and quinoa were also collected from the same region.

### *Isolation and identification of the fungal isolates*

Inocula were extracted from the infected tomatoes (leaves and fruits) and were streaked on Sabouraud dextrose agar plates. After incubation at 30°C±0.2 for 5 days, the morphologically different fungal colonies were collected, purified, and maintained on the same medium used for isolation. The fungi were examined microscopically and identified to the genus level, based on their morphological characteristics,

using fungal slide culture techniques (Betancur et al., 2012; Campbell et al., 2013).

### *Isolation of bacterial isolates*

One gram of intact tomato root system was gently washed to remove all the loose adhering soil without damaging the rhizosphere. The root portions were crushed, suspended in 100mL of sterile and distilled serial dilutions of the root extracts, and the collected rhizospheric soils were prepared using sterile saline solution (Xu & Kim, 2014). From each dilution, inocula were streaked on nutrient agar (NA), MacConkey's agar, mannitol salt agar, and cetrimide agar plates. After incubation at 30°C±0.2°C for 48h, the morphologically different bacterial colonies were collected, purified, and maintained on the same medium used for isolation.

### *Preparation of cell-free supernatant from bacterial culture and identification of selected isolates*

Only the bacterial isolate that showed potential antagonists against *Fusarium* sp. and *Aspergillus* spp. (named B3) was selected. Inocula from the bacterial culture (24 h old, grown in NA slant) were placed into 250mL Erlenmeyer flasks containing 50 ml of nutrient broth (NB) and were used to initiate growth ( $A_{600nm} \leq 1$ ). Standard inocula of 2% (v:v) were collected from the NB after growth for 18h at 30°C±0.2°C on a reciprocal shaker (120rpm), and they were introduced into 250mL Erlenmeyer flasks filled with 50mL of NB. The cultures were incubated under shaking conditions (120rpm) at 30°C±0.2°C for 24h. Then, they were centrifuged at 6000rpm for 20min. The supernatants were filtered sterilized using 0.2µm syringe bacterial filters to ensure their sterility. The sterile supernatants were used for antagonistic bioassays (Spadari et al., 2013).

The bacterial isolate selected for the production of biocontrol agents was gram stained and identified using API 20 NE. Identification was performed using the online database apiweb™ (API 20 NE V4.1).

### *Bioassay of bacterial antagonism against fungi*

#### *Agar plug method*

Using a sterile cork borer, plugs of about 0.5cm in diameter were obtained from the bacterial cultures (24h old) grown on NA medium, and were then transferred to glucose peptone agar plates inoculated with the fungal pathogens.

Fungal inocula were prepared by growing the isolates on glucose peptone agar slants for 5 days. Each slant was flooded with 10 ml of sterile distilled water containing 1% Tween 20, and the suspensions were adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.3 at 530 nm (82% to 60% transmittance). The stock suspension contained mostly conidia at  $5 \times 10^6$  CFU/ml (Aberkane, 2002). The inhibition zones around the plugs were measured after incubation at  $30^\circ\text{C} \pm 0.2^\circ\text{C}$  for 2 days (Balouiri et al., 2016).

#### *Disc diffusion method*

Several glucose peptone agar plates were streaked with stock fungal spore suspensions. Then, sterile filter paper disks (about 6 mm in diameter), were loaded with 25  $\mu\text{l}$  of the prepared bacterial cell-free supernatant and placed on the agar surface. In control plates, the filter paper disk was loaded with 25  $\mu\text{l}$  of sterile NB. The Petri dishes were incubated at  $30^\circ\text{C} \pm 0.2^\circ\text{C}$  for 48 h. After the incubation period, they were checked for inhibition zones, whose diameter was then measured (Spadari et al., 2013; Milijasevic-Marcic et al., 2018).

#### *Preparation of plant extracts*

Ten grams of fresh leaf material was collected from the four tested plant species was washed with water and crushed in a mortar with a pestle as sterile distilled water was added at the ratio of 10mL/g of plant tissue—and was then placed in a refrigerator at  $4^\circ\text{C}$  for 48h. Subsequently, the material was centrifuged for 20min at 6000rpm and filtered using bacterial filters. The extracts were stored at  $4^\circ\text{C}$  for 24h in a refrigerator, and were then tested (Sallam & Kamal, 2012; Swamy Gowda et al., 2018).

#### *Food poison assay*

One ml of each tested aqueous plant extract was incorporated into 20 ml of molten glucose peptone agar, it was mixed well, and the contents were poured in a Petri dish. After overnight pre-incubation, plugs of about 25mm were obtained from fungal cultures (5 days old) using a sterile cork borer, and were deposited in the center of the plates. Control plates were prepared without the addition of the plant extracts. After further incubation at  $30^\circ\text{C} \pm 0.2^\circ\text{C}$  for 48h (Ramaiah & Garampalli, 2015), the diameters of fungal growth in both the control and tested plates were measured, and the antifungal effect was estimated using the following formula (Marques et al., 2018);

Mmbaga et al., 2018):

$$\% \text{ Growth inhibition} = [(D_c - D_s) / D_c] \times 100$$

where,  $D_c$ = Diameter of fungal growth in the control plate,  $D_s$ = Diameter of fungal growth on medium mixed with plant extract

#### *Fungal dry weight loss*

Fungal growth was determined after incubation with the cell-free supernatant prepared from the selected bacterial isolate (B3), plant extracts, or commercial chemical fungicides (Walker et al., 2001). Ten ml of fungal stock spore suspensions was introduced into 250mL Erlenmeyer flasks filled with 30 ml of glucose peptone broth. An aliquot (10, 20, 30, or 40mL) of sterile cell-free supernatant previously prepared from the B3 bacterial culture, or an aliquot of each sterile plant extract previously prepared, or an aliquot from each commercial chemical fungicide, was added to each flask inoculated with the fungal spore suspension. The cultures were then incubated at  $30^\circ\text{C} \pm 0.2^\circ\text{C}$  under static conditions for 5 days; and after this period, they were centrifuged at 6000rpm for 20min. Control flasks were prepared by substituting the antifungal agent with sterile distilled water. The fungal mats were washed with distilled water and recentrifuged, and were then oven-dried at  $60^\circ\text{C}$  until constant weight was obtained (Walker et al., 2001). Finally, the fungal dry weights were determined, and the percent fungal weight loss was calculated using the following formula:

$$\% \text{ Weight loss} = [(M_0 - M_1) / M_0] \times 100$$

where,  $M_0$  = Mass of fungus grown in the control flask,  $M_1$  = Mass of fungus treated with the biocontrol agent.

#### *Phytochemical screening of Inula (Dittrichia viscosa) plant extract*

The phytochemical screening of Inula extract was determined using the standard procedures described in Tiwari et al. (2011).

*Test for alkaloids (Mayer's test):* 0.5mL of the extract was added with a drop or two of Mayer's reagent on the side of the test tube. The formation of a white or creamy precipitate indicated the presence of alkaloids.

*Test for flavonoids (Ammonia test):* Ammonia

solution was added (1:5) to 1mL of the extract, followed by the addition of few drops of concentrated sulfuric acid. The appearance of a yellow color and its disappearance on standing indicated the presence of flavonoids.

*Test for glycosides (Keller Kiliani test):* Two mL of glacial acetic acid was added to 5mL of the extract, followed by the addition of few drops of ferric chloride solution and 1mL of conc. sulfuric acid. The formation of a brown ring at the interface confirmed the presence of glycosides.

*Test for phenols (Ferric chloride test):* Few drops of neutral ferric chloride (0.5%) solution were added to 0.5mL of the extract. The formation of a dark green color indicated the presence of phenolic compounds.

*Test for saponins (Froth test):* Two mL of distilled water was added to 1mL of the extract. The test tube was then placed in a boiling water bath and was shaken vigorously. Froth formation during heating confirmed the presence of saponins.

*Test for steroids (Liebermann-Burchard's test):* Two mL of acetic anhydride was added to 0.5mL of the extract, then 2mL of conc. sulfuric acid was added slowly along the side of the test tube. A change of color to violet blue or green indicated the presence of steroids.

*Test for tannins (Ferric chloride test):* One mL of the extract was added to 5mL of distilled water and the test tube was then placed in a boiling water bath. After boiling, the sample was left to cool down, and was then supplemented with 0.1% ferric chloride solution. The appearance of a brownish green or blue-black coloration confirmed the presence of tannins.

*Test for terpenoids (Salkowski test):* Two mL of chloroform was added to 5mL of the extract, followed by the addition of 3ml of conc. sulfuric acid. The formation of a reddish brown layer at the junction of the two solutions confirmed the presence of terpenoids.

#### *Determination of the minimal inhibitory concentration (MIC)*

The broth microdilution method was used to determine the MICs for two filter-sterilized tested chemical antifungals, one plant extract, and one bacterial extract (Pujol et al., 1996). Serial two-

fold dilutions were prepared with GPB in 96-well sterile microtiter plates. Each well was inoculated with 100 $\mu$ L of prepared fungal inoculum, and trays were covered and incubated at 30°C $\pm$ 0.2°C for 48h. The lowest concentration inhibiting visible growth was recorded as the MIC (NCCLS, 2000; Sangeetha & Nirmala, 2012).

#### *Determination of the minimal fungicidal concentration (MFC)*

The MFC of the tested antifungal agents were determined by taking 100 $\mu$ L aliquots from each clear well and streaking them over the glucose peptone agar plate. The plates were incubated at 30°C $\pm$ 0.2°C, and were checked after 48h for fungal growth. Then, the MFCs were determined, resulting in no fungal growth (Hammer et al., 2002).

#### *Determination of the MIC index*

The MIC index (MFC/MIC) was calculated to determine whether each biocontrol agent was fungicidal (MFC/MIC <4) or fungistatic (MFC/MIC >4). MIC index values higher than 4 and lower than 32 were considered to be fungistatic (Sharma et al., 2012).

#### *Determination of the fractional inhibitory concentrations (FICs)*

The of the combination of commercial chemical antifungal agents and plant extracts were determined according to the checkerboard test (Choi et al., 2009). Stock solutions of chemical antifungal agents (named Between and Logico) were prepared using 1750 $\mu$ g/mL of distilled water. The MICs of the chemical agents and the selected plant extract which exhibited the highest antifungal activity were -fold serially diluted, inoculated with 50 $\mu$ L of fungal suspension (equivalent to 0.5 McFarland), and incubated for 48 h at 30°C $\pm$ 0.2°C; then the MIC values were determined. The fractional inhibitory concentration index (FICI) was calculated as:

FICI = FIC of antifungal chemical agent + FIC of the plant extract.

The results were interpreted as follows:  $\leq$ 0.5: denoted synergy; 0.5–0.75 denoted partial synergy; 0.76–1 denoted an additive effect; 1–4 denoted indifference; and >4 denoted antagonism. The FIC value was calculated for each chemical agent, where FIC = MIC of the chemical agent used in combination with the promising plant extract divided by its MIC alone.

Also, the FIC of the tested plant extract was calculated as follows: FIC= MIC of the plant extract used in combination with the chemical agent divided by its MIC alone (Jayaraman et al., 2010; Singh & Kumar, 2017). Interactions were assessed algebraically according to the following relationship:

$$\text{FIC (index)} = \text{FIC (chemical agent)} + \text{FIC (plant extract)}$$

$$\text{FIC (chemical agent)} = \frac{\text{MIC of chemical agent in combination with plant extract}}{\text{MIC of chemical agent alone}}$$

$$\text{FIC (plant extract)} = \frac{\text{MIC of plant extract in combination with chemical agent}}{\text{MIC of plant extract alone}}$$

*In vivo testing of antifungal agents on infected tomato plants*

Three weeks old tomato seedlings were planted in plastic pots. A completely randomized block design with three replicates was set up. The seedlings (7 weeks old) received six different treatments: **T1**: non-infected control, **T2**: infected control, **T3**: chemical treatment (Logico), **T4**: chemical treatment (Between), **T5**: biocontrol treatment (plant extract), **T6**: biocontrol treatment (bacterial metabolites). Treatments with antifungal agents (1.5 ml) were performed using a sterile atomizer at intervals of 15 days to the 60th day after planting. After two days from the second spraying, each tomato plant was inoculated with 1 ml of fungal spore suspension (containing  $5 \times 10^2$  CFU/mL) (Sallam, 2011). Disease development was recorded 15 days after fungal inoculation. The intensity of disease was recorded in each treatment following a score chart based on a 0 to 9 scale (0=

healthy; 1= 1%–5%; 2= 6%–10%; 3= 11%–25%; 5= 26%–50%, 7= 51%–75%, and 9= >76% of the leaf area infected) (Latha et al., 2009). The disease severity was calculated as follows:

$$\text{Disease severity (\%)} = \frac{[\text{Sum of all disease rating} / (\text{Total number of rating} \times \text{maximum grade})] \times 100}$$

$$\text{Reduction in disease severity (\%)} = \frac{[(\% \text{ of disease severity in infected control plant} - \% \text{ of disease severity in infected treated plant}) / \% \text{ of disease severity in infected control plant}] \times 100}$$

**Results**

*Isolated fungi from infected tomatoes*

Three different fungal strains were isolated from infected tomatoes. Their microscopic examination indicated that two of them belonged to the genus *Aspergillus*, and were named *Aspergillus* sp. 1 and *Aspergillus* sp. 2, while the third fungus belonged to the *Fusarium* genus.

*Isolated bacteria producing antifungal substances*

Ten bacterial strains were isolated from tomato roots and rihzospheric soil: three gram-negative rod-shaped bacteria were grown on MacConkey agar, four gram-positive staphylococci were grown on mannitol salt agar, and three Gram-negative rod-shaped were grown on cetrimide agar plates.

*Determination of the antifungal activity of bacterial isolates using the agar plug method*

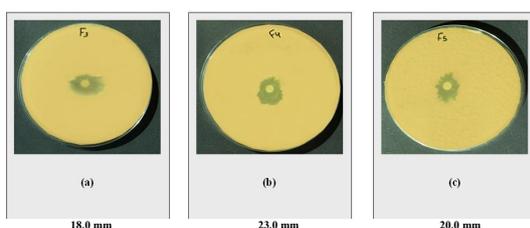
Only six isolates out of ten (namely isolates 2, 3, 5, 7, 8, and 9) showed variable degrees of antagonism (Table 1). The B3 bacterial isolate had the greatest inhibition zone and it was the most potent among all tested strains therefore, it was used for further experiments.

**TABLE 1. Antifungal activity of bacterial isolates using agar plug method**

Bacterial isolates	Mean Inhibition zone diameter (mm)		
	<i>Aspergillus</i> sp. 1	<i>Aspergillus</i> sp. 2	<i>Fusarium</i> sp.
B1	0.0	0.0	0.0
B2	12.0	11.0	10.0
<b>B3</b>	<b>18.5</b>	<b>23.5</b>	<b>19.0</b>
B4	0.0	0.0	0.0
B5	9.5	11.0	13.0
B6	0.0	0.0	0.0
B7	14.5	13.0	15.0
B8	12.5	11.0	15.0
B9	11.0	13.0	12.5
B10	0.0	0.0	0.0

### Determination of the antifungal activity of B3 using the disk diffusion method

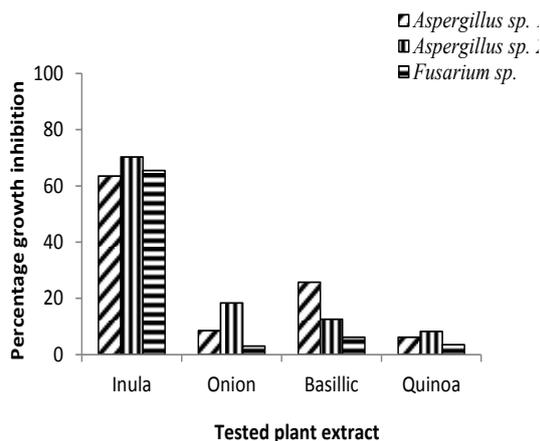
The disk diffusion method was performed to confirm the antifungal activity of the most potent bacterial isolate detected (i.e., B3). Figure 1 shows the inhibition zones formed due to the effect of isolate B3 metabolites against tested fungi. This strain—isolated on MacConkey agar—is a Gram-negative bacterium, short rod-shaped cells, and it was identified using API 20 NE as *Achromobacter xylosoxidans* with 97.3% similarity.



**Fig. 1.** Antifungal activity of B3 against *Aspergillus* sp. 1 (a), *Aspergillus* sp. 2 (b) and *Fusarium* sp. (c) evaluated using disk diffusion method

### Antifungal activity of plant extracts using the food poison method

The antifungal activities of Inula, onion, basil, and quinoa extracts were evaluated using the food poison method. Figure 2 shows that the Inula extract was the most potent among the tested plant extracts, and it was found to inhibit fungal growth by 63.52%, 70.35%, and 65.48% for *Aspergillus* sp. 1, *Aspergillus* sp. 2, and *Fusarium* sp., respectively. The other plant extracts exhibited a lesser percentage of inhibition.



**Fig. 2.** Antifungal activity of plants extracts using food poison method

### Loss of fungal dry weight due to different concentrations of the *Achromobacter xylosoxidans*

#### cell-free supernatant and tested plant extracts

The effect of different concentrations of the *A. xylosoxidans* cell-free supernatant and tested plant extracts on the % loss of fungal dry weight are shown in Table 2. The percentage of fungal dry weight loss recorded for *Aspergillus* sp. 1, *Aspergillus* sp. 2, *Fusarium* sp. increased as the concentration of the *A. xylosoxidans* cell-free supernatant increased from 20% to 50%. *Aspergillus* sp. 2 was the most affected, and a 99% loss in dry weight was achieved when 50% of the culture was represented by the *A. xylosoxidans* cell-free supernatant. A progressive increase in the antifungal activity of plant extracts was also observed with increasing concentration of bacteria. It is interesting to note that the Inula extract showed the highest antifungal activity among the tested plants. The percentages of fungal dry weight loss attained at the Inula concentration of 50% were 78.23%, 97.56%, and 88.63%, for *Aspergillus* sp. 1, *Aspergillus* sp. 2, and *Fusarium* sp., respectively.

### Qualitative phytochemical screening of the Inula extract

The results of the Inula extract qualitative phytochemical screening are shown in Table 3. It was found that this plant contained alkaloids, phenols, glycosides, tannins, and terpenoids.

### MIC, MFC, and MIC index values for the tested antifungal agents

Table 4 shows the MIC, MFC, and MIC index values for the tested antifungal agents. The MIC value of the *Achromobacter xylosoxidans* cell-free supernatant obtained against both *Aspergillus* sp. 1 and *Aspergillus* sp. 2, was 500 $\mu$ L/mL, while it was 700 $\mu$ L/mL against *Fusarium* sp. The Inula extract MIC values were 3750 $\mu$ g/mL, 7500 $\mu$ g/mL, and 15000 $\mu$ g/mL against *Aspergillus* sp. 1, *Aspergillus* sp. 2, and *Fusarium* sp., respectively. Logico showed MIC value of 27.34 $\mu$ g/mL against both *Aspergillus* sp. 1 and *Aspergillus* sp. 2, and 6.83 $\mu$ g/mL against *Fusarium* sp. Also, Between showed a MIC value of 54.68 $\mu$ g/mL against both *Aspergillus* sp. 1 and *Aspergillus* sp. 2, and 13.67 $\mu$ g/mL against *Fusarium* sp. The *A. xylosoxidans* cell-free supernatant showed an MFC value of 600 $\mu$ L/mL against both *Aspergillus* sp. 1 and *Aspergillus* sp. 2, and 800 $\mu$ L/mL against *Fusarium* sp. On the other hand, the Inula extract showed an MFC value of 15000 $\mu$ g/mL against *Aspergillus* sp. 2 and *Fusarium* sp., and 7500 $\mu$ g/mL against *Aspergillus* sp. 1. In regards to the

chemical agents, Logico showed MFC values of 27.34µg/mL, 54.68µg/mL, and 6.83µg/mL against *Aspergillus* sp. 1, *Aspergillus* sp. 2, and *Fusarium* sp., respectively; while Between showed an MFC value of 54.68µg/mL against both *Aspergillus* sp. 1, and *Aspergillus* sp. 2, and 13.67µg/mL against

*Fusarium* sp. In relation to the MIC index, both biocontrol agents were found to be fungicidal against *Aspergillus* sp. 1, *Aspergillus* sp. 2, and *Fusarium* sp. showing MIC index values below 4.

**TABLE 2. Loss of fungal dry weight affected by different concentrations of cell-free supernatant of *Achromobacter xylosoxidans* and tested plants extracts**

Concentration (%)	Fungal dry weight loss (%)		
	<i>Aspergillus</i> sp. 1	<i>Aspergillus</i> sp. 2	<i>Fusarium</i> sp.
<i>A. xylosoxidans</i>			
20	56.0	85.0	57.0
30	70.0	97.0	75.0
40	72.0	98.0	76.0
50	73.0	99.0	77.0
<i>Inula</i>			
20	68.75	80.38	70.21
30	75.52	95.32	86.32
40	76.25	96.41	87.57
50	78.23	97.56	88.63
<i>Onion</i>			
20	9.67	22.03	3.89
30	20.60	43.50	15.25
40	23.00	45.75	17.90
50	25.00	47.25	20.50
<i>Basillic</i>			
20	34.23	16.25	8.85
30	50.25	43.50	25.25
40	52.00	45.54	27.90
50	53.50	47.25	30.50
<i>Quinoa</i>			
20	9.29	10.72	3.64
30	16.82	25.80	13.50
40	18.50	26.00	15.36
50	19.00	27.69	16.89

**TABLE 3. Phytochemical analysis of *Inula* extract**

	Phytochemical analysis							
	Alkaloids	Flavonoids	Glycosides	Phenols	Saponins	Steroids	Tannins	Terpenoids
Inula extract	✓	O	P	✓	×	×	P	✓

TABLE 4. MIC, MFC and MIC index values for antifungal agents

Antifungal agent	<i>Aspergillus</i> sp. 1			<i>Aspergillus</i> sp. 2			<i>Fusarium</i> sp.		
	MIC	MFC	MIC index	MIC	MFC	MIC index	MIC	MFC	MIC index
<b>Biocontrol agent</b>									
<i>A. xylosoxidans</i> ( $\mu\text{L/mL}$ )	500	600	1.2	500	600	1.2	700	800	1.14
Inula ( $\mu\text{g/mL}$ )	3750	7500	2.0	7500	15000	2.0	15000	1500	1.00
<b>Chemical agent</b>									
Between ( $\mu\text{g/mL}$ )	54.68	54.68	1.0	54.68	54.68	1.0	13.67	13.67	1.00
Logico ( $\mu\text{g/mL}$ )	27.34	27.34	1.0	27.34	54.68	2.0	6.83	6.83	1.00

*Fractional inhibitory concentration (FIC) of the combination of commercial antifungal drugs and plant extracts*

The FIC was calculated to test the combined effects of Inula extract and commercial antifungal drugs used against fungal diseases. The addition of Inula did not affect the MIC values of the tested fungicides (Logico and Between) recorded against *Aspergillus* sp. 1, *Aspergillus* sp. 2, and *Fusarium* sp. The effects of the drug combinations are shown in Table 5. The FIC indices of the combinations (Inula + Logico and Inula + Between) recorded in the checkerboard test ranged from 1.25 to 3, showing indifferent activity for all the fungal pathogens under investigation.

*In vivo testing of antifungal agents on infected tomato plants*

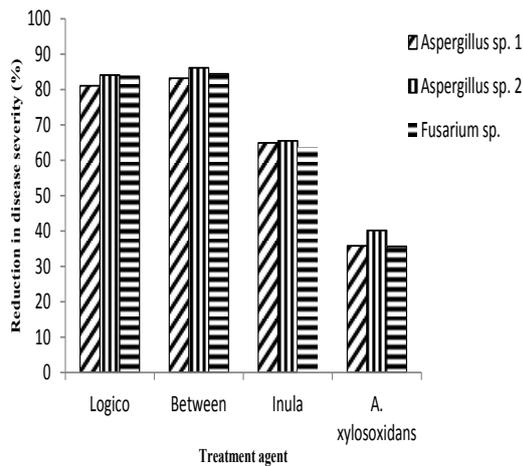
Tomato plants were cultivated in vivo, and growth parameters were monitored in order to assess the relative importance of Inula and *A. xylosoxidans* as biocontrol agents, and to compare these treatments with the two chemical antifungal

agents, Logico and Between. The treatment concentrations were derived from the MIC values previously determined. The percentages of reduction in disease severity in each treatment are shown in Figure 3. For *Aspergillus* sp. 1, the greatest reduction in disease severity was achieved by Between (83.18%). The most effective biocontrol treatment was recorded for the Inula treatment (64.91%), and the least reduction in disease severity was recorded for diseased tomatoes treated with *A. xylosoxidans* metabolites (35.80%). With respect to *Aspergillus* sp. 2 the greatest reduction in disease severity was obtained by Between (86.14%). The treatment with Inula (65.44%) and *A. xylosoxidans* (40.13%) showed the least reduction in disease severity. Finally, in *Fusarium* treatments, the highest reduction in disease severity was attained by Between (85.39%). The most effective biocontrol agent was Inula (63.65%), and the least reduction in disease severity was achieved by *A. xylosoxidans* (35.63%).

TABLE 5. Combined effect of Inula and commercially chemicals against fungal pathogens

Fungi	<sup>(a)</sup> MIC Inula		MIC Logico		<sup>(b)</sup> FICI	Outcome
	Alone ( $\mu\text{g/mL}$ )	With Logico ( $\mu\text{g/mL}$ )	Alone ( $\mu\text{g/mL}$ )	With Inula ( $\mu\text{g/mL}$ )		
<i>Aspergillus</i> sp. 1	3750	7500	27.34	27.34	3.00	Indifferent
<i>Aspergillus</i> sp. 2	7500	7500	27.34	27.34	2.00	Indifferent
<i>Fusarium</i> sp.	15000	3750	6.83	6.83	1.25	Indifferent
Fungi	<sup>(a)</sup> MIC Inula		MIC Between		<sup>(b)</sup> FICI	Outcome
	Alone ( $\mu\text{g/mL}$ )	With Between ( $\mu\text{g/mL}$ )	Alone ( $\mu\text{g/mL}$ )	With Inula ( $\mu\text{g/mL}$ )		
<i>Aspergillus</i> sp. 1	3750	3750	54.68	54.68	3.00	Indifferent
<i>Aspergillus</i> sp. 2	7500	3750	54.68	57.68	2.00	Indifferent
<i>Fusarium</i> sp.	15000	3750	13.67	13.67	1.25	Indifferent

<sup>(a)</sup>MIC: Minimum inhibitory concentration; <sup>(b)</sup>FICI: Fractional inhibitory concentration index.



**Fig. 3. Reduction in the severity of tomato diseases caused by *Aspergillus* sp. 1, *Aspergillus* sp. 2, and *Fusarium* sp.**

*In vivo* testing of antifungal agents on infected tomato plants

Tomato plants were cultivated *in vivo*, and growth parameters were monitored in order to assess the relative importance of *Inula* and *A. xylooxidans* as biocontrol agents, and to compare these treatments with the two chemical antifungal agents, Logico and Between. The treatment concentrations were derived from the MIC values previously determined. The percentages of reduction in disease severity in each treatment are shown in Figure 3. For *Aspergillus* sp. 1, the greatest reduction in disease severity was achieved by Between (83.18%). The most effective biocontrol treatment was recorded for the *Inula* treatment (64.91%), and the least reduction in disease severity was recorded for diseased tomatoes treated with *A. xylooxidans* metabolites (35.80%). With respect to *Aspergillus* sp. 2 the greatest reduction in disease severity was obtained by Between (86.14%). The treatment with *Inula* (65.44%) and *A. xylooxidans* (40.13%) showed the least reduction in disease severity. Finally, in *Fusarium* treatments, the highest reduction in disease severity was attained by Between (85.39%). The most effective biocontrol agent was *Inula* (63.65%), and the least reduction in disease severity was achieved by *A. xylooxidans* (35.63%).

**Discussion**

The management of plant diseases is a significant cost component of crop production.

Compared to chemical pesticides, which pose significant risks to the environment, the application of biological control agents specific for certain pathogenesis considered harmless to non-target species, and destroy only the etiological of plant disease (O’Brien, 2017). Our results revealed that, *Achromobacter xylooxidans* showed a promising antifungal activity against *Fusarium* and *Aspergillus* strains isolated from infected tomatoes. This finding is in line with results reported in Yan et al. (2004) and Moretti et al. (2008), which showed that *A. xylooxidans* was able to inhibit species belonging to these two genera. Harashita et al. (2018) reported that *Trichoderma harzianum*, *Bacillus subtilis*, and *Pseudomonas fluorescens* had an antagonistic activity against *Fusarium oxysporum* f.sp. *lycopersici*. Recently, Attia et al. (2020) demonstrated the antagonistic effect of *Achromobacter xylooxidans* against *Alternaria solani* which causes early blight disease in tomato plants. This bacterium was proved to produce a wide range of secondary metabolites, including antibiotics, volatile organic compounds, iron-chelating siderophores, antifungal agents, and several enzymes that are most often associated with fungal suppression (Palumbo et al., 2007). In addition, Moretti et al. (2008) found that *A. xylooxidans* produced inhibitory substances that can control the pathogenesis of *Fusarium* sp. and *Aspergillus* spp. Biocontrol by *Achromobacter xylooxidans* depends on competition for iron. This element is abundant in the Earth’s crust, but most of it is found in the highly insoluble form of ferric hydroxide. Thus, due to the limited presence of soluble iron in the rhizosphere, microbes and plants scavenge for this element using highly sophisticated iron binding and uptake mechanisms, which include the production of siderophores (Yan et al., 2004). The siderophores of *Achromobacter* sp., in particular, may act as biocontrol through the induction of systemic resistance in plants. Similar results reported in Guo et al. (2019), indicated that *Bacillus amyloliquefaciens* Ba13 induced tomato systemic resistance against tomato yellow leaf curl virus disease. Another biocontrol mechanism of *A. xylooxidans* the production of chitinolytic enzymes. The expression and secretion of these enzymes can result in the suppression of pathogenic fungal activities (Moretti et al., 2008).

The use of biologically-based compounds extracted from plants may be an alternative to the fungicides currently used to control

phytopathogenic fungi, because they virtually constitute a rich source of bioactive chemicals, such as phenols, flavonoids, quinones, tannins, alkaloids, saponins, and sterols. The results of the present study show that *Dittrichia viscosa* (Inula) had the highest antifungal activity among all the tested plants. Inula plants contain compounds with antiviral, antibacterial, antifungal, nematocidal, and cytotoxic activities, and have a protective effect against oxidative stress and genotoxicity (Al-Masri et al., 2015; Koc et al., 2018). The major classes of phytochemicals present in the tested Inula were alkaloids, glycosides, phenols, tannins, and terpenoids. This result was similarly reported in Mahmoudi et al. (2016). Alkaloids are significant for the protection of plants because they ensure their survival against microorganisms, insects, and herbivores, and also against other plants by means of allopathically active chemicals (Saxena et al., 2013). The antimycotic properties of spirostanol glycosides are attributed to their ability to complex with the sterols contained in fungal membranes, and to cause leakage of cytoplasmic materials, which leads to cell death (Khan et al., 2017). The antifungal activity of *Hyocymus niger* L. seeds is due to the action on the sterols in fungal membranes, which cause the formation of pores and a loss in membrane integrity. Phenolics have also been proved as effective antifungal agents (Ansari et al., 2013). Tannins have been isolated from a number of plants, and they were shown to exhibit antimicrobial properties; specifically, Jesudoss et al. (2014) reported that these compounds have an antifungal effect against *Fusarium* and *Aspergillus* sp.. Terpenoids were shown to disrupt the fungal cell membrane and to destroy fungal mitochondria (Jäger and Freiesleben, 2014).

MIC determination is mainly used to determine the potency of antagonistic agents. According to Al-Saadi (2016), bacterial extracts with MIC values of up to 500 µL/mL, and between 600 and 1500 µL/mL, are considered as strong and moderate inhibitors, respectively, while those with values above 1600 µL/mL are considered as weak. The present study showed that the cell-free supernatant obtained from the *A. xylosoxidans* culture containing extracellular metabolites, had MIC values of 500 µl/ml against *Aspergillus* spp., and 700 µl/ml against *Fusarium* sp.. These data show the strong inhibition of *Aspergillus* spp., and the intermediate effect on *Fusarium* sp.

The MIC values of Inula (*Dittrichia viscosa*) extract against *Aspergillus* spp. and *Fusarium* sp.; recorded in the current investigation were between 3750 and 15000 µg/mL (3.7–15 mg/mL), which are higher than those reported by Abou-Jawdah et al. (2004). Previous studies have shown that *Artemisia herba-alba*, *Cotula cinerea*, *Asphodelus tenuifolius*, and *Euphorbia guyoniana* aqueous extracts are effective at both 10% and 20% concentrations in inhibiting the growth of *Fusarium* mycelia (Salhi et al., 2017).

The MIC and MFC values determined the bacterial cell-free supernatant and Inula extract in the present study were comparable. *Aspergillus* spp. were more susceptible to the *A. xylosoxidans* metabolites than *Fusarium* sp. was. The MFC value recorded against *Fusarium* sp. was 800 µL/mL, while it was only 600 µL/mL against *Aspergillus* spp. The MFC values of the Inula extract were in the range of 7500–15000 µg/mL. Al-Rahmah et al. (2013) mentioned that *Thymus vulgaris* and *Zingiber officinale* extracts showed fungistatic and fungicidal activities against phytopathogenic fungi with a MIC value of 4 mg/ml and MFC value of 8 mg/ml, and the MFC of *Z. officinale* extract tested against *F. oxysporum* reached 16 mg/ml. An estimation of the MIC index was performed to determine the potency of the best two biocontrol agents obtained in the present investigation. The calculated MIC index of both tested *A. xylosoxidans* metabolites and Inula extract were below 4, which confirmed their fungicidal effect.

The Lebanese Ministry of Agriculture recommended the use of two fungicidal chemicals Between and Logico to control plant fungal pathogens. The combined action of these commercial products with Inula extract against *Aspergillus* spp. and *Fusarium* sp., was explored. The index values showed that the combination had an indifferent effect (FIC index  $\geq 1 \leq 4$ ) against all the plant pathogens tested. Shinde et al. (2012) classified the FIC values follows: FIC  $\leq 0.5$ , was noted as synergism and FIC  $> 4$  was noted as antagonism, but FIC  $> 0.5$  and  $< 4$  was considered as indifferent.

In vivo testing of the antifungal agents on infected tomato plants was performed. Tomato seedlings that were just infected with the fungal pathogens showed the maximum disease severity, while those infected with pathogens and treated

with bacterial metabolites exhibited a reduction in disease severity by 35, 40, and 35% for diseases caused by *Aspergillus* sp. 1, *Aspergillus* sp. 2 and *Fusarium* sp., respectively. On the other hand, seedlings infected with fungal pathogens and treated with Inula extract displayed considerably smaller reduction in disease severity, namely by 64, 65, and 63% for the same three above-mentioned pathogens, respectively. These results clearly demonstrate the ability of botanical pesticides to control these pathogenic fungi. Sallam (2011) used six plant extracts (*Ocimum basilicum*, *Azadirachta indica*, *Eucalyptus chamadulonis*, *Datura stramonium*, *Nerium oleander*, and *Allium sativum*) to reduce the severity of early blight disease of tomato caused by *Alternaria solani*, obtaining disease reductions ranging between 15.3 and 35.2%. Neela et al. (2014) found that *Piper betel*, *Lowsonia inermis*, *Psidium guajava*, *Carica papaya*, *Moringa oleifera*, *Mimosa pudica*, *Catharanthus roseus*, *Adhatoda vasica*, and *Andrographis paniculata* inhibited *Fusarium oxysporum*, which causes *Fusarium* wilt disease in tomato. Tijjani et al. (2014) suggested four plant extracts (*Moringa oleifera*, *Allium sativum*, *Carica papaya*, and *Azadirachta indica*) for the control of tomato fruit rot caused by *Aspergillus flavus*. Recently, Mossa (2021) reported that *Thymus vulgaris* showed antifungal activity against *Aspergillus parasiticus*.

### Conclusion

The current investigation led to the suggestion that the antifungal agents produced by *Achromobacter xylosoxidans* and Inula (*Dittrichia viscosa*) might be good candidates to control the growth of *Aspergillus* sp. 1, *Aspergillus* sp. 2, and *Fusarium* sp., which are capable of causing several plant diseases. Plant growth-promoting rhizobacteria are known to produce a variety of different antimicrobial agents including lytic enzymes, volatile organic compounds, antibiotics, phenazines, and siderophores, which act through a combination of several different mechanisms that make them highly effective in controlling plant diseases. Moreover, plants contain phytochemicals which show antimicrobial activity and serve as plant defense mechanisms against pathogenic microorganisms. These compounds called botanical pesticides or botanicals are toxic to fungal pathogens when applied on infected crops. Further research is needed to isolate their active constituent(s) and to elucidate the exact

mode of action of antifungal compounds. The practical significance of the present study will be fully realized when the biocontrol agents will eventually replace the environmentally harmful chemical fungicides after the determination of their cytotoxic effects on the environment and on the health of consumer.

*Conflicts of interest:* No conflicts of interest have been declared.

*Authors contribution:* Dr. Salwa: Writing the article and following up its publication, in addition to participating in selecting the research point, planning the experiments and supervising their conduct. She Carries out the experiment concerning the *in vivo* testing of the antifungal agents on infected tomato plants and Phytochemical screening of Inula extract. Dr. Hoda: Helping in the revision of the article and in revising the results of experiments. Jana Halawi: Conducting the practical experiments of microbiology.

*Ethical approval:* Not applicable

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### المكافحة البيولوجية لأمراض الطماطم الفطرية في لبنان

سلوى محمود عبد الرحمن<sup>(1)</sup>، هدى حسن يوسف<sup>(1)</sup>، جنى محمد حلاوي<sup>(2)</sup>  
<sup>(1)</sup>قسم النبات والميكروبيولوجي - كلية العلوم جامعة الإسكندرية - الإسكندرية- مصر، <sup>(2)</sup>قسم العلوم البيولوجية والبيئية - كلية العلوم جامعة بيروت العربية - بيروت - لبنان.

نظرا للاستخدام المتزايد لمضادات الآفات الزراعية الكيماوية وما تسببه من آثار ضارة بالبيئة وصحة الانسان، زاد الاهتمام بتقنيات التحكم البيولوجي والتي تهدف إلى استخدام الكائنات الدقيقة او مستخلصات النباتات في القضاء على الآفات المسببة لأمراض النباتات. لذا فقد كان الهدف الرئيسي من البحث الحالي هو تقييم فاعلية المبيدات البيولوجية في السيطرة على الأمراض الفطرية للطماطم في لبنان ومقارنة النتائج مع نتائج المعاملات الكيماوية المستخدمة فعليا في علاج الأمراض الفطرية. تتلخص نتائج البحث الحالي في الآتي:

1 تم عزل ثلاث عازلات فطريات من نبات الطماطم المصاب و 1 بفحصها مجهريا سميت *Fusarium sp.* و *Aspergillus sp.1* و *Aspergillus sp.2* ،

2- تم عزل عشر سلالات بكتيرية من التربة المحيطة بجذر نباتات الطماطم، و استخدمت لعمل مسح لنشاطها المضاد للفطريات المعزولة و تم اختيار السلالة الأكثر فعالية وتعريفها ب *Achromobacter xylosoxidans*

3 تم دراسة تأثير بعض مستخلصات النباتات كمضادات للفطريات وتبين ان مستخلص *Inula* سجل أعلى معدل لكبح النمو الفطري.

4- على *Inula* تم تقييم *FIC index* و *MIC index* ، *MFC* ، *MIC* للمستخلص المجهز من *A. xylosoxidans* و مستخلص *Inula* و اثنين من المبيدات الفطرية الشهيرة في لبنان.

5- امتدت الدراسة لتقييم عملية العلاج البيولوجي لشتلات الطماطم المصابة بالفطريات. وتبين أن المعالجة البيولوجية لها نساءج مرضية في تقليص أعراض الأمراض الفطرية.