Evaluation of the Biological Control Agent's Efficiency Against the Causal Agent of Early Blight of *Solanum melongena*

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Abstract

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Eggplant, an important vegetable crop in Iraq, faces yield losses due to early blight disease caused by *Alternaria solani*. Two antagonistic fungi (*Aspergillus niger* and *Purpureocillium lilacinum*) isolated from eggplant rhizosphere were tested *in vitro* (dual confrontation technique) and *in vivo* (preventive treatments) as potential bioagents against the most virulent *A. solani* isolates. *A. niger* and *P. lilacinum* at 30% conidia/ml exhibited high inhibitory rate (55.11%) against *A. solani* mycelial growth. Furthermore, these two antagonistic species when applied preventively at 30% of the stock conidial suspension *in vivo*, reduced significantly disease severity index (<28.41%). Moreover, *A. niger* (11.98 units/g of soluble protein) and *P. lilacinum* (8.11 units/g of soluble protein) enhanced the polyphenol oxidase activity. Based on the results obtained by this study, it appears that *A. niger* and *P. lilacinum* could be employed as foliar treatments against *A. solani* to promote eggplant growth and development and induce plant systemic resistance. To control *A. solani*, it is encouraged to use natural enemies as components in integrated disease management.

Keywords: *Alternaria solani*, biological control, polyphenol oxidase, *Solanum melongena*, antagonistic fungi.

Introduction

Eggplant (*Solanum melongena* L.) belongs to the family *Solanaceae*, it is an important vegetable crop and it is among the most important vegetable crops. *S. melongena* has high nutritive values, rich in proteins, carbohydrates, fats, minerals and vitamins. Eggplant contributes quite a bit in the agricultural economy of Iraq. Recently, it is produced throughout the year in greenhouses (off season and early crops) as well as in the field (seasonal culture). The eggplant annual production in Iraq was reported to be around 102,452 thousand tons with an average of 12,261 kg/ha (Saeed Omar & Mohammed, 2020).

In Iraq, the eggplant crop is being affected by several diseases, amongst them is early blight caused by *Alternaria solani*. This pathogen causes severe damage to plant parts such as stems, leaves, fruits and stalks leading to serious yield losses. Worldwide annual economic yield losses due to early blight have been estimated around 35-78% decrease in yield. Early blight disease can occur and survive over a varied range of certain climatic conditions but it is most dominant in areas with high rainfall, dew and very high relative humidity (El-Debaiky, 2018; Matrood & Rhouma, 2021a).

The most appropriate strategy used to control this *Ascomycota* is by methods that reduce spores' density (El-Debaiky, 2018). Control of *A. solani* is currently based on integrating different approaches. Unfortunately, the most common strategy employed to manage early blight by Iraqi farmers is through the application of fungicides. Around $32-$45 million is spent annually on fungicides worldwide to control *Alternaria* sp. (El-Tanany *et al.*, 2018). Nevertheless, this approach caused human health hazards and increased environmental pollution. Therefore, alternative strategies to control this disease is needed. Biological control is the best alternative and eco-friendly approach for this purpose, and it is defined as total or partial destruction of *A. solani* spores by naturally occurring other organisms (Atia, 2005; Esh *et al.*, 2010; Rhouma *et al.*, 2016, 2018; Taghian *et al.*, 2008). Many genera of antagonistic fungi such as; *Trichoderma* spp., *Penicillium* spp., *Aspergillus* spp. and *Purpureocillium* spp. proved to have high efficacy against early blight when tested under *in vitro* and *in vivo* conditions (El-Debaiky, 2018; Fontenelle *et al.*, 2011; Leelasuphakul *et al.*, 2008). D'Souza & Devaraj (2011), Ramamoorthy *et al.* (2002), and El-Tanany *et al.* (2018) revealed that efficacy of these biological control agents (BCA's) also induced plant defence mechanisms.

The objectives of this investigation were to: (i) evaluate the pathogenicity of some *A. solani* isolates under greenhouse condition, (ii) isolate and identify antagonistic fungi from eggplant rhizosphere samples, and (iii) screen certain soil-borne fungal antagonist's for their abilities to reduce *A. solani* growth under *in vitro* and *in vivo* conditions.

Materials and Methods

Eggplant sample collection and *Alternaria solani* isolation

Eggplant leaves were collected from three greenhouses (9 m x 60 m) located in Basra Iraq (Hartha, Safwan and Fayhaa). Diseased plants samples showing typical symptoms of early blight caused by *A. solani* were

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collected. Eggplant small leaf pieces (0.5-1 cm) were sterilized by soaking in 3% solution of sodium hypochlorite (NaOCl) for 2 min and washed three times with sterilized distilled water. The samples were dried and placed on the surface of Petri dishes (9 cm in diameter) containing potato dextrose agar (PDA) medium amended with streptomycin (60 µg/ml). In each Petri dish, seven fragments were placed (total of 30 Petri dishes). The plates were incubated in the dark at 25±2°C for 5-7 days, and then examined to identify fungal colonies. The fungal species identification was carried by observing the macroscopic (growth, color, aspect of the colony) and microscopic characteristics (mycelium, conidiophore, conidia, resistance structures, sexual form), after a series of sub-culturing until purification of the fungus. The fungal species were identified using the blue cotton as a mounting liquid and by using different reference identification keys.

Pathogenicity of *Alternaria solani* isolates

This experiment was carried out in the greenhouse and concerned only with eggplant (cv. *Barcelona*) plants. The seeds were placed on the surface of Petri dishes (9 cm in diameter) containing cotton balls soaked in sterilized distilled water. In each Petri dish, 25 seeds were placed (with total of 30 Petri dishes). Germinated eggplant seeds were placed in a pot (50 cm in diameter) containing a mixture of peat and vermiculite (1:1) which was autoclaved twice at 120°C. The assay was carried out by spraying the eggplant leaves of each seedling (15 days old) by the same amount (10 ml) of conidial suspension (10^6 cfu/ml) of each *A. solani* isolate. The plants were covered with a transparent plastic to ensure high humidity of 70-90% during 3 days after inoculation to ensure infection. A negative control was used by inoculating eggplant plants with sterilized distilled water (negative control). The pots were then placed in a greenhouse for 15 days. The experimental design was a randomized complete block design (RCBD) with 10 plants per replicate (3 replicates), and the entire experiment was repeated three times (Boughalleb-M'Hamdi *et al.,* 2018). Different parameters were evaluated 15 days after inoculation. To measure the disease index for the different treatments, the following 0-4 scale was used (Mostafa *et al.,* 2013): 0 = no lesions; 1 = lesions covering about 1-10% of the leaf surface; 2 = lesions covering about 11-25% of the leaf surface; 3 = lesions covering about 26-50% of the leaf surface; 4 = lesions covering about 51-100% of the leaf surface. The severity data were processed by McKinney's formula, which generates a numeric disease severity index (DSI): DSI (%) = (Σvn)/(NV)×100, where v represents the numeric value of the disease index scale, n is the number of plants assigned to the disease index scale, N is the total number of the plants and V is the numeric value of the highest disease index scale (Rhouma *et al.,* 2018).

Fungal community in eggplant rhizosphere

Soil samples were collected from the same locations in Basra Iraq (Harth, Safwan and Al-Faiha) cultivated with eggplants. Soil samples were taken using a 7-cm-diameter soil auger from the rhizosphere of eggplant randomly in a zigzag fashion at 15 cm depth, and each sample was composited by ninety soil cores for each greenhouse. For each greenhouse, samples were mixed together into a single one. Nine soil samples (200 g) per replicate (3 replicates) were collected from each greenhouse (Rhouma *et al.,* 2019). Samples were collected in sterile polythene bags and brought to the laboratory to isolate and identify fungal communities in different greenhouses. The isolation of soil-borne fungi was determined by the dilution-plate method according to Boughalleb-M'Hamdi *et al.* (2017). 10 g of soil was diluted in 90 ml sterile distilled water. Serial dilutions were made from 10^{-1} to 10^{-7}. A volume of 0.1 ml of each dilution was transferred aseptically into Petri dishes containing MEA (Malt extract agar). The plates were incubated in the dark at 25°C for 3 days. The fungal species identification was carried out by observing the macroscopic and microscopic characteristics, after a series of sub culturing until purification of the fungus, using cotton blue as a mounting liquid and by using different reference identification keys.

In vitro* and *in vivo* antagonism potential of fungal bioagents against pathogenic *Alternaria solani

Two antagonistic fungi isolated from eggplant rhizosphere were used in this study. For routine use, *Purpureocillium lilacinus* and *Aspergillus niger* were grown on potato dextrose agar (PDA) and conidia filtrate were prepared as described by Rhouma *et al.* (2018). The two fungal species were routinely grown on Sabouraud dextrose yeast agar at 25±1°C under a 16-h light photoperiod, separately. Conidia were harvested from 7-days-old cultures by adding 10 ml of sterilized distilled water supplemented with 0.02% Tween-80. The conidial suspension was filtered through 2 layers of sterile muslin into a sterile 25 ml plastic universal bottle and then agitated for 5 min using a horizontal shaker with 40 mm horizontal movement and 300 oscillations per min (conidia stock suspension). The conidial stock suspension was prepared in different dilutions (10, 20, 30%) using sterilized distilled water. Conidial viability was evaluated for each batch and only lots found to be >95% viable were used in this study.

The *in vitro* antifungal activities of the two fungal antagonists (*P. lilacinus* and *A. niger*) on radial mycelial growth of *A. solani* were determined by the dual confrontation technique on PDA according to Matrood & Rhouma (2021b). A volume of 100 µl with different conidia filtrate concentrations (10, 20 and 30% conidia/ml) for each antagonistic fungus was transferred aseptically into Petri dishes containing PDA. One disc plug (0.5 cm) of pathogen (4-days-old culture) was placed separately on a single PDA plate (9 cm) in the same Petri dishes containing different conidia filtrate concentrations of each antagonistic fungus. The pathogen plug was placed in the center of the plate. A plug of pathogen was used as control treatment (without conidia filtrate of antagonistic fungus). Three replicates (five plates/replicate) for each individual treatment were conducted and the Petri dishes were incubated at 25±2°C for 5 days. The inhibition percent of pathogen radial mycelial growth (I) was assessed according to the formula of Rhouma *et al.* (2018): I (%) = (1- C_n/C_0) x 100; where: C_n represents the diameter of pathogen radial growth in the presence of the indicated conidia filtrate

concentrations of antagonist, whereas, C_0 represents the diameter of pathogen radial growth in the control treatment.

The *in vivo* experiment was concerned only with preventive treatments. Eggplant (cv. *Barcelona*) leaves were sterilized by soaking them in 3% solution of sodium hypochlorite (NaOCl) for 2 min and washed three times with sterilized distilled water. The preventive assay was carried out by spraying leaves of eggplant (10 ml) with indicated concentrations of conidia filtrate (10, 20 and 30% conidia/ml) separately for each antagonistic fungus. 10 ml (10^6 cfu\ml) of *A. solani* (the most virulent isolate isolated from Hartha) was applied 7 days later. Two controls were used; one by inoculating the leaves with the pathogen only (positive control), while the other with sterilized distilled water (negative control). The treated eggplant leaves were placed on the surface of Petri dishes (9 cm). Four leaves were placed per plate, with a total of 10 Petri dishes for each replicate, with three replications. The experimental design used was RCBD, and the entire experiment was repeated twice. The plates were incubated in the dark at 25±2°C for 10 days, and then examined for disease severity index (DSI) (Matrood *et al.*, 2021; Rhouma *et al.*, 2018). Polyphenol oxidase activity (units/g of soluble protein) was determined by the method described by D'Souza & Devaraj (2011) and measured only for the eggplant leaves treated with 30% conidia/ml (10 days after inoculation) at 420 nm wavelength.

Statistical analysis

The data were analyzed by ANOVA using SPSS version 20.0 statistical software (SPSS, SAS Institute, USA), to evaluate parameter values differences. Differences between treatments were determined by least significant difference (LSD) test at P=0.05.

Results and Discussion

Pathogenicity of *Alternaria solani* isolates

A. solani isolates exhibited high degree of pathogenicity according to the measured disease severity index (DSI) parameter. All *A. solani* isolates were virulent with DSI values varying from 59.08 (*A. solani* isolated from Safwan) and 71.97% (*A. solani* isolated from Hartha) (control = 0%) (Table 1). Symptoms initially appeared on the leaves in the form of small yellow spots as concentric rings then turned brown surrounded by a halo, and spots coalesce with time. These results are in agreement with previous investigations reported by Singh *et al.* (2017). Typical symptoms of *A. solani* to those produced in natural field conditions were observed on many inoculated plants (Alsafadi *et al.*, 2012; Matrood *et al.*, 2020; Poly & Srikanta, 2013).

Fungal community in the eggplant rhizosphere

The identification of fungal species recorded in different samples rhizosphere soil samples collected from three experimental fields cultivated with eggplant are presented in Table 2. Data obtained showed that *Aspergillus* spp. were the most frequent (isolated from three greenhouses located in Basra), followed by *Penicillium* spp. and *Mucor* spp. However, the lowest frequency was found for *Alternaria* spp. The prevalence of *Aspergillus* and *Penicillium* spp. in the soil samples was consistent with what has been reported by Boughalleb-M'Hamdi *et al.* (2017), Gaddeyya *et al.* (2012), and Onyimba *et al.* (2014). De Lucca (2007) pointed out that variation in fungal species diversity and conidia dispersion was attributed to various factors (wind, relative humidity, moisture, temperature and air pollution) and varied from one season to another.

Table 1. Pathogenicity of *A. solani* isolates collected from different locations in Iraq.

Treatments	Disease severity index (%)
Negative control	0.00 d
A. solani (Hartha)	71.97 a
A. solani (Safwan)	59.08 c
A. solani (Fayhaa)	64.50 b

Values followed by the same letters are not significantly different at P=0.01

Table 2. Fungal isolates obtained from eggplant rhizosphere collected from different locations in Iraq.

Locations	Fungal isolates
Hartha	*Aspergillus niger*
	Fusarium spp.
	Penicillium spp.
	A. flavus
Safwan	*A. niger*
	A. flavus
	Purpureocillium lilacinus
Fayhaa	*Fusarium* spp.
	Penicillium spp.
	A. niger
	Alternaria spp.
	Mucor spp.
	P. lilacinum

In vitro* and *in vivo* antifungal potential of fungal bioagents against *Alternaria solani

The two antagonistic fungal species at different conidia concentrations exerted high significant reduction ($P < 0.01$) on radial mycelial growth of *A. solani*, five days after incubation. Statistical analysis revealed high significant interactions between *A. solani* and the conidia filtrate concentrations of antagonists ($P < 0.01$). The linear decrease of growth of the pathogenic fungi ranged from 63.66 (*A. niger* at 30% concentration of the stock conidial suspension) to 17.0% (*P. lilacinum* at 10% of stock conidial concentration). *A. niger* showed a good ability to limit the mycelial growth of *A. solani in vitro*. In fact, the mycelial growth decreased in presence of *A. niger* with values of 63.66 and 40.86% at 30 and 20% concentration, respectively. In the same sense, *in vitro* assay revealed that *P. lilacinum* possessed a good antifungal potency with mycelial inhibition rate of 55.11% at 30% of stock conidial concentration (Table 3).

The effect of four treatments on the disease severity index under *in vivo* conditions is shown in Table 4. Statistical analysis showed significant differences between the treatments ($P < 0.01$). All treatments with different conidia filtrate concentrations decreased significantly the disease severity index (DSI) as compared with the positive control (DSI Mean = 53.11%). Results showed that *A. niger* (DSI Mean = 33.69%) and *P. lilacinum* (DSI Mean = 31.15%) were found effective to reduce the disease severity index. Among the treatments with the different conidia filtrate concentrations of the two fungal antagonists, *P. lilacinum* at 20% of stock conidial concentration (DSI = 18.61%) exhibited significant DSI reduction in eggplant leaves followed by the 30% concentration of *A. niger* (DSI= 28.41%) (Table 4).

Table 3. Effect of direct confrontation of two fungal antagonists (*Aspergillus niger* and *Purpureocillium lilacinum*) on mycelial growth inhibition of *Alternaria solani* five days after incubation at 25°C.

Fungal antagonists	Mycelial growth inhibition rate (%) Dilutions of stock conidial suspension (%) 10	20	30	Mean
A. niger	29.17 aC	40.86 B	63.66 aA	44.56
P. lilacinum	17.01 bC	42.96 B	55.11 bA	38.36
Mean	23.09	41.91	48.79	

Values followed by the same capital letters in the same row (P=0.01) or small letters in the same column (P=0.05) are not significantly different.

Table 4. The effect on eggplant leaves treated with fungal antagonists as measured by disease severity index.

Treatments	Disease severity index (%) Dilutions of stock conidial suspension (%) 10	20	30	Mean
Positive control	57.98 aA	49.34 aC	51.98 aB	53.11
Negative control	0.00 d	0.00 d	0.00 d	0.00
A. niger	33.87 cB	38.80 bA	28.41 cC	33.69
P. lilacinum	42.98 bA	18.61 cC	31.86 bB	31.15
Mean	33.71	26.69	28.06	

Values followed by the same capital letters in the same row or small letters in the same column (P=0.01) are not significantly different at P=0.01.

The results reported in this study are in agreement with previous findings reported by El-Debaiky (2018), and Fontenelle *et al.* (2011). These authors reported that the using of several fungi such as *Trichoderma* spp., *Penicillium* spp., *Aspergillus* spp. and *Purpureocillium* spp. as biological control, agents (BCAs) against the *A. solani* isolates, revealed that *Trichoderma* spp. and *Aspergillus* spp. inhibited the growth of these pathogenic isolates under *in vitro* and *in vivo* conditions. Microorganisms reportedly produce toxic substances via antibiosis which are very effective in the inhibition of *A. solani* growth (Leelasuphakul *et al.*, 2008; Matrood & Rhouma, 2021c). *A. niger* has the ability to produce cellulase and other enzymes including B-1,3 glucanase, which destroys the glucan found in the *A. solani* cell wall, a substance which is the main component of the multiple sugars that are involved in the synthesis of the fungal cell wall of *Ascomycota* (Leelasuphakul *et al.*, 2008).

Polyphenol oxidase (PPO) activity on eggplant leaves treated with two antagonistic fungi at 30% concentration of stock conidia suspension (as compared with the positive and negative controls) are presented in Figure 1. Statistical analysis revealed a significant difference in PPO activity ($P < 0.01$). Data obtained showed that the highest value of 11.98 units/g of soluble protein was recorded for eggplant leaves treated with *A. niger*. However, the lowest activity was found for eggplant leaves treated with *P. lilacinum* (8.11 units/g of soluble protein) ($PPO_{Positive\ control}$ = 9.87 units/g of soluble protein; $PPO_{Negative\ control}$ = 6.12 units/g of soluble protein). El-Tanany *et al.* (2018) indicated that some BCAs plays an important role in stimulating systemic resistance in plants treated with *Trichoderma* spp. against *A. solani* due to increased polyphenol oxidase enzyme activity. Furthermore, increased PPO activity was also reported due to drought stress in *Dolichos lablab* leaves (D'Souza & Devaraj, 2011). Ramamoorthy *et al.* (2002) pointed out that the induction of defence enzymes involved in phenylpropanoid pathway accumulation of phenolics and PR-Proteins (phenylalanine ammonia-lyase, peroxidase and polyphenol oxidase) may have contributed in the blocking of invasion of *Fusarium oxysporum* f. sp. *lycopersici* to tomato roots.

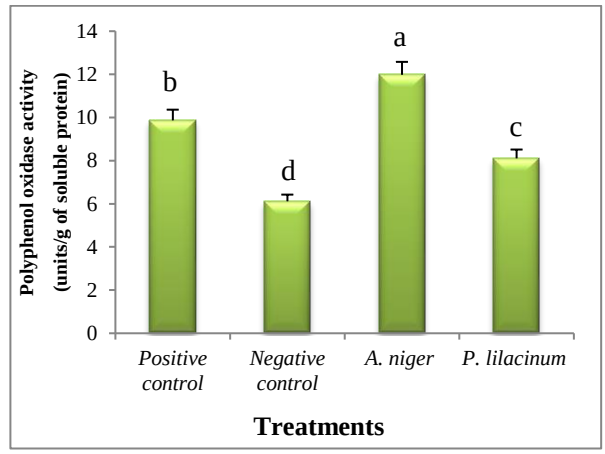

Figure 1. The effect of eggplant leaves treated with fungal antagonists as measured by polyphenol oxidase activity.

A. niger and *P. lilacinum* applied preventively showed significant effect on eggplant leaves infected with *A. solani*, and could be recommended for biocontrol. These two soilborne antagonistic fungi allowed not only the protection of plants, but also enhanced the ability to inhibit the mycelial growth of *A. solani* under *in vitro* conditions. Based on the results of this study, it is concluded that *A. niger* and *P. Lilacinum* isolates used could be employed as foliar treatment to induce *Solanaceae* systemic resistance, through a specific signal transduction cascade. The systemic resistance induction mechanism of *Solanaceae* by *Aspergillus* spp. and *Purpureocillium* spp. against *A. solani* is a subject of future research.

الملخص

مطرود، عبد النبي عبد الأمير، عبد الحق رحومة واكون جودوين اكون. 2021. تقويم كفاءة عوامل المكافحة البيولوجية ضد العامل المسبب لمرض اللفحة المبكرة لـ *Solanum melongena*. مجلة وقاية النبات العربية، 39(3): 204–209. https://doi.org/10.22268/AJPP-039.3.204209

يعد نبات الباذنجان من محاصيل الخضر المهمة في العراق. يتعرض هذا المحصول لخسائر اقتصادية كبيرة نتيجة إصابته بمرض اللفحة المبكرة المتسبب عن الفطر *Alternaria solani*. تم عزل فطرين من فطور المكافحة الحيوية (*Aspergillus niger* و *Purpureocillium lilacinum*) من التربة المحيطة بجذور نبات الباذنجان. تم اختبار هذان الفطران في المختبر (تقنية المواجهة المزدوجة) وفي الجسم الحي (المعالجات الوقائية)، وهي فطور متحملة القدرة لمكافحة المسببات المرضية الفطرية وخصوصاً الفطر الممرض *A. solani*. أدى استعمال الفطرين *A. niger* و *P. lilacinum* عند استخدام تخفيف 30% من المستحضر الكونيدي ظهور نشاط مثبط عالي ضد نمو فطور *A. solani* بنسبة تثبيط 55.11%. علاوة على ذلك، أدى استعمال هذين النوعين من الفطرين الأحيائيين بشكل وقائي عند هذا التركيز في الجسم الحي، إلى تقليل مؤشر شدة الاصابة بالمرض بشكل ملحوظ (إلى أقل من 28.41%). كما عزز الفطر *A. niger* (11.98 وحدة/غ من البروتين القابل للذوبان) والفطر *P. lilacinum* (8.11 وحدة/غ من البروتين القابل للذوبان) نشاط الإنزيم بولي فينول أوكسيداز. بناءً على نتائج هذه الدراسة تبين أن استخدام الفطرين الأحيائيين *A. niger* و *P. lilacinum* أدى إلى تعزيز نمو وتطور نبات الباذنجان وزيادة كفاءته في مقاومة الفطر الممرض *A. solani* وذلك من خلال زيادة كفاءة دفاعات العائل. للسيطرة على *A. solani* ضمن استراتيجيات الإدارة المتكاملة للأمراض، ينصح باستخدام الأعداء الطبيعية كأحد عناصر المكافحة.

كلمات مفتاحية: *Alternaria solani*، المكافحة البيولوجية، إنزيم البوليفينول أوكسيداز، الباذنجان.

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