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Original Article

Formulations as biocontrol and role of pectinase and cellulose in pathogenicity

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A B S T R A C T

The eight virulent strains of *Fusarium oxysporum* which cause wilt pepper disease were screened for their abilities to produce pectinase and cellulase enzymes. *F. oxysporum* AUMC 11424, which was the most virulent strain, gave the highest activity of pectinase and cellulase therefore, which showed a positive correlation between the disease severity and both pectinase and cellulase enzymes. Moreover, the carrier formulations of antagonistic fungi (talc-based powder and rice-bran) were tested under greenhouse and field conditions and, the results revealed that the formulations of *P. oxalicum* AUMC 11419, *A. verrucaria* AUMC 11414, *C. rosea* AUMC 11442 and *T. harzianum* AUMC 11422 showed a highly reduction of disease severity (up to 80%).

Introduction

F. oxysporum AUMC 11424 is a most virulent strain causes vascular wilt diseases in a pepper plants in Egypt (Ismail *et al.,* 2017). The plant pathogens produce enzymes that degrade the plant cell wall components (Moreira *et al.,* 2005, Maina *et al*., 2016). The majority of pathogenic microorganisms invade host plant epidermis with the help of pathogenic enzymes, which help cross the plant cell wall barrier and penetrate the host. Before this, there is an interaction between the pathogen and the host. Pectinases are the first enzymes secreted by most fungal pathogens when attacking plant cell walls, followed by hemicellulases and cellulases (Vallejo Herrera *et al.,* 2004). The enzymes diffuse from the vessels and hydrolyze the pectic substances of the middle lamellae and primary cell walls of adjacent xylem parenchyma cells. The large increase in depolymerase activity in diseased plants may cause wilting by clogging of the vessels with degradation products caused by fungal pectic enzymes acting on constituents of the cell walls (Groenewald, 2006). Moreover, many plant pathogenic organisms are capable of degrading cellulose by producing a cellulose complex which involves the synergistic action of three main enzymatic complexes, endoglucanase, exoglucanase that releases either glucose or cellobiose, and β-1,4-glucosidase that hydrolyzes cellobiose and cellodextrins to glucose (Okunowo *et al.,* 2010). A positive correlation has been established between the production of enzymes, virulence and disease symptoms in several pathosystems (Ramos *et al.,* 2016). Different formulations have been used in control of soil-borne pathogens, these are, fungal spores, and powdery preparations of fungal mycelium (Abdel-Kader *et al*., 2012; Nashwa *et al.,* 2014). A biocontrol formulation with agricultural potential should possess several desirable characteristics such as: easy preparation and application, stability, adequate shelf life, abundant viable propagules, and low cost (Kumar *et al.,* 2014). The formulation should be amenable for application to both phylloplane and rhizosphere, depending on the pathogens and plants to be controlled (Abdel-Kader *et al*., 2012).

Therefore, the objective of the present study was to find relation between the virulent and enzyme production. The efficacy of the application of formulation contained the antago-

nistic fungi against *Fusarium oxysporum* under greenhouse and field conditions were also determined.

Materials and Methods

1. Assay of some enzymes that may be involved in pathogenicity

1.1. Colorimetric assay of pectinase and cellulase activity

1.1.1. Extracellular pectinase production

Growth medium and conditions

The highly virulent eight *Fusarium oxysporum* strains (previously tested for their pathogenicity) were grown in 250 ml Erlenmeyer flask containing 100 ml of pectin broth (pH 7.0), which contains 0.2% NaNO3; 0.1% K2HPO4; 0.05% MgSO4.7H2O; 0.05% KCl; 0.01% FeSO4.7H2O; 0.03% Sucrose; 0.001% ZnSO4; 0.001% CuSO4 and 1% of citrus pectin. This medium was used for pectinase production. The inoculum (one agar plug of four mm diam./flask from the growing edges of cultures of 3 days old were used). Culture flasks were incubated in the shaking incubator operating at 120 rpm at 28 ± 1 ºC for 5 days. At the end of incubation period, the culture broth was filtrate and the culture filtrate was used as a source of crude enzyme (Banakar and Thippeswamy, 2012).

Colorimetric assay of pectinase activity

The pectinase activity was measured in the culture supernatant using the method of Miller (1959). Thus, 1 ml of the cell-free supernatant was mixed with an equal volume of 1% (w/v) citrus pectin in 0.2 M Tris-HCl buffer (pH 8) as the substrate. The mixture was incubated at 40°C for 15 min. Dinitrosalicylic acid reagent (3 ml) was then added and the reaction mixture was boiled in water bath for 15 min. Immediately after boiling, 1 ml sodium potassium tartrate (40% w/v) was added into the mixture for colour stability. The mixture was cooled to room temperature and the absorbance of the reaction mixture was measured at 540 nm (JascoV-630 spectrometer) against a blank .

The standard curve was established using glucose as a reducing sugar. One unit (IU) of pectinase activity was defined as the amount of enzyme that releases 1 µmol of glucose per min under the assay conditions. The enzyme activity was calculated (Chaiyasut *et al.,* 2013) following the formula

$$\text{Enzyme activity (IU/ml)} = \frac{\text{Absorbance of enzyme solution x Standard factor}}{\text{Time of incubation (min)}}$$

$$\text{Where, Standard factor} = \frac{\text{Conc (µMol/ml) of standard (glucose)}}{\text{Absrobance at 540 nm}}$$

1.1.2. Extracellular cellulase production

Growth medium and conditions

The Eight *Fusarium oxysporum* strains were grown each in 250 ml Erlenmeyer flask containing 100 ml of cellulase broth (pH 7.0), which contains carboxymethyl cellulose CMC, 1g; (NH4)2SO4 0.14g; K2HPO4 0.6 g; KH2PO4 0.20g; and MgSO4. 7H2O 0.01 g in distilled water. One agar plug (four mm diam.) from the growing edges of cultures of 3 days old culture was used for inoculation of 250 ml conical flask containing 100 ml broth. The flasks were incubated in shaking incubator operating at 120 rpm at 28 ± 1 ºC for 5 days. At the end of incubation period, the culture broth was separated by filtrations and the culture filtrate was used as a source of crude enzyme (Gilkes *et al.,* 1992).

Colorimetric assay of cellulase activity

Cellulase activity was assayed following the method of Miller (1959). The assay mixture contained 1 ml of 0.5% pure cellulose (Sigma Co.) suspended in 50 mM phosphate buffer (pH 5.0) and 1 ml of culture filtrate of different *Fusarium oxysporum* strains. The reaction mixture was incubated for 30 min at 40 0C. The blank was made in the same way using distilled water in place of culture filtrate. The absorbance was measured at 540 nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit of cellulase activity is defined as the amount of enzyme that catalyzed 1.0 µ mol of glucose per minute during the hydrolysis reaction.

2. Effect of carrier formulation of antagonistic fungi (talc-based powder) and (rice bran) on the disease severity of pepper wilt under greenhouse and field conditions

2.1 Preparation of formulated antagonistic fungi

The highly antagonistic strains of *A. nidulans* AUMC 11418, *B. atrogriseum* AUMC 11415 , *C. rosea* AUMC 11442, *Albifimbria verrucaria* AUMC 11414, *P. oxalicum* AUMC 11419 and *T. harzianum* AUMC 11422 were selected. For mass production, after sterilization 1000-ml flasks containing 500 ml potato dextrose broth (100 g potato, 10 g dextrose in 500 ml water), one agar plug (4 mm diam.) of 3 day old cultures were used for inoculation. The cultures of antagonistic fungi were incubated at 28°C for 7 days in shaking incubator at 150 rpm. The cultures were blended at a low speed (1000 rpm) for 20 seconds and the suspensions were used for preparing formulations (Jayaraj *et al.,* 2006).

2.1.1 Preparation of talc-based formulation:

Fine grade-talc powder (one kg talc, pH was adjusted to 7.0 by adding 15 g $CaCO_3$) was taken in a sterilized metal tray and then 10 g of carboxy methyl cellulose (CMC) was added to talc powder and mixed well then the mixture was autoclaved twice for 30 min. in two consecutive days. 500 ml of the spore suspension was mixed with the sterilized talc under aseptic conditions. The talc-based powder formulation was dried at 28°C for 24 h in sterile plastic trays. The dried formulation was homogenized at a low speed (1000 rpm) for 20 seconds and packed in polyethylene bags (50 g/bag) (Jayaraj *et al.,* 2006).

2.1.2 Preparation of rice bran formulation

For mass production, 1000-mL conical flasks each containing 250 gm vermiculite, 250 gm rice bran and 250 ml Cz medium, were autoclaved for 20 min at 121°C, in two consecutive days. The flasks were inoculated with antagonistic fungi (one agar plug, 4 mm diam) and incubated at 28°C. After 15 days of incubation, contents of flasks were transferred to sterilized plates under sterile conditions, left to dry at 28°C for 24 hours then mixed in a blender to become powder and kept in polyethylene bags.

2.2. Greenhouse experiment

This experiment was carried out in 2016 growing season in the greenhouse of Plant Pathology Department, Faculty of Agriculture, Assiut University. Inocula of *F. oxysporum* f. sp. *capsici* AUMC 11424 was prepared as described previously. Sterilized pots (25 cm in diam) were filled with 3 kg sterilized loamy clay soil pre-infested with *F. oxysporum* at the rate of 3% (w/w) two weeks before sowing. The formulated antagonistic fungi were added (at the rate of 1.5 % (w/w)) to infested soil at the time of the planting. Each pot was sown with 3 seedlings of balady pepper cultivar. Three replicate-pots were used for each treatment and untreated pots without antagonists were used as the control. The per-

centages of disease incidence were calculated after 8 weeks of sowing and severity of wilt was also determined.

2.3. Field experiment

This experiment was carried out in the 2016 growing season. Balady pepper cultivar seedlings were sown in 3 x 3.5 m plots contained 2 rows (1.5 m length). Each row contained 5 hills 20-cm spaced. Each hill was sown with 5 seedlings and three replicates were used for each treatment. 25 g of *F. oxysporum* f. sp. *capsici* AUMC 11424 inoculum was placed in each hill two weeks before planting and each formulation of antagonistic fungi was added to the infested soil (approximately 17 g / hill) at the time of planting. Plots containing inocula of *F. oxysporum* without antagonistic formula were used as controls. Percentages of survival plants were recorded after 8 weeks from sowing and severity of wilt was determined.

3. Statistical analysis

Analysis of variance (ANOVA) was carried out using MSTAT-C program. The least significant difference (LSD) at $p \leq 0.05$ was applied to detect differences among treatments.

Results

1. Colorimetric assay of pectinase and cellulase activities of virulent Fusarium oxysporum strains

Results presented in Table (1) revealed that the eight virulent strains of *Fusarium oxysporum* assayed showed pectinase activities in a range of 2-3.8 IU/ml and AUMC strain 11424 gave the highest activity (3.8 IU/ml) which was the most virulent strain which means a positive correlation between the disease severity and pectinase activity.

Results presented in Table (1) revealed that cellulase activities obtained for *Fusarium oxysporum* strains were less than those of pectinases ranging from (0.4-2.1 IU/ml). The highest cellulase activity 2.1 IU/ml was detected also, as in case of pectinase, by the most virulent strain of *F. oxysporum* AUMC 11424, which also means a positive correlation between the disease severity and cellulase activity.

Table 1. The pectinase and cellulase activities of 8 virulent strains of *F. oxysporum*.

Strain No.	Pectinase IU/ml	Cellulase IU/ml
AUMC 11423	3.5	1.6
AUMC 11424	3.8	2.1
AUMC 11425	3.5	1.6
AUMC 13080	2.9	1.2
AUMC 13081	3.4	1.6
AUMC 13084	2.4	1.06
AUMC 13085	2	0.4
AUMC 13086	2.4	1.7

2. Effect of carrier formulations of some antagonistic fungi on the disease severity of pepper wilt under greenhouse and field conditions

2.1. Effect of talc formulation

Results presented in Table 2 and Fig. 1 indicate that the highest reduction of wilt severity was induced by *P. oxalicum*, *Albifimbria verrucaria* and *T. harzianum* (by more than 80%) followed by *Clonostachys rosea* in case of greenhouse conditions, while *P. oxalicum*, *Albifimbria verrucaria* and *C. rosea* followed by *T. harzianum* in case of field conditions compared to the untreated control. The lowest percentage of reduction of disease severity was obtained by application of *B. atrogriseum* formulation.

Table 2. Effect of talc-formulated antagonistic fungi on the disease severity of pepper wilt under greenhouse and field conditions.

Talc based powder	AUMC No.	Greenhouse conditions		Field conditions	
		Disease Severity	% inhibition	Disease Severity	% inhibition
Albifimbria verrucaria	11414	8.6 ± 2.3^{a}	87.2	6.6 ± 1.1^{a}	89.7
Aspergillus nidulans	11418	20.7 ± 1.1^{c}	69.4	35.3 ± 12^{b}	45.4
Botryotrichum atrogriseum	11415	28.7 ± 2.3^{d}	57.6	42 ± 0^{b}	35
Clonostachys rosea	11442	15.3 ± 2^{b}	77.4	8.6 ± 8^{a}	86.7
Penicillium oxalicum	11419	8.3 ± 4^{a}	87.7	6.3 ± 0.3^{a}	90.2
Trichoderma harzianum	11422	10.7 ± 4^{a}	84.1	9.3 ± 3^{a}	85.6
Control (pathogen alone)		67.7 ± 2.5^{e}	-	64.7 ± 5.7^{c}	-

Mean with different superscripts in a column indicate significant difference (P>0.05)

Fig. 1. Effect of talc-formulated antagonistic fungi on the disease severity of pepper wilt under field conditions-Control (the pathogen infesting soil with no antagonist) (A), Soil treatments with talc-formulated *T. harzianum* (B), *Albifimbria verrucaria* (C) and *Penicillium oxalicum* (D).

2.1.1. Effect of rice bran-formulation

Results in Table 3 and Fig. 2 showed the effect of the rice bran powder formulations of fungal isolates on the severity of wilt disease of pepper. The highest reduction in wilt disease severity was induced by *B. atrogriseum*, *P. oxalicum*, *C. rosea*, and *T. harzianum* by more than 60% compared to the untreated control under greenhouse conditions and by more than 80% under field conditions. The lowest percentage of disease severity was achieved by application of *A. nidulans* formulation.

Table 3. Effect of rice bran-formulated antagonistic fungi on the disease severity of pepper wilt under greenhouse and field conditions.

Rice-bran	AUMC NO.	Greenhouse conditions		Field conditions	
		Disease Severity	% inhibition	Disease Severity	% inhibition
A. verrucaria	11414	21.7±7.5[a]	67.4	8.7±6.4[a]	77.4
A. nidulans	11418	46±0[b]	31	21.6±2[b]	56.8
B. atrogriseum	11415	26±0[a]	61	8.7±0.5[a]	82.6
C. rosea	11442	24±10.1[a]	64	11.3±3[a]	82.6
P. oxalicum	11419	22±2[a]	67	8±4[a]	84
T. harzianum	11422	24.3±7.5[a]	63.5	4.7±3[a]	90.6
Control		66.7±2.8[c]	-	50±6.9[c]	-

Mean with different superscripts in a column indicate significant difference (P>0.05)

Fig 2. Soil treatments with rice bran-formulated *Albifimbria verrucaria* (A), *P. oxalicum* (B), *Trichoderma harzianum* (C), Control (pepper plant infested with the pathogen only) (D).

Discussion

Results revealed that the eight virulent strains of the *Fusarium oxysporum* assayed showed pectinase and cellulose activities and the most virulent strain *Fusarium oxysporum* AUMC 11424 gave the highest activity of pectinase (3.8 IU/ml) and cellulase (2.1 IU/ml) and therefore, which means a positive correlation between disease severity and pectinase and cellulase activities. A positive correlation has been established between the production of pectinolytic enzymes, virulence and disease symptoms in several pathosystems (Kikot *et al.*, 2009; Ramos *et al.*, 2016).

The effect of carrier formulations of antagonistic fungi (talc-based powder and rice bran) on wilt of pepper were tested under greenhouse and field conditions and the results revealed that the formulations from *T. harzianum* AUMC11422, *P. oxalicum* AUMC 11419, *C. rosea* AUMC11442 and *A. verrucaria* AUMC 11414 showed high percentages of inhibition against *F. oxysporum*. Similar results were observed for *T. viride* and *T. harzianum* which showed the best performance in biological control of *F. oxysporum* f. sp. *capsici*, the cause of wilt disease in pepper plants in Egypt (Madbouly and Abdel Backi, 2017), Pakistan (Sahi and Khalid, 2007), and India (Sastiya *et al*., 2016). This effect may be due to mycoparasitism or secretion of extracellular lytic enzymes and other compounds like harzianien and viridin which enhanced their antagonistic activity against *Fusarium* wilt of chilli pepper (Ozbay and Newman, 2004). Harman *et al.* (2004) and Vinale *et al.* (2008a,b) reported also that root colonization by *Trichoderma* spp. Frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients. *T. harzianum* was also the most promising biocontrol agent of *F. oxysporum* in tomato plants (Morsy *et al.*, 2009, Barari, 2016), sugarcane plants (Sabalpara *et al*., 2009) and chickpea (Ramezani, 2009). However, all isolates of *Trichoderma* spp. are not equally effective in control of the pathogen *in vitro* and *in vivo* conditions (Sahi and Khalid, 2007, Ramezani, 2008).

Penicillium oxalicum is reported also as a promising fungal agent for the biological control of *Fusarium* wilt of tomato plants (De Cal and Melgarejo, 2001). *P. oxalicum* formulations have been reported to reduce tomato wilt caused by *Fusarium* spp. under greenhouse conditions and other crop diseases (Santamarina *et al*., 2002, Ahmad *et al*., 2014). *Emericella* spp. (*Aspergillus nidulans* group) have been also reported as antagonistic fungi against *F. oxysporum* f. sp. *lycopersici* (Madbouly, 2016, Anuragi and Sharma, 2016).

Conclusion

F. oxysporum AUMC 11424, which was the most virulent strain, gave the highest activity of pectinase and cellulase therefore, which showed a positive correlation between the disease severity and both pectinase and cellulase enzymes. The effect of carrier formulations of antagonistic fungi (talc-based powder and rice bran) on wilt of pepper revealed that *P. oxalicum* AUMC 11419, *A. verrucaria* AUMC 11414, *C. rosea* AUMC11442 and *T. harzianum* AUMC11422 showed highly reduction of disease severity under greenhouse and field conditions.

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