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Biological Control of Root Rot Complex of Pea (*Pisum sativum* L.)

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Pea root rot complex, caused by several soil borne fungi is a global major yield-limiting factor for pea production. *Fusarium oxysporum, Rhizoctonia solani Kühn, Sclerotinia sclerotiorum,* and *Thielaviopsis basicola* were isolated, identified and confirmed in pathogenicity test as root rot pathogens involved. Thin layer chromatography (TLC) technique revealed that the filtrates of *R. solani, F. oxysporum, S. sclerotiorum* and *T. basicola* contained a variety of toxins that were collected from the chromatogram bands. *Fusarium oxysporum* (one fraction) produced fusaric acid. *Sclerotinia sclerotiorum* (two fractions) produced Sclerotinin A. Control of root rot complex of pea using biological agents (BCAs) as *Trichoderma harzianum, Rhizobium sp* and *Bacillus subtilis* was studied to elucidate their disease control efficacy. Soil treated with *B. subtilis*, *T. harzianum, Rhizobium sp* significantly decreased the disease severity after treatment. The treatments gave the highest valued of plant growth parameters. Results showed that the enzyme activities of PG and Cx decreased in treated root pea plants compared to untreated ones such enzymes are supposedly involved in pathogenesis. Further studies are needed to define the best application methods to achieve the most effective disease control.

Keywords: *Bio-agents, disease complex, enzyme, pea, root rot, toxins and wilt.*

Pea (*Pisum sativum* L.) is one of the most important and popular legume vegetable crops grown in Egypt and many countries all over the world. It has many nutritional values such as high content of protein, carbohydrates, phosphorus, iron, calcium and vitamins A and B (Hassan, 1997). The winter plantation of pea Egypt in 2014 was 43995 feddans that produced 184032 ton with an average yield 4.183 ton/fed. Pea is highly susceptible to pre-emergence damping off and to post-emergence root and foot rots caused by soil and seed borne fungi. Such diseases are often considered as a major limiting factor of production (Grünwald *et al*., 2004). The most common fungi involved are different species of *Aphanomyces, Pythium, Fusarium, Thielaviopsis, Ascochyta ,Phoma, Rhizoctonia, and Sclerotinia* (Kraft and Pfleger 2001).

Microbial toxins are metabolites produced by plant pathogens (fungi, bacteria), which play a role in host-pathogen interactions and important roles in inhibiting the physiological processes in cells surrounding the point of infection, enabling the spread of the disease (Feys and Parker. 2000). Some disease syndromes suggest the

possible involvement of phytotoxins in disease inception and development (Horbach *et al*., 2011). Toxins are secreted by micro-organism host complex that acts on living host protoplast to affect disease symptoms (Meena *et al.,* 2017). Chemical fungicides are commonly sought in control of *Rhizoctonia* root-rot of pea (Khan *et al*., 1998), however their field application may not always be desirable. The persistent and injudicious use of chemicals were discouraged owing to their toxic effects on non-target organisms, the undesirable changes they inflict upon the environment (Arcury and Quandt 2003), the development of resistant strains of pathogens against various chemical fungicides were also considered (Deising *et al*., 2008).

Biological control of soil and seed-borne plant pathogenic fungi has been addressed using bacterial and fungal antagonists. Strains of *Pseudomonas* spp., *Bacillis* spp., *Trichoderma* spp. and non-pathogenic isolates of *F. oxysporum*, isolated from the rhizospheres of crop plants, were shown effective not only to control plant pathogens but also in helping the plants to mobilize and acquire nutrients (Perner *et al.,* 2006). Both *Trichoderma* spp. and *Bacillis* spp. use are widespread throughout the world and have been recognized as the most successful biocontrol agents for soil borne pathogens. Several modes of action have been described, including competition for nutrients, space, antibiosis, induced resistance, mycoparasitism, as well as plant growth promotion and rhizosphere colonization capability (Bailey *et al*., 2008).

Rhizobium spp., not only control soil borne pathogens but produce beneficial effect on plants by improving nutrient and water uptake of plants besides N_2 fixations (Seuk Bae *et al*.,2000) Plant growth promoting Rhizobacteria improve plant growth through direct stimulation in the plant either producing growth regulators or suppressing pathogens (Weller *et al*., 2002). Pathogenic fungi usually occupy the host through colonization process by production a wide range of cell wall degrading enzymes (CWDEs), including pectinases, β-1, 3 glucanases, glycosidases, cellulases, xylanases and cutinases (Annis and Goodwin 1997).

The objectives of this study were: (1) evaluation the role of some bioagents in decreasing disease severity in open field (2) studying the role of phenols in pathogenicity (3) characterizing toxins produced by recovered isolates (4) studying the activities of PG, and Cx produced in field treated plants.

Materials and Methods

Isolation and identification of the associated fungi

Root rot affected pea plants were collected from Sharkia, El Nobaria and Giza governorates. Associated fungi were isolated on potato dextrose agar medium from discolored root tissues. Purification of the isolated fungi was done using the hyphal tip and single spore techniques (Dhingra and Sinclair, 1995). Cultural properties, morphological and microscopical characteristics were taken into consideration in identification of the isolated fungi according to Booth (1971) and Barnett, and Hunter (1972). The total number of fungal colonies and the frequency of each fungus were determined. Pure cultures were preserved on PDA at 4±1°C.

Preparation of inoculum:

The fungi tested were separately grown on PDA medium 7 day at 25°C. Two discs (5 mm) of agar with mycelium taken from 7 day old culture of each fungus tested were transferred onto the surface of autoclaved cornmeal sand medium (75 g grinded corn meal, 25 g fine washed sand and 50 ml tab water) in glass bottles (500 ml)and inoculated bottles were incubated at 28°C for 15 days.

Pathogenicity test in greenhouse:

Seeds of Victory and Master B cultivars obtained from Veg. Res. Dept., Hort. Res. Inst., Agric. Res. Center, Giza, Egypt were used. Soil infestation was made by mixing the fungal inoculum of each isolate at the rate of 3% (w/w) active inoculum, with the upper layer in pots (25 cm) contain 5 kg sterilized soil 3:1 (sand + peat moss) . Pots used as control were filled with soil mixed its upper layer with autoclaved cornmeal and medium (3%). Pots were distributed under the greenhouse conditions in complete randomized design. Ten seeds were sown per pot, and three replicates were used for each treatment.

Disease assessment:

Percentage of pre- and post-emergence damping-off as well as healthy survived plants in each treatment were determined15,30 and 45 days after sowing respectively using the formula according to El- Helaly *et al*. (1970).

Pre- emergence damping-off % =

No. of non-germination seeds / Total no. of sown seeds X 100

Post-emergence damping-off % =

No. of dead seedlings / Total no. of sown seeds X 100

Survived plants % =

No. of survived plants / Total no. of sown seeds X 100

Determination of Phenolic compounds:

After 45 days of cultivation in greenhouse, five gram roots of each treatment, phenolic content (free, conjugated and total phenols) were colorimetrically assayed at 540 nm following the technique described by Snell and Snell (1953). The results were expressed as mg phenolic content / g fresh weight.

Toxins evaluation and Identification:

Identification of the toxins were carried out by comparing R_f value and colors with those given in Suzuki *et al*. (1968) and the analysis was carried out using CAMAG TLC system.

Instruments used:

CAMAG TLC system with:
Linomat 5 applicator
ADC2 developing system
TLC scanner 3 UV/ Vis detector

Condition:

TLC plates: silica gel G -60 aluminum sheet, Merck, Germany
Application volium: 25 microlitere
Migration Distance: 95 millimeter
Developing system: Toluene, Ethylacetate, 90% (w/v) Formic acid (5:4:1,v,v,v)
Detection Wavelength: 254 nm, 365 nm.

Evaluation of thin- layer chromatograms was done in Regional Center for Mycology and Biotechnology, Azhar University, Egypt. Method of Paterson (1986) and Suzuki *et al.* (1968) for extraction and identification of toxins produced in culture filtrates of *R. solani, F oxysporum, S. sclerotiorum* and *T. basicola. w*as applied. The fungal isolates were grown on Cazpek.s Dox medium and incubated for 14 days at 25 ^{o}c ± 2. Culture filtrates of the tested isolates were extracted three times with equal volume of chloroform:methanol(2:1). The extracts were evaporated until dryness and residues were dissolved in 1 ml of chloroform: methanol (2:1). TLC plates 20x20 cm (Merck aluminum sheet, silica gel 60 F254, layer thickness 0.2 mm) were used by spotting chloroform:methanol fraction of each isolate (25 ul)at the same distance from the base and 2 cm apart from edges . The plate was developed for 17 cm in toluene- ethyl-acetate - 90% formic acid (5:4:1) (TEF) in a solvent saturated atmosphere. After evaporation the solvents, the Rf values of greater intensity spots were recorded when TLC plate was exposed to uv light (365 nm and 254 nm) ; color / shape visualization was scanned by(365 nm) UV light . The TLC plates were scanned under scanner 3 at 254 and 365 nm using CAMAG TLC scanner 3. A distance peaks of separated metabolites were observed at 365 nm for *R.solani*, *F.oxysporum*, *S. sclerotiorum* , and *T. basicola.* Peak area (and / or width of spot vs. quantity) for standard and metabolites were measured and quantities of metabolites were obtained according to amount and peak area of fusaric acid. Identification of toxins was appointed through comparing color intensity and Rf values to that in the documentation system, CAMAG TLC Scanner Digi Store 2 with win CATS Software for densitometry.

Antagonists:

Cultures of *T. harzianum*, *B.subtilus* and *Rhizobium* sp. were obtained from Mycology Res. and plant disease survey department, the Plant. Pathol. Res. Inst. Agric. Res. Center, Giza, Egypt and Microbiology Dept., Soil, Water and Environ. Res.Ins., Agric. Res. Center, Giza, Egypt. Antagonistic fungi (*T. harzianum*) was grown on PDA medium whereas antagonistic bacteria *B. subtilis* and *Rhizobium* sp. were grown on nutrient broth medium (Salfinger and Tortorello, 2001) and yeast extract mannitol agar (YEMA) (Subba Rao 1977),respectively Incubation was carried at 28-30°C for 24-96 hours.

Field experiments:

The experiments were conducted at Anshas, Sharkiya Governorate, to study the effect of bio-agents, *Bacillus subtilis*, *T. harzianum, Rizopium* sp and fungicide Flutolanil 25% in controlling pea root rots and wilt (Table 1). A complete randomized block design with three replicates for each treatment. The plot area was 12 m^2 included 5 ridges each of 0.6 m width and 4.0 m length. Pea seeds (*Pisum sativum*, L.) cvs. Victory and Master B were sown in pits on one side of ridges at 10 cm apart. Sowing was made on 21st of October 2016. Farming practices such as irrigation, fertilization were carried out as recommended. Soil irrigation were performed one week before sowing the seeds. Sterilized seeds of pea cultivars were sown in each pit, and covered with thin layer of soil. Treatments were applied after sowing and 20 days at the rate of 50 and100 mL/pit of the prepared bioagent and fungicide suspension.

Table 1. List of pea seed treatments used

Treatments	Source	Concentration/L
T. harzianum	Plant. Pathol. Res. Inst., Agric.Res.Center, Giza,Egypt	(3×10^7 cfu/ml)
Rhizobum sp	Microbiology Dept., Soil, Water and Environ.Res.Ins.,Agric.Res.Center, Giza,Egypt	20 ml/ L
B. subtilis	Microbiology Dept., Soil, Water and Environ.Res.Ins.,Agric.Res.Center,, Giza,Egypt	(30×10^6 CFU /ml).
Flutolanil 25% wp	Shoura Chemicals	1.5-2.5 g / L
Tap water (Control)	-	-

Disease severity assessment:

The plants are scored for disease severity using the root damage scale from 0–5 (Kraft *et al,*. 1994).whereas,

0 = healthy (without any damage)
1 = weak damage (1–10%)
2 = medium damage (10–25%)
3 = medium strong damage (26–50%)
4 = strong damage (51–75%)
5 = very strong damage to total destruction (76 to 100%)

$$\text{Disease severity \%} = \Sigma (fv) / nx \times 100$$

F=number of roots tested in each grade.
V= numerical rating of the scale (1-5), grade.
nX=total number of roots tasted multiplied by (5) *i.e.*, the highest grade

Preparation of enzyme extract:

After 50 days of cultivation one gram roots of each treatment was freezed then blended in 10 ml 0.1 M phosphate buffer (pH 7.0) in a previously chilled .The homogenate was then centrifuged at 10,000 rpm for 20 min in a refrigerated centrifuge at 0–4°C. The supernatant obtained was referred to as crude extract and stored in freeze for enzyme assays.

Polyglactoronase assessment:

Polyglactoronase (PG) activity was determined by measuring loss in viscosity of the reaction mixtures according to the method of Mahadevan and Sridhar (1982). Mixtures containing 2.5 ml of enzyme preparations (sample extract), 2.5 ml citrus pectin1.5% solution and 5 ml in 0.1 M phosphate buffer at pH 6. The reaction mixtures were incubated at 28 ^{o}C and the loss in viscosity of the mixture was measured after 30 minutes against control containing heat inactivated tissue extract instead of the active one.

Cellulase assessments:

Cellulase (Cx) activity was also assayed viscometrically in mixtures containing 2.5 ml carboxymethyl cellulose1.5% (CMC) solution , 5 ml 0.1 M phosphate buffer at pH 6 mixed with 2.5 ml enzyme preparations (sample extract). The mixtures

were incubated at 28°C and the percentage loss in viscosity was estimated after 30 min against control containing heat inactivated tissue extracts instead of the active one. The enzyme activity was determined in terms of loss of viscosity (%) using the following formula (Tolbays and Busch, 1970).

$$\text{Loss in viscosity \%} = T_0 - T_1 / T_0 \times 100$$

T_0 = time of flow of blank
T_1 = time of flow treated sample

Statistical analysis:

Data obtained were analyzed by the analysis of variance according to the procedures of Snedecor and Cochran (1980). Means of all treatments were compared by the least significant difference LSD at 5% level.

Results

Isolation and identification of the associated fungi:

Isolation of fungal pathogens from diseased samples collected from Sharkia, El Nobaria and Giza governorates are shown in Table (2). The isolated fungi were identified and their frequency percentage were calculated. *Rhizoctonia solani* Kühn, showed the highest percentage followed by *Fusarium oxysporum* ,*Sclerotinia sclerotiorum* (Lib.) and *Thielaviopsis basicola* (Berk. & Br.) being, 30.89; 25.22, 20.69 and 15.11%, respectively. Very low frequency of minor fungi (8.09 %) was also scored and neglected.

Table 2. Frequency percentage of fungi isolated from the rotten roots and wilted pea plants

Isolate fungi	Frequency (%)
Rhizoctonia solani (Kühn)	30.89
Fusarium oxysporum	25.22
Sclerotinia sclerotiorum (Lib.)	20.69
Thielaviopsis basicola (Berk.& Br.)	15.11
Other fungi (neglected)	8.09

Pathogenicity test:

Results in Table 3 clearly indicate that all the fungi tested either individually or in dual, triple and quadruple combinations caused pre and post emergence damping off in both cultivars tested *i.e.,* Master B and Victory. In a single soil inoculation with the fungi tested the highest percentage of pre emergence damping off in seedlings of Master B cv was obtained from *S. sclerotiorum* (30.0%) followed by those indicated by *T.basicola* (23.3%), *R. solani* (16.7%) and *F. oxysporum* (13.3%) respectively. Meanwhile in case of Victory cv the highest percentage of pre – emergence damping off was recorded from soil inoculated with *S. sclerotiorum* (26.7%) followed by that incited by growing in soil infested singly by *T.basicola* (13.3%). Data also indicated that dual, triple and quadruple soil inoculation by the fungi tested significantly increased the percentages of pre-emergence damping off in

both cultivars tested. Between dual combinations, *S. sclerotiorum with T. basicola* gave the highest percentage being 50% in Master B cv. and 40% in Victory cv. The highest percentages referred to triples soil infestation by fungi tested in both cultivars was that contained *R. solani , F. oxysporum* and *T. basicola*.

Regarding the post–emergence damping off incidence, data in Table 3 show that single soil infestation with *F. oxysporum* gave the highest percentages in both cultivars tested, being 80%. On the other hand, dual, triple and quadruple combinations caused great increases in the incidence of post emergence damping off in combination with the single soil infestation with any fungi tested (Table 3).

Table 3. Effect of single and different combinations of fungi tested on disease incidence of damping off in two pea cultivars under greenhouse conditions

Treatment	Cultivar					
	Master B			Victory		
	% Damping off		Survival seedlings %	% Damping off		Survival seedlings %
	Pre	Post		Pre	Post	
R.solani	16.7	76.7	6.66	23.3	66.6	10.0
F.oxysporum	13.3	80.0	6.70	13.3	80.0	6.70
S.sclerotiorum	30.0	60.0	10.0	26.7	63.3	10.0
T.basicola	23.3	70.0	6.70	13.3	43.3	10.0
R+F	30.0	66.7	3.30	36.7	56.7	6.70
R+S	46.7	46.7	6.70	50.0	46.7	3.30
R+T	40.0	56.7	3.30	36.7	53.3	10.0
F+S	43.3	50.0	6.70	40.0	43.3	16.7
F+T	36.7	56.7	6.70	26.7	66.7	6.70
S+T	50.0	46.7	3.30	40.0	46.7	13.3
R+F+S	60.0	36.7	3.30	63.3	30.0	6.70
R+S+T	53.3	36.7	10.0	50.0	46.7	3.30
F+S+T	63.3	33.3	3.30	53.3	36.7	10.0
R+F+T	66.7	23.3	10.0	63.3	30.0	6.70
R+F+S+T	53.3	43.3	3.30	46.7	50.0	3.30
Control	0.0	0.0	100	0.0	0.0	100
LSD at 5%	3.08	5.66	0.96	3.16	2.49	1.01

Pre. = 15 days Post. = 30 days Survival = 45 days

Determination of Phenolic compounds:

The level of total phenolic amount significantly increased in cv. Master B than cv. Victory (Table 4). Few exceptions may be recognized in case of multiple infection being more pronounced in R+F, R+T , F+S, S+T and F+S+T treatments. In a single soil inoculation with the fungi tested the highest percentage of the level of free phenolic amount in Master B cv was obtained from *F. oxysporum* followed by *R. solani, S. sclerotiorum* and *T. basicola,* respectively. Meanwhile, in case of Victory cv the highest level of free phenolic in single treatments was recorded from rot infected by *F. oxysporum* followed by that infected by *R. solani, T. basicola and*

S. sclerotiorum . Data also indicated that dual, triple and quadruple soil inoculation by the fungi tested significantly decreased the free phenolic amount in both cultivars tested. Between dual combinations, *S. sclerotiorum with T.basicola* gave the lowest determine free phenol amount being 12.0 in Master B cv and 19.0 in Victory cv. The highest free phenolic amount to triples soil infestation by fungi tested in Master B cv was due to soil infestation with fungi tested being 70.0 (R+F+T), 45.0(R+S+T), and 24.0 (F+S+T) respectively. While the level phenolic amount significantly in Victory cv. soil infestation by fungi tested being 30.0 (F+S+T) and 22.0 (R+S+T), also the highest level of conjugated phenolic amount significantly increased treatments of tested fungi single, dual, triple and quadruple being 340.0 (F), 290.0 (T), 298.0 (R+S), 201.0(F+T), 245.0(R+S+T), 200.0(R+F+T) and 293.0(R+F+S+T) in cv. Master B, respectively.

Table 4. Phenol contents of pea plants grown in soil artificially infested with the fungi tested 45 days after sown under greenhouse conditions

Treatment	Free phenols (µg/g f.w)		Conjugated phenols (µg/g f.wt)		Total phenols (µg/g f.w)	
	Master B	Victory	Master B	Victory	Master B	Victory
R. solani	211.0	45.0	51.0	140.0	262.0	185.0
F. oxysporum	569.0	59.0	340.0	183.0	909.0	242.0
S. sclerotiorum	100.0	20.0	34.0	85.0	134.0	105.0
T. basicola	15.0	36.0	290.0	10.0	305.0	46.0
R+F	60.0	77.0	107.0	9.0	167.0	184.0
R+S	37.0	56.0	298.0	141.0	335.0	197.0
R+T	88.0	16.0	25.0	16.0	113.0	32.0
F+S	17.0	100.0	120.0	120.0	137.0	220.0
F+T	22.0	15.0	201.0	142.0	223.0	157.0
S+T	12.0	19.0	79.0	10.0	91.0	29.0
R+F+S	17.0	10.0	91.0	19.0	108.0	29.0
R+S+T	45.0	22.0	245.0	52.0	290.0	74.0
F+S+T	24.0	30.0	91.0	107.0	115.0	137.0
R+F+T	70.0	7.0	200.0	20.0	270.0	27.0
R+F+S+T	7.0	14.0	293.0	31.0	300.0	45.0
Control	6.0	2.0	16.0	5.0	22.0	7.0
LSD at 5%	179.5	6.5	17.9	8.5	8.8	9.3

Toxins evaluation and identification:

The results reported herein confirmed that the tested pathogens produced bioactive metabolites into culture filtrate. Accordingly, toxins were successfully isolated and identified by using thin layer chromatography, compounds were detectable under UV light (365nm) in the culture filtrate extracts of *R. solani*., *F. oxysporum, S. sclerotiorum,* and *T. basicola* (Tables 5&6 and Fig. 1). These compounds were identified based on the comparison of their R_f values with the R_f value of the standard fusaric acid in addition to the data base in the TLC scanner. The R_f values of metabolites isolated from the culture filtrates of *R.solani* are presented in Tables (5,6) and Fig (1). A total of 2 metabolites were detected after scanning TLC plate under UV at 365. The metabolite numbers;1 and10 with R_f values were 0.03 and 0.06 were detected in the culture filtrate.

The R_f values of metabolites isolated from the culture filtrates of *F.oxysporum* presented in Tables (5,6) and Fig (1). A total of 9 metabolites were detected after scanning TLC plate under UV at 365. The metabolite numbers;3,5,7,8,12,14,16,18 and 22 with R_f values 0.10,0.22,0.31,0.36,0.47,0.65,0.73,0.83 and 0.91 were detected in the culture filtrate. The metabolite number 3 with R_f value 0.10 was identified as fusaric acid. at the concentrations of 0.03mg/ml. The R_f values of metabolites isolated from the culture filtrates of *S. sclerotiorum* presented in Tables (5,6) and Fig (1). A total of 4 metabolites were detected after scanning TLC plate under UV at 365. The metabolite numbers; 2,14,20 and 23 with R_f values 0.09,0.65,0.86 and 0.92 were detected in the culture filtrate. The metabolite number 2 with R_f value 0.65 was identified as sclerotinin A. at the concentrations of 0.14mg/ml. The R_f values of metabolites isolated from the culture filtrates of *T. basicola* presented in Tables (5,6) and Fig (1). A total of 9 metabolites were detected after scanning TLC plate under UV at 365. The metabolite numbers;4,6,9,11,13,15,17,19 and 21with R_f values 0.15,0.30,0.40,0.45,0.52,0.66,0.78,0.84 and 0.90 were detected in the culture filtrate.

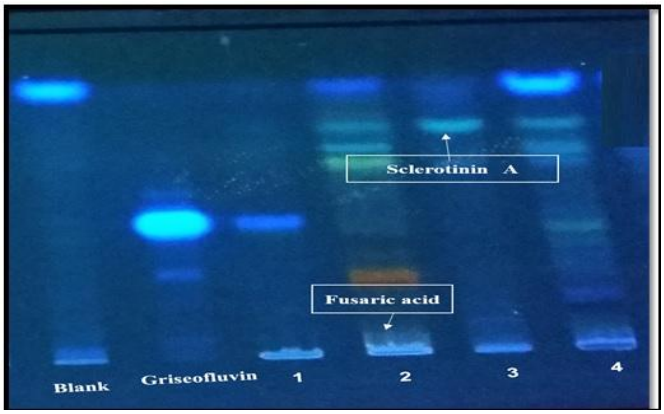

Fig 1. Fractions in culture filtrate (14 days) of isolates under UV at 365 nm

Table 5. Toxins produced by the fungal isolates tested

Metabolites No	R_f	Identification of toxin	Area (unit)			
			R. solani	*F.oxysporum*	*S.sclerotiorum*	*T. basicola*
1	0.01		150.0			
2	0.09				538.1	
3	0.10	Fusaric acid		1528.7		
4	0.15					137.7
5	0.22			16009.6		
6	0.30					137.7
7	0.31			5061.2		
8	0.36			4697.1		
9	0.40					2590.2
10	0.43		2428.1			
11	0.45					3111.7
12	0.47			1203.4		
13	0.52					3246.5
14	0.65	Sclerotinin A		8368.5	5615.7	
15	0.66					11292.8
16	0.73			11394.0		
17	0.78					6162.3
18	0.83			18497.7		
19	0.84					14124.7
20	0.86				7890.8	
21	0.90					5774.4
22	0.91			2234.1		
23	0.92				4238.6	
Standard fusaric acid	0.10			82845		

Table 6. R_f values and concentrations of the isolated metabolites from the culture filtrates of the fungal isolates tested

Metabolites No	R_f	Concentration of metabolites (mg/ml)			
		Isolate			
		R. solani	*F. oxysporum*	*S. sclerotiorum*	*T. basicola*
1	0.01	0.03			
2	0.09			0.01	
3	0.10		0.03		
4	0.15				0.03
5	0.22		0.39		
6	0.30				0.04
7	0.31		0.12		
8	0.36		0.11		
9	0.40				0.06
10	0.43	0.06			
11	0.45				0.08
12	0.47		0.03		
13	0.52				0.08
14	0.65		0.20	0.14	
15	0.66				0.27
16	0.73		0.28		
17	0.78				0.15
18	0.83		0.45		
19	0.84				0.34
20	0.86			0.19	
21	0.90				0.14
22	0.91		0.05		
23	0.92			0.10	

Field-experiment:

Data in Table 7 indicate that all treatments significantly decrease in disease severity. The fungicide was more effective at (50,100 ml) in two cultivars Master B (11.6%,10.7%) and Victory (9.8%,8.9%) respectively. The disease severity decrease in cultivars Victory and Master B treatments with *Rhizobium* sp at (50,100 ml), that gave the best control (16.9 %,13.1%) and (17.8%,14.2%) respectively, followed by treatments with *B. subtilis* (15.4%,13.3%) and (16.9%,14.7%) and treatments with *T. harzianum* (15.1%,13.8%) and (17.8%,15.6%) compared to control treatment. The obtained results showed that in two cultivars Master B and Victory, all biocontrol agents used in this study caused significant increase in growth parameters when applied with at 100ml.

Table 7. Effect of soil treatment with biocontrol agents on disease severity and plant parameters of pea (*Pisum sativum* L.) raised in open field

Treatment	ml/ pit	Cultivars							
		Master B				Victory			
		% Disease severity	Plant length (cm)	Plant fresh weight (g)	Plant dry weight (g)	% Disease severity	Plant length (cm)	Plant fresh weight (g)	Plant dry weight (g)
T..harzianum	50	17.8	42.2	54.0	1.4	15.1	51.6	66.0	1.3
	100	15.6	43.6	61.0	1.9	13.8	71.2	78.5	1.9
B. subtilis	50	16.9	43.1	44.0	1.7	15.1	57.2	57.0	1.4
	100	14.7	45.0	62.0	2.2	13.3	71.0	64.0	1.9
Rhizobium sp	50	17.8	42.5	51.0	1.4	16.9	51.4	61.5	1.3
	100	14.2	46.8	65.0	1.7	13.1	74.3	74.5	1.6
Flutolanil	50	11.6	61.4	87.0	2.0	9.8	79.2	100.0	2.3
(Fungicide)	100	10.7	66.9	107.0	2.5	8.9	82.4	137.0	2.5
Control (infested)	---- -	22.2	41.0	36.0	1.1	20.5	46.2	37.0	1.3
LSD at 5%									
T= Treatments		0.98	1.67	1.86	0.20	1.28	2.30	2.22	0.38
C= Concentration		0.62	1.06	1.18	0.13	0.81	1.46	1.40	0.24
TxC= Treat. x Conc.		1.39	2.36	2.64	0.29	1.81	3.26	3.13	0.53

Assessment of Polyglactoronase and Cellulase enzymes:

Table 8 shows that the production of Polyglactoronase and Cellulase enzymes was increased at low concentration used. Maximum Polyglactoronase activity was observed in the control in two Cultivars Master B and Victory. The cultivar Victory produce enzymes PG (57.3%) and Cx (39.8%) viscosity loss at 100 ml /pit, plants treatment with *Rhizobium* sp . On other hand increased loss in viscosity at (50ml) produce PG (65.5 %) and Cx (55.2 %). When plants treatments with *subtilis* and *T. harzianum*. At 100 ml / pit produce PG (38.8 %) and Cx (57.7%) viscosity loss, PG (39.6 %) and Cx (58.2%) viscosity loss respectively. The cultivar Master B produce enzymes PG (70.4%) and Cx (57.9%) viscosity loss at 100 ml /pit with plant treatment *Rhizobium* sp. On other hand increased loss in viscosity at (50ml) prduce PG (87.9%) and Cx (68.6 %). When used *B. subtilis* and *T. harzianum*. At

100 ml / pit PG (40.3%) and Cx (73.7%) viscosity loss , PG (41.5%) and Cx 66.8% viscosity loss respectively. The chemical fungicides inhibited on produce PG and Cx enzymes of two cultivars at (50 ,100) ml/pit. Cultivar Master B viscosity loss produce enzymes PG (23.1%,34.9%) and Cx (30.9%, 35.4%) and cultivar Victory produce PG (28.4%,29.9%) and Cx (17.2%,20.9 %) compared with control. The cultivar Master B samples treatments showed more produce Polyglactoronase and Cellulase enzymes (PG,Cx) than the cultivar victory.

Table 8. Polyglactoronase and Cellulase activates in roots of two pea cultivars received different treatments by bio control agents under open field conditions

Treatment	ml/ pit	Cultivars			
		Master B		Victory	
		% loss in viscosity after (30min)		% loss in viscosity after (30min)	
		PG	Cx	PG	Cx
T. *harzianum*	50	52.9	69.4	68.0	59.4
	100	41.5	66.8	39.6	58.2
B. subtilis	50	52.4	74.7	59.9	64.5
	100	40.3	73.7	36.8	57.7
Rhizobium sp	50	87.9	68.6	65.6	55.2
	100	70.4	57.9	57.3	39.8
Flutolanil 25% (fungicide)	50	34.9	35.4	29.9	20.9
	100	23.1	30.9	28.4	17.2
Control (infested)	-----	91.3	82.3	80.7	78.3
LSD at 5%					
T=Treatments C=Concentration TxC= Treat. X Conc.		T= 2.99 C= 1.89 TxC= 4.23	T=0.79 C=0.49 TxC=1.11	T=1.48 C=0.94 Txc=2.94	T=0.95 C=0.59 TxC=1.34

Discussion

The present work investigation indicated that isolated fungi that cause root rot complex of pea plants were identified as *F. oxysporum, R. solani, S. sclerotiorum* and *T. basicola* according to Barnett and Hunter (1972). Similar results were documented that damping off and root rots are indicated by soil bone fungi Grünwald *et al*. (2004). In pathogenicity tests, all the isolated fungi were pathogenic to two pea cultivars Master B and Victory plants with different degrees of disease severity. Phenols, were accumulated in plants under the influence of the pathogens. The level of total phenolic content increased in cv. Master B more than cv. Victory. Few exceptions may be recognized in case of dual and triple infection being more pronounced in R+F, R+T, F+S, S+T and F+S+T treatments. Phenolic compounds are the most numerous group of secondary metabolites with many different properties. Especially phytoalexins are of great importance in the immune responses of plants to abiotic and biotic stresses (Stoessl, 1981). Moreover, plants resistant and susceptible to the pathogen should differ in the phytoalexin formation rate and defense reaction after the pathogen attack. The present work confirmed that pathogenicity and disease severity caused by the tested isolates coincided with number and concentration of toxins produce functional metabolites into culture filtrate. Accordingly, toxins were successfully isolated and identified using thin layer chromatography in the culture filtrate extracts of *R. solani*., *F. oxysporum, S. sclerotiorum,* and *T. basicola* . The chromatographic behavior of the 2,9,4 and 9 fractions, respectively was detected. These compounds were identified based on the comparison of their R_f values with the R_f value of the standard fusaric acid.

The R_f values of metabolites isolated from the culture filtrates of *R.solani* , total of 2 metabolites, the metabolite numbers; 1 and10 with R_f values 0.03 and 0.06 were detected in the culture filtrate. Brooks, (2007) stated that host-selective toxin (HSTs) from *R. solani* could increase the virulence of the pathogen as HC-toxin on maize. Bartz *et al*, (2013) had shown that *R. solani* also produces phenyl acetic acid and its derivatives. The R_f values of 9 metabolites isolated from the *F. oxysporum* culture filtrates were detected. The metabolite numbers; 3,5,7,8,12,14,16,18 and 21 with R_f values 0.10,0.22,0.31,0.36,0.47,0.65,0.73,0.83 and 0.91 were detected in the culture filtrate. The metabolite number 3 with R_f value 0.10 was identified as fusaric acid at the concentrations of 0.03mg/ml.

In infected cereal grains, *F. graminearum* can produce several mycotoxins, including trichothecene derivatives *e.g*., deoxynivalenol (DON), polyketide zearalenone (ZEA), fusarin C (Kimura *et al*., 2007). The interrelation between plant phenols and toxin was intensively investigated by many investigators Dambolena *et al*. (2011) studied the capacity of 10 natural phenolic compounds to inhibit FB1synthesis by *F.verticillioides* and revealed that thymol, carvacrol, isoeugenol as well as eugenol were the most active. Beekrum *et al*.,(2003) who stated that the plant phenol chlorophorin was found effective in decreasing production of fumonisin B1 (FB1) toxin with 94%, followed by caffeic acid (hydroxycinnamic acid), ferulic acid, vanillic acid and iroko .

The R_f values of 4 metabolites isolated from *S. sclerotiorum* culture filtrates were detected in the metabolite numbers; 2,14,20 and 23 with R_f values 0.09,0.65,0.86 and 0.92. The metabolite number 2 with R_f value 0.65 was identified as sclrotinin A. at the concentration of 0.14mg/ml . These results are in agreement of Suzuki *et al.* (1968) who isolated from *Sclerotinia sclerotiorum* culture filtrate several plant growth regulators, sclerin, sclerloide, sclerotinin A and B. This finding confirmed that pathogenicity and culture extract toxicity of the tested isolates coincided with number and concentration of phytotoxins (Vidhyasekaran, *et al.,* 1997).

Toxins are different from enzymes in the fact that they do not attack the structural integrity of the tissues but affect the metabolism in a subtle manner (Kumar and Hayward, 2005). In recent classification, toxins are divided into two categories (Ravichandra, 2013). Slow accumulation of similar chemicals has been reported in susceptible host plant tissues (Pusztahelyi *et al*., 2015). These substances include; phenolic compounds, phytoalexins, new protein synthesized, in active of enzymes and toxins and altered biosynthetic pathways (Pusztahelyi *et al*., 2015).

Pea seeds of two cultivars treated with *B. subtilis, T. harzianum, Rhizobium* sp, showed reduction in disease severity, and increased in plant height, fresh weight, and dry weight under field conditions, compared with control treatment. Enzyme production by the infected cultivar Master B samples showed more activities polyglactoronase and cellulase enzymes (PG, Cx) than that recorded from the cultivar Victory and this might be attributed to susceptibility. These enzymes play a key role in fungal metabolism and may be involved in fungal pathogenesis, causing damage to the host tissues and overcoming the host immune system and strongly contribute to fungal pathogenicity (Karkowska-Kuleta *et al* 2009).

Bertagnolli *et al*. (1996) had suggested production of antagonistic substances which can play an important role in lysis of the cell wall components of the pathogenic fungi. Whipps, (2001) found that the modes of action include: parasitism, antagonism, competition for nutrients and space and induction of plant defense. The plant growth promoting effect of the biocontrol agent *Trichoderma harzianum* has been suggested to be based on the production of antagonistic compounds against root pathogens, which also serve as plant hormones, which can increase root growth (Harman *et al*., 2004) and (Vinale *et al*., 2008).

Saharan, and Nehra (2011) found that the decrease of disease incidence and severity by biocontrol agents may be directly due to activation of plant defense mechanism and induce systemic resistance in plant against *R. solani* lead to produce proteins in the plant acts as antifungal agents.

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المكافحة البيولوجية لمرض عفن الجذور المركب فى البسلة

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يُعتبر مرض عفن جذور البسلة من الأمراض المركبة التى تسبب عن العديد من الفطريات الكامنة فى التربة وقد عزلت الفطريات المسببة ودرس تأثير إفرازتها من السموم والتعرف عليها وتقدير الانزيمات المحللة للنباتات المعاملة وكانت النتائج كالتالى: تم عزل الفطريات وتعريفها والتأكد من قدرتها المرضية كمسببات مرضيه لعفن جذور البسلة – كما أكدت الدراسة لاختبار مرشحات الفطريات الممرضة انها تحتوى على أعداد من السموم حيث وجد أن فطر الفيوزاريوم أوكسي سيورم يعطى (رقم ١) فيوزاريك اسد وفطر سكليروتينيا سكلورشيم يعطى (رقم ٢) سكلورتينين A . واكدت الدراسه باستخدام المكافحة البيولوجية لمقاومة المسببات المرضية مثل التريكودريما هيرزيانم والباسلس ساتلس والريزوبيم عند معاملة النباتات بالتربة ادى الى انخفاض شدة الاصابة مقارنة بالكنترول - كما أعطت صفات خضرية للنبات اعلى مقارنة بالكنترول. كما أدى استخدام المكافحة البيولوجية إلى انخفاض النشاط الانزيمي البولي جلاكتورينيز والسليوليز فى النباتات المعاملة مقارنة بالنباتات غير المعاملة حيث أن هذه الانزيمات لها دور فى حدوث الاصابة.