ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Studies on the effect of olive mill waste on pre-harvest and plant fungal diseases.

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ABSTRACT

Olive mill waste is one of the most enviromental problem in Egypt. OMW act as a good biofertilizer as it used for reduction of cellulase and pectinase of soil born fungi *(Fusarium solani*, *Sclerotinia sclerotiorum, Sclerotium rolfsii* and *Rhizoctonia solani*) when used as carbon source at concentration 100 g/l after incubation from 5-15 days. Olive mill waste affect on reducing linear growth of soil born fungi at concentration 100 g/l reduction of growth was 83.3 and 88.9% comparing with control. *Trichoderma harzianum* and *Trichoderma viride* isolated from healthy bean plants inhibit the growth of soil born fungi grown on different concentrations of OMW. *Trichoderma harzianum* and *Trichoderma viride* reduce phenol content of olive mill waste and increase its C/N ratio after incubation 15-30 day. Field experiment at El –Saf village Giza was determined on bean plant result in reduction of root rot disease caused by soil born fungi when treated with *T. harzianum* and *T. viride* grown on olive mill waste.

Keywords: olive mill waste, biofertilizer, fungi.

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ISSN: 0975-8585

INTRODUCTION

Olive mill waste (OMW) has been concerned as a big environmental problem for all countries produce olive oil. This compound has low pH, salinity, high organic load and amount of phytotoxical compounds, such as polyphenol (Di Bene *et al*.2013; Chaari *et al*. 2013). The polyphenols, protein, polysaccharides, etc .,responsible for the high chemical oxygen demand (COD) values and minerals salts, represent a significant problem for the treatment of wastes (Borja *et al*. 1997)

The interest in the recovery, recycling and upgrading of residues from plant food processing gained attention in the last years has been increased (Laufenberg *et al*. 2003). The classic production of olive oil generates three phases and two wastes: olive oil (20 %), solid wastes(30 %) and aqueous liquor (50 %). The solid waste (olive oil cake (OOC) or "orujo") is a combination of olive pulp and stones (Amal and Mahmoud 2012).

OMW have antimicrobial and phytotoxic properties oing to their content of various simple and complex phenolic compound such as flavonoids (Hachicha *et al*.2009). Depending on environmental conditions polyphenol can be degraded and transformed into humic substances . (Sierra *et al*.2007).

Different organic matter, nitrogen, potassium, phosphorus, magnesium, considerable amount of nutrient and non negligible water source which found in rich quantity in OMW make it good biofertilizer for agricultural application (Ammar *et al*.2005; Mechri *et al*.2000;|Chaari *et al*. 2013).

Several strains of filamentous fungi have been revealed a great capacities for the removal of several problems present in OMW compounds (Dias *et al*. 2004). Different studies proved that wastes have a high fertilizer value when mixed with soil for the high content of organic matter (OM) and nutrient content ,soil donation of dry residue of olive(DOR) play an important role in the increase the soil OMW and the inorganic elements essential for plant growth (Paredes *et al*. 1999).

Bean plants (Phaseolus vulgaris L.) are one of the most important leguminous crops in Egypt. Root rot disease caused by Fusarium solani and Rhizoctonia solani is a serious and persistent disease problem of bean plants during growing season (Filion et al. 2003; Harveson et al. 2005; Wen et al. 2005)

Velickovic *et al*.(2008)stated that plant material and solid waste residue may contain biological compounds which could be exploited as secondary raw material for obtaining different bioactivities.

The main objective of this work is to study the effect of OMW as a source of enzymes production and biofertilizer for growth promotion of bean plant by controlling root rot disease.

MATERIALS AND METHODS

Microorganisms:

Soil born fungal strains (*Fusarium solani*, *Sclerotinia sclerotiorum, Sclerotium rolfsii* and *Rhizoctonia solani*) which are the causative agents of bean damping-off and root-rot diseases, were isolated from bean roots that showed the disease symptoms. The fungi in pure culture were identified after pathogenicity test according to the keys given by Barnett and Hunter (1972) and Nelson *et al*. (1983) affiliated to the Plant Pathology Department, National Research Centre, Giza, Egypt. *Trichoderma harzianum* and *Trichoderma viride* were isolated from healthy bean plants and identified in Plant Pathology department at(NRC). Cultures were kept on potato dextrose agar media (DIFCO) PDA slants and at 4 ◦C.

Fermentation media:

Fermentation was carried out in Erlenmeyer flask each containing 50 ml of fermentation media consist of (g/l): olive mill waste (OMW) (20.0), $NaNO_3$ (2.0), K_2HPO_4 (1.0), $MgSO_4$ (0.5) NaCl (0.5), $FeSo_4$ (0.01), the pH was adjusted at 6.5 and autoclaved at 121 ◦C for 15 minutes. One ml of10^6 spore suspension of the selected fungal strain was inoculated in each flask and incubated at 28-30 ◦C for 7 days at 200 rpm.

Enzymatic activity:

Determination of the released reducing sugars due to cellulolytic activity was done by the method reported by Neish (1952) and based on those described by Somogyi (1945) and Nelson (1944). Pectinase activity was assayed according to Miller (1959).

Antimicrobial activity:

In vitro: evaluation of polyphenols compounds:

To determine the antifungal activity of OMW (polyphenols) against fungal phyto-pathogens species .Treatments were PDA plates with OMW added into the medium with different concentrations (20, 40, 60, 80, and 100 g/l) and autoclaved. Disks from each isolate of soil born fungi (5mm. in diameter were inoculated on the center of PDA medium with different concentrations. Three replicates were used for each isolate. The inoculated plate with pathogens without OMW were used as control. All plates were incubated for 7 days at 28 ◦C. The linear growth of pathogens was recorded.

In vitro: antagonistic effect of *T. harzianum* and *T. viride* grown on OMW on the mycelium growth of pathogenic fungi:

The effect of T. harzianum and T. viride grown on OMW against soil borne plant pathogens (Fusarium solani, ,Rhizoctonia solani, Sclerotinia sclerotiorum and Sclerotium rolfsii) were tested. Treatments were PDA plates with OMW added into the medium with different concentrations (20,40,60,80and100 g/l) and autoclaved. Disks from each isolates of T. harzianum and T. viride (5mm. in diameter) were inoculated on PDA medium with different concentration of OMW at one end of petri plate and with each pathogenic fungus at the other end. Three replicates were used for each isolate. The inoculated plate with only pathogens were used as control and incubated for 7 days at 28◦C (Anees et al.,2010). The linear growth of pathogens was recorded. Inhibition percentage of growth was calculated using the following formula:

$$\text{Growth reduction \%} = \frac{\text{Growth in control} - \text{Growth in treatment}}{\text{Growth in control}} \times 100$$

Field Experiment

The field experiment was carried out at El-Saf village, Giza government during the 2016 growth season to evaluate the efficacy of *T. harzianum* and *T. viride*, which was formulated on olive mill waste.

The field experiment consisted of plots of 7 x 6 m2; each comprised 12 rows and 30 holes per row, which were arranged incompletely. Randomized block design with five plots served as replicates for each particular treatment as well as for untreated controls. Bean seed cv. Giza 3 was sown in all treatments at the rate of three seeds per hole. All plots received the traditional agricultural practices. The average percent of root-rot infection at pre-emergence stage was recorded 15 days after sowing. The root-rot symptoms shown by the bean plants were recorded at the post-emergence stage, and average accumulated disease incidence was calculated three times, i.e. after 30, 45 days sowing and throughout the growing season. At harvest time, the average accumulated yield was calculated on all the applied treatments and on the control(. Hamed et al., 2012) and(Nadia et al. 2016).

Chemical analysis

Determination of total organic carbon (Jackson,1958), total Nitrogen (Bremner and Mulvaney,1982).

Determination of total phenol content using a modification of the method of (Romero et al. 2002), five gram compost was dried then addition of 30 ml of methanol: water (80:20 v/v) in Erlenmeyer conical flask .The flasks were incubated for 24 hours at 200 rpm . The supernatants was kept at -20◦C. The

process repeated in 30 ml methanol :water (80:20 v/v) for 48 hours under the same conditions discussed before . The supernatants were evaporated to dryness, re-suspended in 4 ml of distilled water (4ml), and placed in a glass vial. One ml of sample were transferred to glass test tubes and 1ml of 20% sodium bicarbonate was added , followed by 0.5 ml of Folin-Ciocalteau's phenol reagent. After 60 minutes incubation at room temperature, the tubes were mixed again and the absorbance at 750nm was measured. A standard curve was constructed gallic acid.

Physiological factors:

Different concentrations of OMW (20-100) g/l and different incubation period (5, 7 and 14) days was determined for optimization of culture conditions.

Statistical analysis:

Tukey test for multiple comparisons among means was utilized(Neler *et al*. 1985).

RESULTS AND DISCUSSION

Effect of different olive mill concentrations (OMW)on enzymes production of soil born fungi:

Different concentrations of OMW were tested for their ability to increase or reduce the ability of soil born fungi for enzyme production . Results in Table (1) showed that pectinase and cellulase activity decrease with increasing OMW concentration till reached 100 g /l, minimum activity recorded for both enzymes in comparison with control. This means that OMW act as good biofertilizers as it reduce pathogenicity of soil born fungi . Olive mill dry waste can be used as biofertilizers due to their high organic content ,(Nogales *et al*. 1999). OMW characterized by particular characters, in addition to fat and triglycerides, sugars, phosphate, polyphenols, polyalcohols, pectins and metals, could provide microorganisms with biotechnological potential and low-cost fermentation substrates (Darvishi ,2012).

Table (1): Effect of different OMW on cellulase and pectinase production by soil born fungi

Olive mill concentrations(g/l)	*F. solani*		*S. sclerotiorum*		*S. rolfsii*		*R. solani*	
	Pectinase (U/ml)	Cellulase (U/ml)	Pectinase (U/ml)	Cellulase (U/ml)	Pectinase (U/ml)	Cellulase (U/ml)	Pectinase (U/ml)	Cellulase (U/ml)
Control	29.42	6.00	19.93	11.98	21.52	2.57	21.91	2.48
20.0	16.21	4.18	9.72	8.24	13.61	1.32	14.80	0.27
40.0	9.31	4.09	7.29	7.15	12.04	1.13	15.90	0.18
60.0	8.20	3.07	6.06	5.10	11.66	0.17	13.32	0.13
80.0	7.10	2.12	6.01	2.10	10.87	0.16	11.85	0.11
100.0	6.90	1.10	2.13	1.01	10.28	0.10	6.66	0.10
120.0	6.80	1.10	2.10	1.01	10.26	0.10	6.64	0.10

Effect of different incubation period

The productivity of cellulase and pectinase affected with varying incubation period . Results in Table (2) showed that with increasing incubation period from 5-15 days the activity of both enzymes produced by soil born fungi were reduced. *F. oxysporum* produce lower enzymes activity after 15 days incubation period in presence of olive mill wastes Aranda *et al*.(2014). Jose *et al*.(2014) found that *Aspergillus* sp. differ in their capability for production lignocelluloytic enzymatic on growing on OMW as carbon source from 7-14 days incubation period.

ISSN: 0975-8585

Table (2): Effect of different incubation period on cellulase and pectinase production by soil born fungi

Different incubation periods (days)	*F. solani*		*S. sclerotiorum*		*S.rolfsii*		*R. solani*	
	Pectinase	Cellulase	Pectinase	Cellulase	Pectinase	Cellulase	Pectinase	Cellulase
5	26.63	2.97	18.51	11.03	4.90	11.98	23.00	4.60
7	24.61	2.68	16.10	9.54	10.57	6.17	15.63	4.40
10	14.17	2.78	14.90	7.78	2.88	5.04	13.34	3.78
12	12.01	1.12	13.44	7.01	2.28	4.13	12.98	2.28
15	11.41	0.12	12.44	6.71	1.98	1.73	12.28	1.98

Effect of different concentration of OMW on the linear growth of soil born fungi

Data in Table (3) showed that all concentration of OMW have significant effect on reducing linear growth of all soil born fungi .The effect increase by increasing the concentration of OMW. At 100 g/l of OMW causes completely inhibition of linear growth of *F. solani* and *S. sclerotiorum* while in *R. solani* and *S. rolfsii* causes reduction in linear growth 83.3 and 88.9% comparing with control. Many phenolic compounds, free fatty acids and aromatic compounds have been detected to consist as waste in the production process of olive oil and linked phytotoxic and antimicrobial properties inhibited microbial growth (Obied *et al*. 2005; Bisignano *et al*. 1999 and Bonanomi *et al*.2006).

Table (3): Effect of different concentration of olive mill waste on the linear growth of soil born fungi causing root bean diseases.

Different Conc. of Olive mill waste (g/l)	*F.solani*		*S.sclerotiorum*		*S. rolfsii*		*R. solani*	
	L.G	%R.	L.G.	%R	L.G.	%R.	L.G.	%R.
20	44.0	61.1	55.0	38.9	65.0	33.3	70.0	22.2
40	39.0	72.2	50.0	44.4	52.5	41.7	50.0	44.4
60	33.0	77.8	35.0	61.1	42.5	52.8	40.0	61.1
80	20.0	88.9	22.0	75.6	20.0	77.8	33.0	68.9
100	0.0	100.0	0.0	100.0	10.0	88.9	15.0	83.3
Control	90.0	-----	90.0	-----	90.0	-----	90.0	-----

Abbreviation: L.G.: linear growth , R.: reduction

Antagonistic effect of *T. harzianum* and *T.viride* on the bean soil born fungi grown on different concentrations of olive mill waste

Data in Table (4) showed that the highest inhibitory effect on the growth of all soil born fungi with *T. harzianum* at 100 g/l of OMW followed by *T. viride* and control. Leontropoulos *et al*. (2015) observed that liquid polyphenolic compound especially in three fungi *Botrytis cinerea*, *S. sclerotiorum* and *Ascochyta lentis* showed inhibitory effect of fungus spore germination and mycelium growth. In contrast, oleuropain was less effective for fungi *Alternaria alternata*, *Fusarium oxysporum* and *Rhizopus* sp. compared with the growth of *Botrytis cinerea*, *Colletotrichum higginsianum* and *Phytophthora parasitica*.

Table (4): Antagonistic effect of *T .harzianum* and *T.viride* on the bean soil born fungi grown on different concentrations of olive mill waste.

Treatments / Conc.	*T. harzianum*					*T. viride*				
	20	**40**	**60**	**80**	**100**	**20**	**40**	**60**	**80**	**100**
F. solani	35.0	25.0	20.0	15.0	0.0	49.0	40.0	34.0	30.0	25.0
S.sclerotiorum	40.0	37.0	30.0	25.0	0.0	45.0	40.0	35.0	30.0	25.0
S. rolfsii	53.0	45.0	35.0	30.0	10.0	50.0	44.0	36.0	3.1	27.0
R. solani	53.0	46.0	35.0	28.0	15.0	47.0	42.0	37.0	31.0	18.0
Control	90.0	90.0	80.0	65.0	55.0	90.0	90.0	70.0	60.0	50.0

ISSN: 0975-8585

Chemical characterization

Determination of total carbon and nitrogen:

Carbon and nitrogen were most important component of OMW, soil fungi plays an important role in decomposing carbon and nitrogen .In presence of microorganisms different ratios of carbon and nitrogen were measured after 15 and 30 days . Results in Table (5) showed that *T. harzianum* produce higher C/N ratio after 15 days incubation period while *T. viride* give higher ratio after 30 days incubation period . This results were coincided with Amal and Mahmmoud (2012) who found that in presence of olive mill wastes *Azotobacter vinelandii* increase C and N %. Olive mill wastes used as substrate for the synthesis of biotechnological application of high-value metabolites (Mafakher *et al*. 2010).

Table (5):Chemical characterization

Microorganisms / Days	Total carbon %		Total nitrogen %		C/N Ratio	
	15	30	15	30	15	30
T. harzianum	49.20	45.60	0.77	o.85	37.15	34.88
T. viride	53.40	49.60	0.89	0.73	31.19	39.50
S.sclerotiorum	39.10	19.38	2.38	2.13	9.55	5.29
F. solani	33.32	34.34	1.98	0.96	9.78	20.77
R. Solani	23.80	35.02	2.22	1.60	6.23	12.73
S.rolfsii	33.32	26.86	2.13	0.99	9.09	14.62

Determination of total phenol:

From the Results obtained in Fig (1) the total percentage of phenol produced by OMW in presence of *T. harzianum* and *T. viride* was reduced gradually after 30 days of incubation in comparing with soil born fungi Sampedro *et al* .(2004) illustrated that *Paecylomyces farinosus* hydrolyzed polyphenols after two weeks of composting. Linares *et al*. (2003) found that *Phanerochaete flavido-alba* decrease 70 % of the total polyphenols in olive pomace treated for 10 weeks , while Aggelis *et al* .(2003)recorded that *Pleurotus ostreatus* reduce polyphenols after 2 weeks, which need to hydrolysed most phenols content at the first stage(2weeks) of growth and then eliminate all monomeric phenols.

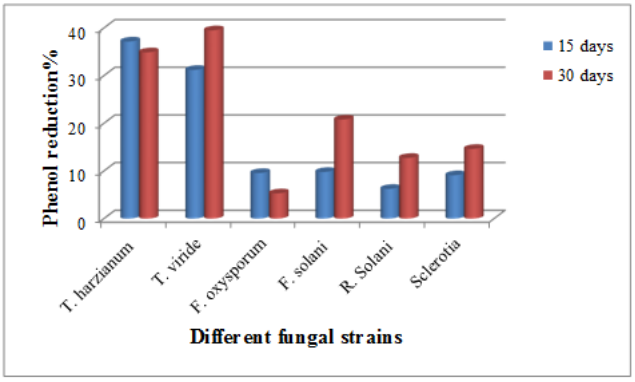

Fig.(1): Total phenol reduction

Field experiments:

The effect of treatments with *T. harzianum* and *T. viride*, which were grown on OMW has influenced the percentage of infection with root-rot disease and also shows the average effective amount of yield at harvested stage.

Treatment soil with *T. harzianum* and *T. viride* grown on OMW. Before sowing bean plants resulted in reducing root rot disease under field conditions. Results in Table (5) showed that all applied soil treatment

with bioagent alone or in formulation with OMW reduced the incidence of root rot caused by *F.solani, R.solani, S.sclerotiorum* and *S.rolfsii* .The highest reduction in disease incidence was observed with treatment by *T. harzianum* and *T. viride* grown on OMW which reduced the pre-emergency by 50 and 37.5%.Also at post-emergency were by 73.3 and 63.5% respectively compared with bioagent alone or control without treatment .

OMW contains phenolic compounds Ramos *et al*.(1995) polysaccharides, lipids ,proteins and a number of monocyclic and polymeric aromatic molecules, which significantly reduced the growth of important soil born plant pathogens as *Fusarium oxysporum , Fusarium sp., lycopersici, Pythium* sp*., S. bsclerotiorum* and *Veticillium dahliae* Ethaliotis *et al*.(1999)

The same trend the most significant increasing in yield with treatment by bioagents which formulated on OMW.63.6 and 48.0 followed by treatment with *T. harzianum* or *T. viride* alone 31.6 and 28.9 respectively comparing with control.

Using agricultural waste as substrates for *T. harzianum* and *T. viride* growth formulation and directly delivery in soil for controlling soil born pathogens on some crops were recorded Vagelas *et al*.(2009)

Table (5): Bean root rot disease incidence in response to applied treatments different bio-agents which formulated on olive mill waste under field conditions.

Treatments	Root rot disease %				yield (Kg/plot	Increased
	Pre-emergence	Reduction	Post-emergence	Reduction		
T. harzianum	12.5c	37.5	28.6c	54.2	29.6b	31.6
***T. harzianum+* OMW**	10.0d	50.0	16.6e	73.4	36.8d	63.6
T. viride	17.5b	12.5	36.4b	41.8	29.0b	28.9
***T.viride* + OMW**	12.5c	37.5	22.8 d	63.5	33.3c	48.0
Control	20.0a	-------	62.5a	-----	22.5a	-----

Figures with the same letters are not significant (P ≤ 0.05)

CONCLUSION

OMW act as a good biofertilizer used for reduction of cellulase and pectinase of soil born fungi when used as carbon source at concentration 100 g/l after incubation from 5-15 days. Olive mill waste affect on reducing linear growth of soil born fungi at concentration 100 g/l. *T. harzianum* and *T. viride* isolated from healthy bean plants inhibit the growth of soil born fungi grown on different concentrations of OMW.

Carbon and nitrogen content and phenol of olive mill waste varied in presence of fungi .Field experiment at El –Saf village Giza on bean plant result in reduction of root rot disease .

ACKNOLWLEDGMENTS

This work was supported by National Research Centre, Dokki, Cairo, Egypt by project no. P100223. The author express her sincere gratitude and gratefully acknowledge for Department of Chemistry of Natural and Microbial Products.

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