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Antifungal potential of extracts produced from decomposed agricultural wastes by *Bosea thiooxidans*

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Abstract

This work aimed to produce antifungal bioactive compounds from agro-wastes using cellulolytic bacteria, and to evaluate their *in vitro* and *in vivo* antifungal activities against faba bean damping off disease caused by *Rhizoctonia solani*. To achieve this purpose, a total of 34 rhizospheric bacterial isolates were screened for their abilities to degrade cellulosic compounds. The bacterial isolate which gave the best cellulolytic activity was identified as *Bosea thiooxidans*, according to its morphological, biochemical characteristics, and according to its 16S rRNA sequencing. Five native plants remains including; Pomegranate peel, chili waste, prickly peel, olive leaves and garlic stalks, were hydrolyzed by the selected cellulolytic *B. thiooxidans* strain UAB7, and their extracts were tested for *in vitro* antifungal potential against a number of fungal phytopathogens mainly; *Rizoctonia solani*, *Fusarium moniliform*; *Pythium* sp. and *Phytophothora* sp. Extracts of the decomposed garlic and olive wastes gave inhibition percentages ranging from 25-100 %, against the tested fungal pathogens. Transmission electron microscopy (TEM) showed cyto-morphological alterations and empty cavities in hyphae of the treated *R. solani*. Moreover, qualitative and quantitative analyses of the phenolic compounds present in the tested extracts were carried out. Results showed that among the tested phenolic compounds, kaempferol and phenanthrene were more abundant in the decomposed garlic and olive extracts, respectively. The fermented garlic and olive extracts were evaluated for their *in vivo* potency to suppress the damping off disease of faba bean caused by *R. solani*. Faba bean plants treated with the fermented garlic and olive extracts demonstrated significantly better disease suppression, compared to the infested control. Furthermore, bands recovered through the electrophoretic patterns of the superoxide dismutase and peroxidase isozymes in the infested control plants, showed wide differentiation in their intensities and forms, compared to the healthy and the treated plants.

Keywords: Solid state fermentation, Cellulose decomposers, Antifungal activity, Bioactive compounds, *Rhizoctonia solani*, Ultrastructure

1. Introduction

In recent times, the bio-conversion of cellulosic resources has become very significant as an environmentally sustainable cost-effective approach to solve the problems of waste disposal. Indeed, such wastes could be regarded as low cost raw materials for bioactive compounds extraction, which can be reused in many industrial applications. From both of the economic and environmental overviews, there is a great interest on the reuse of these wastes, due to their existence in large quantities, and their rich composition of bioactive compounds (Bilal *et al*., 2017; Sarsaiya *et al*., 2019). Biodegradation of agricultural wastes by cellulose decomposers has great importance, because it results in the bioconversion of discarded wastes into useful products.

In Egypt, the agricultural wastes have increased because of the exhaustive agricultural practices to meet the demand of the increasing populations, thus an estimate of 33.4 million tons of agro-wastes are produced every year (Elfeki *et al*., 2017). A previous study conducted by Chandra *et al*., (2012) reported that most of the lingocellulosic wastes are either burned in the field or are allowed to rotting, resulting in an environmental pollution through emission of CO_2 and methane gases.

According to Ignat *et al*., (2011), several methods of solvent extraction (i.e. ultrasound, high hydrostatic pressure extraction and Soxhlet) have been used to extract the bioactive compounds from the waste materials. Previous results of Adom and Liu, (2002) highlighted that the bioactive components including; flavonoids, terpenoids, alkaloids and other phenolic compound, are present in joint forms with other molecules such as sugars, fatty acids or amino acids, so their extraction using organic solvents would be hard.

Enzymatic treatment of lignocellulotic wastes could serve as a useful technique for natural bioactive compounds extraction. Banjo and Kuboye, (2000) added that cellulose degrading microorganisms are of great importance, due to their abilities to utilize lignocellulosic materials and convert them into valuable and effective substances mainly; organic acids and antibiotics. Glycosidic bonds in cellulose, hemicellulose, lignin, and glucans are hydrolyzed by a set of enzyme proteins called cellulases. A previous study conducted by Makoi and Ndakidemi, (2008) reported that there are three major types of cellulases enzymes mainly; endo-1,4-β-glucanase (attacks the cellulose chains randomly), exo-1,4-β-glucanase (eliminates glucose or cellobiose from the end of the cellulose chains), and β-D-glucosidase (converts the disaccharide cellobiose to glucose). Agro-wastes could be used as effective nutrient sources in solid-state fermentation (SSF) processes, for the production of different bioactive compounds. According to Pandey, (2003), the solid plant wastes were used as substrates for growth and metabolic activities of the various microorganisms. Recently, Sarsaiya *et al*., (2019) reported that SSF is the best way to convert inexpensive and useless agro-wastes into numerous valuable compounds.

Phytopathogenic fungi that infect fruit and vegetable crops have induced various plant diseases, which have caused great harm to the crop production and food safety. According to Yoon *et al*., (2013), the synthetic fungicides are commonly used as a measure of controlling plant diseases. Nevertheless, due to their adverse effects on the environment and public health, and also the increase in pathogen resistance with long-term use, the wide use of these chemical substances is greatly limited. Hence, there is an inevitable necessity to search for alternative control agents to overcome these disadvantages. *R. solani* is well recognized as a soil-borne pathogen capable of affecting a wide variety of plant species causing damping off, root rot, and stem rot diseases. Matloob and Juber, (2013) added that *R. solani* is the causative agent of bean disease under both greenhouse and field conditions, resulting in serious economic losses. Currently, faba bean is used in developing countries for human consumption,

as an alternative source of animal protein. It also used as animal feed in many countries.

The objectives of this study were to produce antifungal bioactive compounds from the agro-wastes by solid state fermentation, and to evaluate the *in vitro* and *in vivo* antifungal potential of the decomposed plant extracts against damping off disease of faba bean, caused by *R. solani*.

2. Material and methods

2.1. Isolation of the cellulose decomposing bacteria

Twenty rhizospheric soil samples were collected from Wadi Um Ashtan basin (31° 23` 06`` N and 27° 04` 33`` E), north-western coast, Egypt. For isolation of the cellulolytic bacteria, the collected soil samples were serially diluted, and then 1 ml of the soil suspension was inoculated into tubes of a Filter paper basal salt medium (FPBM) (Mann, 1968), composed of; $NaNO_3$: 2.0 g, KH_2PO_4: 1 g, $MgSO_4$: 0.5 g, KCl: 0.5 g per liter of dist. water, and Whatman filter paper strip (1×7 cm) was added as a sole carbon source. The inoculated tubes were incubated at 30°C± 2 for 10 d. The growing bacterial colonies that were capable of utilizing the filter paper as a sole source of carbon were cultured on carboxymethylcellulose (CMC) agar medium (Hankin and Anagnostakis, 1977), composed of; $NaNO_3$: 2.0 g, KH_2PO_4: 1 g; $MgSO_4$: 0.5 g, KCl: 0.5 g, carboxymethylcellulose (CMC): 5.0 g, peptone: 0.5 g, agar: 20 g, in a liter of dist. H_2O, at a pH of 6.8-7.2 (HiMedia, India).

2.2. Confirmation of cellulose decomposing activity of bacterial isolates

The cellulose decomposing activity of the bacterial isolates was confirmed by spotting the colonies on CMC agar, and then incubation at 28°C for 48 h. After incubation, the plates were either flooded with 0.1% Congo red for 20 min. and then with 1 M NaCl for other 20 min. (Teather and Wood, 1982), or were flooded with Gram's iodine (2.0 g potassium iodide and 1.0 g iodine in 300 ml dist. H_2O) for 5 min. (Kasana *et al*., 2008). Colonies showing discoloration of the Congo-Red or Gram's iodine were considered as positive cellulose decomposers, and were selected for further study.

2.3. Enzyme assay

The bacterial isolates were cultured on FPBM and\or CMC broth medium, and then incubated at 28°C in a shaker incubator (150 rpm) for 72 h. After incubation, the broth cultures were centrifuged at 6000 rpm for 15 min. at 4°C. The Filter paperase (FPase), and endoglucanase or carboxymethyl-cellulase (CMCase) activities were evaluated by determining the quantity of reducing sugars released from filter paper or from amorphous cellulose (CMC), using the di-nitrosalicylic (DNS) method, according to Zhang *et al*., (2009). One unit of enzyme activity is evaluated as the quantity of enzyme that frees 1 μmol reducing sugars (glucose), for each one ml per hour.

2.4. Identification of the most potent isolates

The selected isolate was characterized morphologically and biochemically according to Cappuccino and Sherman, (2005), and the identification was confirmed using a molecular technique. Briefly, the genomic DNA of the selected promising isolate was extracted and amplified by using universal 16S rRNA primers, according to modified method of Ishikawa *et al*., (2000). The amplified PCR product was analyzed in 1% agarose gel, purified by QiaQuick gel extraction kit (Qiagen, Germany), and then sequenced. The phylogenetic analysis of the 16S rRNA sequence was conducted using BLAST program, and aligned with multiple nucleotide sequences using Clustal W program. The phylogenetic tree was built using the neighbor joining (NJ) method of Saitou and Nei, (1987).

2.5. Agricultural wastes and solid state fermentation

Pomegranate peel, chili waste, prickly peel, olive leaves and garlic stalk were used as substrates for antifungal agent's production under solid state fermentation, according to the method of Pan *et al*.,

(2012), with major modification. About 200 g of each fresh agro waste was washed, cut into very small pieces, put into glass jars, and then autoclaved at 121°C for 15 min.

The selected cellulolytic bacteria were grown in nutrient agar medium (0.5 % peptone, 0.5% yeast extract, 1 % glucose, and agar 2 %) at 30°C for 24 h. After incubation, a bacterial suspension was prepared in sterile dist. water, and then adjusted to 2×10^6 cells/ ml. About 100 ml of the bacterial suspension was inoculated aseptically into each glass jar containing 200 g of sterile agro waste, and then incubated at 30°C for 21 d. The jars were shaken every 2 d. After incubation, the fermented wastes were filtrated through cotton wool, and centrifuged for 10 min. at 6000 rpm to get a clear supernatant. The obtained stock supernatant of each agro waste was used individually for evaluating its antifungal potency, and designated as 100 %.

2.6. *In vitro* antifungal potential of the wastes extract's

Isolates of *Rizoctonia solani*, *Fusarium moniliform*, *Pythium* sp. and *Phytophothora* sp. were kindly provided by Plant Pathology Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. These pathogenic fungi were maintained on potato dextrose agar (PDA) medium at 4°C. The antifungal potential of the fermented waste extracts was determined using the poisoned food technique (Nene and Thapliyal, 1993). Each extract was added individually and aseptically into the PDA medium at final concentrations of 25, 50 %, while PDA medium mixed with sterile water served as control. The prepared PDA media were poured into Petri plates and allowed to solidify. A 5 mm agar disc was cut from 5 d old culture of each test fungus using a sterile cork borer, and then placed individually at the center of the seeded Petri plates. Finally, the plates were incubated at 28± 2°C for 5-7 d. All extracts were examined in triplicates, and the assay was repeated twice. After incubation, the antifungal potency of the different extracts was determined by measuring the diameter of mycelial growth, compared with the untreated control plates. The percentage of growth inhibition was calculated using the formula suggested by Vincent, (1947):

Growth inhibition (%) = (Mycelial growth diameter of control - Mycelial growth diameter of treatment) / Mycelial growth diameter of control × 100

2.7. Transmission electron microscopy (TEM) studies

The effect of decomposed garlic and olive extracts on the morphology of *R. solani* hyphae was studied using transmission electron microscope (TEM) (JEOL - JEM 1010), at 70 kV. The samples were prepared according to Bianchi *et al*., (1997). The stained sections of fungal mycelia were observed with TEM, at The Regional Center for Mycology and Biotechnology, Al- Azhar University, Cairo, Egypt.

2.8. Chemical analysis of the fermented garlic and olive extracts

2.8.1. Total phenols and total flavonoids content

The total phenols and total flavonoids content in the extracts of garlic and olive wastes were evaluated by the Folin–Ciocalteu method according to Madrigal-Carballo *et al*., (2009), and the $AlCl_3$ colorimetric method in reference to Chang *et al*., (2002), respectively.

2.8.2. High performance liquid chromatography (HPLC)

The phenolic components of the decomposed garlic and olive extracts were analyzed by HPLC (Ultimate 3000 Thermo). The system Thermo (Ultimate 3000) consisted of: pump, automatic sample injector, and associated DELL-compatible computer supported with Cromelion7 interpretation program. A diode array detector DAD-3000 was used. The Thermo-hypersil reversed phase C18 column 2.5× 30 cm was operated at 25ºC. The UV absorption spectra of the standards as well as the samples were recorded in the range of 230-400 nm. Samples and standards

solutions as well as the mobile phase were degassed, and then filtered through 0.45 μm membrane filter (Millipore). Identification of the compounds was carried out through comparison of their retention's time and UV absorption spectrum, with those of the standards (Biswas *et al*., 2013).

2.9. Greenhouse assay

A pot experiment was conducted to investigate the effect of the two fermented wastes (garlic stalk and olive leaves) on severity of faba bean root rot disease, under greenhouse conditions. Soil was collected from Ismailia governorate, Egypt, having pH 7.27, EC 1.61 dS/m, clay texture and organic matter content of 0.59% (Yaseen *et al*., 2020). The soil was air dried, sieved, and then 10 Kg soil was placed in each pot with five replicates. The mass production of *R. solani* culture was performed on sand sorghum medium according to Scholten *et al*., (2001). Soil infestation was carried out by mixing the fungal inoculum with soil (30 g/ pot) and watered for two weeks to enhance fungal growth. After 2 weeks, 200 ml of waste extract of each of the decomposed garlic stalk and olive leaves, was added to the pots a week before sowing, so that it did not affect the seed germination, and added again after 15 d of sowing. The mineral fertilizer used was NPK 19-19-19. Five healthy seeds of faba bean (cultivar Sakha 1) were sown in each pot. Pots containing infested soil without treatment with the extract served as positive control, whereas pots containing untreated and non-infested soil served as negative control. Pots were distributed in complete randomized design. After 45 days of sowing, faba bean plants were carefully removed; the fresh weight, dry weight and dry matter content of the plants were recorded according to Brouwer, (1962). Moreover, the disease severity of *R. solani* damping off were recorded using the scale of Cárdenas *et al*., (2015) that ranged from 0 to 4, where: 0= healthy roots, 1= 1-25 % of the taproot is infested, 2= 26–50 % of the taproot is infested, 3= 51–75 % of the taproot is infested, and 4= 76-100 % of the taproot is infested. The disease severity data were used to calculate the disease incidence (DI), disease severity index (DSI) and the biological control efficacy (BCE), according to the following formula of Mckinney, (1923):

DI = No. of infected plants/ total no. of plants × 100

DSI = ∑ (disease scale × No. of plants within this scale)/ total no. of plants.

BCE = (disease incidence of control - disease incidence of treatment) / disease incidence of control × 100

2.10. Electrophoretic analysis of the antioxidant enzymes

Variations of the different isozymes were determined using polyacrylamide gel electrophoresis (Native PAGE). The isozymes of Peroxidase (POD) were performed according to Guikema and Sherman, (1981), and the superoxide dismutase (SOD) isozymes were estimated according to Beauchamp and Fridovich, (1971).

2.11. Statistical analysis.

Analysis of variance (ANOVA) was carried out using computer program of Statistics version 9. Treatment means were compared at 0.05 level of significant using Duncan's multiple range tests.

3. Results and Discussion

3.1. Isolation and screening of cellulose decomposing bacteria

A total of 34 bacterial isolates were obtained from the rhizosphere of different plants in different landforms of wadi Um Ashtan basin (Table 1.). These isolates were selected as they could degrade the filter paper in the broth medium. Qualitative screening was done by Congo red and Gram's iodine test, where 17 out of the 34 isolates showed clear zones indicating an extracellular cellulase production (Fig. 1.). The halo zones formed around the colonies are due to the action of cellulases, because the dye remains attached to the regions where β-(1,4)-linked glucose units are still present.

Table 1. Isolation of the cellulolytic bacteria from the rhizosphere soils of different plants

Site No.	Rhizosphere plant soil	Bacterial isolates
1	Olive trees	UAB1, UAB2
2	Fig trees	UAB3
3	Olive and Fig trees	UAB4,UAB5, UAB6, UAB7
4	Cereal crops	UAB8,UAB9, UAB10
5	Fig trees	UAB11, UAB12
6	Natural plants	UAB13
7	Natural plants	UAB14, UAB15, UAB16
8	Natural plants	UAB17, UAB18, UAB19
9	Cereal crops	UAB20, UAB21
10	Cereal crops	UAB22, UAB23, UAB24
11	Cereal crops	UAB25
12	Natural plants	UAB26
13	Cereal crops	UAB10, UAB27
14	Natural plants	UAB28
15	Cereal crops	UAB29, UAB30, UAB31, UAB32
16	Fig trees	UAB33, UAB34

Where; The UAB symbol refers to the first three letters of Um Ashtan, bacteria (isolation area)

(a) **(b)**

Fig. 1. Zones of hydrolysis created by the cellulolytic bacteria on: Congo red (a) and Gram's iodine (b) agar plates

However, the diameter of the clear zone could be an effective measure in the selection of isolates having great cellulase activity (Florencio *et al*., 2012).

The selected seventeen isolates were screened quantitatively for total cellulase (FPase) and endoglucanases (CMCase) production. Data shown in Table (2) demonstrated that among the 17 cellulolytic bacterial isolates an isolate designed as UAB7 exhibited the highest FPase and CMCase enzyme activities, and thus was selected for further study. A study conducted by López-Mondéjar *et al*., (2016) reported that certain limited groups of bacteria have the ability to degrade cellulose and hemicellulose, whereas others could only attack amorphous cellulose.

3.2. Identification of the potent cellulolytic bacteria

The cellulolytic bacterial isolate that exhibited the highest FPase and CMCase enzyme activity designed as UAB7, is Gram negative short rods, rounded, smooth and creamy colonies on Nutrient agar, positive for catalase and indole tests. The 16S rRNA sequence analysis revealed that the closest sequence similarity of the isolate UAB7 is *Bosea thiooxidans*, with 93 % blast identity. The construction of a phylogenetic tree with closely related genera indicated the alignment of UAB7 isolate with *Bosea thiooxidans*, with 83 % bootstrap value (Fig. 2). The 16S rRNA gene nucleotide sequence of *Bosea thiooxidans* UAB7 strain was submitted to NCBI Genbank, and assigned an accession number of MN904936.

3.3. The *in vitro* antifungal efficacy of the decomposed agricultural wastes

In the current study, five native plants were selected and hydrolyzed by the selected cellulolytic *Bosea thiooxidans* strain UAB7. These plants were selected according to some criteria such as; having antimicrobial properties, low commercial value and availability in large quantities. The extracts of the decomposed wastes were evaluated for their *in vitro* antifungal activities. In general, their performance as efficient antifungal agents could be arranged as follows; garlic stalk > olive leaves > pomegranate peel > chili waste, while the extract of decomposed prickly peel did not show any antifungal activity against the tested fungal pathogens. Data also revealed that, the percent of inhibition of fungal growth positively increased with increasing the corresponding concentration of the fermented plant extract. The highest inhibition percentage (100 %) is recorded by the fermented garlic stalk (50 %) against all the tested fungi. This is followed by the fermented olive leaves (50 %) recording; 55.6 %, 36%, 55% and 66.7%, against *R. solani*, *F. moniliform*, *Pythium* sp. and *Phytophothora* sp., respectively (Fig. 3, 4). Several recent studies of Cherkupally *et al*., (2017); Sanaullah *et al*., (2018) reported the antimicrobial activities, and control of several plant diseases by the different plant extracts under both *in vitro* and *in vivo* conditions. A recent study conducted by Nivedha *et al*., (2019) reported that the mycelial growth of *Alternaria* sp. was suppressed by garlic extract recording 100 % inhibition, followed by Datura leaf extract (68.44%), and neem oil (59.88 %). According to Tocmo *et al*., (2015), the significant activity of garlic extract as an antimicrobial agent may be attributed to its high contents of organosulfides, which are known for their broad biological activities, including; antioxidant, antibacterial, and antifungal effects. Similarly, Rocha *et al*., (2019) reported that olive leaves are rich in flavonoids that have efficient inhibitory potency against a variety of microorganisms.

3.4. Detection of alterations in the mycelial morphology of *R. solani*

The TEM was used to detect alterations in the mycelial morphology of *R. solani* treated with extracts of the decomposed garlic and olive wastes.

Table 2. Cellulase activity of the isolated bacteria

Site No.	FPase activity mg glucose/ ml/ h	CMCase activity mg glucose/ ml / h
UAB3	0.011	0.09
UAB4	0.042	0.13
UAB5	0.078	0.22
UAB7	0.122	0.45
UAB12	0.055	0.19
UAB14	0.049	0.16
UAB15	0.07	0.21
UAB16	0.053	0.18
UAB19	0.01	0.09
UAB20	0.044	0.14
UAB21	0.086	0.33
UAB23	0.047	0.16
UAB24	0.053	0.18
UAB27	0.074	0.25
UAB28	0.078	0.26
UAB31	0.081	0.31
UAB32	0.069	0.19

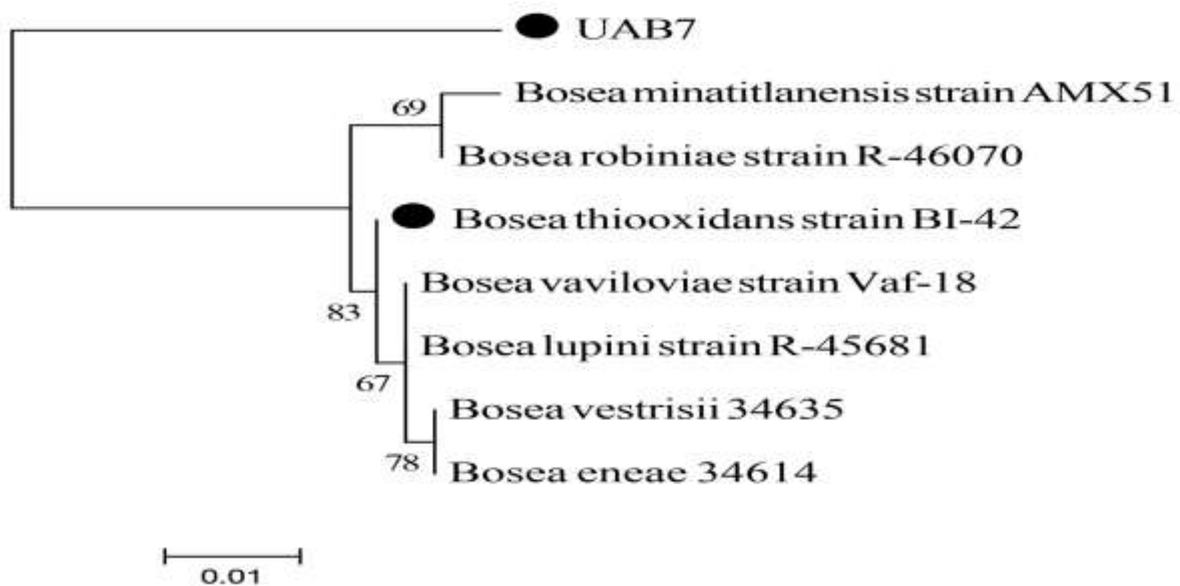

Fig. 2. Phylogenetic tree of partial 16S rRNA sequence of the bacterial isolate UAB7. Numbers at the node represent the percentages of bootstrap values recovered from 1000 replications

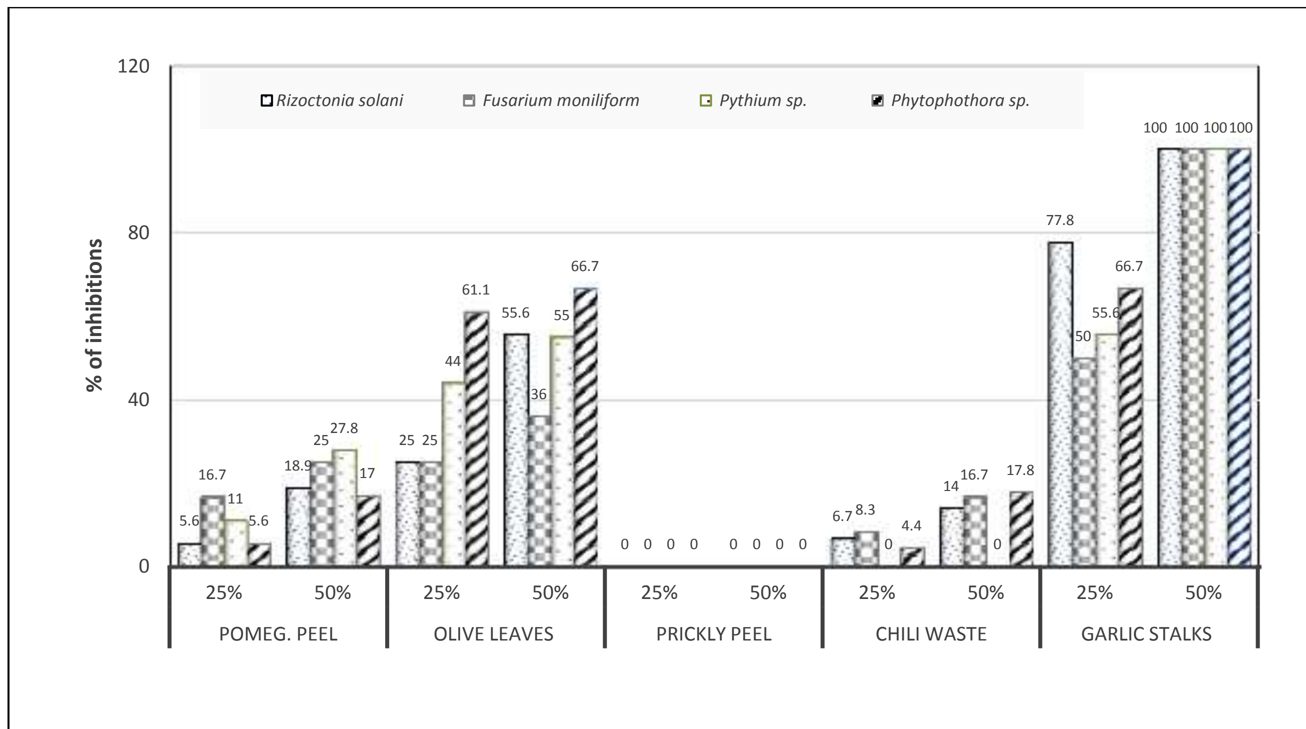

Fig. 3. Percentage of inhibition (%) of mycelial growth of the tested pathogenic fungi induced by the five extracts of the decomposed wastes. Values above the columns represent the percentages of inhibition of each fungus. Results are averages of three replicates.

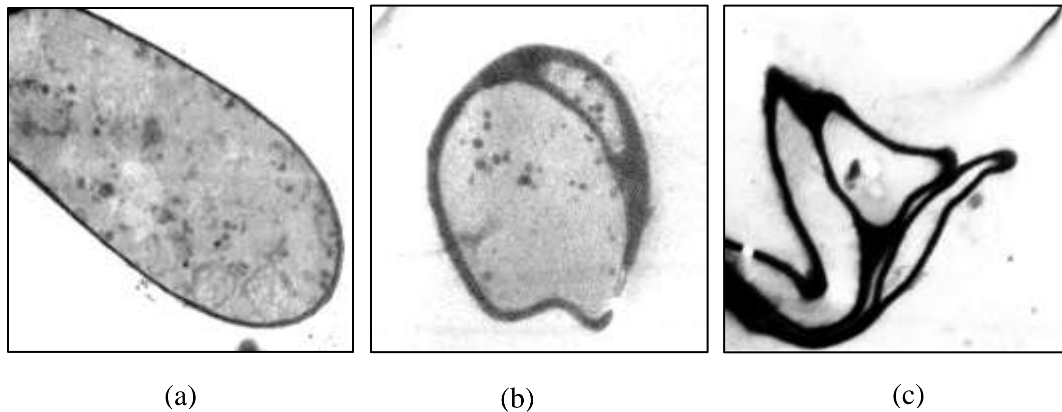

Fig. 4. Transmission electron microscope (TEM) micrographs of *Rhizoctonia solani* hyphae. (a) Control hyphae showing normal cell wall constituents, (b) Hyphae treated with the fermented olive leaves extract, and (c) Hyphae treated with the fermented garlic stalks extract. Scale bar for a–c = 0.5 mm.

From TEM analysis, it is obvious that the cells of untreated fungus have regular cell walls, plasma membranes, nuclei, and cell biomolecules as shown in Fig. (4a). After treatment with the decomposed waste extracts, evidences of morphological damage occur on the cells of *R. solani* (Fig. 4b, c). This damage includes disorganization of cellular organelles, leakage of intracellular components, and increased frequency of the cytoplasmic vacuolation. Furthermore, a marked thickening in the hyphal cell walls, approximately two fold thicker than the control, in addition to changes in the cytoplasmic membrane that are partially separated from the cell wall. Cells of *R. solani* that were treated with decomposed garlic extract showed complete autolysis of most organelles. The occurrence of thickening in the cell wall of treated fungal mycelium means that the systems of the cell wall integrity have been destructed. According to Rella *et al*., (2016), cell membrane alterations of the treated fungal mycelium could be related to modifications that happened in the composition of the lipid microdomains. A recent study of Rashad *et al*., (2018) reported the induction of cell wall thickening in *R. solani* as a response to exposure to Khella extract. According to Arana *et al*., (2009), cell wall thickening inhibits fungal growth by limiting the nutrients exchange between the outside and the inside of the fungal cells. Previous study of Bianchi *et al*., (1997) reported that ultrastructural changes of the cytoplasmic membrane were detected on some phytopathogenic fungi, following treatment with the garlic extract. These alterations resulted in modifications in the activity of some enzymes involved in the formation of the hyphal cell wall.

3.5. Chemical analysis of the fermented garlic and olive extracts

The total phenolics within the decomposed garlic stalks and olive leaves extracts are found to be 0.888 and 2.056 mg/ g respectively, gallic acid equivalents per milliliter of the crude extract. While the total flavonoids are 0.259 and 1.282 mg/ g respectively, rutin equivalents per gram of the crude extract. Phenolic acids represent a wide range of secondary metabolites found in the cells of plants and microbes including fungi. They are regarded to impart active defense responses (Nicholson and Hammerschmidt, 1992). However, Chen *et al*., (2013); Özcan *et al*., (2019) reported that the concentration of these natural compounds could differ depending on the developmental phase, cultivar type, environmental conditions including abiotic and biotic factors, and the method of extraction. The HPLC was used to detect and quantify coumarin, ferulic acid, resorcinol, quercetin, kaempferol, naphthaline, phenanthrene and caffeic acid in extracts of the decomposed garlic and olive wastes. Extract of the decomposed garlic stalks showed higher concentration of kaempferol (12.89 mg/ g of the crude extract), whereas the decomposed olive extract showed higher concentration of phenanthrene (7.04 mg/ g of the crude extract), as shown in Table (3). Similar results were obtained by Dziri *et al*., (2012), who reported that kaempferol and its glycosylated derivatives were more abundant in the methanolic extract of rosy garlic. Moreover, the study of Kuley *et al*., (2017) revealed that the main phenolic compounds recorded in the olive leaves extract were palmitic acid, phenanthrene, and linoleic acid. Recently, Ribes *et al*., (2018) attributed the antifungal activity of the phenolic compounds to several mechanisms including i) cell rupture and release of intracellular proteins and carbohydrates that prevent fungal growth; ii) inhibition of mitochondrial respiration causing reduction of ATP production; and iii) oxidative lesions; and chelation of iron ions.

3.6. Evaluation of the *in vivo* antifungal potential of the decomposed garlic and olive extracts in the greenhouse

In the present assay, an attempt was made to evaluate the ability of the most active antagonistic

Table 3. Concentration of the detected phenolic compounds (mg/ ml of crude extract) in extracts of the decomposed garlic and olive wastes

Phenolic compound	Phenolic compounds (mg/ ml) of the garlic stalks extract	Phenolic compounds (mg/ ml) of the olive leaves extract
Coumarin	ND	0.15
Ferulic acid	2.6	0.07
Resorcinol	0.012	ND
Quercetin	ND	1.47
kaempferol	12.89	ND
Naphthaline	ND	ND
Phenanthrene	ND	7.04
Caffeic acid	0.93	0.54

Where; ND: not detected. Values are means of three replicates of each extract

extracts to suppress the damping off disease of faba bean caused by *R. solani*, under greenhouse conditions. Data presented in Table (4) revealed that application of the decomposed garlic and olive extracts resulted in significantly better disease suppression, compared with the infested control as shown in Fig. (5). The highest disease incidence (DI, 100%) and disease severity index (DSI, 2.49) are observed in the infested faba bean control plants. The DI of faba bean damping off is reduced to 21.5 and 31.4 % in plants treated with extracts of the decomposed garlic and olive wastes, respectively. The fresh and dry weights of plants treated with extracts of the decomposed garlic and olive wastes increased by 33, 18 % for shoot, and 107, 84 % for root, respectively, compared with the infested control plants. Results also revealed that the infestation reduced the dry matter content from 13.51 to 8.66 %, compared to non-infested treatments. The biological control efficacy of the extracts of the decomposed garlic and olive wastes is 78.41 and 68.51 %, respectively, indicating that they were effective in protecting faba bean seedlings from *R. solani* damping-off. Ajayi-Oyetunde and Bradley, (2018) highlighted that *R. solani* is a soil-borne pathogen with a wide host range and worldwide distribution. It attacks the roots and lower stems of plants at early stages of growth (seeds and seedlings). Moreover, this pathogen causes various plant diseases such as; root rot, hypocotyl rot, damping off, and wire stem, resulting in serious losses in the plant yield and quality. Traditionally, synthetic fungicides were used as common means for controlling plant diseases. However, previous study of Aktar *et al*., (2009) documented that these chemical fungicides have great harmful effect on the environment and human health; in addition, the long-term use of these chemicals resulted in development of more resistant pathogens. Results obtained in current study demonstrated that, the bioactive compounds (phenolics, flavonoids) released from fermented garlic and olive wastes could serve as alternative control agents, to overcome the disadvantages of chemical fungicides. Similar results were obtained by Sanaullah *et al*., (2018); Varo *et al*., (2017), who concluded that the application of some aqueous plants extract such as cinnamon, moringa and clove, inhibited the damping off and wilt disease caused by *R. solani* and *Verticillium dahlia*, respectively.

Table 4. Disease-related parameters of Faba bean plant growing in artificially infested soil with *R. solani*, and treated with extracts of the decomposed garlic and olive wastes

Treatment	Disease incidence (DI %)	Disease severity index (DSI)	Biological control efficacy (BCE %)	Fresh weight	Dry weight	Dry matter content %
C	0^{c}	0^{c}	-	7.46^{a}	1.008^{a}	13.51^{a}
IP	100^{a}	2.49^{a}	-	5.92^{b}	0.513^{b}	8.66^{b}
DG	21.5^{b}	0.39^{b}	78.41^{a}	7.87^{a}	1.066^{a}	13.54^{a}
DO	31.4^{b}	0.5^{b}	68.51^{a}	7.00^{a}	0.944^{a}	13.48^{a}
LSD at 0.05	10.6	0.36	23.3	8.7	2.04	1.3

Where; C: Un-infested control plant, IP: Infested positive control plant, DG: Plants treated with the decomposed garlic stalks extract, DO: Plants treated with the decomposed olive leaves extract. LSD: Least significant difference. Values in the same column followed by different superscript letters are significantly different according to Duncan's multiple range test. These values are means of five replicates of the treated plants

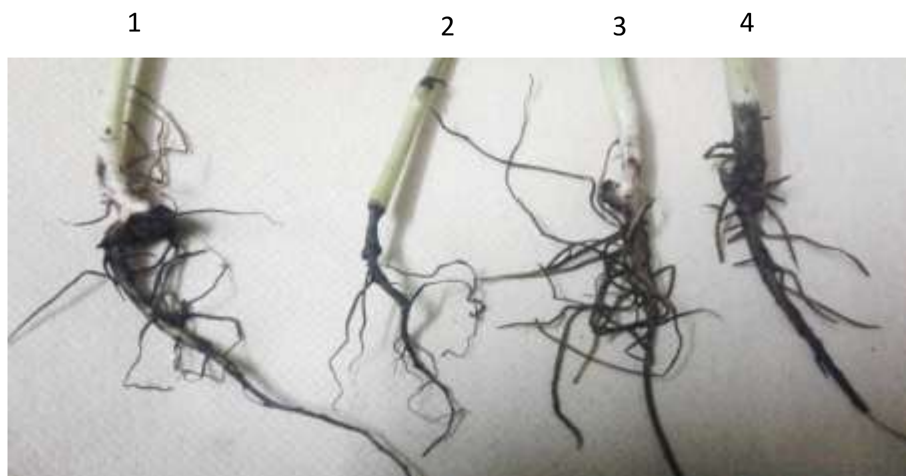

Fig. 5. Infested faba been root, arranged from left to right: (1) Uninfected control plant, (2) Infested positive control plant, (3) Plant treated with the fermented garlic stalks extract, (4) Plant treated with the fermented olive leaves extract

3.7. Electrophoretic analysis of the antioxidant enzymes

The Reactive oxygen species (ROS) are reduced forms of atmospheric oxygen. ROS exist in the form of a single oxygen atom, superoxide radical, hydrogen peroxide or hydroxyl radical, which are highly reactive molecules that cause serious oxidative damage to the plant cell. Sharma and Dubey, (2007) reported that the level of ROS of in the plant cells is regulated by the enzymatic activities of several antioxidants enzymes such as; superoxide dismutase (SOD) and peroxidase (POD). In this study, the isozymes of both SOD and POD were evaluated in the faba bean leaves. Using PAGE, the electrophoretic patterns of SOD isozyme illustrate the appearance of five bands. Bands no. SOD-1, SOD-2 and SOD-5 are present in all treatments (common bands); whereas, bands no. SOD-3 and SOD-4 are unique bands, which appeared only in the untreated infested plants (Tables 5, Fig. 6).

Table 5. Polyacrylamide gel electrophoresis (PAGE) protein pattern of the Superoxide dismutase and the Peroxidase isozymes, detected in leaves of Faba bean plants

Treatments	Dismutase (SOD)		Peroxidase (POD)	
	Band no.	Intensity (KDa)	Band no.	Intensity (KDa)
Un-infested negative control plant	1	112	1	2
	2	283	2	152
	3	ND	3	120
	4	ND	4	21
	5	1188		
Infested positive control plant	1	138	1	114
	2	362	2	224
	3	195	3	148
	4	185	4	21
	5	258		
Plants treated with extract of fermented garlic stalks	1	37	1	15
	2	246	2	98
	3	ND	3	96
	4	ND	4	22
	5	1124		
Plants treated with extract of fermented olive leaves	1	44	1	24
	2	307	2	195
	3	ND	3	124
	4	ND	4	20
	5	1054		

Where; ND: not detected, KDa: Kilo-dalton

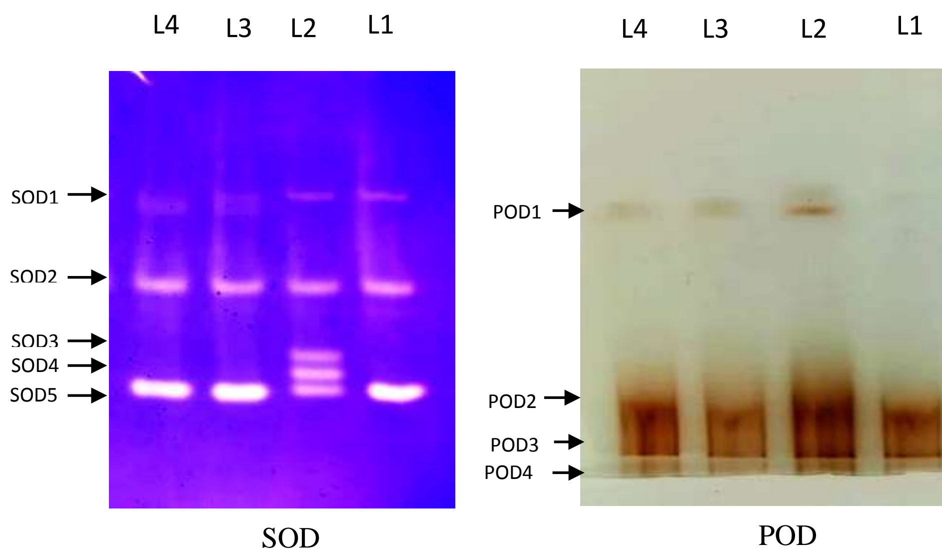

Fig. 6. Electrophoretic patterns of Superoxide dismutase (SOD), and Peroxidase (POD) isozymes of faba bean (cv. Sakha 1) leaves, in response to various treatments with the waste's extracts. Lane 1: Healthy control plants, Lane 2: Infested positive control plants, Lane 3: Infested plants treated with the extract of decomposed garlic stalks, and Lane 4: Infested plants treated with the extract of decomposed olive leaves. The lateral black arrows point to the main SOD (5) and POD (4) separated protein bands.

Infection with *R. solani* led to the formation of new SOD bands (SOD-3 and SOD-4) that are not detected in the healthy or in plants treated with the extracts of the decomposed wastes. Accordingly, the maximum number of bands (five bands) is recorded in the infested control faba bean leaves. Moreover, infection caused an increment in the activities of some SOD isozymes, i.e. the intensity of SOD-1 and SOD-2 bands in the infested control plants is higher than that present in the other treatments.

On the other hand, the isozyme profile of POD from faba bean leaves is composed of four bands; all these bands are common bands that appeared in all the plants under study. It is deduced from the current results that infection with *R. solani* increased POD activity in faba bean leaves, since the intensity of POD-1, POD-2 and POD-3 are higher than those present in the healthy control plants, and in the infested plants treated with extracts of the decomposed wastes. Also, we observed that POD activity in the infested plants treated with extracts of decomposed wastes is similar to its corresponding activity in the healthy control plants, indicating that treatments with the decomposed waste extracts reduced the oxidative stress induced by *R. solani* (Table 5 and Fig. 6).

Similar to the current results, Dodds and Rathjen (2010); Swami *et al*., (2018); Bharathi *et al*., (2019) recorded the subsequent accumulation of the antioxidant enzymes such as SOD and POD, which have a vital role in induction of plant resistance against several biotic and abiotic stresses. Previous study of Sharma and Dubey, (2007) reported that the induction and changes in the isozyme profile are considered to play an important role in the cellular defense against the oxidative stresses. In this respect, several studies have suggested that pathogen infection is strongly correlated with increasing the antioxidant enzymes activity. In this context, a positive correlation between the peroxidase activity

and resistance to barley leaf stripe disease caused by *Drechslera graminea* was reported by Goel *et al*., (2018). In addition, Akladious *et al*., (2019) observed a rapid induction of the peroxidase and polyphenol oxidase activities in faba bean plants infected with *R. solani* AG-4.

Conclusion

It can be concluded that, the agricultural wastes such as garlic and olive wastes could be very useful for the production of valuable products through solid state fermentation, using cellulolytic bacteria. The recorded antifungal potency of the fermented garlic and olive wastes make them proper alternatives to the chemical fungicides, as they are cheaper and eco-friendly bio-compounds for plant disease control. They could be used as promising control agents in management of faba bean damping off disease. Accordingly, the use of extracts from different agro-wastes through solid state fermentation represents an economic alternative to their unmanaged disposal and gives highly enriched extracts with secondary metabolites that have potent antimicrobial activity.

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Ethical approval

Non-applicable.

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