Evaluation of the Pleurotin Efficacy Extracted from *Pleurotus* spp. in Controlling Leaf Spot Disease caused by *Alternaria alternata* on Broad Bean

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Abstract

This study was conducted at the College of Agriculture, Al-Qasim Green University, to evaluate the efficacy of Pleurotin extracted from Pleurotus spp. mushroom in controlling leaf spot disease caused by *Alternaria alternata* on broad bean. The results of this study revealed that treatment with Pleurotin induced systemic resistance, which included both peroxidase and chitinase activities. The highest peroxidase and chitinase activities were 3.188 and 2.725 units/mL for the spanish and local bean varieties, respectively, compared to 0.32 and 0.057 units/mL for the turkish variety in the control treatment for these enzymes, respectively. Furthermore, healthy plants treated with pleurotin showed the highest peroxidase and chitinase activities reached 2.931 and 2.604 units/mL, respectively. The treatment with Pleurotin also stimulated the vegetative growth of all tested broad bean varieties, with the highest plant height recorded in the spanish variety, reaching 108.24 cm/plant, compared to 63.77 cm/plant in the turkish variety infected with *A. alternata*. Moreover, the dry weight of the plants increased to 115.33 g/plant in the healthy plants treated with pleurotin, for the Spanish variety, compared to 45.53 g/plant in the Turkish variety infected with *A. alternata.* The results indicated a reduction in disease severity for all studied broad bean varieties infected with *A. alternata* and treated with pleurotin, reached to 24.08, 21.09, and 15.57% for the turkish, local, and spanish varieties, respectively, compared to 78.86, 74.07, and 67.55% for the same respective varieties in the *A. alternata* control treatment. As a result of the reduced disease severity and stimulated vegetative growth due to the elicitor effect of Pleurotin, this was reflected in the increased plant productivity indices. The highest weight of fresh grains was 221.70 g/plant in the healthy plants treated with pleurotin for the spanish variety, compared to 18.01 g/plant in the turkish variety infected with *A. alternata*.

Keywords: Pleurotin, *Pleurotus* spp., *Alternaria alternata*, leaf spot, Bean plants, Biological control.

Introduction

Broad Bean (*Vicia fabae L.*) is a widely cultivated winter annual crop belonging to the Fabaceae family, which ranks second in importance after the Poaceae family. Faba beans are known for their high protein content, ranging from 30 to 40%, making them valuable for their nutritional value and abundant carbohydrates [10]. The Fabaceae family stands out from other plant families due to its ability to improve soil properties by nitrogen fixation through root nodules in symbiosis with *Rhizobium* spp. bacteria present in the rhizosphere [4]. Due to the importance of this crop, it is susceptible to various fungal, viral, nematode, and parasitic flowering plant diseases. Among the significant fungal diseases affecting faba beans are early blight, late blight, ascochyta blight, root and stem base rot, and rust[7] ، [25] ،[30]. *Alternaria alternata* leaf spot disease is one of the most important and widespread fungal diseases affecting faba beans worldwide, including Iraq. It causes economic losses in this crop and is also a major cause of early blight in many agricultural crops, leading to devastating leaf, flower, and fruit spot lesion [9], [33]. The fungus destroys plant tissues by impairing their photosynthetic ability. Additionally, this

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fungus remains latent in stored products, causing hidden infections that penetrate tissues and remain dormant until favorable conditions for infection occur[24] ,[32]. Methods of controlling this disease include various physical, chemical, and biological approaches. The most common method is the use of chemical fungicides. However, it has been found that these methods have negative effects on the environment, human health, and non-target organisms due to their intensive and improper use, as well as their high cost. Therefore, there is a need to explore safer alternatives, with one of the most prominent being biological control [8].

Biological control agents (BCAs) have been used against leaf spot fungi, where living organisms or their products are utilized as effective biological control factors. These fungi possess several mechanisms that make them effective BCAs, such as rapid growth, competition for nutrients and space, secretion of numerous antibiotics and enzymes, as well as stimulation of systemic resistance in plants and disruption of host cells through parasitism [28]. Induced systmic resistance included some Enzymes such as chitinase, protease, glucanase, peroxidase, and polyphenol oxidase, and proved effective in combating fungal root rot diseases in broad bean plants[18].

Among the cultivated mushrooms with wide commercial scope, Oyster mushroom *(Pleurotus* spp.) is one of the most important after *Agaricus bisporus* in terms of production and consumption [27], [29]. Some species of the *Pleurotus* have shown the ability to produce antibacterial and antifungal agents, used to control of fungal and bacterial diseases. Additionally, certain P*leurotu*s spp. such as *P. ostreatus*, *P. florida*, and *P. sajor caju* have been found to be effective biopesticides against plant pathogens such as nematodes and soil-borne fungi, particularly the pathogenic fungi *F. oxysporum* and *R. solani* [17]. Pleurotin, derived from the Oyster mushroom *Pleurotus* spp., is an antibiotic compound belonging to the naphthoquinone class. It is one of the important secondary metabolites extracted from certain species of Oyster mushrooms such as *Pleurotus griseus*, *Hohenbruehelia geogenius*, and *Hohenbruhelia atrocaerulea*. Pleurotin has been found to be effective against various fungi and bacteria, in laboratory tests, it demonstrated inhibitory activity against fungi *Trichophyton mentagrophytes* and *Candida albicans* [6]. Due to the significance of pleurotin since its discovery, numerous studies have been dedicated to its chemical synthesis, which involves complex and costly stages [23], [31].

For the importance of leaf spot disease and the widespread economic losses it causes, particularly in grain crops such as faba beans, and to exploit the antifungal compounds produced by *Pleurotus* spp., including pleurotin, there is a need for research on its potential use in plant disease management. To the best of our knowledge, no previous study has investigated the application of pleurotin for plant disease control. Therefore, this study, conducted for the first time in our country, aims to evaluate the efficacy of purified pleurotin in combating leaf spot disease, promoting plant growth and induced resistance in three faba bean varieties.

Materials and Methods

The laboratory experiments were conducted at the laboratories of the College of Agriculture, Tikrit University.

The pathogenic fungus *A. alternata*

A highly pathogenic isolate of *A. alternata*, previously identified both morphologically and molecularly (Accession number ON834662.1) [20].was used.

Culture Media

Potato Dextrose Agar (PDA)

The medium was prepared by dissolving 39 grams of PDA powder (Himedia, India) in one liter of distilled water. The medium was then sterilized using an autoclave at 121°C and a

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pressure of 15 pounds per inch^2. After sterilization, The medium was cooled to 40°C, and chloramphenicol was added at 250 mg/liter. The medium was poured into Petri dishes and allowed to solidify.

Extraction of Pleurotin

Pleurotin was extracted from *Pleurotus* spp., and its concentration was determined using High-Performance Liquid Chromatography (HPLC) following the standard method described by [19].

Field Experiment

A randomized complete block design (RCBD) was used for the field experiment in the agricultural season of 2022-2023. The experiment was conducted at the Research Station of the Department of Field Crops, College of Agriculture, Al-Qasim Green University. The experiment included the cultivation of three faba bean varieties (local, Turkish and Spanish). All necessary agricultural operations, including plowing, leveling, and soil sterilization using a 5% commercial formalin solution, were carried out. The fields were covered with plastic sheeting for seven days and then ventilated for further 3 days. The field was divided into three blocks, each containing 18 treatments with equal spacing between them. Each treatment was further divided into 54 experimental units, each measuring 1x1 m^2. Each experimental unit was divided into two rows, with five plants in each row, spaced 60 cm apart between rows and 40 cm apart between plants. One square meter was left as a control treatment. The field was fully irrigated before planting, and the soil was fertilized with superphosphate, urea, and potassium sulfate according to the recommended rates. After sterilization, faba bean seeds were surface-sterilized with a 3% sodium hypochlorite solution at a rate of three seeds per hole. After three weeks of faba bean planting, the fungus suspension of *A. alternata* was applied at 10^8 cfu/ml using a manual sprayer, with each leaf receiving 12 ml of the fungal suspension. The plants were covered with polyethylene bags during the first three days after fungal application to ensure humidity. Control treatments without fungal application were included for comparison. 10 ml/seedlings of Pleurotin at 0.2% and 10 ml/seedlings of the fungicide Othilotop at 0.1% were sprayed, while for the treatment of the (fungicide + Pleurotin) according the previous study [20]. 10 ml/seedling was sprayed with a mixture of both materials (1:1).

Estimation of Pathogenesis-Related Proteins (PRPs)

Preparation of enzyme extract

The enzyme extract was prepared according to the method described by [18].One gram of roots was thoroughly washed with running water, then washed with distilled water. The washed roots were dried with filter paper and cut into 1 cm lengths. They were crushed using a porcelain mortar within an ice bath. To the crushed roots, 10 mL of a phosphate buffer solution with a pH of 6 was added. The mixture was filtered with filter paper and centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant, which represents the enzyme extract, was collected in a new test tube.

Estimation of Peroxidase activity

The peroxidase activity was estimated using the method described by [11] After 15 days of treatment with *A. alternata* ,The enzyme activity was determined by measuring the absorbance at 470 nm wavelength of a reaction mixture containing 2.5 mL of (guiacol 0.025% + hydrogen peroxide 0.35%) solution and 0.1 mL of the enzyme extract for each treatment. The enzyme unit was defined as the change in absorbance per minute by 0.01.

Estimation of Chitinase activity

The reaction mixture for estimating chitinase activity was prepared by adding 0.5 mL of the enzyme extract to 0.5 mL of chitin solution (1%) for each treatment. The mixture was then incubated in a water bath at 37°C for two hours. Afterward, the samples were

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centrifuged at 2000 rpm for two minutes. Then, 1 mL of the supernatant was mixed with 1 mL of dinitrosalicylic acid (DNS) solution. The samples were further heated in a water bath at 100°C for five minutes and then cooled to room temperature. The absorbance was measured at 540 nm using a spectrophotometer. The enzyme unit (unit/mL) was defined as the amount of enzyme required to release 1 micromole of the substrate (chitin) per minute per milliliter of the enzyme [34].

Vegetative growth markers

Estimation of plant height

The plant height was estimated by randomly selecting three plants from each treatment and each replicate. The height of the shoot system was measured using a measuring tape .

Estimation of Dry Shoot system weight

The vegetative parts were washed, then, dried in an electric oven at 60°C until a constant weight was achieved. The dry shoot weight (grams) was determined using a sensitive balance.

Estimation of infection severity by *A. alternata*

The severity of infection was assessed for all treatments by evaluating disease symptoms on a five-point scale: 0 = no lesions, 1 = 1-3 lesions, 2 = 4-6 lesions, 3 = 7-9 lesions, 4 = more than 9 lesions. Disease severity was calculated using the equation provided by [26],) as cited in [5].

Disease Severity (%) = (Sum of (Lesion Grade × Number of leaves)) / (Highest Grade × Total Number of leaves) × 100.

Faba bean productivity

Productivity was estimated as wet grains weight (g plant-1)

Statistical Analysis

The data were analyzed using analysis of variance (ANOVA) in the SPSS software, following a randomized complete block design (RCBD) experimental design. Mean comparisons were performed using the Least Significant Difference (LSD) test at a significance level of 0.05 [3].

Results

Effect of pleurotin on Peroxidase activity of Three faba bean varieties under *A. alternata* infection conditions

The results presented in Table (1) showed the effect of pleurotin on peroxidase activity of three faba bean varieties. The highest peroxidase activity was observed in the infected Spanish variety (with *A. alternate*) treated with pleurotin reaching 3.188 unit/mL, followed by infected local variety (with *A. alternate*) treated with pleurotin, with a peroxidase activity of 3.103 unit/mL compared with the lowest peroxidase activity was recorded in the control treatment of the Turkish variety reaching 0.325 unit/mL.

Table (1): Effect of Pleurotin on peroxidase activity (unit/mL) of three faba bean varieties under *A. alternata* infection conditions

Treatments	Faba bean varieties			Average of treatments
	Turkish	Spanish	Local	
Control (Healthy plants)	0.325	0.498	0.491	0.44
Alternaria alternata (A. al.)	2.116	2.735	2.659	2.50
A. al. + Pleurotin (Pl)	2.555	3.188	3.103	2.95
Healthy plants +Pl	2.304	2.931	2.854	2.69
A.al + Fungicide Othilotop (Oth)	1.992	2.624	2.548	2.39
A.al + (Pl:Oth, 1:1)	2.267	2.825	2.749	2.61
Average of varieties	1.93	2.47	2.40	
LSD, 0.05	Treatments= 0.11 ,Variety= 0.08, Treatments×variety=0.17			

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Effect of pleurotin on chitinase activity of Three faba bean varieties under *A. alternata* infection conditions

Table (2) illustrated the highest chitinase activity was observed in the infected local faba bean variety (with *A. alternate*) treated with pleurotin reaching 2.725 unit/mL. This was followed by infected Spanish variety treated with pleurotin reaching 2.688 unit/mL. Healthy plants of the local variety treated with pleurotin exhibited a chitinase activity of 2.604 unit/mL, while the Spanish variety in the same treatment showed a chitinase activity of 2.567 unit/mL. The lowest chitinase activity was 0.057 unit/mL in the control of the Turkish variety.

Table (2): Effect of pleurotin on chitinase Aactivity of Three faba bean varieties under *A. alternata* infection conditions

Treatments	Faba bean varieties			Average of treatments
	Turkish	Spanish	Local	
Control (Healthy plants)	0.057	0.091	0.095	0.08
Alternaria alternata (A. al.)	2.026	2.285	2.322	2.21
A. al. + Pleurotin (Pl)	2.329	2.688	2.725	2.58
Healthy plants +Pl	2.108	2.567	2.604	2.43
A.al + Fungicide Othilotop (Oth)	1.616	1.975	2.012	1.87
A.al + (Pl:Oth, 1:1)	2.102	2.479	2.516	2.37
Average of varieties	1.71	2.01	2.05	
LSD, 0.05	Treatments= 0.11 ,Variety= 0.05, Treatments×variety=0.13			

Effect of pleurotin on plant height (cm /plant) of three faba bean varieties under *A. alternata* infection conditions

The results presented in Table (3) showed the highest plant height was recorded in the healthy spanish, local and turkish variety treated with pleurotin, reaching 108.24 , 96.06 and 88.15 cm/plant, respectively. Under the *A. alternata* infection, these variety treated with (pleurotin+ othilotop) gave the highest height reaching 99.58, 87.76 and 79.65 cm/plant. The results also showed the lowest chitinase activity was 63.77 in the infected turkish variety.

Table (3): Effect of pleurotin on plant height (cm /plant) of Three faba bean varieties under *A. alternata* infection conditions

Treatments	Faba bean varieties			Average of treatments
	Turkish	Spanish	Local	
Control (Healthy plants)	82.44	102.34	90.54	91.77
Alternaria alternata (A. al.)	63.77	86.66	71.68	74.04
A. al. + Pleurotin (Pl)	77.78	97.68	85.88	87.11
Healthy plants +Pl	88.15	108.24	96.06	97.48
A.al + Fungicide Othilotop (Oth)	74.72	94.62	82.82	84.05
A.al + (Pl:Oth, 1:1)	79.65	99.58	87.76	88.10
Average of varieties	77.75	98.19	85.79	
LSD, 0.05	Treatments=3.17, Variety=5.7, Treatments×variety=7.05			

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Effect of pleurotin on dry vegetative weight(g/plant)of three faba bean varieties under *A. alternata* infection conditions

The effect of pleurotin on the dry vegetative weight of studied faba bean was illustrated in the table (4). It can be observed that the healthy spanish variety treated with pleurotin gave the highest dry vegetative weight, reaching 115.33 g/plant. The highest plant height, under the *A. alternata* infection, was 111.06 in the spanish variety treated with (pleurotin+ othilotop) , compared with lowest plant height (45.53 g/plant) in the infected Turkish faba bean variety.

Table (4): Effect of pleurotin on Dry Biomass Weight (g/plant) of Three Lentil Varieties under *Alternaria alternata* Infection Conditions

Treatments	Faba bean varieties			Average of treatments
	Turkish	Spanish	Local	
Control (Healthy plants)	85.24	111.95	94.47	97.22
Alternaria alternata (A. al.)	45.53	60.24	52.78	52.85
A. al. + Pleurotin (Pl)	81.65	109.36	91.89	94.3
Healthy plants +Pl	88.62	115.33	97.87	100.61
A.al + Fungicide Othilotop (Oth)	78.04	107.71	90.25	92
A.al + (Pl:Oth, 1:1)	83.44	111.06	93.69	96.06
Average of varieties	77.09	102.61	86.83	
LSD, 0.05	Treatments=3.08, Variety=4.82, Treatments×variety=6.31			

Effect of pleurotin on disease severity of three faba bean varieties infected with *A. alternata*

The results presented in Table (5) showed the disease severity increased significantly in the treatment with *A. alternata* infection for all studied varieties, reaching 78.86, 74.07, and 67.55% for the turkish, local, and spanish varieties, respectively, while the disease severity increased significantly in these infected varieties when treated with (pleurotin+ othilotop) reaching 13.77, 19.29 and 23.08%, respectively.

Table (5): Effect of pleurotin on disease severity of three faba bean varieties infected with *A. alternata*

Treatments	Faba bean varieties			Average of treatments
	Turkish	Spanish	Local	
Control (Healthy plants)	0	0	0	0
Alternaria alternata (A. al.)	78.86	67.55	74.07	73.49
A. al. + Pleurotin (Pl)	24.08	15.57	21.09	20.25
Healthy plants +Pl	0	0	0	0
A.al + Fungicide Othilotop (Oth)	24.87	16.43	23.95	21.75
A.al + (Pl:Oth, 1:1)	23.08	13.77	19.29	18.71
Average of varieties	25.15	18.89	23.07	
LSD, 0.05	Treatments=2.07, Variety=2.14, Treatments×variety=3.15			

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Effect of pleurotin on wet grain weight of three faba bean varieties under *A. alternata* infection conditions

The results presented in Table (6) elucidate the effect of pleurotin on the wet grain weight in the faba bean plants. The highest wet grain weight was recorded in the healthy plants of spanish, local and Turkish varieties treated with pleurotin reaching 221.7, 208.33 and 186.49 g/plant, respectively. Under the *A. alternata* infection, these variety when treated with (pleurotin+ othilotop) gave the highest wet grain weight reaching 218.32, 203.95 and 183.11 g/plant, compared with the lowest wet grain weight (23.44, 20.65 and 18.01 g/plant) in the infected these varieties, respectively.

Table (6): Effect of pleurotin on wet grain weight of three faba bean varieties under *A. alternata* infection conditions

Treatments	Faba bean varieties			Average of treatments
	Turkish	Spanish	Local	
Control (Healthy plants)	185.15	219.36	205.99	203.5
Alternaria alternata (A. al.)	18.01	23.44	20.65	20.7
A. al. + Pleurotin (Pl)	180.03	215.21	200.84	198.69
Healthy plants +Pl	186.49	221.70	208.33	205.51
A.al + Fungicide Othilotop (Oth)	179.11	213.32	200.09	197.51
A.al + (Pl:Oth, 1:1)	183.11	218.32	203.95	201.79
Average of varieties	155.32	185.23	173.31	
LSD, 0.05	Treatments=2.08, Variety=3.13, Treatments×variety=4.07			

Discussion

The results indicate an increase in the activity of peroxidase and chitinase in the infected faba bean varieties with *A. alternata* compared with control, this may be due to each pathogenic fungus acts as an elicitor, stimulating the production of defense factors in plants. Furthermore, there was an increase in the activity of peroxidase and chitinase in healthy plants treated with pleurotin, this can be attributed to the fact that pleurotin acts as an elicitor, inducing plant resistance. In response to infection, plants exhibit various resistance factors, including pathogenesis-related proteins (PRPs), which are natural defense responses to plant diseases. The high activity of these enzymes is associated with a high level of plant resistance. These results are agree with other similar studies [12]،[13] . ،[2]،Peroxidase interacts with hydrogen peroxide to break down pathogenic enzymes, including chitinase, responsible for breaking down the chitin in the cell wall of the host plant. This inhibits the penetration of the pathogenic agent into the plant tissue. Peroxidase also interacts with certain cell wall proteins to form cross-links and multiple compounds, thereby increasing cell wall strength [22]، [35] . Chitinase is one of the key proteins associated with pathogenesis and is involved in the degradation of fungal hyphal walls. It is considered one of the important components of biological control agents that break down these essential compounds of the fungal hyphae, leading to weakening and death of the pathogenic fungus [1] ، [15]، [16]. The chitinase breaks the linkage between carbon 1 and carbon 4 in the N-acetylglucosamine molecule, which represents the structural basis of chitin in the fungal hyphal wall [21]. In contrast, healthy plants showed low levels of these enzymes as they were free from elicitors. These results are consistent with the study of[20], which proved that pleurotin has an inhibitory role against pathogenic fungi , *A. alternata* and *R. solani* in the laboratory, when the inhibition zone reached 86.3 and 92.7 mm , respectively.

The stimulatory effect of the pleurotin elicitor, as demonstrated by the reduced disease

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severity, it was reflected in the indicators of vegetative growth and productivity and noted the superiority of the indicators of vegetative growth such as plant height and vegetative dry weight, possibly due to the inhibitory effect of pleurotin on the pathogen, leading to higher values of these indicators in healthy plants treated with the pleurotin compared to the pathogenic fungus treatment. This is because the pathogenic fungus possesses degradative enzymes that break down plant tissues and produce fungal toxins that inhibit many vital processes in plants. Therefore, when resistance is induced and the disease is suppressed, there is an improvement in plant growth parameters and an increase in vegetative growth values. This is further manifested in the productivity traits, such as wet grain weight. This serves as another evidence of the inhibitory effect of pleurotin on the disease and its ability to stimulate plant productivity. The successful elicitation of plant resistance using environmentally safe, cost-effective, and easy-to-use factors leads to disease resistance and avoids the economic losses associated with it, while avoiding the use of expensive chemical pesticides that have negative effects on humans and the environment [14].

Through the results, in the field level, pleurotin showed compatibility with fungicides Othilotop, , this explains the existence of a synergistic effect between pleurotin and this fungicides, thus, which leads to increase in the activities of each.The high effectiveness of the inhibitory effect, in the field, of the fungicides Othilotop with pleurotin in this study is consistent with the role of their inhibitory effect against the pathogenic fungi, *R. solani* and *A. alternata* in the laboratory, in which, Othilotop with pleurotin lead to a complete inhibition of 100% [20].

Conclusion

Pleurotin is a natural substance that was extracted from oyster mushrooms *Pleurotus* spp. as an antifungal agent that inhibited the pathogenic fungus *A. alternata* and reduced the severity of leaf spot disease of bean varieties. As well as the potential roles of the pleurotin in inducing plant systemic resistance and encouraging vegetative and productive growth.

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