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Influences of some biotic and abiotic factors on protein production and as inducers of *Fusarium* wilt disease resistance in lupine (*Lupinus albus* L.)

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Two bacterial species; *Pseudomonas fluorescens* and *Pseudomonas putida* and three different chemical compounds; copper sulphate (CuSO₄), Indole butyric acid (IBA) and potassium chloride (KCl) were tested for their ability to induce resistance in lupine plants against wilt disease caused by *Fusarium oxysporum* f. sp. *lupini*. Treatment of seeds with the selected bacterial species and chemical compounds significantly reduced wilt disease incidence of *Fusarium oxysporum* f. sp. in *Lupine* under greenhouse conditions. Potassium chloride and *Pseudomonas fluorescens* were most effective. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS- PAGE) analysis of lupine seedlings revealed that seeds treated with biotic and abiotic inducers resulted in a rapid induction of different novel Pathogenesis-related protein (PR) in shoot and root of lupine seedlings upon infection with the pathogen. These new proteins were not detected in untreated healthy or infected controls. This study aimed to use bacterial species and chemical compounds to decrease *Fusarium* wilt disease.

Key words: *Pseudomonas fluorescens*, *Pseudomonas putida*, copper sulphate, Indole butyric acid, potassium chloride.

INTRODUCTION

White lupine (*Lupinus albus*) is a legume field crop with high protein content. It has many benefits for human and animal nutrition. Green plants are useful as green-manuring because of the high nitrogenous content (Chiej, 1984). Soil borne fungal diseases are among the most important factors limiting the yield production of grain legumes in many countries, resulting in serious economic losses. Pathogens such as *Fusarium oxysporum* can have significant negative effects on the growth of lupine plants (Zian, 2005). White lupine is attacked by many different soil borne pathogens. *Fusarium* wilts disease caused by *Fusarium oxysporum* f. sp. *Lupini* is one of the most destructive diseases causing severe losses in

seed yield and quality (Zian, 2005).

The disease can be controlled by certain fungicides (El-Awadi et al., 1997). However, disease management using fungicides is not economically practical or environmentally safe. Therefore, the induction of disease resistance in plant may be an alternative approach to diminish the hazardous side effects of chemical fungicides.

Both biotic and abiotic inducers are known to eliciting a variety of defense reactions in host plants in response to microbial infection, including the accumulation of pathogenesis related PR- proteins (Nafie and Mazen, 2008). SDS- PAGE analysis revealed rapid induction of novel PR-proteins in root and shoot of the induced seedlings

in response to pathogen infection. Such proteins are not detected in untreated healthy or infected control.

PR proteins are inducible proteins implicated in active defense against disease and they could play a key role in restricting pathogen development and spread in plant through their antifungal activity (Van Loon et al., 2006).

The objective of this investigation was to evaluate the ability of some biotic and abiotic compounds to induce resistance in lupine plants against *Fusarium* wilt disease under greenhouse and the relationship between resistance and protein pattern changes in induced plants.

MATERIALS AND METHODS

Source of fungal pathogen

The fungal pathogen (*F. oxysporum* f. sp. *lupini*) was isolated from diseased lupine plants collected from Ismailia governorate and identified according to Barnett and Hunter (1986). The isolate proved its pathogenic capability in the pathogenicity test.

Preparation of fungal inoculum

Isolate of *F. oxysporum* f. sp. *lupini* was grown in Petri dishes containing sterilized (PDA) medium at 25°C for 12 days. Then conidia were harvested in sterilized distilled water using sterile brush and filtered through four layers of cheesecloth to separate the mycelium from the culture. Spore suspension was adjusted to 1×10^6 spore/ml using a hemocytometer (Sharma et al., 2005).

Preparation of *Pseudomonas* strains

Strains of *P. fluorescens* and *P. putida* were provided from Department of Microbiology, Soil, Water and Environment Res. Inst., ARC, Giza. *P. fluorescens* and *P. putida* were cultured individually in nutrient broth medium in 250 ml flasks and incubated at 28°C for 48 h then a cell suspension of each strain was adjusted to provide 10^9 cfu/ml.

Seed treatment

Surface sterilized seeds of lupine (cv. Giza 1) were soaked in twice their volume in cell suspension of the biotic inducers [*P. fluorescens* and *P. putida* (10^9 cfu/ml)] individually or solution of the abiotic inducers including IBA (2.0 mM), KCl (5 mM) and CuSO_4 (0.5 mM)] individually for 18 h under laboratory temperature. The seeds were then allowed to dry in air. Untreated seeds were used as controls. Seeds treated with fungicide (Rizolex- T at the rate of 3g/Kg seeds) were used as comparison treatment.

Greenhouse experiment

Pots (35 cm diameter and 25 cm in depth) containing sterilized soil were sown with lupine seeds pretreated with *P. fluorescens*, *P. putida*, IBA, KCl, CuSO_4 and fungicide as well as untreated seeds (serve as control plant). This experiment was conducted under natural conditions (day length 12- 14 h, temperature 25-27°C and humidity 70%). Twenty days after sowing, potted soil was infested with spore suspension of *F. oxysporum* f. sp. *lupini* 1×10^6 spore/ml (100 ml/ pot). Pots containing un-infested soil sown with untreated seeds served as healthy control for further studies. Three replicates

were used for each treatment and seven seeds were sown in each pot. The growing seedlings were examined periodically and disease incidence was recorded 21 days after inoculation with the pathogen (Sharma et al., 2005).

Disease assessment

Disease incidence (percent wilting) was recorded after 21 days of inoculation according to the following formula:

$$\text{Disease incidence \%} = \frac{\text{Number of wilted plants}}{\text{Number of total plants}} \times 100$$

Protein electrophoresis

Protein extraction

Samples of lupine seedlings (shoot and root) pretreated with biotic or abiotic inducers were collected after 2 and 5 days of inoculation with the pathogen. Also, untreated healthy or infected seedlings were used as the control. For SDS-PAGE, 0.5 g of shoots and roots of lupine seedlings were ground to powder under liquid nitrogen and melted in ice-cold extraction buffer (0.5 M Tris-HCl, pH 6.5, 1% SDS, 5% 2-mercaptoethanol, 20% sucrose, 0.4% bromophenol blue), followed by centrifugation at 10,000 g at 4°C for 15 min. Extracts were stored at -20°C until used.

One-dimensional (SDS-PAGE)

Proteins (50 µg of each sample) were separated by SDS-PAGE according to the method of Laemmli (1970). The separation was performed with 12.5% separating gel and 5% stacking gel using protein vertical electrophoresis unit. Electrophoresis was started at 80 V constant current until the tracking dye entered the separating gel and continued at 125 V until the tracking dye reached the end of the gel. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Sigma) in 50% (v/v) methanol and 10% (v/v) acetic acid for 2 h and destained with 50% (v/v) methanol and 10% (v/v) acetic acid until the background was clear. Relative molecular weight of each protein was determined using a standard protein marker. The gel was scanned using Gel Pro-Analyzer.

Statistical analysis

Analysis of variance was carried out using MSTAT-C program version 2.10 (1991). Least significant difference (LSD) was employed to test for significant difference between treatments at $P \leq 0.05$ (Gomez and Gomez, 1984).

RESULTS

Under greenhouse conditions

Lupine seeds pretreated with fungicide, biotic or abiotic agents significantly reduced wilt disease incidence compared with untreated infected control (Table 1). Nevertheless, efficiency of the tested agents varied.

Fungicide treatment ranked as the most effective one (84.62% reduction in disease incidence), followed by *P. fluorescens*, KCl, *P. putida* and CuSO_4 (76.93, 76.93, 46.15, 38.46% reduction respectively), while IBA gave the lowest protection against the disease (23.06%).

Table 1. Effect of seed treatment with biotic and abiotic substances on *Fusarium* wilt disease incidence in lupine plants 21 days after inoculation under greenhouse conditions.

Treatment	Disease incidence %	Reduction over control %
Indole butyric acid (2.0 mM)	47.62±2.02a	23.06
Copper sulphate (0.5 mM)	38.09±1.33a	38.46
Potassium chloride (5 mM)	14.28±0.99a	76.93
<i>P. fluorescens</i> 10 ⁹ cfu/ml	14.28±0.87a	76.93
<i>P. putida</i> 10 ⁹ cfu/ml	33.33±1.14a	46.15
Fungicide (Rhizolex-T) 3g/Kg	9.52±0.54a	84.62
Control	61.90±2.11	-
L.S.D. at 5%	16.33	-

Means ± SD (n=10) of measurements on each ten plants. Means are significantly different, P≤0.05, according to least significant difference (LSD) test.

Protein electrophoresis

Seed treatment with biotic and abiotic agents induced new pathogenesis related proteins with various molecular weights in shoots and roots of lupine seedlings upon infection with the pathogen (Tables 2, 3; Figures 1 and 2). These new proteins were not detected in untreated healthy or infected control.

Two days after inoculation with the pathogen (Table 2 and Figure 1), new proteins with different molecular weights (58, 51 and 38 KDa) were expressed only in the shoot of lupine seedling pretreated with biotic or abiotic inducers. At the same time, new proteins with 138, 110, 88, 69, 44 and 24 KDa were restricted only in the root of seedlings pretreated with biotic or abiotic inducers.

Five days after challenge with the pathogen, another group of novel proteins were detected only in the shoot and root of seedlings induced by biotic or abiotic inducers (Table 3 and Figure 2). Proteins of 64, 43, 26, and 15 KDa were detected in the shoot of induced seedlings, whereas, proteins of 88, 36 and 32 KDa were detected in the root of induced seedlings.

DISCUSSION

Resistance inducers provide an additional option to manage plant diseases while maintaining sustainable production. The results obtained in this investigation indicated that application of the biotic and abiotic inducers as seed treatment significantly reduced wilt disease incidence under greenhouse conditions compared with untreated controls. A comparative evaluation showed that the tested inducers varied in their effectiveness against lupine wilt disease. *P. fluorescens* (biotic inducer) and KCl (abiotic inducer) were the most effective treatments.

Strains of *Pseudomonas* spp. have been reported to induce resistance against *Fusarium* wilt disease on several plant species. Some *Pseudomonas fluorescens* strains present biocontrol properties, protecting the roots of some plant species against plant pathogens. These bacteria induce systemic resistance in the host plant, so it

can better resist attack by a true pathogen. The bacteria outcompete other (pathogenic) soil microbes, example, by siderophores, giving a competitive advantage at scavenging for iron. The bacteria produce compounds antagonistic to other soil microbes, such as phenazine - type antibiotics or hydrogen cyanide (Faraji et al., 2013). In addition, Saikia et al. (2003) used different isolates of *P. fluorescens* as seed treatment to induce resistance against *F. oxysporum* f. sp. *ciceri* in chickpea. They found that the isolates significantly reduced wilt disease incidence by 26-50% compared to the control. In addition, *P. fluorescens* and *P. putida* successfully reduced *Fusarium* wilt disease incidence in induced tomato seedlings (Maina et al., 2008). Similarly, induced resistance by abiotic inducers is another promising approach to decrease *Fusarium* wilt diseases. Application of potassium chloride (KCl) as seed treatment effectively suppressed *Fusarium* wilt disease in sesame plants under greenhouse and field conditions (Shalaby, 1997). Cotton seedlings induced by methyl jasmonate showed the lowest wilt disease incidence caused by *F. oxysporum* f. sp. *Vasinfestum* (Couto et al., 2009). Also, Sarwar et al. (2010) showed that chickpea seed treatment with bion, salicylic acid and potassium phosphate reduced *Fusarium* wilt disease by 63, 40 and 30% respectively.

Plant growth promotion is another beneficial effect of *Pseudomonas* spp. The mechanisms by which these bacteria affect plants involve the production of phytohormones (Indole acetic acid, gibberellin and cytokinin and other associated activities which include phosphate solubilization in soil resulting in stimulation of sunflower plant growth (Bhatia et al., 2005). Our results were consistent with those reported by Govindappa et al. (2010) who found that application of *P. fluorescens* as seed treatment enhanced safflower plant growth and increased seed yield of induced plants. As for abiotic inducers, Shalaby (1997) indicated that seed treatment with KCl significantly increased seed yield of sesame plants. Similarly, Abdel-Monaim (2008) showed that application of abiotic inducers as seed treatment was accompanied with pronounced increase of crop parameters and seed yield

Table 2. The amounts (%) of soluble proteins extracted by SDS PAGE from shoot and root of lupine seedlings from seeds treated with biotic and abiotic inducers two days after challenge with *Fusarium oxysporum* f. sp. *lupini*.

Marker (Mol. Wt.)	Shoot							Root						
	Untreated healthy	Untreated infected	IBA	CuSO ₄	KCl	<i>P.</i> <i>fluorescens</i>	<i>P.</i> <i>putida</i>	Untreated healthy	Untreated infected	IBA	CuSO ₄	KCl	<i>P.</i> <i>fluorescens</i>	<i>P.</i> <i>putida</i>
166	5.23	2.78					4.39	2.6						
147	2.77	3.01	1.6	2.48	2.73	3.03		1.99	1.8	2.4			2.69	
138	1.52	1.41			1.88	1.93	1.8				2.62			2.54
131	4.65	5.04	3.85	2.08	3.88	6.07	4.81	4.33	2.77	3.04		2.21		
110		2.37			2.52	1.84						2.14		
103	2.68	2.12	2.48	2.97	1.92	1.64	2.02	4.25	4.97	6.47	4.68		3.91	4.25
88	1.56	2.74	3.83	1.84	2.26	2.65	2.84					4.12	2.17	2.78
78	2	2.3			1.78		1.15							
69	2.65		2.53	10.5	1.72	2.7				1.71				2.8
58					1.96			3.48	2.85	5.21	7.32	5.58		4.54
51				2.23	10.8		11.5	3.08	1.84				7.16	
44	16.3	12.6	9.44	2.36			8.17				4.87			1.23
42													3.56	1.97
38							1.97	1.95	2.29	2.13				
35		1.6	1.9	4.04	2.76	2.13	4.61	2.2	2.84	3.22		3.42	3.2	3.72
32	8.17		3.14	2.88	3.6	5.32			3.87		4.11			
30		1.8		3.84				4.03		5.39	5.25	3.49	4.62	6.42
26		1.31	3.33			1.85	4.42							
24	2.01			2.46	6.67	1.72					6.24	3.62	3.14	2.95
23	4.04				3.86									
22		2.35			2.55		4.23	4.77					6.8	7.21
21	4.43		2.65	2.35	5.58	3.2			6.5	7.24	6.84	5.9		
19		4.2					7.29							
18	2.49	2.52	3.38	6.35	2.81	4.73		8.31	4.24	4.81	4.15	5.43	6.05	4.99
Sum	24.1	60.5	48.2	38.1	46.4	59.2	47	51	41	34	41.6	46.1	35.9	43.3
Total Number of bands	14	15	11	13	17	14	12	11	10	10	9	9	10	12

Table 3. The amounts of soluble proteins extracted by SDS PAGE from shoot and root of lupine seedlings from seeds treated with biotic and abiotic inducers after five days challenge with *Fusarium oxysporum* f. sp. *lupini*.

Marker (Mol. Wt.)	Shoot							Root						
	Untreated healthy	Untreated infected	IBA	CuSO ₄	KCl	<i>P.</i> <i>fluorescens</i>	<i>P. putida</i>	Untreated healthy	Untreated infected	IBA	CuSO ₄	KCl	<i>P.</i> <i>fluorescens</i>	<i>P. putida</i>
138	5.54	5.27	3.75	3.81	3.11	3.47	2.69	2.9		3.02	3.98	6.16		
113		2.92	3.08	1.63	3	2.74	1.12	2.58					6.39	5.34
102	2.93	1.51					2.03		1.88	6.69	6.56	3.69		
88	1.63	2.37	2.99	2.61	3	3.54	3.53						6.4	7.72
72	3.17	2.43	2.74	2.52	4.55									
64			4.48	4.25		3.06		2.27		6.38	8.38			
60							6.86	5.05	5.1			7.87	8.08	7.02
53	15	9.35	14.3	14.5	12.8	9.38								
45							2.28							
43			7.66				2.66							
38				4.96			4.35	6.76	8.41	6.81	8.57			9.11
36	6.87	5.77			6.75	5.38	3.34					7.75	7.64	
32	1.81						3.65			4.08	3.25	5.2	8.08	8.82
29	2.37	2.66	3.07											
26				3.76	4.15	5.41	8.19	3.76	6.96	4.9	5.15	5.76	4.2	6.34
20							3.91	5.25	6.78	10.3	9.27	14.1	5.66	5.96
17	17.1		14.5					6.34						
15				12.5	1.44	1.19			5.27					
13		9.73												
Sum	56.5	42	56.6	50.6	38.8	34.2	44.6	34.9	34.4	42.1	45.2	50.6	46.4	50.3
Total Number of bands	9	9	9	9	8	8	12	8	6	7	7	7	7	7

of lupine plants. Generally, it could be concluded that *P. fluorescens* and/ or KCl can be used to induce resistance to control soil borne disease. This may provide a practical supplement to environmentally friendly disease management when combined with appropriate integrated agronomic practices.

SDS- PAGE analysis of lupine seedlings revealed that seed treated with biotic and abiotic inducers resulted in a rapid induction of different novel

PR- protein in shoot and root of lupine seedlings upon infection with the pathogen. These new proteins were not detected in untreated healthy or infected control. Two days after inoculation with the pathogen, KCl (which recorded maximum protection against wilt disease incidence) induced expression of 58 and 110 KDa proteins in shoot and root of seedlings. Similarly a 88 KDa protein was detected only in shoot of seedlings induced by KCl, *P. fluorescens* and *P. putida* (the most eff-

ective treatments). Furthermore 24 KDa protein syntheses were recorded in response to all biotic and abiotic treatments except IBA (the least effective inducer). Such protein may have critical role in plant resistance mechanism.

Woloshuk et al. (1991) reported that protein of 24 KDa is related to osmtin, a member of the pathogen related protein (PR-5) and have antifungal effect. Thus, it could be suggested that in resistance induced plants the accumulation of PR-

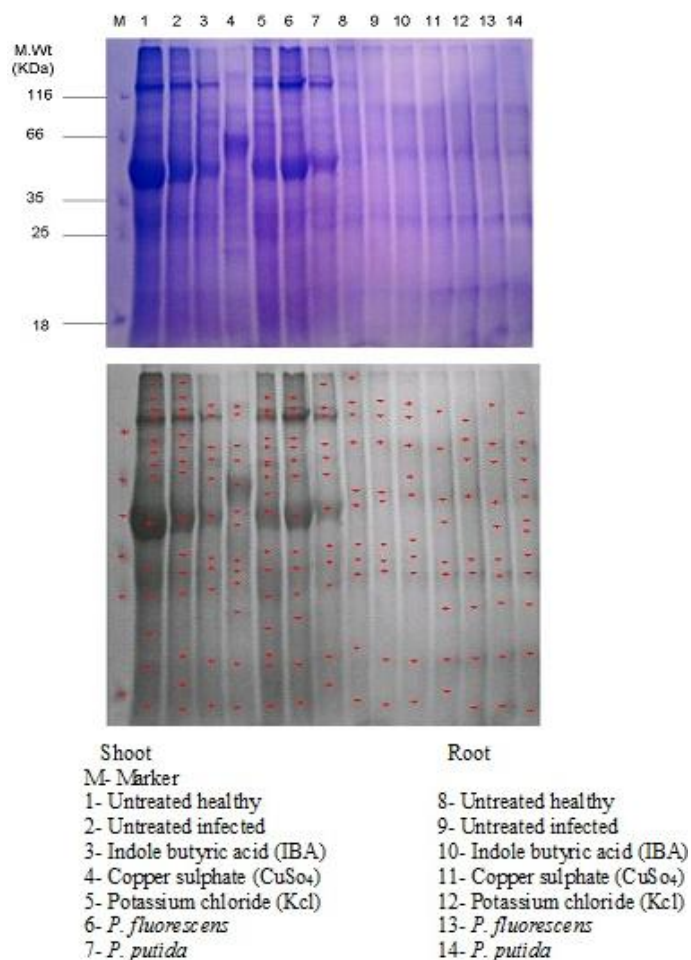


Figure 1. SDS PAGE of soluble proteins extracted from shoot and root of lupine seedlings from seeds treated with biotic and abiotic inducers and challenged with *Fusarium oxysporum* f. sp. *lupini* (two days after challenge).

proteins forms the first line of defense to a challenging pathogen and they are implicated in plant defense because of their antifungal activity (Van-Loon, 1997).

Synthesis of another group of novel proteins with different molecular weights was only induced five days after seedlings were infection with the pathogen. Among them, is a 15 KDa protein expressed in the shoot of lupine seedling induced by KCl, CuSO₄ and *P. fluorescens* (which recorded high protection against wilt disease incidence). In addition, is a 26 KDa protein one of (PR-3) members which belong to endochitinases. Chitinases have the potential to hydrolyse chitin (a major component of fungal cell walls) resulted in suppressing disease development (Radjacommare et al., 2004).

On the other hand, a 32 KDa protein was expressed in root of lupine seedlings upon infection with the pathogen in both biotic and abiotic inducers, but was not detected in untreated healthy or infected control. Nafie and Mazen (2008) stated that such protein expression may enhance

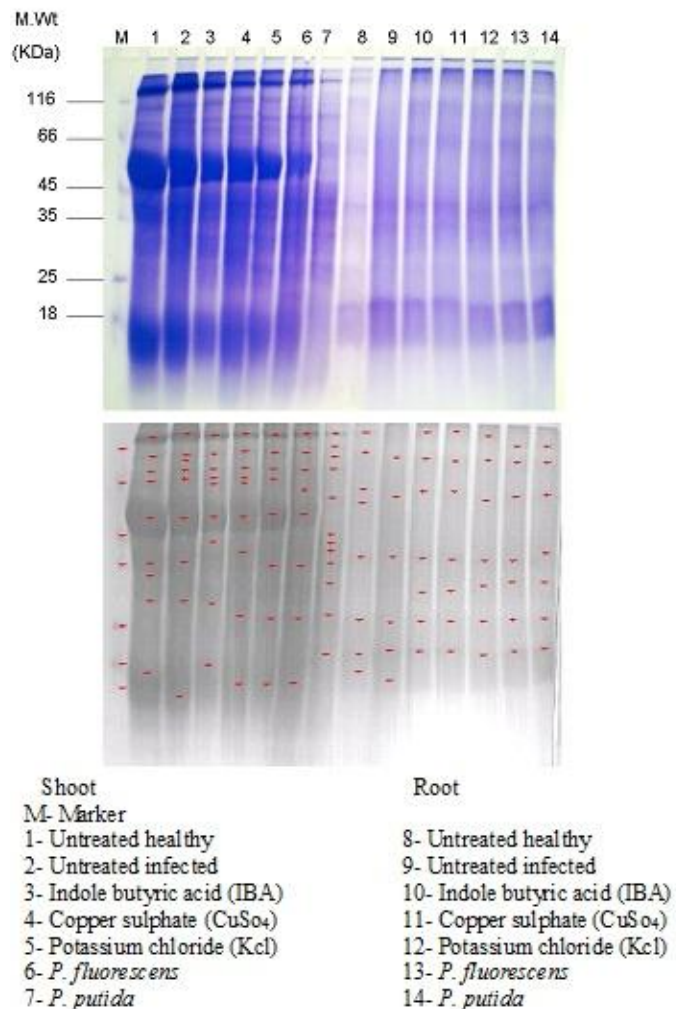


Figure 2. SDS PAGE of soluble proteins extracted from shoot and root of lupine seedlings from seeds treated with biotic and abiotic inducers and challenged with *Fusarium oxysporum* f. sp. *lupini* (five days after challenge).

plant responses to overcome further pathogen invasion. In addition, a new 36 KDa protein was detected only in root of seedling treated with the most effective inducers; (KCl and *P. fluorescens*). The 36 KDa protein belonging to PR-2 was identified as β -1,3 glucanase.

Generally, it could be concluded that induction of resistance by some abiotic and/ or biotic especially by *P. fluorescens* and/ or KCl to control soil borne disease may provide a practical supplement to environmentally friendly disease management when they are combined with appropriate integrated agronomic practices.

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