Influence of some biotic and abiotic inducers on *Fusarium* wilt disease incidence of lupin (*Lupinus albus*) on disease resistance and protein pattern

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Two biotic inducers (*Pseudomonas fluorescens* and *P. putida*) and three abiotic inducers (copper sulphate, indole butyric acid and potassium chloride) were tested for their ability to induce resistance in lupin plants against wilt disease caused by *Fusarium oxysporum* f. sp. *lupini*. Application of the inducers, as seed treatment, significantly reduced wilt disease incidence under greenhouse conditions. Potassium chloride and *Pseudomonas fluorescens* were superior. SDS-PAGE analysis of lupin 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 lupin seedlings upon infection with the pathogen. These new proteins were not detected in untreated healthy or infected control seedlings.

Key words – copper sulphate – Indole butyric acid – potassium chloride – *Pseudomonas fluorescens* – *Pseudomonas putida*

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Introducation

White lupin (*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. White lupin 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).

Fusarium wilt can be controlled by 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 have eliciting activities leading to a variety of defense reactions in host plants in response to microbial infection, including the accumulation of pathogenesis related PR-proteins (Nafie & Mazen 2008). PR-proteins

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

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 were not detected in untreated healthy or infected control.

The objective of this investigation was to evaluate some biotic and abiotic inducers to induce resistance in lupin 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 lupin plants collected from Ismailia Governorate and identified according to Barnett & Hunter (1986). The isolate proved its pathogenic capability in the pathogenicity test.

Preparation of fungal inoculum

F. oxysporum f. sp. *lupini* was grown on potato dextrose agar (PDA) medium at 25 °C for 12 days. Conidia were then harvested in sterilized distilled water using a sterile brush and filtrated through four layers of cheesecloth to remove the mycelium. The spore suspension was adjusted to 1 x 10^6 spores/ml using a haemocytometer (Sharma et al. 2005).

Preparation of Pseudomonas strains

Strains of *Pseudomonas fluorescens* and *P. putida* were provided from Department of Microbiology, Soil, Water and Environment Research Institute, ARC, Giza. The bacteria 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 lupin (cv. Giza 1) were soaked in twice their volume of a cell suspension of each of the bacterial inducers or in the individual abiotic inducers [indole butyric acid (IBA) (2.0 mM), potassium chloride (KCl) (5 mM) and copper sulphate ($CuSO_4$) (0.5 mM)], for 18 h at laboratory temperature. The seeds were then allowed to air dry. Untreated seeds were used as a control. Seeds treated with fungicide (Rizolex- T at the rate of 3g/Kg seeds) were used as a comparison treatment.

Greenhouse experiment

Pots (35 cm diameter and 25 cm in depth) containing sterilized soil were sown with lupin seeds pretreated with *P. fluorescens*, *P. putida*, IBA, KCl, $CuSO_4$ and fungicide as well as untreated seeds (served as control plants). This experiment was conducted under natural conditions (day length 12-14 hrs, temperature 25-27° C and humidity 70%). Twenty days after sowing, potted soil was infested with a spore suspension of *F. oxysporum* f. sp. *lupini* (1 X 10^6 spore/ml, 100 ml/pot). Pots containing un-infested soil were sown with untreated seeds as a healthy control. 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 at 21 days after inoculation with the pathogen (Sharma et al. 2005).

Disease assessment

Disease incidence (percent wilting) was recorded 21 days after 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 lupin seedlings (shoot and root) pretreated with biotic or abiotic inducers were collected 2 and 5 days after inoculation with the pathogen. Untreated healthy or infected seedlings were used as controls. For SDS-PAGE, 0.5 g of shoots and roots of lupin seedlings were ground to powder under liquid nitrogen and melted in ice-cold extraction

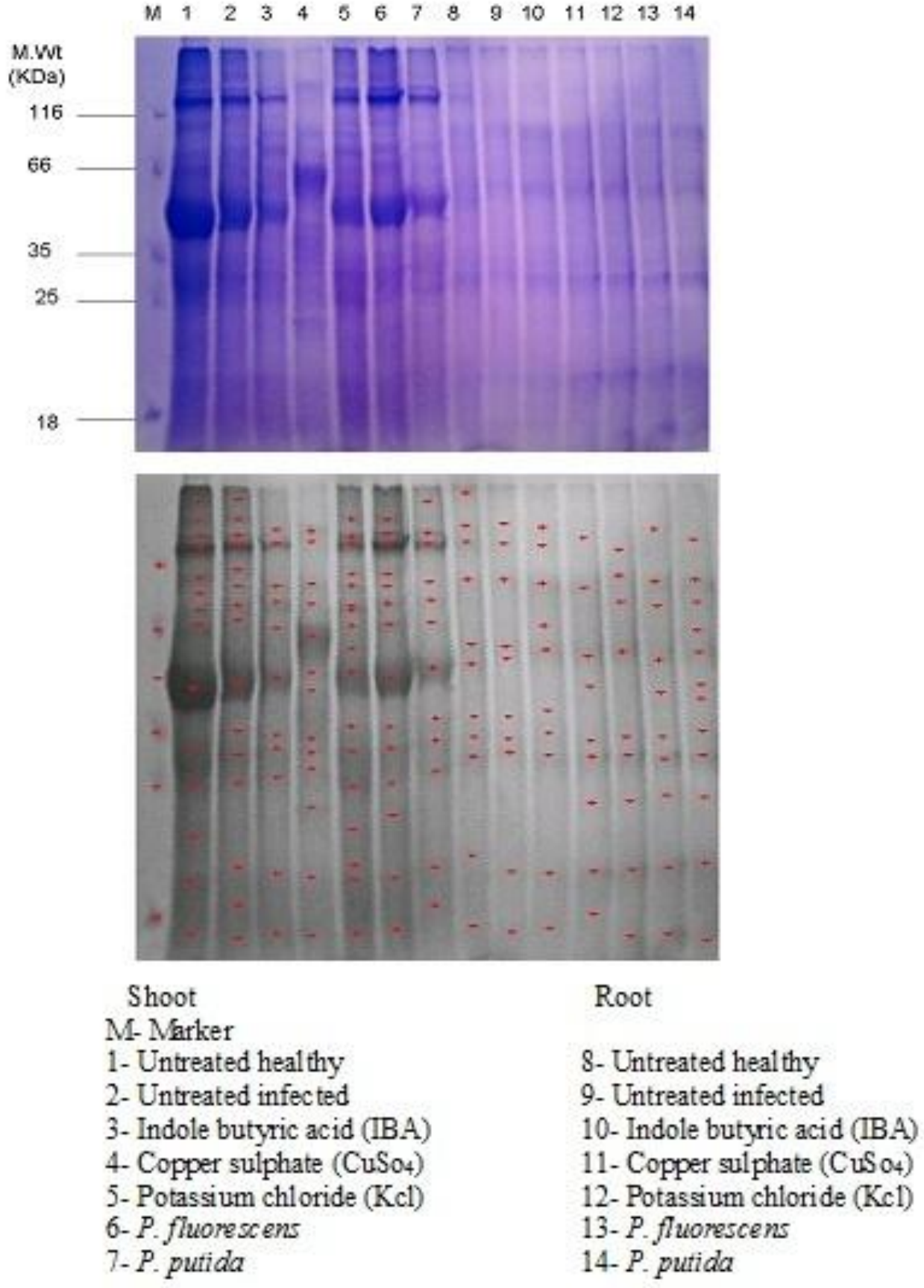

Fig. 1 – SDS PAGE of soluble proteins extracted from shoot and root of lupin seedlings from seeds treated with biotic and abiotic inducers and challenged with *Fusarium oxysporum* f. sp. *lupini* (two days after challenge).

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 & Gomez 1984).

Results

Under greenhouse conditions

Lupin seeds pretreated with fungicide, biotic or abiotic agents significantly reduced wilt disease incidence compared with untreated infected control (Table1). Nevertheless, efficiency of the tested agents varied. Fungicide treatment ranked as the most effective (84.6% reduction in disease incidence), followed by *P. fluorescens*, KCl, *P. putida* and $CuSO_4$ (76.9, 76.9, 46.2, 38.5% reduction, respectively), while IBA gave the lowest protection against the disease (23.06%).

Protein electrophoresis

Seed treatment with biotic and abiotic agents induced new pathogensis related proteins with various molecular weights in shoot and root of lupin seedlings upon infection with the pathogen (Table 2, 3, Figs. 1, 2). These new proteins were not detected in untreated healthy or infected control.

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

Five days after challenging 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, Fig. 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. Results obtained in this investigation indicated that application of 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 lupin 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. 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

Table 1 Effect of seed treatment with biotic and abiotic inducers on Fusarium wilt disease incidence in lupin plants 21 days after inoculation under greenhouse conditions. Means ± SD (*n=10*) of measurements on each ten plants. Means followed by a are significantly decrease at $P \leq 0.05$, according to least significant difference (LSD) test.

Treatment	Disease incidence %	Reduction over control %
Indole butyric acid (IBA) 2.0 mM	47.6±2.02a	23.1
Copper sulphate ($CuSO_4$) 0.5 mM	38.1±1.33a	38.5
Potassium chloride (KCl) 5 mM	14.3±0.99a	76.9
P. fluorescens 10^9 cfu/ml	14.3±0.87a	76.9
P. putida 10^9 cfu/ml	33.3±1.14a	46.2
Fungicide (Rhizolex-T) 3g/Kg	9.5±0.54a	84.6
Control	61.9±2.11	-
L.S.D. at 5%	16.33	-

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. *vasinfectum* (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, gibberallin and cytokinin) and other associated activities which include phosphate solubilization in soil resulting in stimulation of sunflower plant growth (Bhatia et al. 2005). Obtained results were consistent with those reported by Govindappa et al. (2010). They 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 of lupin plants.

Generally, it could be concluded that induction of resistance by some abiotic and/or biotic inducers, especially *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.

SDS- PAGE analysis of lupin 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 lupin 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, proteins of 58 and 110 KDa expressed only in shoot and root of seedlings, respectively, induced by KCl (which recorded maximum protection against wilt disease incidence). Similarly protein of 88 KDa detected only in shoot of seedlings induced by Kcl, *P. fluorescens* and *P. putida* (the most effective treatments). Furthermore, protein of 24 KDa synthesis in response to all biotic and abiotic treatments except IBA (the least effective inducer). Such protein may have a 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-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).

Another group of novel proteins with different molecular weights synthesis only in induced seedlings five days after infection with

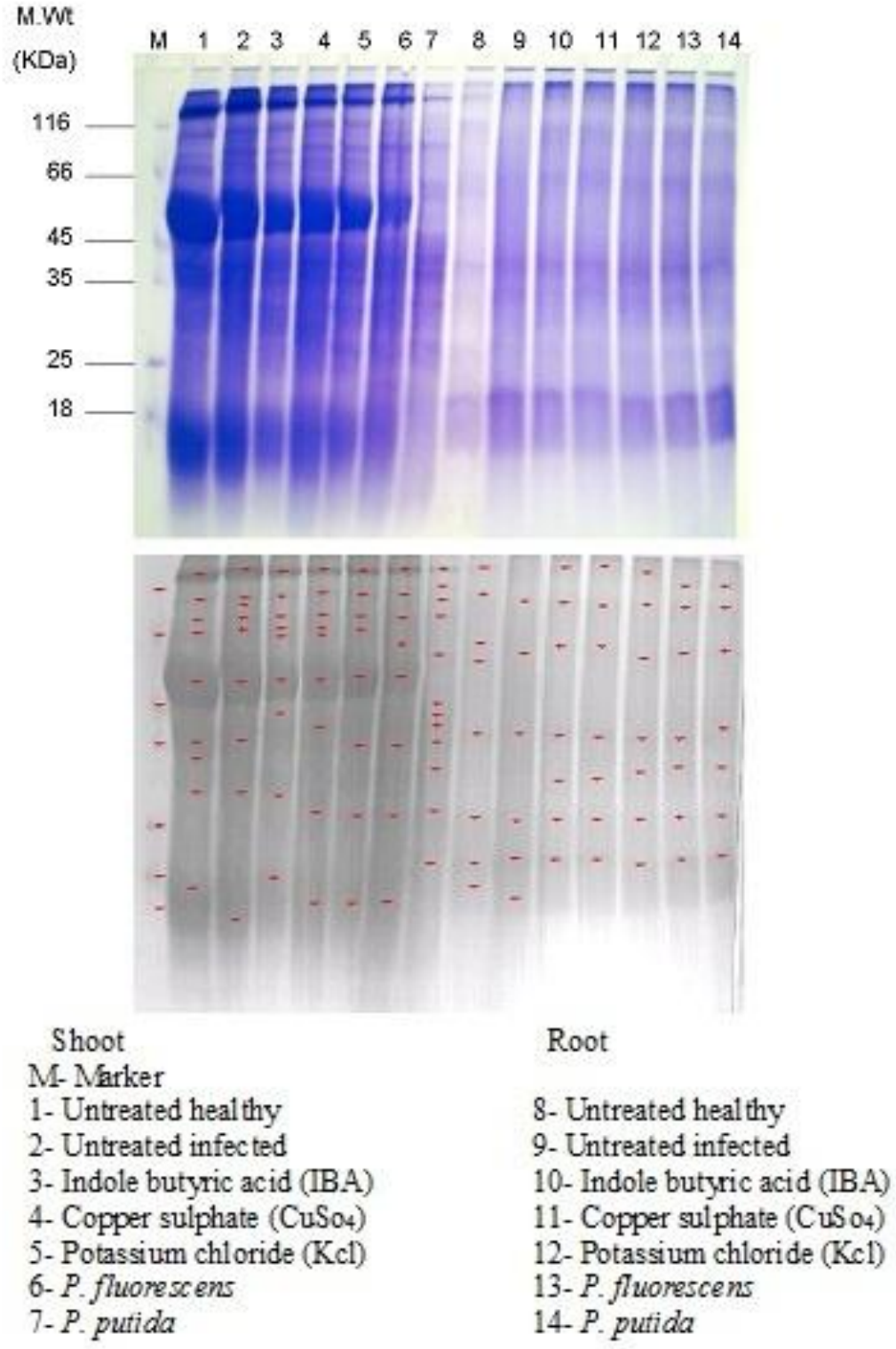

Fig.2 – SDS PAGE of soluble proteins extracted from shoot and root of lupin seedlings from seeds treated with biotic and abiotic inducers and challenged with *Fusarium oxysporum* f. sp. *lupini* (five days after challenge).

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

Marker (Mol.wt.)	shoots							roots						
	Untreated healthy	Untreated infected	IBA	$CuSO_4$	Kcl	*P. fluorescens*	*P. putida*	Untreated healthy	Untreated infected	IBA	$CuSO_4$	Kcl	*P. fluorescens*	*P. putida*
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**
No. 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.)	shoots							roots						
	Untreated healthy	Untreated infected	IBA	$CuSO_4$	Kcl	*P. fluorescens*	*P. putida*	Untreated healthy	Untreated infected	IBA	$CuSO_4$	Kcl	*P. fluorescens*	*P. putida*
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**
No. of bands	**9**	**9**	**9**	**9**	**8**	**8**	**12**	**8**	**6**	**7**	**7**	**7**	**7**	**7**

the pathogen. Among them, protein of 15 KDa expressed in the shoot of lupin seedling induced by KCl, $CuSO_4$ and *P. fluorescens* (which recorded high protection against wilt disease incidence). In addition, protein of 26 KDa is 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 side, in root of lupin seedlings, protein of 32 KDa expressed upon infection with the pathogen in all biotic and abiotic inducers and not detected in untreated healthy or infected control. In this respect, Nafie & Mazen (2008) consider such protein expression may enhance plant responses to overcome further pathogen invasion. In addition, new protein of 36 KDa was detected only in root of seedling induced by the most effective treatments (Kcl and *P. fluorescens*). Protein of 36 KDa belonging to PR-2 and was identified as β- 1,3 glucanase.

References

Abdel-Monaim MF. 2008 – Pathological studies of foliar and root diseases of lupine with special reference to induced resistance. Ph.D. Thesis, Faculty Agricultural Minia University.

Barnett HL, Hunter BB. 1986 – Illustrated genera of imperfect fungi, 4th edition. St. Paul, M.N., APS Press. Macmillan Publishing, New York. pp.217.

Bhatia S, Dubey RC, Maheshwari DK. 2005 – Enhancement of plant growth and suppression of collar rot of sunflower caused by *Sclerotium rolfsii* through *Pseudomonas fluorescens*. Indian Phytopathology 58, 17–24.

Chiej R. 1984 – The Macdonald Encyclopedia of Medicinal Plants. MacDonald, London. 447 pp.

Couto EF, Coelho RSB, Suassuna ND, Oliveira SMA. 2009 – Evaluation of induced resistance to *Fusarium* wilt in cotton: elicited enzymatic activity and disease index. Tropical Plant Pathology 34, 352–356.

El-Awadi FA, Ismail IA, Essmat NEA. 1997 – Evaluation of lupin entries and fungicides against root rot and wilt disease of lupin. 8th Congress of the Egyption Phytopathology Society Cairo, 393–401.

Gomez KA, Gomez AA. 1984 – Statistical Procedures for Agricultural Research. A. Lviley Interscience, New York. pp.678.

Govindappa M, Lokesh S, Rai VR, Nail VR, Raju SG. 2010 – Induction of systemic resistance and management of safflower *Macrophomina phaseolina* root rot disease by biocontrol agents. Archives Phytopathology Plant Protection 43(1-3), 26–40.

Laemmli UK. 1970 – Cleavage of structural proteins during assembly of head bacteriophage T_4. Nature 227, 680–685.

Maina M, Hauschild R, Sikora R. 2008 – Protection of tomato plants against fusaric acid by resistance induction. Journal of Applied Biosciences 1, 18–31.

MSTAT-C. 1991 – A software program for the design, management and analysis of agronomic research experiments. Michigan State University, pp. 400.

Nafie E, Mazen MM. 2008 – Chemical induced resistance against brown stem rot in soybean: the effect of benzothiadiazole. Journal of Applied Sciences Research 4(12), 2046–2064.

Radjacommare R, Kandan A, Nandakumar R, Samiyappan R. 2004 – Association of the hydrolytic enzymes chitinase against *Rhizoctonia solani* in *Rhizobacteria* treated rice plants. Journal of Phytopathology 152, 365-370.

Saikia R, Singh T, Kumar R, Srivastava J, Srivastavaa AK, Singh K, Arora D K. 2003 – Role of salicylic acid in systemic resistance induced by *Pseudomonas fluorescens* against *Fusarium oxysporum* f. sp. *ciceri* in chickpea. Microbiological Research 158, 203–213.

Sarwar N, Zahid MHC, Haq I. 2010 – Seed treatments induced systemic resistance in chickpea against fusarium wilt in wilt sick field. Pakistan Journal of Botany 42(5), 3323–3326.

Shalaby SIS. 1997 – Induction of systemic resistance in sesame plants for controlling fusarium wilt disease. Al-

Azhar Journal of Agriculture Research 25, 17–29.

Sharma KD, Chen W, Muehlbauer FJ, 2005 – Genetics of chickpea resistance to five races of fusarium wilt and a concise set of race differentials for *Fusarium oxysporum*f sp. *ciceri*. Plant Disease 89, 385–390.

Van-Loon LC, 1997 – Induced resistance in plants and the role of pathogenesis related proteins. European Journal plant Pathology 103,753–765.

Van-Loon LC, Rep M, Pieterse CMJ, 2006 – Significance of inducible defense related proteins in infected plants. Annual Review of Phytopathology 44, 135–162.

Woloshuk CP, Meulenhofb JS, Sela-Buurlage M, VandenElzen PJM, Comelissen BJC, 1991 – Pathogen induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell 3, 619–628.

Zian AH, 2005 – Studies on lupine root rot and wilt disease. M.Sc. Thesis, Faculty of Agriculture, Suez Canal University, Egypt.