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Full Length Research Paper

Induction of systemic acquired resistance against Leaf blight of lupine (*Lupinus termis* Forsk) caused by *Bacillus megaterium* pv *lupine*

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Four chemical inducers *viz.* Benzoic, ferulic, malic, and oxalic acids at different concentrations (50, 100 and 200 ppm) were applied on lupine plants in the frame of leaf blight caused by *Bacillus megaterium* management study. All the tested chemicals clearly retarded disease development on lupine plants treated with chemical inducers (especially in case of higher concentration, 200 ppm) compared with the untreated plants. Oxalic and malic acids were the most effective chemical inducers as they greatly reduced disease severity, while ferulic and benzoic acids were the least effective compounds. Also, the *in vitro* effect of these chemicals on bacterial growth was weak or moderate at higher concentration when compared with the control. Assessment of peroxidase, polyphenoloxidase, phenylalanine ammonia lyase and phenol compound contents in lupine treated plants indicated great increase in their activities in chemical inducers treated plants. Lupine plants treated with oxalic and malic acids recorded the highest of all the tested enzymes and total phenol compounds, while ferulic and benzoic acid gave the lowest ones in this respect.

Key words: Chemical inducers, leaf blight, lupine, induce resistance, oxidative enzyme, phenol contents.

INTRODUCTION

Lupine (*Lupinus termis* Forsk) is one of the oldest field crops grown in Egypt. Lupine is used as fodder crop and green manure for sandy and poor soils to reclaim new lands. The seeds of lupine contain great ratio of proteins, fibers and carbohydrates. It is also used for medical and industrial purposes (Maknickiene, 2001). Lupine plants are prone to many diseases which attack the foliar parts causing downy mildew, rust and leaf blight diseases (Paulitz and Cote, 1991; Yang and Sweetingham, 2002, Abdel-Monaim et al., 2012). Such diseases are destructive and cause great reduction in seed yield and quality (Osman et al., 1986; Muller et al., 1999). Hosford (1982) found that a form of *Bacillus megaterium* causes a white to very light tan blotching and streaking of wheat (*Triticum aestivum* L.) leaves. The name *B. megaterium* de Bary 1884 pv. *cerealis* pv. Nov. is proposed for this pathovar; and the halopathotype strain is WB 28.

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There is no adequate chemical control for such disease. However, management practices, such as the use of bacteria-free seed, rotation and ploughing of infested straw, has not been studied until now in this disease. Limited success has been achieved with application of fungicides, such as Bordeaux mixture, copper oxychloride, copper sulphate, and antibiotics (Saettler, 1989 and Schwartz & Galvez, 1989). Cost, potential chemical residues, and resistance among bacterial strains are the known drawbacks of chemical applications. Thus, the use of resistant lupine cultivars to *Bacillus megaterium* is economically and technically the most practical method for effective management of leaf blight (Abdel-Monaim, 2008).

A new technology for plant disease control is based on the activation of the plant's own defense system with the aid of low molecular weight synthetic molecules that induce systemic acquired resistance in plants against a wide range of microbial pathogens. The systemic acquired resistance (SAR) is an important component of plant defense against diseases where initial infection provides systemic resistance to subsequent infection by a

variety of bacterial, fungal and viral pathogens (Gaffney et al., 1993). Induced resistance depends on recognition of a pathogen or stress by the plant. This generates a cascade of events, eventually leading to the expression of defense mechanisms, which include physical barriers, metabolites and proteins that interfere with spread of the invading microorganism. In recent years, a large number of chemicals, (*viz.,* benzoic, ferulic, oxalic and malic acids etc.) which mimic the function of the pathogen in induction of systemic acquired resistance have been reported (Ryals et al., 1996; Ragab et al., 2009). Induced resistance is expressed locally at the site of infection or systemically at the sites remotely located from the initial infection. The induced systemic resistance (ISR) sensitizes the plant to respond rapidly after treatment. These responses include activation of peroxidase, polyphenoloxidase and phenylalanine ammonia lyase and phenols (Panina et al., 2005; Ragab et al., 2009; Abdel Aal et al., 2012).

This research aimed at studying the effect of benzoic, ferulic, oxalic and malic acids as chemical inducers on lupine plants against leaf blight disease and identifying some associated biochemical changes in treated plants.

MATERIAL AND METHODS

In vitro effect of chemical inducers on bacterial growth

In this study two pathogenic bacterial isolates (B2 and B6) of *B. megaterium* isolated from lupine plants that caused leaf blight previously isolated by Abdel-Monaim (2008) and four chemical inducers, i.e., benzoic acid ($C_7H_6O_2$), felulic acid ($C_{10}H_{10}O_4$), malic acid ($C_4H_6O_5$) and oxalic acid ($C_2H_2O_4$) that are known to function as antioxidants were used.

Stock solutions containing different chemical inducers to be tested were prepared and volumes of which were incorporated into nutrient agar media to obtain concentrations of 50, 100 and 200 ppm. The hydrogen concentrations of the prepared media were adjusted to pH 7 before autoclaving. Sterilized media were then poured in sterile Petri dishes and left to solidify. Plates were then inoculated by screening a film of cell suspensions of selected bacterial isolates onto the surface of the nutrient agar medium. Plates containing nutrient agar media without any chemicals were similarly inoculated with the same bacterial isolates to be taken for comparison. All plates were incubated for 48 h at 27 ºC.

The inhibitory effect of the aforementioned chemicals on two bacterial isolates was evaluated after 48 h by calculating the decrease in colony numbers of the subjected isolates at all concentrations as compared with the control treatment **(**Graham and Leite, 2004).

Effect of chemical inducers on disease resistance development under greenhouse conditions

The efficacy of the aforementioned chemical to be used as inducer for resistance in lupine plants against *B. megaterium* (isolates B2 and B6) was studied. This was accomplished by spraying foliar lupine plants (30-day-old) with the chemical solutions at 50, 100 and 200 ppm concentrations. Treated plants were then inoculated with two bacterial isolates after 24 h. from plant spraying with chemical inducers. The inoculated plants were covered with plastic bag for 24 hr. There were 3 replicates for each treatment and each was sown with 8 seeds. Untreated plants with any chemical were similarly inoculated with the same pathogens to be used for control treatment.

All experiment units were irrigated as needed and manure as usual with ammonium sulphate. The plants were kept under daily observation. After the incubation period (15 days), disease severity was evaluated using the disease index (DI) as follows: 0 = no symptoms, 1=1–9%, 2=10–24%, 3=25-49%, 4=50-74%, and 5=75-100% of blighted leaf area. The mean of disease index (DI) and disease severity index (DSI) for each replicate was calculated by the formula suggested by Liu *et al*. (1995) as follows:

$$DSI = \frac{\sum d}{d\ \max \times n} \times 100$$

Where, d is the disease rating of each plant, d max is the maximum disease rating, and n is the total number of plants examined in each replicate.

Effect of lupine foliar treatment with chemical inducers on oxidative enzymes

Activities of polyphenol oxidase, peroxidase and phenylalanine ammonia lyase enzymes were studied in tissue extracts of lupine plants treated with chemical inducers and untreated plants.

Preparation of tissue extracts

This was carried out by grounding 0.5 g fresh tissue in a pre-cooled mortar containing 10 ml solution of 10 mM potassium phosphate buffer, pH 7.0, 1% (W/V) polyvinylpyrrilidon (PVP), and 0.1% (V/V) triton –x 100 (Croft *et al*., 1990). The homogenate was centrifuged for 25 min at 8000 rpm and 4^{o}C and the supernatant was used for assay immediately.

Enzyme determination

Polyphenoloxidase activity was assayed in the prepared extracts as reported by Jennings *et al*. (1969). The reaction mixture consisted of 0.5 ml tissue extract, 1.5 ml of 25 mM citrate phosphate buffer, pH 6.0, plus 0.5 ml proline (5 mg proline / ml buffer solution) and 0.5 ml catechol (2 mg catechol /ml buffer solution). Activity of PPOX (define) was determined colorimetrically at 485 nm and then expressed as enzyme unit mg^{-1} protein min $^{-1}$

Peroxidase activity was determined with guaiacol as an enzyme substrate using a modified procedure of Maehly and Chance (1954) as describes by Moerschbacher *et al*. (1988). The reaction mixture consisted of 3 ml of phosp-

phosphate buffer (0.1 M, pH 5.8) containing guaiacol (40 mM) and 200 µl crude enzymes extract. The reaction was started by adding 50 µl H_2O_2 solution (250 µl H_2O_2/10 ml distilled water). The activity was expressed as increase in absorbance at 470 nm mg^{-1} protein min^{-1}

Phenylalanine ammonia layse activity was measured using a modified method of Green *et al*. (1975). The reaction mixture consisted of 1.5 ml of L-phenylalanine (2 mM in borate buffer pH 8.8) and 0.5 ml crude extract. The reaction mixture was incubated at 40°C for 2 h. The activity was expressed as increase in absorbance at 290 nm mg^{-1} protein min^{-1}.

The control treatment contained inactivated enzyme by boiling for 10 min. All colorimetrical measurements were conducted using Milton Roy Spectrophotometer (Milton Roy spectronic 1201).

Protein assay

Protein of extracted samples was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard solution.

Effect of lupine foliar treatment with chemical inducers on phenol contents

Total phenols were assayed by method of Mahadevan and Sridhar (1982). Well expanded lupine leaves were detached, air dried at laboratory, crushed and extracted in 95% ethanol (1:20 w/v). One microliter of Folin Demis reagent was added to 1 ml extract. After 3 min, 1 ml of saturated sodium carbonate solution was added to the reaction mixture and mixed thoroughly. After 1 h, the developing blue colour was measured colorimetrically at 735 nm against blank. Phenol concentration was calculated using a standard curve prepared with catechol.

Statistical analysis

All experiments were performed twice. Analyses of variance were carried out using MSTAT-C, 1991 program version 2.10. Least significant difference (LSD) was employed to test for significant difference between treatments at P≤0.05 (Gomez and Gomez, 1984).

RESULTS

Effect of chemical inducers on bacterial growth *in vitro*

Data in Table (1) show that all tested chemical inducers had no or little effect on growth of the two bacterial isolates B2 and B6. However, the lowest tested concentration of some chemical inducers (50 ppm) stimulated the growth of *B. megaterium* isolate B2, but the highest concentration (200 ppm) exhibited little inhibited growth of both bacterial isolates.

Effect of chemical inducers on disease severity under greenhouse conditions

All the tested chemicals significantly reduced the disease severity caused by either bacterial isolates B2 or B6 but with slight variation (Table 2). Benzoic and oxalic acids were the most active compounds retarding disease development especially when applied through plant spring method at higher concentration (200 ppm). The means of disease severity in plants treated with solutions of the aforementioned compounds (200 ppm) were 8.5 and 10% for isolate B2 and 11.5 and 15.5%, for isolate B6, respectively. On the other hand, ferulic acid and malic acid recoded the lowest ones in this respect.

Effect of chemical inducers on some oxidative enzymes

Changes of the activities of some oxidative enzymes peroxidase, polyphenol oxidase, and phenylalanine ammonia lyase were known to play a great role in plant defense mechanism against the invading microbes was investigated.

Peroxidase activity

All the tested chemical inducers increased peroxidase activity in foliage tissues as compared with untreated ones (Fig. 1). Oxalic acid and malic acid recorded the highest POX activity, whereas recorded 0.977 and 0.908 enzyme unit mg^{-1} protein min^{-1} compared with 0.274 enzyme unit mg^{-1} protein min^{-1} in control. While benzoic acid and ferulic acid recoded the lowest ones.

Polyphenoloxidase activity

In general, a significant increase in the activity of PPO (define) was observed in lupine plants treated with any chemical inducers more than control treatment (Fig. 2). PPO accumulated more markedly in plants treated with oxalic and malic acid (0.524 and 0.498 enzyme unit mg^{-1} protein min^{-1}) than the plants treated with ferulic acid and benzoic acid (0.430 and 0.435 enzyme unit mg^{-1} protein min^{-1}).

Phenylalanine ammonia lyase activity

Data in Figure 3 show that the levels of PAL activity in

Table 1. Effect of chemical inducers on growth of two *Bacillus megaterium* isolates B2 and B6 pathogenic to lupine foliage.

Treatments	Concen. (ppm)	*B. megaterium* isolates B2		B6	
		Number of colony / plate	Inhibition (%)	Number of colony / plate	Inhibition (%)
Benzoic acid	50	172.00	2.27	153.33	9.45
	100	171.33	2.65	150.67	11.02
	200	163.67	7.01	146.00	13.78
	Mean	169.00	3.98	150.00	11.42
Ferulic acid	50	183.00	-3.98	161.67	4.52
	100	181.00	-2.84	152.33	10.04
	200	180.00	-2.27	149.67	11.61
	Mean	181.33	-3.04	154.56	8.72
Oxalic acid	50	185.67	-5.49	176.67	-4.33
	100	176.33	0.19	164.33	2.95
	200	160.33	8.90	160.33	5.32
	Mean	174.11	1.07	167.11	1.31
Malic acid	50	187.67	-6.63	163.33	3.54
	100	172.00	2.27	157.33	7.09
	200	170.33	3.22	150.33	11.22
	Mean	176.67	-0.38	157.00	7.28
Control		176.00	-	169.33	-

LSD at 0.05 for:

Treatments (A)	=	2.53
Concentrations (B)	=	1.79
Isolates (C)	=	1.23

Table 2. Effect of chemical inducers on lupine foliage (cv. Balady) against to *Bacillus megaterium* isolates B2 and B6 under greenhouse conditions

Treatments	Concen. (ppm)	Disease severity caused by *B. megaterium* (%) Isolate B2		Isolate B6	
		Disease severity (%)	Protection (%)	Disease severity (%)	Protection (%)
Benzoic acid	50	11.4	88.38	18.4	80.73
	100	9.6	90.21	12.8	86.60
	200	8.5	91.33	11.5	87.96
	Mean	9.8	90.01	14.2	85.13
Ferulic acid	50	33.6	65.75	36.5	61.78
	100	23.6	75.94	19.2	79.89
	200	19.9	79.71	16.5	82.72
	Mean	25.7	73.80	24.1	74.76
Oxalic acid	50	13.4	86.34	17.3	81.88
	100	13.1	86.65	17.1	82.09
	200	10.0	89.81	12.2	87.22
	Mean	12.2	87.56	15.5	83.77
Malic acid	50	34.2	65.14	31.5	67.02
	100	17.2	82.47	23.4	75.50
	200	17.4	82.26	21.5	77.49
	Mean	22.9	76.66	25.5	73.30
Control		98.1	-	95.5	

LSD at 0.05 for:

Treatments (A)	=	1.32
Concentrations (B)	=	2.28
Isolates (C)	=	1.33
Interaction (AxBxC)	=	5.22

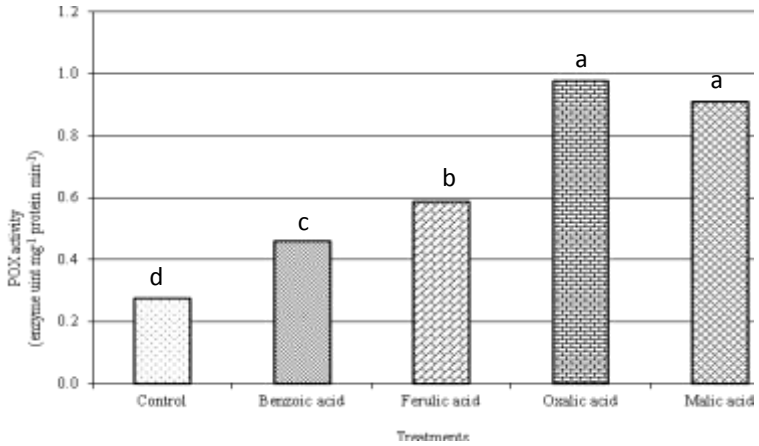

Figure 1. Peroxidase (POX) activity in extracts prepared from foliar lupine tissues treated with chemical inducers as compression untreated tissue. Different letters indicate significant differences among treatments according to least significant difference test (P = 0.05).

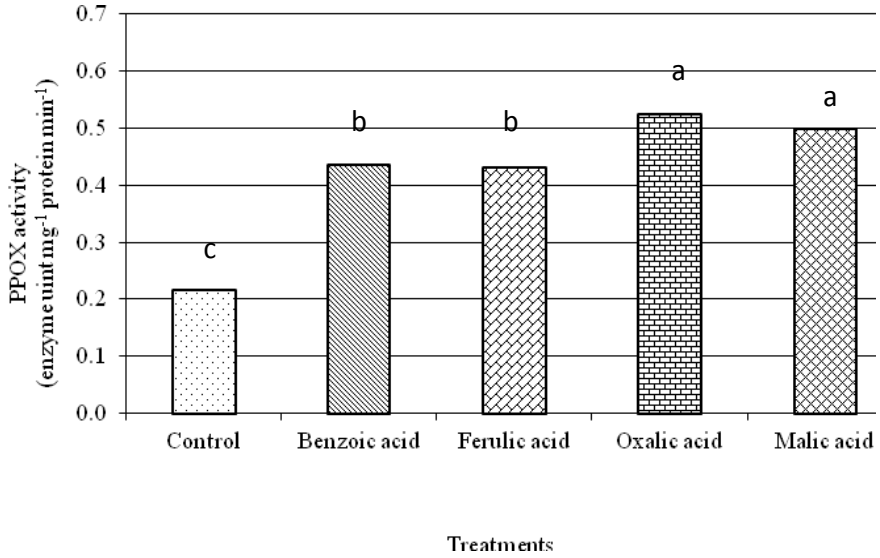

Fig. 2. Polyphenol oxidase (PPOX) activity in extracts prepared from foliar lupine tissues treated with chemical inducers as compression untreated tissue. Different letters indicate significant differences among treatments according to least significant difference test (P = 0.05).

treated plants with any tested chemical inducers were highly increased than in untreated plants (control). On the other hand, data show that PAL activity was highly

Total phenol content

The total phenols were measured in lupine plants treated with chemical inducers (Fig. 4). It is obvious that all chemical inducers show significant role to accumulation

significant increase in treated plants with oxalic acid and malic acid than plants treated with ferulic acid and benzoic acid.

of phenolic compounds than untreated plants. However, oxalic acid and malic acid pre-treated lupine plants challenge inoculated with the pathogen showed rapid increase in the accumulation of phenol compounds. While lupine plants treated with ferulic acid and benzoic acid recorded the lowest increased of accumulation of total phenol compounds.

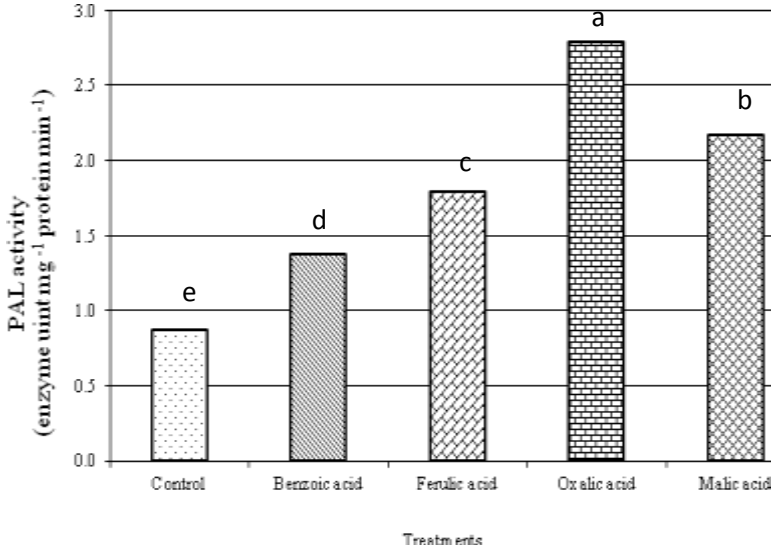

Figure 3. Phenylalanine ammonia lyase (PAL) activity in extracts prepared from foliar lupine tissues treated with chemical inducers as compression untreated tissue. Different letters indicate significant differences among treatments according to least significant difference test (P = 0.05).

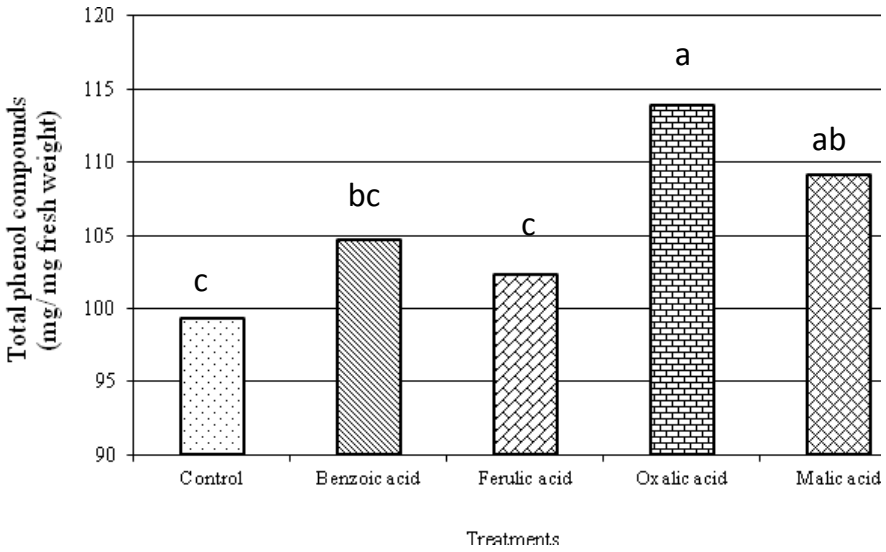

Figure 4. Total phenol compounds (TPC) content in extracts prepared from foliar lupine tissues treated with chemical inducers as compression untreated tissue. Different letters indicate significant differences among treatments according to least significant difference test (P = 0.05).

DISCUSSION

Induced resistance in plants treated with chemicals is now in current use in disease management. These compounds are accessible and not harmful to the environment. During the present work four chemical inducer compounds were tested for their potentiality as resistance inducers in the treated plants. From which four

compounds namely benzoic, ferulic, malic and oxalic acids proved the most active inducers for disease resistance in lupine plants against bacterial leaf blight caused by *Bacillus megaterium* pv. *lupini*. However, lupine plants treated with solutions of the four chemical inducers achieved great protection against this disease under greenhouse conditions.

Such resistant inducers against plant diseases was first reported by White (1979) who reported salicylate treatment to cause dramatic reduction in TMV lesion number in tobacco leaves. Subsequently, many reports concerning the use of these chemicals as resistance inducers have been published (Abdel-Kareem et al., 2004; Obradovic et al., 2005; Hilal et al., 2006; Maggie et al., 2006; Hassan et al, 2007).

Induced resistance achieved in plants treated with inducer compounds appears to be the result of several mechanisms, including synthesis of protein inhibitors, release of oxidative enzymes and increase of total phenol compounds and probably other mechanisms (Nicholson, 1992).

In the present study, assessment of the oxidative enzymes that is, peroxidase, polyphenyl oxidase and phenylalanine ammonia layse and total phenolic compounds were performed in healthy foliage lupine tissues treated with the aforementioned four chemical inducers at concentration 200 ppm. The results obtained showed great increase in all tested enzymes in healthy plant foliage treated with the four chemicals over those of the control untreated plants. This simple experiment carried out in healthy treated tissues apart of any pathogen invasion clearly indicates a strict response of the increase of oxidative enzymes and total polyphenols with application of chemical inducers compounds and this in turn causes the resistance to be induced. Polyphenoloxidase and peroxidase are naturally present in plant tissues. Polyphenoloxidase converts polyphenolic compounds to the oxidized form quinines which are thought to be inhibitory to pathogens invasion. Also it was found that higher peroxidase activity was usually more active in resistant cultivars. On the other hand, increase in PAL enzyme due to the chemical treatment was found to be correlated with increase in polyphenol accumulation (Chen et al., 1993; Milosevic & Slusarenko, 1996 and Faize et al., 2004).

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