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Full Length Research Paper

Pathological and physiological studies on root rot disease in white lupine (*Lupinus termis* Forsik)

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***Fusarium solani* and *Macrophomina phaseolina* is common fungal pathogens on lupine plants causing damping-off and root rot diseases resulting in serious economic losses. In this study, six isolates of *F. solani* and four isolates of *M. phaseolina* were isolated from lupine plants. The obtained resulted indicate that, all isolate able to attack lupine plants causing damping-off and root rot symptoms. Tissue extracts prepared from experimentally diseased root systems showed great pectolytic and cellulolytic activities while healthy tissue extracts of both organs showed slight activities of the enzymes. In varietal response test, four lupine cultivars i.e. Australian, Balady, Giza 1 and Giza 2 were tested for their susceptibility to *F. solani* (FR2) and *M. phaseolina* (MR1) and the cultivar Balady was most sensitive to both pathogens. Under field conditions, Balady cultivar was highly susceptible towards damping-off and root rot diseases and produced the least seed yield followed by Australian cultivars. On contrary, Giza 2 recorded the least damping-off and root rot severity and gave the highest seed yield followed by Giza 1. The first of December was more suitable to minimize the infection with damping-off and root rot diseases in field. Sowing lupine at 1st November gave the highest seed yield in both growing seasons.**

Key words: Lupine, damping-off, root rot, cultivars, sowing dates, pectolytic and cellulolytic activities.

INTRODUCTION

White lupine (*Lupinus termis* Forsik) is one of the oldest agricultural crops widely used in the world not only as a protein source in fodder production but also for soil improvement (Maknickiene, 2001). Lupine belongs to the genus Lupinus in the Legume family. Lupine seeds contain considerable nutrition due to its high protein (35-45%) and oil content (10-15%). Many soilborne fungi, including *Rhizoctonia solani* Kühn, *Fusariumsolani* (Mart.) Sacc, *F. oxysporum* Shelct. and *Macrophomina phaseolina* (Tassi) Goid infect lupine plants causing damping-off, root rot and wilt diseases. Such diseases cause great decrease in seed yield (Infantin *et al*., 2006; Abdel-Monaim, 2008; Ali et al., 2009). Plant diseases caused by plant pathogens are a complicated process because a number of factors play a part. However, direct involvements of pectic and cellulitic enzymes produced by the pathogen in pathogensis were reported (Gaber et al., 1990).

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Several agricultural affecting spread of these diseases in lupine fields and most important of which are selected of resistance cultivars, sowing date. Generally, effects of these factors on lupine damping –off and root rot diseases are complex and often interrelated, because they affect both the host lupine and root pathogens. Some factors may affect the lupine negatively and the fungus positively, leading to a clear-cut increase in lupine damping-off and root rot, while other may affect both the lupine and the fungus positively, leaving the resultant disease increase or decrease of damping-off and root rot in a matter of speculation (Sallam and Abdel-Monaim, 2012). Many authors studied the response of lupine cultivars to infection with *M. phaseolina* and *F. solani*, they found that the most cultivars susceptible to infection with the diseases (Christiansen, et al., 1999; Hassan et al., 2002; Atalay, 2007*; Zian* et al., 2013). Also, Sowing dates were found to play a vital role in the development of lupine damping-off, root rot diseases and seed production. In a controlled-environment study of the impact of temperature on infection of lupine plants by soil

brorne pathogens was severe at warm temperatures and declined in warmer or cooler soils.

This study aimed to isolate and identify the pathogens that cause damping-off and root rot diseases of lupine, furthermore, the cell wall degradation enzymes in pathogenesis were discussed. Evolution of available lupine cultivars to susceptibility of infection and effect of sowing dates on these diseases were also investigated.

MATERIAL AND METHODS

Isolation of pathogenic organisms

Diseased lupine plants showing root rot were collected from different fields located at Minia Governorate for isolation.

The infected roots were first separated from the plants and washed thoroughly using running tap water then cut into small parts, surface sterilized using 0.1% mercuric chloride solution for 2 minutes then washed several times in sterilized distilled water. Small pieces of the infected tissues of roots were deposited onto the surface of PDA medium in Petri dishes containing 20 Iu / ml penicillin. All the inoculated plates were incubated at about 25 ^{o}C and examined daily for fungal growth. The developing growth was transferred separately to Petri dishes containing water agar or PDA medium and incubated for 5 days at room temperature (25^{o}C). Pure cultures were obtained from the developing fungal colonies using hyphal tip and / or single spore isolation techniques. Subcultures of the obtained isolates were then kept on PDA slants and stored at 5^{o}C for further studies. The purified cultures were then tested for their pathogenicity and those proved pathogenic were submitted to identification, and to some pathological studies schemed during the course of this thesis.

Pathogenicity tests

The purified fungal isolates secured from diseased lupine plant organs were tested for their pathogenicity on healthy lupine plants cv. Balady grown in pots (25 cm in diameter) containing sterilized soil and sown with disinfested seeds. Soil sterilization was carried out using formalin solution 5%. The disinfested soil before sowing the seed was left to aeration for 3 weeks to get rid of the chemical remains. The obtained isolates (10 isolates) were grown separately on barley grain medium in conical flasks for 7-10 days to be used as a source of inoculum. Inocula of these tested fungi were applied separately at the rate of 5% of the soil weight (El-Awadi, 1997 and Ragab *et al.*, 1997), mixed thoroughly with the soil then irrigated and left 7 days for establishment. Disinfested lupine seeds cv. Balady was sown in the infested pots at the rate of 10 seeds/pot. Three pots were used for each isolate, (which were considered as replicates). Pots containing sterile soil mixed with barley grains free of any fungi were sown similarly with disinfested lupine seeds at the same rate to be used as control treatment. Pots were kept under observation and irrigated as needed.

Results were recorded after 30 days of planting for damping- off and after 90 days for rot root and wilt. The percentage of pre and post emergence damped-off seedlings was estimated per each replicate. The root rot and wilt, plants of each replicate were removed from the soil after the inoculation period, washed thoroughly to remove soil debris then disease severity was estimated according to the percentage of root discoloration as follows:

0 = roots without discoloration (no infection), 1= 1-20%, 2= 21- 40%, 3= 41-75%, 4= 75-100% and 5= completely dead plants. Disease severity index (DSI) for each replicate was calculated by the formula suggested by Liu et al. (1995) and calculated as follows:

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

Whereas: 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.

Reisolation was carried out from some of the experimentally diseased plants to fulfill Koch's postulations and the developing fungi were compared with the original isolates.

Identification of the causal organisms

Identification of the obtained pathogenic fungal isolates was carried out according to Nelson, et al. (1983) and Rotem (1994). Representative of these isolates were sent to Assuit University Mycological Center (AUMC) for verification.

Assessment of some hydrolytic enzymes i.e. cellulase and pectinase in diseased and healthy lupine tissues

Assessment of pectinase and cellulase enzymes were assayed in tissue extracted prepared from diseased and healthy root system taken from the subjected plants during the pathogenicity test.

Preparation of tissue extracts

Half gram of leaf and/or root tissues (either healthy and/or infected) were existed and separately macerated with clean mortar and pestle containing 5 ml of 0.05 M phosphate buffer (pH6). Samples of infected and healthy roots with *F. solani* isolate FR2 and *Macrophomina phase-*

olina isolate MR1 were used in this study. The homogenated tissue extracts were filtered though several layers of cheese cloth, cooled to temperature near zero then centrifuged at 5000 rpm for 20 min. The clarified enzyme preparations of diseased and healthy tissues were directly subjected to the Viscometrical assessment.

Assessment of pectic enzymes

Assessment of pectic enzyme was employed Viscometrically according to the method of Mahadevan and Sridhar (1982). This was carried out by measuring the reduction in viscosity of the reaction mixtures containing 2 ml of crude enzyme preparations (tissue extract), 5 ml, 1.5% citrus pectin solution in 0.1 M phosphate buffer at pH 5 and/or 8 adjusted by 0.3 M NaOH or HCl. The reaction mixtures were incubated at 28 ^{o}C and the loss in viscosity of the mixture was measured after 90 minutes against blank containing boiled inactivated extracts instead of the active ones.
Reduction in viscosity of the substrate was calculated using Fenske- Ostwan Viscosimeter according to the formula:

$$V = \frac{T_0 - T}{T_0 - T\,H_2O} \times 100$$

Where V= percent of loss in viscosity.
T_0= Flow time in seconds at of blank (boiled enzyme)
T = Flow time in seconds after incubation (90 minutes).
T H_2O = Flow time of distilled water.

Cellulase Assessments

Cellulase activity was assayed viscometrically in mixtures containing 5 ml 1.5% carboxymethylcellulose (CMC) in 0.05 M phosphate buffer at pH 6 mixed with 2 ml crude enzyme preparations. The mixtures were incubated at 28 ^{o}C and the percentage loss in viscosity was estimated after 90 min against control containing heat inactivated tissue extracts instead of the active ones.

Varietal response

Response of four lupine cultivars namely cvs. Balady, Australian, Giza 1 and Giza 2 to infection with the infection isolates were investigated using some selected root fungal isolates *viz. F. solani* isolate FR2 and *M. phaseolina* isolate MR1 which showed high pathogenic property through the pathogenicity test. The tested cultivars were grown in pots containing sterile soil and the inocula of the fungal and/or bacterial isolates were prepared and applied similarly as was done in the pathogenicity test.

Data were recorded for damping –off (pre- and post-) and root rot after 15, 30 and 90 days of sowing, respectively. However, in case of foliar diseases data were recorded after 15 days as above mentioned.

Field Experiments

In this study, two experiments were conducted during 2006/2007 and 2007/2008 growing seasons under field conditions. The experiments were carried out in a field naturally infested with the causal organisms of damping-off and wilt diseases of lupine located at experimental farm of El-Kharga Agriculture Satiation, New Valley governorate. The experimental design was Randomized Complete Block Design (RCBD) with four replicates. The experiments were conducted in plots 10.5 m2, 3.5 m in length and 5 rows, between both of them 0.60 m. two seeds/hill were sown with 20 cm apart between hills on one side from row. Damping-off and wilt was recorded 30 and 90 days after sowing. At the end of the growing season, seed yield was harvested, weighted and calculated as (kg fed.-1) (feddan=4200 m^2). Experimental that have been studied were as follows:
Varietal response: Response of four lupine cultivars, namely Giza 1, Giza 2, Australian, Balady were used in this study.
Effect of sowing dates: Lupine seeds cv. Balady were planting at three different dates i.e. 1 st October, 1 st November and 1 st December in both growing seasons.

Statistical Analysis

All experiments were performed at least twice. Analyses of variance were carried out using MSTATC. Least significant difference (LSD) was employed to test for significant difference between treatments at P≤0.05 (Gomez and Gomez,1984).

RESULTS

Isolation, Purification and Identification of the Fungi Associated with Lupine Diseased Plants

Ten fungal isolates were isolated from lupine plants collected from different locations in El-Minia governorate, Egypt, that show root rot symptoms. Hyphal tip cultures of grown fungi were maintained on PDA medium. All fungi were purified using single spore technique cultures, then they were identified.

The fungal isolates were microscopically examined and tentatively identified at the generic rank as *Fusarium solani* (6 isolates from FR1 to FR6) and *Macrophomina phaseolina* (4 isolates from MR1 to MR4) secured from diseased roots.

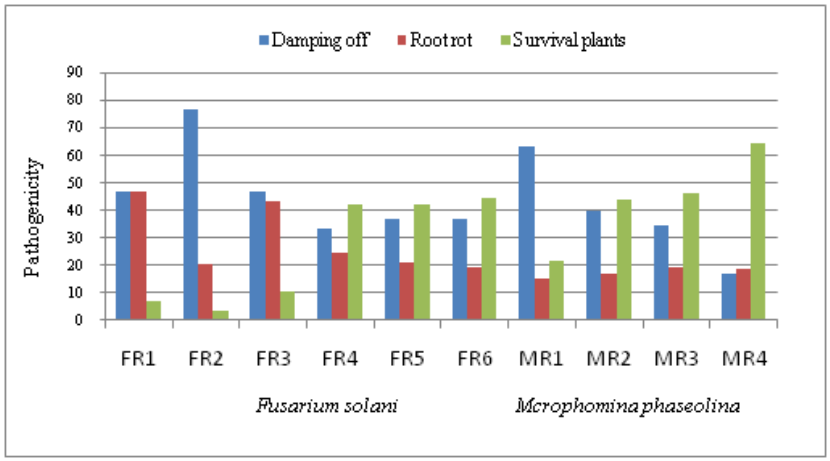

Figure 1. Pathogenicity tests of fungal isolates obtained from naturally diseased lupine roots cv. Balady.

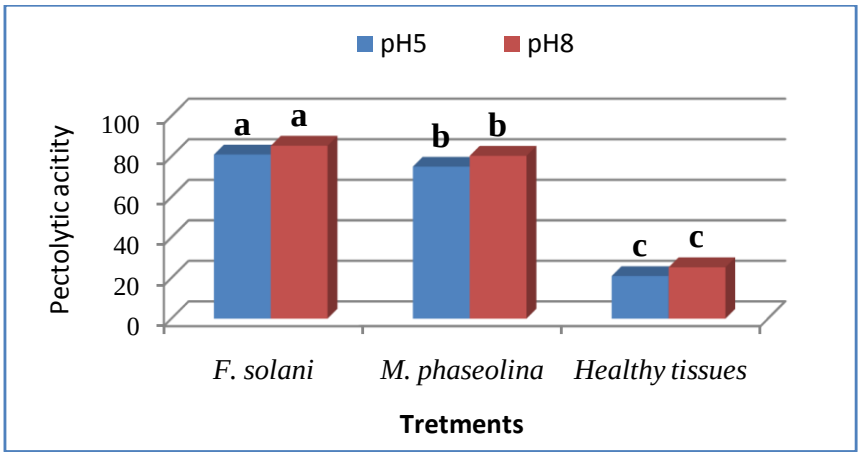

Figure 2. Pectolytic activity of extracts prepared from diseased lupine root tissues infected with *F. solani* isolate FR2 and *M. phaseolina* isolate MR1 compared with healthy tissue extract. Different letters indicate significant differences among treatments within the same color column according to least significant difference test (P ≤ 0.05).

Pathogenicity tests

Purified fungal isolates (ten isolates) secured from diseased lupine roots were tested for their virulence on root system of lupine plants grown in pots as mentioned before.

Data in Figure 1 reveal that all the six *Fusarium solani* and the four *Macrophomina* isolates proved virulent on the subjected plants causing damping-off and root rot to the root system. *Fusarium* isolates proved slightly more pathogenic than *Macrophomina phaseolina* isolates however varied considerably in their virulence being isolates FR1, FR2 and FR3 the most active ones as they causes respectively 46.67, 76.67 and 46.67% damping-off and 46.67, 20.33 and 43.0 % root rot/ wilt diseases. On the other hand, *Macrophomina* isolates came next after *Fusarium* isolates in their reaction towards lupine roots and greatly varied in their virulence. They caused about 16.67-63.33% damping-off and 15-19.08% root rot diseases being isolate R7 the most virulent isolate which caused 63.33% damping – off and 15% root rot and isolate R10 the least virulent one caused 16.67% damping-off and 18.67% root rot.

Assessment of pectolytic enzymes activity

In diseased root extracts, the pectolytic activities means exhibited by the two pathogens *F. solani* isolate FR2 and *M. phaseolina* isolate MR1 were 81.0 %, 85.3 % and 75.0 %, 80.3 % at two pHs, respectively and the enzyme activity was slightly higher at pH8 (Figure 2).

Also extracts prepared from healthy foliage and/or roots showed marked pectolytic activity when estimated at either pHs. The foliar extracts recorded 19.0 % and 23 % loss in viscosity while healthy root extracts caused 21.0 % and 25.3 % loss in viscosity at the two pHs.

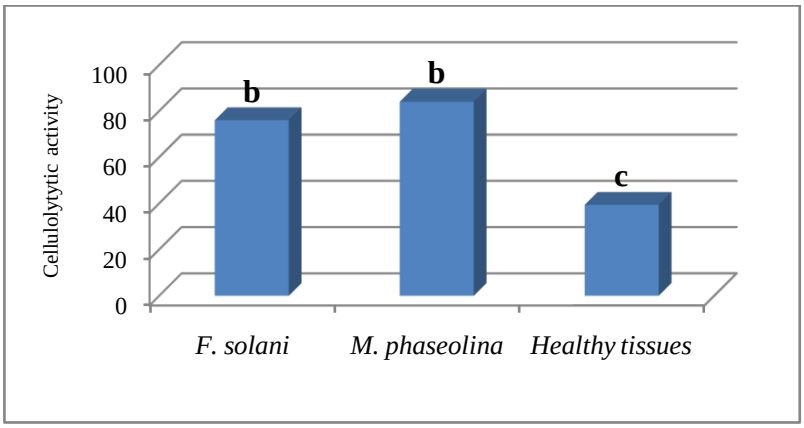

Figure 3. Cellulolytic activity of extracts prepared from diseased and healthy lupine root tissues infected with *F. solani* isolate FR2 and *M. phaseolina* isolate MR1 compared with healthy tissue extract. Different letters indicate significant differences among treatments within the same color column according to least significant difference test (P≤ 0.05).

Table 1. Cultivars response towards *F. solani* isolate FR2 and *M. phaseolina* isolate MR1 secured from diseased lupine roots.

Cultivars	Pathogens Isolates	% Damping-off		% Root rot	% Survival plants
		Pre-	Post-		
Australian	*F. solani*	30.00	9 .67	33.25	27.08
	M. phaseolina	20.00	20.33	40.29	19.38
	Mean	25.00	15.00	36.77	23.23
Balady	*F. solani*	40.00	26.67	32.25	1.08
	M. phaseolina	50.33	16.00	29.65	4.02
	Mean	45.17	21.34	30.95	2.55
Giza 1	*F. solani*	15.00	15.00	32.65	37.35
	M. phaseolina	15.00	18.33	30.25	36.42
	Mean	15.00	16.67	31.45	36.88
Giza 2	*F. solani*	10.00	16.67	30.89	42.44
	M. phaseolina	20.00	12.33	31.35	36.32
	Mean	15.00	14.50	31.12	39.38
Control		0.00	0.00	0.00	100.0
LSD at 0.05 for:					
Cultivars (A)	=	2.84	2.47	3.38	3.26
Isolates (B)	=	3.49	4.02	2.39	2.30
Interaction (AxB)	=	2.01	4.94	4.78	4.60

Assessment of cellulolytic enzyme activity

Data in Figure 3 show that diseased root tissue extracts showed marked cellulase activity means of 76.0 % and 80.0% for *F. solani* and *M. phaseolina* isolates, respectively. However, extracts prepared from healthy root tissues showed cellulolytic activity of 39 %.

Reaction of Different Lupine Cultivars to Root Infection under Greenhouse Condition

Four lupine cultivars were tested for their relative susceptibly towards two fungal root isolates. The tested isolates were, *F. solani* isolate FR2 and *M. phaseolina* isolate MR1. Data in Table (1) show that, the four tested cultivars were susceptible towards the two tested pathogens however there were some variations among them. Both fungi severity attacked the subjected lupine cultivars causing damping-off and root rot diseases. The cultivar Balady was the most susceptible towards both fungi and recorded the highest disease severity means 98.92 % and 95.98 % by *F. solani* and *M. phaseolina* isolates, respectively. Giza 1 recorded 62.65 % and 63.58 % disease severity by *F. solani* and *M. phaseolina* respectively and similarly reacted Giza 2 with slight variation. The Australian cultivar was also so sensitive but recorded disease severity mean slightly less than that recorded for the most susceptible one cv. Balady.

Table 2. Varietal response of four lupine cultivars towards damping-off, root rot and seed yield under natural infection in field during seasons.

Cultivars	% Damping-off	% Root Rot	Seed yield fed.$^{-1}$ (Kg)
Season 2006-07			
Australian	27.33 b	30.47 b	459.36 c
Balady	35.33 a	37.59 a	416.67 d
Giza 1	20.67 c	28.28 bc	502.54 b
Giza 2	18.67 c	23.67 c	562.36 a
Season 2007-08			
Australian	24.33 b	33.67 b	439.67 c
Balady	36.67 a	40.25 a	412.25 d
Giza 1	22.67 bc	29.36 c	486.39 b
Giza 2	19.00 c	20.19 e	529.36 a

Table 3. Effect of sowing dates on damping-off, root rot and seed yield of Giza 1 lupine variety under field conditions during seasons 2006-07 and 2007-08.

Showing dates	% Damping-off	% Root Rot	Seed yield fed.$^{-1}$ (Kg)
Season 2006-07			
1 st October	29.33 a	30.87 a	406.36 c
1 st November	23.67 b	25.25 b	482.25 a
1 st December	15.33 c	17.35 c	457.25 b
Season 2007-08			
1 stOctober	27.33 a	32.00 a	420.36 c
1 st November	22.33 b	23.36 b	514.36 a
1 st December	16.00 c	18.36 c	473.36 b

Different letters indicate significant differences among treatments within the same column according to least significant difference test (P≤ 0.05).

Field Studies

Rection of Certain Lupine Cultivars

Data in Table (2) indicate that all the tested luoine cultivare were sesceptible to infection root rot pathogens causing damping-off and root rot diseases under field condtions.

Balady and Australian cultivares were highly sesceptible than Giza 1 and Giza 2. Balady cv. recorded the highfffest damping-off (35.33 and 36.67%) and root rot (37.59 and 40.25%) in both growing seasons, respectively. While, Giza 2 cv. recorded the lowest damping-off (18.67 and 19.00%) and root rot (23.67 and 20.19%) in both growing seasons, respectively. On the other hand, Giza 2 cv. resulted highly seed yield (562.36 and 529.36 Kg fed. $^{-1}$) in both seasons, respectively compared with the other cultivars followed with Giza 1 (502.54 and 486.39 Kg fed.$^{-1}$), whereas Balady cv. gave less seed yield (416.67and 412.25 Kg fed.$^{-1}$) followed by Austeralian cv. (459.36 and 439.67 Kg fed^{-1}) in both growing seasons.

Different letters indicate significant differences among treatments within the same column according to least significant difference test (P≤ 0.05).

Effect of Sowing Dates

Damping-off and root rot of lupine plants cv. Giza 1 was significantly affected by sowing dates (Table 3). The highest average of damping-off and root rot severity of lupine plants was occureed at the sowing date 1 st October (29.33, 27.33 % damping –off and 30.87 , 32.00 % root rot) in both growing seasons, respectivily, while planting at 1 st Decmber recorded the lowest damping-off (15.33 and 16.00%) and root rot (17.35 and 18.36%). On the other hand, seed yield significantly affected by sowing

dates wheras the highest seed yield were recorded when lupine seeds sowing at 1st November in both seasons, where produced (482.25 and 514.36 Kg fed.$^{-1}$) followed by date at 1 st Decewmber (457.25 and 473.36 Kg fed. $^{-1}$). While lupine seeds sowing at 1st October gave the lowest seed yield (406.36 and 420.36 Kgfed^{-1}) in both seasons. Gnarly, lupine seeds sowing at 1 st December recorded the highest survival plants and sowing date at 1 st November recorded the hightest seed yeild during both growing seasons.

DISCUSSION

Several soil-borne fungi attack lupine plants during its various growth stages from seedling till maturity causing damping-off and root rot diseases. The present work showed that damping-off and root rot diseases are incited by the soil borne fungi, *Fusarium solani* and *Macrophomina phaseolina*. These results are in agreement with these obtained by Hassan et al., (2002); Abdel-Kareem et al., (2004); Infantin, et al., (2006); Abdel- Monaim (2008) and Ali, et al., (2009).

Tissue extracts prepared from experimentally diseased root systems showed great pectolytic and cellulolytic activities while healthy tissue extracts of both organs showed slight activities of the enzymes. It has long been known that some plant pathogens secreted tissue macerating enzyme i.e. pectinase and cellulose in the invaded tissues during the pathogenesis processes. These enzymes cause disintegration and collapse to the subjected tissues, hence facilitates the development of disease processes (Holz and Knox-Davies, 1986).

In varietal response test four lupine cultivars namely cvs. Australian, Balady, Giza 1 and Giza 2 were tested for their susceptibility to *F. solani* (RF2) and M. phaseolina (RM1) pathogens. All the tested cultivars were susceptible to *F. solani* and *M. phaseolina* under greenhouse and field conditions being cv. Balady the more sensitive than the other tested cvs. Also, Balady cv. production the lowest seed yield under field condition, however Giza 2 recorded the highest protection against root pathogens and seed yield under field conditions. These results are in agreement with those obtained by Hassan et al. (2002) reported the Australian lupine cultivar more susceptible than Giza 1 and Giza 2. Also, *Zian* et al., (2013) indicated that the differences among tested lupine cultivars in their susceptibility to *F. oxysporum* f. sp. *lupini*, Dijon 2 cultivar was less susceptible to *F. oxysporum* followed by Giza 1 and Giza-2 cultivars.

Sowing date is considered as one limiting factor for disease incidence and onset and effect seed production in field (Akem et al., 2004; Landa et al., 2004; Tahir et al., 2004 and Sallam and Abdel-Monaim, 2012). In the study revealed that sowing dates have significant effects on lupine damping-off, root rot diseases and seed production. Sowing date of 1 st December was more suitable to minimize the disease severity but sowing at 1 st November produced the highest seed yield therefore preferred of sowing at the first of November and may have to see that the impact of weather.

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