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Full Length Research Paper

Bacillus megaterium, a new pathogen on lupine plants in Egypt

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The causal agents of bacterial blight in lupine (*Lupinis termis*) were isolated from leaves displaying symptoms in Minia governorate, Egypt. The pathogens were characterized by biochemical and physiological tests, and identified as *Bacillus megaterium*. Tissue extracts prepared from experimentally diseased shoot 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 that is, Australian, Balady, Giza 1 and Giza 2 were tested for their susceptibility to all tested *B. megaterium* isolates and the cultivar Balady was most sensitive to all isolates. However, isolates B2 and B6 were more virulent than the others. Two isolates of *B. megaterium* isolates that caused blight on lupine plants were subsequently tested on other foliar plants representatives of 17 plant species. Both *B. megaterium* isolates could not infect any of the tested plants but produced small necrotic spots on faba bean leaves. These results indicate the host specificity of this bacterium towards its host plant and accordingly justify the suggested forma specials *lupini* to be given to the lupine bacterial pathogen *B. megaterium*. According to the literature review, this is the first report on the occurrence of *B. megaterium* as a causal agent of leaf blight of lupine plants in Egypt.

Key words: *Bacillus megaterium*, lupine, pectolytic and cellulolytic activities, cultivars.

INTRODUCTION

Lupine (*Lupinus termis* Forsk) is one of the oldest field crops grown in Egypt. Lupine uses 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). Such diseases are destructive and cause great loss 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 leaves. The name *B. megaterium* de Bary 1884 pv. Cerealis pv. Nov. is proposed for this pathovar; the halopathotype strain is WB 28 (Dye et al., 1980). 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; Walker et al., 1994).

The objective of this investigation is to isolate and identify the pathogenic agent that caused leaf blight of lupine plant at Minia governorate and test the varietal response to infection with *B. megaterium,* furthermore, the cell wall degradation enzymes in pathogenesis were

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Figure 1. Naturally diseased lupine foliage showing blighted leaves and stem. The affected tissues became winked, weathered and turned grayish – brown.

discussed.

MATERIALS AND METHODS

Isolation of pathogenic organisms

Diseased lupine plants showing spotting and blight symptoms (Figure 1) were collected from different fields located at Minia Governorate for isolation. The infected stems and leaves 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 min then washed several times in sterilized distilled water and maceration in sterilized mortar containing small amounts of sterilized distilled water, left for 15 min. Loopfulls of the obtained homogenates were streaked onto nutrient sucrose agar medium in Petri dishes. All the inoculated plates were incubated at about 25±2°C and examined daily for bacterial and fungal growth.

To obtain pure cultures of the developing growth: in case of bacteria, the developing growths were further streaked onto the same medium and subcultures were prepared from single separated colonies and maintained on nutrient sucrose agar slopes and stored at 5°C for future studies.

Pathogenicity tests

The purified bacterial 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% whereas the seeds were disinfested in 0.1% mercuric chloride for 2 min then washed several times with tap water and sown in the prepared pots (10 seeds/pot). The disinfested soil before sowing the seed was left to aeration for 3 weeks to get rid of the chemical remains. The inocula of the purified bacterial isolates was prepared by streaking the secured purified isolates onto glucose nutrient agar in plates (3 plates/ isolate) and incubated for 48 h. at 25 ±2°C. The developing growth of each isolate was suspended in appropriate amount of water to obtain inocula of 1-1.5 × 10^8 colony– forming units (CFU) /ml (Shoeib, 1997). Foliage of 30 day old lupine plants (cv. Balady) grown in pots was sprayed with the prepared bacterial inocula by hand automizer. Each isolate was used to inoculate 30 days old lupine plants grown in 3 pots as replicates. Check treatment was sprayed with water. Inoculated and uninoculated plants were enclosed within polyethylene bags for 24 h. to maintain adequate humidity. All plants were placed in the greenhouse for 2 weeks, irrigated as needed and their foliage was carefully observed for disease development and the percentage of blighted leaf area was assayed. After the incubation period, disease severity was evaluated using the disease index (DI) according to Abdel-Monaim (2008) as follows: 0 = no symptoms, 1=1–9%, 2=10–24%, 3=25-49%, 4=50-74%, 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) and calculated as follows:

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

Wher d is the disease rating of each plant, and 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 Kock's postulations and the developing bacterial colony was compared with the original isolates.

Identification of the causal organisms

Identification of the bacterial isolates was performed according to Bergey's manual of systematic bacteriology (Sneath et al., 1986).

Assessment of some hydrolytic enzymes that is, 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 shoot system taken from the subjected plants during the pathogenicity test.

Preparation of tissue extracts

Half gram of leaf 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 (pH 6). 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°C and the loss in viscosity of the mixture was measured after 90 min 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 min), T H_2O=Flow time of distilled water.

Cellulase assessments

Cellulase activity was assayed viscometrically in mixtures containing 5 ml and 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°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 bacterial isolates which showed high pathogenic property through the pathogenicity test.

The tested cultivars were grown in pots containing sterile soil and the inocula of the bacterial isolates were prepared and applied similarly as was done in the pathogenicity test. Data were recorded after 15 days as mentioned above.

Host range

The pathogenicity of two bacterial isolates (isolates B2 and B6) were further tested against other plants selected to be representatives of different plant species presented in Table 1. Seeds of the tested plant species were disinfested and sown in pots containing sterile soil (10 seeds / pot). There were three pots per treatment. After 45 days of sowing, the plants were inoculated by spraying their foliage with the inocula of the pathogen bacterial isolates to be tested prepared as described before in pathogenicity test. Control plants were sprayed with tap water. Disease severity index (DSI) was recorded 15 days after inoculation.

Statistical analysis

All experiments were performed twice. Standard deviation (SD) was used for analysis of variances as described by Little and Hills (1972).

RESULTS

Isolation of the causal organisms

Isolation trials carried out from diseased specimens taken from lupine or spotted/ blighted foliar on sucrose nutrient agar media resulted in development of 6 bacterial isolates that was purified and kept in agar slants.

Pathogenicity tests

Lupine plants inoculated with purified bacterial isolates behaved aggressively towards the inoculated plants. The inoculated plants soon became soft discolored grayish-brown bent over and eventually collapsed (Figure 2). Also stem inoculated lupine plant through artificial wound inoculated by an effective *B. megaterium* isolate (B2) causing discoloration and softening to the surrounding tissues (Figure 3). The infection extended to the adhering leaf. Data present in Figure 4 show that bacterial isolate B2 recoded the highest disease severity (100%) followed by isolate B6 (96%), while isolate B3 recoded the lowest ones (84%).

Identification of the causal organisms

The morphological and physiological activities performed on six bacterial lupine isolates that proved pathogenic on

Table 1. List of plant species tested for their reaction to lupine foliar pathogens.

Scientific name	Common name	Variety
Family: Leguminaceae		
Glycine max, Merr.	Soybean	Clark
Medicago sativa, L.	Alfalfa	Giza 1
Phaneolus vulgaris, L.	Bean	Giza 6
Vicia faba, L.	Faba bean	Giza 3
Pisum sativum, L.	Pea	Master peas
Lupinus termis, Forsk.	Lupine	Balady
Lens esculenta, L.	Lentil	Giza 4
Trifolium alexandrinum, L.	Egyptian clover	Giza 1
Trigonellafoenum graecum, L.	Ferugreek	Balady
Vigna unguiculata, L.	Cowpea	Cream 7
Cicer arietinum, L.	Chickpea	Giza 3
Medicago playmorpha,L.	Medic	Balady
Melilotus indica,L.	India melilot	Balady
Family: Garmineae		
Hordeum vulgare, L.	Barley	Giza 125
Triticum sativum, L.	Wheat	Giza 168
Family: Compositae		
Heliathus amnuus, L.	Sunflower	Giza 1
Family: Asteraceae		
Xanthium pungens, Wallr	Cocklebur	Balady
Family: Chenopodiaceae		
Spinacia oleracea	Spinach	Balady

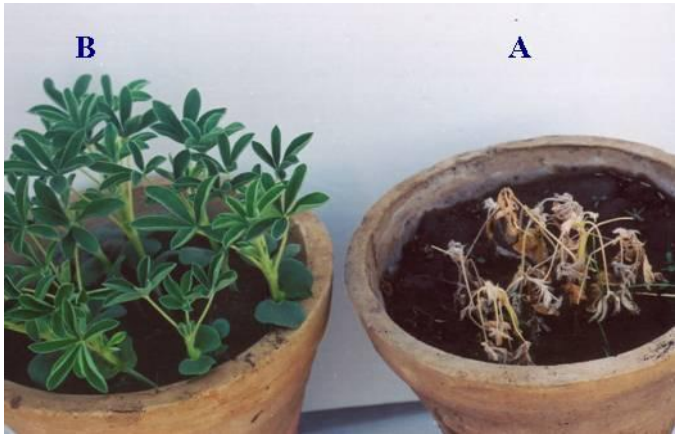

Figure 2. A: Severity attacked lupine plants by *B. megaterium* isolate B2. The attacked plants turned grayish – brown, reduced in size, bent over and collapsed. B: Control healthy plants.

their host and presented in Table 2 show the following: the isolates growth on glucose medium had creamy smooth colonies tended to be yellow on ageing, cells were Gram-positive, rods, spore formers, motile. Sporangia were not swollen within the vegetative cell. They were strict aerobes that could grow in broth medium amended with 5% NaCl, catalase positive, oxidase negative, V.P. negative. Negative results were recorded in each of starch hydrolysis, Indol and H_2S production. Nitrate was not reduced to nitrite expect in case of isolate B5.

All isolates produced acid from glucose, glycerol,

Figure 3. Stem inoculated lupine plant through artificial wound inoculated by an effective *B. megaterium* isolate (B2) causing discoloration and softening to the surrounding tissues. The infection extended to the adhering leaf.

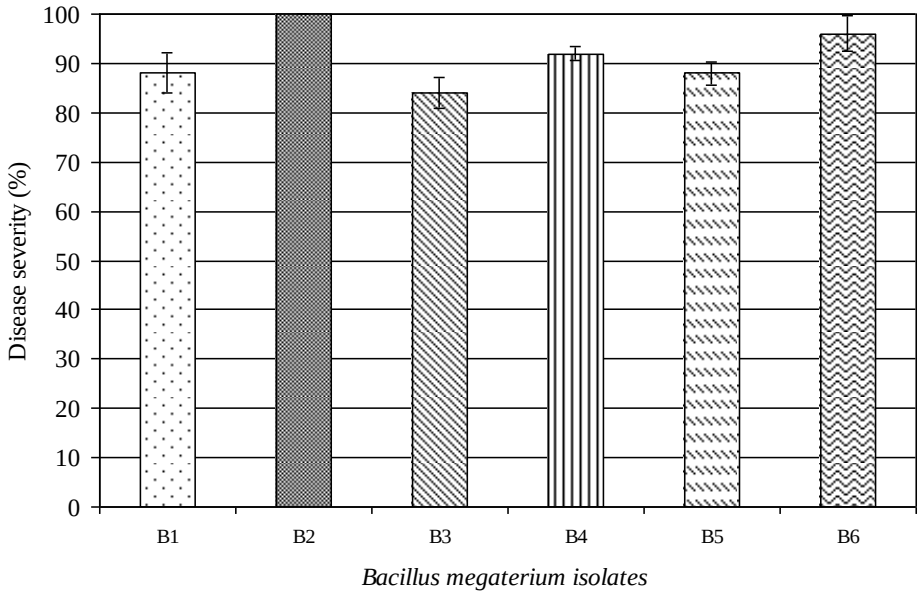

Figure 4. Pathogenicity test of bacterial isolates obtained from naturally diseased lupine foliar cv. Balady. Mean ± SDs for three replicates per treatment are shown.

maltose, raffinose and xylose but not from sorbitol or dextrin. Acid was also produced from sucrose, fructose and mannitol by the growing isolates except isolate B4. The isolates except isolate B5 gave negative results in gluconate and arginine dehydrolyzate tests. All the isolates utilized citrate and fumarate as a sole carbon source but not benzoate or tartarate. However, they varied in utilization of each of malonate, acetate and

Table 2. Morphological, physiological and biochemical characters of *B. megaterium* isolated from lupine foliage plants.

Characters	Reaction					
	B1	**B2**	**B3**	**B4**	**B5**	**B6**
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+
Gram reaction	+	+	+	+	+	+
Spore forming	+	+	+	+	+	+
Growth at 45°C	+	+	+	+	+	+
Aerobiosis	Strict aerobic	Strict aerobic	Strict aerobic	Strict aerobic	Strict aerobic	Strict aerobic
Starch hydrolysis	-	-	-	-	-	-
Nitrate reduction to nitrite	-	-	-	-	+	-
Indole formation	-	-	-	-	-	-
V.P. test	-	-	-	-	-	-
Gelatin liquefaction	+	+	-	+	-	+
Oxidase	-	-	-	(+)	-	-
Catalase	+	+	+	+	+	+
Casein hydrolysis	-	+	-	+	-	+
Growth in						
5% NaCl	+	+	+	+	+	+
7% Nacl	-	-	-	-	-	-
Urease	-	-	-	+	-	-
Rot potato slices	-	+	-	-	-	+
Acid from:						
Glucose	+	+	+	+	+	+
Succrose	+	+	+	-	+	+
Fractose	+	+	+	-	+	+
Mannitol	+	+	+	-	+	+
Xylose	+	+	(+)	(+)	+	+
Maltose	+	+	+	+	+	+
Arabinose	-	-	+	+	-	-
Galactose	+	+	-	-	+	+
Glycerol	+	+	+	(+)	+	+
Lactose	+	-	-	-	+	-
Raffinose	+	(+)	(+)	(+)	+	+
Sorbitol	-	-	-	-	-	-
Mannose	-	+	+	-	+	+
Dextrin	-	-	-	-	-	-
Hypersensitive reaction tobacco leaves	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-
2- Ketogluconate test	-	-	-	-	+	-
Lipolatic activity (tween 80)	-	+	+	+	-	+
Aesculin hydrolysis	+	+	+	+	+	+
Pectate hydrolysis	-	-	-	-	-	-
Utilization of:						
Malonate	-	-	-	(+)	+	-
Citrate	+	+	(+)	+	+	(+)
Acetate	(+)	-	-	(+)	+	-
Benzoute	-	-	-	-	-	-
Tartrate	-	-	-	-	-	-
Fumarate	+	+	+	+	+	+
Succinate	-	-	-	+	+	(+)

+ = Positive reaction, - = Negative reaction, (+) = Weak reaction.

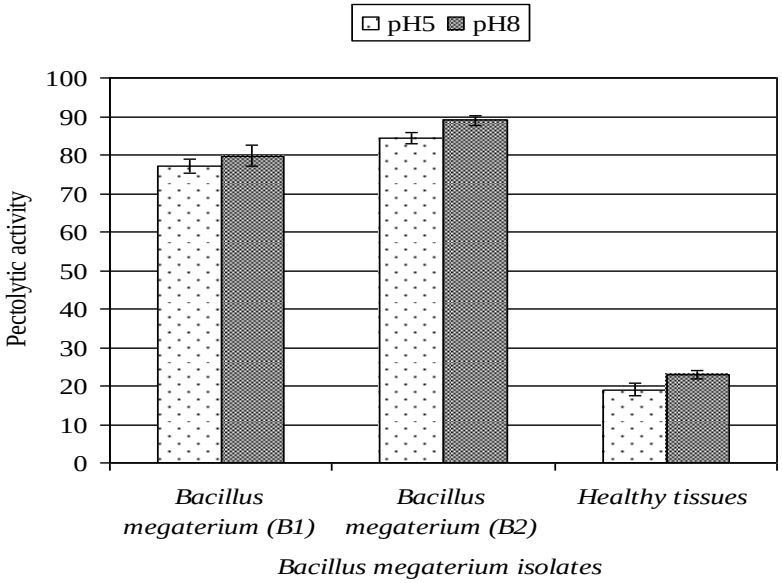

Figure 5. Pectolytic activity of extracts prepared from diseased foliar tissues infected with *B. megaterium* isolates B2 and B6 compared with healthy tissue extract**.** Mean ± SDs for three replicates per treatment are shown.

succenate.

According to Bergey's Manual of Systematic Bacteriology, 1986, these isolates resembles *B. megaterium* in the majority of the determinative tests except in starch hydrolysis hence it could be identified as pathogenic strain of the bacterium *B. megaterium*.

Assessment of pectolytic enzymes activity in diseased shoot and root system

Assessment of pectolytic enzymes secreted in diseased foliage by *B. megaterium* (B2 and B6) was assayed viscometrically. Great loss in viscosity of the pectin solution (enzyme substrate) was recorded according to the effect of the pectolytic enzyme(s) secreted in diseased tissues by bacterial isolates (Figure 5). While, the loss in viscosity took place at the two hydrogen ion concentrations (pH 5 and 8), the activity was higher at pH 8 in case of tissue extracts prepared from diseased shoot systems. The average of pectolytic activity means were 77.0, 79.7% and 84.3, 89.0% at two pHs due to the *B. megaterium* isolates B2 and B6, respectively and the enzyme activity was slightly higher at pH 8.

Assessment of cellulolytic enzyme activity

Diseased shoot system extracts showed marked cellulase activity when assayed at pH 6. Data present in Figure 6 show that the enzyme activity was 74.0 and 81.0% in case of the two bacterial infection (*B .megaterium* isolates B2 and B6) compared with 35.0% cellulytic activity in healthy tissues.

Varietal response

Foliage of the aforementioned cultivars was tested for their susceptibility towards six *B. megaterium* isolates. Data present in Figure 7 show that all bacterial isolates proved pathogenic towards the four cultivars being Balady cultivar the most susceptible. However, isolates B2 and B6 were more aggressive pathogen towards all tested cultivars especially Balady causing 94.5 and 97.7% disease severity. On the other hand, the Australian cultivar despite its susceptibility to the bacterial isolates, isolate B4 was less virulent on this cultivar.

Hast range

Effective isolates of *B. megaterium* isolates B2 and B6 which proved highly pathogenic on lupine plants were subsequently tested on other plant foliages representatives of 18 plant species as mentioned before to inquire about their host range. Both *B. megaterium* isolates B2 and B6 used in this study were avirulent to all inoculated plants, however, produced only small necrotic spots on faba bean leaves of the inoculated plants (Data not shown).

DISCUSSION

Diseased lupine plants showing foliar blight symptoms

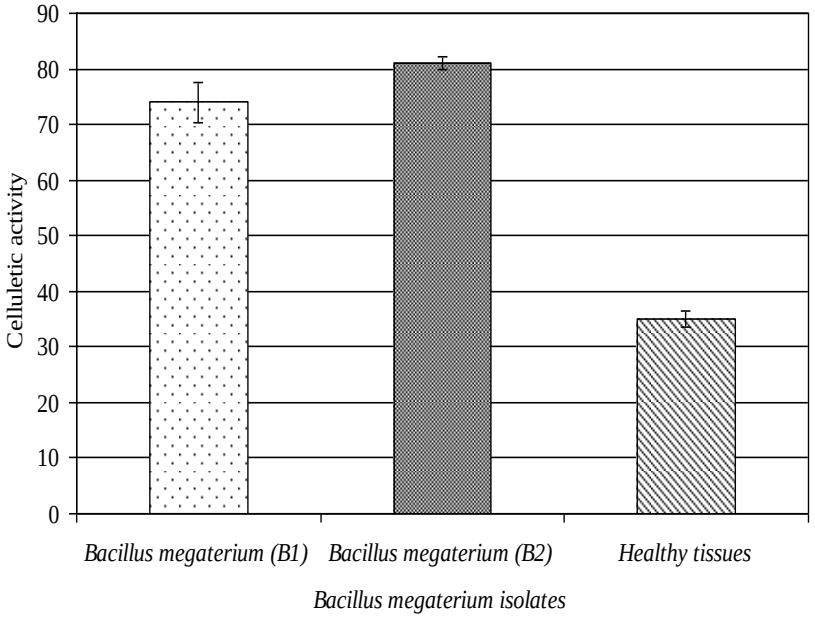

Figure 6. Cellulolytic activity of extracts prepared from diseased lupine foliar tissues infected with *B. megaterium* isolates B2 and B6 compared healthy tissue extract. Mean ± SDs for three replicates per treatment are shown.

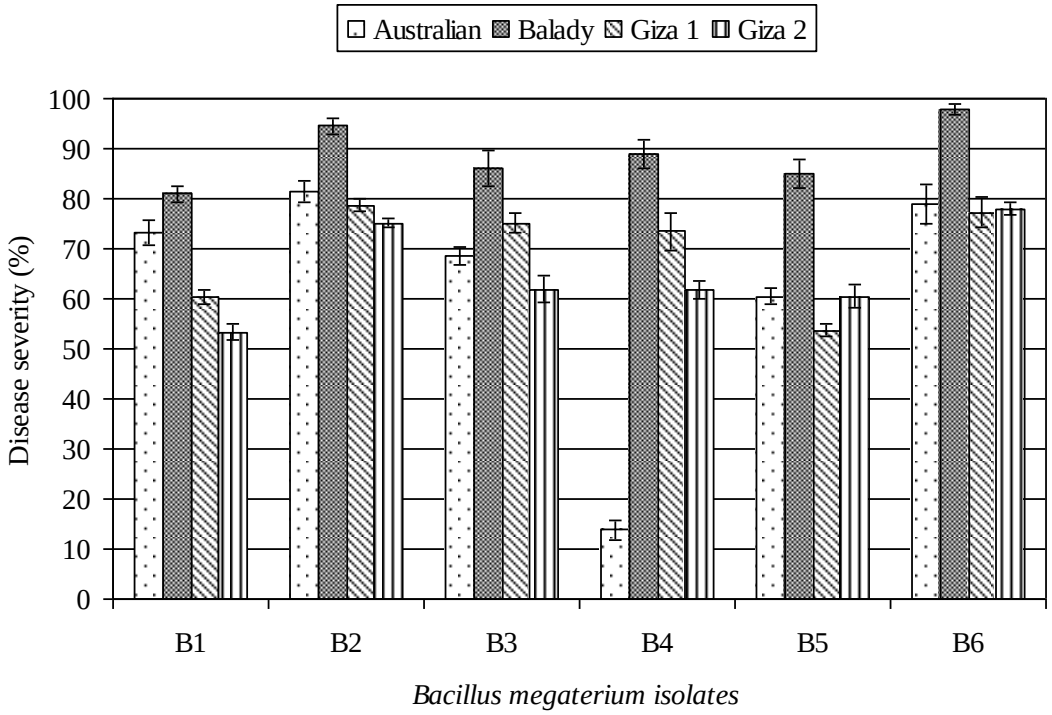

Figure 7. Cultivars response to lupine pathogenic *B. megaterium* isolates on lupine foliage. Mean ± SDs for three replicates per treatment are shown.

were collected from El- Minia Governorate for isolation trials. From diseased lupine foliage, the recovered bacterial isolates proved virulent on lupine plants in the pathogenicity test and incited symptoms on the subjected plants. The infected plants soon became blighted, turned grayish- brown and rather soft. The diseased plants bent

over and eventually collapsed in a shorter period.

The published reports concerning lupine foliar diseases is rather limited as compared with those reported for root diseases. Kalis- Kuznia et al. (1991); Yang et al. (1996), Bolland et al. (2001) and Yang and Sweetingham (2002) reported the fungus *Pleiochaeta setosa* to cause dark brown and sunken lesions on leaves and stems of lupine. Paulitz and Cote (1991) reported *Phoma* sp to incite reddish brown lesions on the lower stems of lupine. Muller et al. (1999) found lupine anthracnose caused by *Colletotrichum* sp has been a major cause of yield losses in Germany.

No bacterial pathogens were reported to be isolated from diseased lupine foliage. Hence the obtained bacterial isolates during the present work could be regarded as hitherto unrecorded bacterial pathogen of lupine. The bacterial isolates were all similar to each other and were spore formers, aerobic, Gram positive motile. The determinative tests were carried out on these isolates and according to Bergey's Manual of Systematic Bacteriology, 1986, the bacteria could be identified as *B. megaterium* according to the majority of the tested characters except starch hydrolysis, nevertheless deviation in one character does not exclude the bacterium from a given species.

It is worth noting that Hosford (1982) isolated a spore forming bacterium causing white blotch to wheat in North Dakota. The author identified the bacterium as *B. megaterium* pv. *cereals*. Also, Ismail (2004) in Egypt isolated *B. megaterium* from affected sunflower leaves. The bacterium could incite black spots on sunflower leaves when experimentally inoculated.

Tissue extracts prepared from experimentally diseased shoot systems showed great pectolytic and cellulolytic activities while healthy tissue extracts showed slight activities of the enzymes. It has long been known that some plant pathogens secreted tissue macerating enzyme that is, 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 (Saleh and Stead, 2003; Ismail et al., 2012).

In varietal response test four lupine cultivars namely cvs. Australian, Balady, Giza 1 and Giza 2 were tested for their susceptibility *B. megaterium*. All the tested cultivars were susceptible to all tested *B. megaterium* isolates. The six bacterial isolates similarity attacked the four tested cultivars being cv. Balady the more sensitive cultivar.

Infective two *B. megaterium* isolates were subsequently tested for their virulence on other foliar plants representatives of 17 plant species. None of the inoculated plants were affected with any of the *B. megaterium* isolates except production of small and limited necrotic spots on inoculated faba bean leaves. These results indicate the host specificity of this bacterium towards its host plant and accordingly justify the suggested forma specials *lupini* to be given to the lupine bacterial pathogen *B. megaterium*.

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