Serological Studies on *Pseudomonas Syringae* Pv. *Syringae* The Leaf Spot Disease Pathogen of Cane-Apple (*Arbutus Pavarii* Pampanini) in Jabal Al-Akhdar Area- Libya

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ABSTRACT

Polyclonal antiserum was produced against *Pseudomonas syringae* pv. *syringae* the causal agent of bacterial leaf spot on Cane-Apple (*Arbutus pavarii* Pampanini) in Jabal Al-Akhdar area- Libya. Results of the slide agglutination and Ouchterlony gel double diffusion tests showed positive reactions between bacterial isolate and its homologous antiserum. Regarding collecting dates of antiserum, indirect ELISA revealed that antiserum of the first collecting date (after 2 days of the last injection) was the best comparing with those of second and the third dates (7 and 14 days respectively) .Titre of the obtained antiserum was $1{:}10.24X10^4$ as determined by indirect ELISA. Indirect ELISA revealed the efficiency of this antiserum for comparing among the different bacterial isolates.

INTRODUCTION

Cane-apple is one of the evergreen trees and shrubs which are important in many countries. It contains 20 species all over the world. In Libya there is only one species (*Arbutus pavarii* Pampanini) that is growing naturally in Jabal Al-Akhdar area- Libya (Siddiqii, 1978). Recently, leaf spot disease was observed on Cane-Apple (Mohamed, 2005) (Fig. 1). Isolates of *Pseudomonas syringae* pv. syringae, the leaf spot pathogen were obtained from naturally infected leaves.

Rapid and accurate method for identification of the pathogen is important for effective control of disease and prevention the distortion of green plantation.

Serological tests are considered as one of the essential, rapid and important methods for identification of bacteria. Specially Enzyme Linked-

Immunosorbent. Assay (ELISA). Hamwieh *et al*. (1999) produced an antiserum to *Pseudomonas syringae* pv. *Pisi*, the causal pathogen of bacterial blight of pea and they mentioned the needs for rapid and sensitive methods to examine a high number of seeds to ensure its free from plant pathogenic bacteria. The produced antiserum gave a negative result when tested with *Pseudomonas syringae* pv. syringae in ELISA or slide agglutination tests as a negative control, although it is considered as a pathotype for the same species. Kayali *et al*. (2004) produced an antiserum to *Xanthomonas translucens* pv. *Undulosa,* the causal organism of bacterial stripe on wheat, they considered ELISA and slide-agglutination were the best serological tests for identification of the pathogen in contaminated seeds and plant samples, for their rapid application and high sensitivity and effectiveness of the antiserum. Al-Ani *et al*. (2004) depended on host range and serological studies to define the biotypes of the bacterium *Ralstomia solanacearum* which cause tomato wilt disease.

The objective of this study aimed to prepare an antiserum for *Pseudomonas syringae* pv. syringae to be used in identification of this bacterium in infected plant parts, define its titer and the best collecting time for antiserum and to study the efficiency of the antiserum in comparing among bacterial isolates from different areas.

MATERIALS AND METHODS

Antiserum production: Method of Al-Ani *et al*. (2004) was used for production of antiserum to *Pseudomonas syringae* pv. syringae which isolated from leaf spot on Cane-Apple trees that growing naturally at Blanje area in El-Gabal Al-Akhtar region. This isolate was supplied by Plant Protection Department, Faculty of Agriculture, Omer Al-Mokhtar University, Elbieda, Libya.

Antiserum was produced in New Zealand white rabbit by five intravenous injections, three days apart. Gradual volumes (0.5, 1.0, 1.5, 2.0, and 2.5 ml) of bacterial suspensions at a concentration of 10^8 cfu / ml prepared in phosphate buffer saline (PBS) were used.

Bleeding was carried out after 2, 7 and 14 days from the last injection. Each time blood was collected from the marginal ear vein then left for 2-3 hours at room temperature to clot and stored in refrigerator overnight. The antiserum was clarified by centrifugation at 5000 rpm for 10 min. and stored in the presence of 0.05% sodium azide at -20ºC till used (Kayali *et al*., 2004). Normal serum was obtained from rabbit before immunization to use as a control in subsequent tests.

Serological tests:

1- Slide agglutination test:

Method of Lyons and Taylor (1990) was used. Bacteria were grown on nutrient agar for 72 hours at 28 ºC. Bacterial growth was washed by PBS and suspension was prepared at a concentration of 10^8 cfu/ml. A drop (5 µl) of bacterial suspension was mixed on microscopic slide with 5 µl of produced antiserum as well as with normal serum for check. Positive agglutination reaction could be observed through 60 s period.

2- Ouchterlony gel double-diffusion test:

Gel mixture was prepared by dissolving 0.7g agarose in 100 ml distilled water, autoclaved and after cooling 0.2g sodium azide added as a preservative. Gel was dispensed in Petri dish, and allowed to dry. One central well and six peripheral wells were punched in the agarose in a defined geometrical arrangement. Bacterial suspension was prepared at a concentration of 10^8 cfu/ml in sterilized distilled water, sonicated for 4 minutes to remove clumps (Hamwieh *et al*. 1999) and 10 µl were placed in the peripheral wells. 10 µl of produced antiserum was placed at the next day in the central well. Plate was maintained at 30°C and observed for precipitin lines after 24 hrs.

3- Indirect ELISA:

3.1. Determination of the optimum antiserum collecting period:

Indirect ELISA was carried out as described by Fegla *et al*., (1997) and Younes (1995). Bacterial suspension was prepared in coating buffer (0.05 M carbonate, pH 9.6) at a concentration of 10^8 cfu / ml and was sonicated for 2 min. Wells were coated by adding 100 µl of the immunogen to the bottom of the well (2 wells per each antiserum dilution) and incubated overight at 4°C. The plates were rinsed five times by flooding wells with PBST for 3 minutes each.

Eight dilutions of double fold up to 1:12800 for normal serum (as control) and the three antisera obtained after 2, 7 and 14 days of the last injection in serum buffer (PBS-Tween 20 containing 2% soluble polyvinylpyrrolidone, 0.2% BSA) were used. 100 µl aliquots from the diluted normal serum and antisera were added to each well, after which the plates were incubated at 37°C for 2 hours, then washed as before.

Goat anti-rabbit gamma globulin conjugated to alkaline phosphatase (Sigma No 2937) was diluted 1:20000 in serum buffer, and 100 µl were added to each well, followed by one hour incubation at 37°C, and then washed as before.

100 µl of the enzyme substrate, 0.5 mg/ml paranitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8 were added to each well and incubated at room temperature (25°C) for about 30 minutes. The enzyme activity was stopped by adding 50 µl of 3 M NaOH. The ELISA values measured by ELISA reader (BioRAD, 550) were expressed as absorbency at 450 nm and absorbency values of at least double that of the healthy control were considered positive.

3.2. Determination of antiserum titer:

The titer of *Pseudomonas syringae* pv. syringae antiserum was determined by using indirect ELISA as described previously. Bacterial suspension was prepared in coating buffer (0.05 M carbonate, pH 9.6) at a concentration of 10^8 cfu / ml and was sonicated for 2 min. Wells were coated by adding 100 µl of the immunogen to the bottom of the well (2 wells per each antiserum dilution). Serial dilutions of double fold up to 1:409600 of antiserum in serum buffer were used. Data were calculated according to O. D. at 450 nm.

3.3. Efficiency of the antiserum for comparing among bacterial isolates from different areas.

Ten isolates of *Pseudomonas syringae* pv. syringae isolated from infected samples of leaf of Cane-Apple (*Arbutus pavarii* Pampanini) collected from different areas of Jabal Al-Akhdar region ; Blanje, Alghariqea, Ras-Etrab,Lamloda, Alkob Abied, Ras halal, Kafanta, Habon, El-zarda and Wasatia were supplied by Plant Protection Department, Faculty of Agriculture, Omer Al-Mokhtar University, Elbieda, Libya. (Mohamed, 2005). Indirect ELISA previously described was used to demonstrate the relation between the ten bacterial isolates through their reaction with the produced antiserum against Blanje isolate. Bacterial suspension was prepared in coating buffer (0.05 M carbonate, pH 9.6) at a concentration of 10^8 cfu / ml for each isolate as well as the associated non-pathogenic isolate (*Xanthomonas* sp) to be used as negative control beside the normal serum. Eight dilutions of double fold up to 1:12800 were used.

RESULTS AND DISCUSSION:

1- Slide agglutination test:

Results showed clear precipitin between the tested isolate (Blanje isolate) and its homologous antiserum, while there is no precipitin appeared between the bacterial isolate and the normal serum such results indicate the efficiency of the produced antiserum in identification of *Pseudomonas syringae* pv. syringae the leaf spot disease pathogen of Cane-Apple. This results were in agreement with recent studies regarding possibility of

producing antiserum for identification of plant pathogenic bacteria as Kayali *et al*. (2004) who produced an antiserum to *Xanthomonas translucens* pv. *undulosa* the bacterial stripe pathogen of wheat, they considered ELISA and slide-agglutination the best serological tests for identification of the pathogen for their rapid application and high sensitivity and those of Hamwieh *et al*. (1999) who produced antiserum to *Pseudomonas syringae* pv. *pisi* the bacterial blight pathogen of pea to examine a high number of seeds for ensuring its free from plant pathogenic bacteria. Slide agglutination test is simple, rapid and easy to apply in the laboratory or in the field. So, these properties make it better than the other methods. Although, it is less sensitive and need bigger amount of antiserum.

2- Ouchterlony gel double-diffusion test:

Results of Ouchterlony gel double-diffusion test showed a relation between the bacterial isolate and its homologous antiserum which appeared in precipitation lines between the antiserum well and the bacterial isolate wells (Fig. 2) after 24 hrs of incubation the plate at 30°C. These results confirmed those of slide agglutination test of this research and in line with those of Kiraly *et al*. (1974), Rath and Addy (1977) and Al-Ani *et al*. (2004) who used Ouchterlony gel double-diffusion test for revealing serological relationship between a bacterium and its specific antiserum.

3- Indirect ELISA:

3.1. Determination of the optimum antiserum collecting period:

Results of indirect ELISA to determine the optimum period to collect the antiserum showed that the first collecting date (2days after the last injection) was the best followed by the second (7 days) and then the third (14 days) (Table 1). From our results it can be concluded that the efficiency of antiserum decreased as the collection period advanced. This is in contract with those of Hamwieh *et al*. (1999) and Kayali *et al*. (2004). This could be attributed to genera, species and types of the pathogenic bacteria involved in the tests.

3.2. Determination of antiserum titer:

Antiserum titer was determined by indirect ELISA. Positive ELISA values were obtained up to dilutions of 1:102400 and not with 1:204800 (Table 2). These results were in agreement with those of Hamwieh *et al*. (1999) and Kayali *et al*. (2004) who mentioned that, it is important to determine the antiserum titer to reduce its consumption.

Table 1. Indirect ELISA absorbance values (E 405 nm) of *Pseudomonas syringae* pv. *syringae* in various dilutions of its antiserum*

Serum dilution	Normal serum	Antiserum collection periods after the last injection		
		2 days	7 days	14 days
1: 10^2	0.864	2.106	1.936	1.825
1: $2X10^2$	0.657	1.596	1.505	1.567
1:$4X10^2$	0.422	1.115	1.017	0.833
1:$8X10^2$	0.166	0.886	0.564	0.559
1:$1.6X10^3$	0.212	0.589	**0.492**	**0.459**
1:$3.2X10^3$	0.189	0.492	0.350	0.364
1:$6.4X10^3$	0.168	0.438	0.252	0.189
1:$1.28X10^4$	0.097	**0.202**	0.182	0.108

*The experiment was repeated twice and ELISA absorbance values at 405 nm are average of two replicates each

*Absorbency values of at least double that of the healthy control were considered positive.

* 100 μl of 10^8 cfu / ml bacterial concentration was added to each well.

3.3. Efficiency of the antiserum for comparing among bacterial isolates from different areas:

Indirect ELISA was used to compare among ten isolates of *Pseudomonas syringae* pv. syringae, the leaf spot pathogen of Cane-Apple (*Arbutus pavarii* Pampanini), from different areas in Jabal Al-Akhdar region. Obtained data were presented in Table (3) which showed that there were a relationship among the five isolates ; Blanje, Alghariqea, Ras-Etrab,Lamloda and Alkob Abied, which differed from the other five isolates Ras halal, Kafanta, Habon, El-zarda and Wasatia. This results were in agreement with those reported by Hamwieh *et al*. (1999) who compared among *Pseudomonas syringae* pv. *pisi* isolates by ELISA. They attributed these differences to the presence of surface antigenic determinant on each isolate.

Table 2. Indirect ELISA absorbance values (E 405 nm) of *Pseudomonas syringae* pv. syringae in various dilutions of extract serum its homologous antiserum*

serum dilution	Normal serum	Antiserum
1: 10^2	0.651	2.103
1:2X10^2	0.347	1.971
1:4X10^2	0.149	1.671
1:8X10^2	0.063	1.325
1:1.6X10^3	0.037	1.079
1:3.2X10^3	0.025	0.648
1:6.4X10^3	0.025	0.297
1:1.28X10^4	0.022	0.179
1:2.56X10^4	0.025	0.068
1:5.12X10^4	0.014	0.066
1:10.24X10^4	**0.017**	**0.043**
1:20.48X10^4	0.017	0.027
1:40.96X10^4	0.021	0.020

*The experiment was repeated twice and ELISA absorbance values at 450 nm are average of two replicates each

*Absorbency values of at least double that of the healthy control were considered positive.

Table (3). Indirect ELISA of ten isolates of *Pseudomonas syringae* pv. *syringae* obtained from different areas at Jabal Al-Akhdar region

Serum dilution	Indirect ELISA absorbance values at 450 nm											
	C1	**C2**	**Iso.1**	**Iso.2**	**Iso.3**	**Iso.4**	**Iso.5**	**Iso.6**	**Iso.7**	**Iso.8**	**Iso.9**	**Iso.10**
1: 10^2	0.617	0.620	2.301	1.581	1.355	2.014	1.335	0.866	0.850	0.902	0.667	0.472
1: $2X10^2$	0.345	0.347	2.106	1.079	0.945	1.823	0.932	0.612	0.533	0.558	0.371	0.280
1:$4X10^2$	0.241	0.245	1.772	0.664	0.583	1.575	0.591	0.410	0.185	0.338	0.211	0.159
1:$8X10^2$	0.130	0.132	1.320	0.425	0.362	1.144	0.373	0.253	0.166	0.206	0.146	0.109
1:$1.6X10^3$	0.070	0.072	1.155	0.250	0.195	0.807	0.204	0.161	0.103	0.136	0.090	0.067
1:$3.2X10^3$	0.045	0.046	0.844	0.151	0.115	0.564	0.127	0.085	0.058	0.078	0.051	0.042
1:$6.4X10^3$	0.026	0.026	0.571	0.084	0.069	0.322	0.069	0.050	0.031	0.041	0.038	0.021
1:$1.28X10^4$	0.015	0.017	0.339	0.064	0.046	0.166	0.013	0.028	0.019	0.024	0.019	0.013

C1= normal serum, C2= *Xanthomonas* sp, Iso.1= Blanje area, Iso.2= Algharigea area, Iso.3= Ras-Etrab area, Iso.4= Lamloda area, Iso.5= Alkob Abied area, Iso.6= Ras halal area, Iso.7= Kafanta area, Iso.8= Habon area, Iso.9= El-zarda area and Iso.10= Wasatia area

Fig. 1. *Arbutus pavarii* naturally infected with bacterial leaf spot

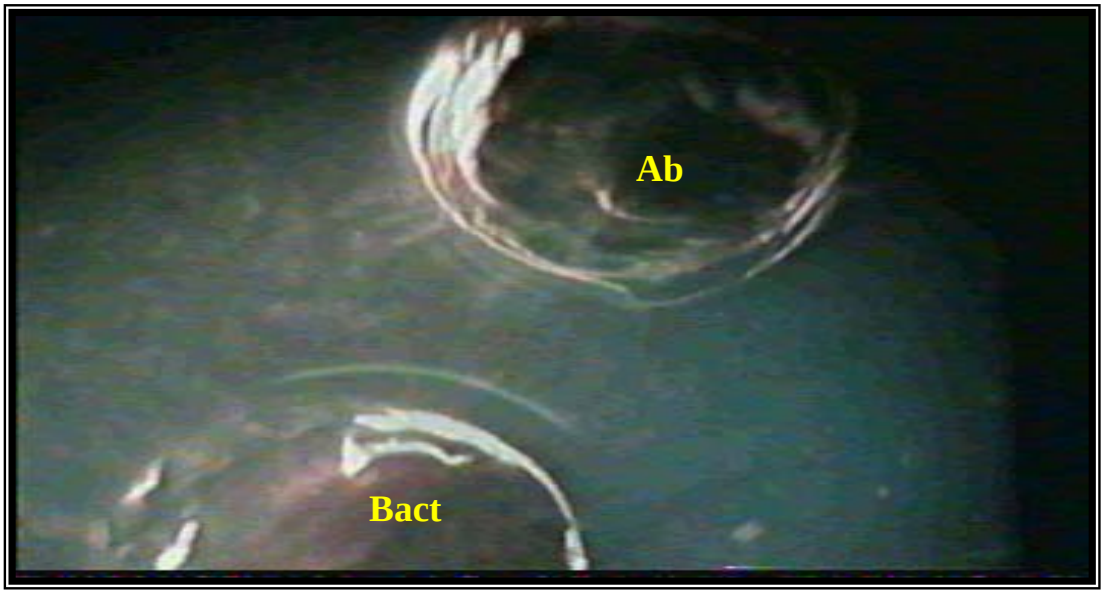

Figure 2. Precipitation lines between the antiserum well and the bacterial suspension well
Ab = Antibody well
Bact = Bacterial suspension well

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الملخص العربي

دراسة مصلية للبكتيريا *Pseudomonas syringae* pv. syringae المسببة لمرض تبقع نبات الشمارى بمنطقة الجبل الاخضر – ليبيا

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تم إنتاج مصل مضاد متخصص للبكتيريا *Pseudomonas syringae* pv. *syringae* المسببة لمرض التبقع البكتيرى على أوراق أشجار الشمارى فى منطقة الجبل الأخضر – ليبيا . أظهرت نتائج أختبارى التلبد على الشريحة والانتشار المزدوج فى الاجار تفاعل موجب بين البكتيريا المعزولة والمصل المضاد المتخصص لها. تبين بأختبار اليزا غير المباشرة أن أول سحبة للمصل المضاد (بعد يومين من اخر حقنة) كانت الأفضل مقارنة بالسحبة الثانية والثالثة (7 و14 يوم على التوالى). كان تركيز 1: $10X10.24^4$ هو أقل تركيز فعال للمصل المضاد تبعا لإختبار اليزا غير المباشرة. ولقد أتضح بأختبار اليزا غير المباشرة ايضا كفاءة المصل المضاد فى التفرقة بين عزلات البكتيريا.