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Morphological and biochemical characterization of *Xanthomonas arboricola* pv. *pruni*

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Abstract

This study was carried out to investigate the bacterial spot disease where the typical symptoms identified and were obtained two isolates from different locations of the Alghabal Alakhder (Al-Marj area, Al-Bayda). In this study, isolated bacteria were identified according to their response to the hypersensitivity reaction as well as the shape of the colonies on the different media (YDC, NA and King's B). In addition to physiological and biochemical tests. All isolates correspond to a mobile, aerobic, gram negative gram negative, positive for levane, and negative result with oxidase and arginine dehydrolase. The ability of isolated bacteria to induce the disease of spotting on almond leaf has been examined and has been determined by final validation of its disease on a variety of almonds. After 9 days of vaccination, a large number of small spots were observed on the fertilized leaves. They are identical with those *Xanthomonas arboricola* pv. *pruni*.

Keywords: *Xanthomonas arboricola* pv. *pruni*, YDC, NA, King B, Biochemical characterization tests.

Introduction

Bacterial spot, caused by *Xanthomonas arboricola* pv. *pruni* (Xap), is a severe disease of *Prunus* spp. across the world.

Particularly, the fruit crops almond, peach cherry, plum, and apricot are the main targets of Xap (12). Bacterial spot was first described in 1902 on plums in North

America (36), and it is referred to as bacterial leaf spot, shot-hole, and black spot. Different disease symptoms were observed on leaves, twigs, and fruits, Almond (*Purnus amygdalus*) is the fifth nut tree in Libya in order of its economic importance it is estimated that about 2,680,000 trees with a production of 8,049 tons were found in Libya the most common varieties in EL-GEBEL AL.AKHDER (11). Almond are not only beautiful deciduous trees, but also nutritious and delicious, leading many gardeners to grow their own. Even with the best care, almonds are susceptible to their share of almond tree diseases. Bacterial spot of stone fruits is a plant disease caused by *Xanthomonas arboricola* pv. *pruni*, which affects a wide range of *Prunus* species, including fruit crops and ornamental species of great economic interest (13,19,22,24,29).

Xanthomonas arboricola pv. *pruni* is a Gram-negative plant-pathogenic. Bacterium that causes spot disease of stone fruits and almond, one of the major threats of *Prunus* fruit crops. Symptoms occur on leaves, fruits, and twigs, ranging from necrotic angular lesions on leaves and sunken lesions on fruits to cankers on twigs. *X. arboricola* pv. *pruni* (36) can be very damaging when severe infections occur on highly susceptible cultivars. This

weakening the vigor of the tree year by year, and decreasing the fruit quality and production severely (34).

bacterium is considered a quarantine pathogen in the European Union phytosanitary legislation and in the European and Mediterranean Plant Protection Organization (12). *X. arboricola* pv. *pruni*, a warm, moderate season with temperatures of 19-28°C and with light, frequent rains accompanied by fairly heavy winds and heavy dews is most favorable for severe infection (3,9,13). The disease tends to appear and spread in the spring, and then makes little progress through the summer, but late infections occur in the autumn. In culture, bacteria have survived ice-box conditions of -2°C to +2°C for 5 months. The disease is not usually found in arid regions. However it is most serious in areas with warm and wet or humid conditions during the growing season (3,9,13). Currently, the disease is known to occur in all continents (2,5,6). The disease mainly causes lesions on leaves and fruits, but also cankers on twigs. The economic impact of the disease consists of a reduced quality and marketability of fruits, reduced orchard, and productivity and increased costs of nursery productions (30).

The purpose of the research was to detection and identification of *Xanthomonas arboricola* pv. *pruni* and provide an overview of *X. arboricola* pv. *pruni* diseases of almond trees

**Material and Methods **

Pathogenic Strains:

Collection of diseased sample: The infected leaves showing typical symptoms on almonds bacterial spot were surveyed and collected during autumn season 2016-2017 from two district areas of AL-Gabel AL.Akhder region.

Isolation of pathogen: The infected leaves of almond showing typical symptoms of bacterial spot were collected. The leaves were washed and pieces of infected tissue were cut aseptically from the edge of typical spots with a little portion of healthy tissue. These tissues were surface sterilized with 0.1 per cent sodium hypochloride solution for 60 sec. It was washed with sterilized distilled water for four times to remove traces of sodium hypochloride solution. On a clean slide 1-2 drops of sterilized distilled water was placed, a piece of diseased tissue was kept on water drop and teased well with a sterilized scalpel. The water drop from slide was taken with a bacterial inoculation loop and streaked on a Petri plate containing Nutrient Agar media (NA). The

inoculated Petri plates were kept for incubation at 28 +/- 1 C incubator for 72 hours (13).

Hypersensitivity reaction:

Pathogenicity of the obtained isolates was tested on tobacco plants (*Nicotiana tabacum*) with the method described by (20). Each bacterial isolate was suspended in sterile distilled water and inoculated on the underside of tobacco leaves by injection. Sterile distilled water was used as a negative control. The plants were kept under greenhouse conditions (28°C +/- 1) and the observations for a hypersensitive reaction were conducted after 24 h.

Pathogenicity tests In vitro: This method determines the pathogenicity of the bacterial isolates and it was done following the standard procedure (23). The tests were performed by immersing leaf of almond into bacterial suspensions calibrated at 1×10^6 CFU/ml. This method allowed the homogeneous and reproducible inoculations and limited inoculum dispersal in the growth chamber. Two leaf were inoculated at 28°C with 16 h of light, 95% of Relative humidity (RH) / 25°C, 8 h of darkness. The symptoms were recorded on the 11th day following the inoculation (8). Disinfested leaves were placed on sterile filter paper. Using a sterile, 3 ml syringe (needle removed), bacterial suspension was loaded and

placed firmly against the abaxial surface. Leaves were infiltrated by applying gentle pressure until a 2-3 mm water soaked spot appeared on the leaf. The soaked spot eventually disappears. Several spots (4 for each leaf) were applied per leaf half, approximately 1 cm apart. Inoculated leaves were placed in Petri dishes on the surface of water agar (1.5%) with the abaxial side up. plates were left at room temperature on lab bench. Development of symptoms on leaves was evaluated periodically up to 21 dpi. Lesion counts were performed at 14 dpi. The leaves were placed randomized in plastic petri supplemented with sterilized water agar media and incubated for 48 h at 28 °C (33).

Identification of the spots bacteria on almonds leaves:

Morphological, physiological and biochemical tests: The identification of the pathogen involved in bacterial spot of almonds was determined by conducting studies on its morphological, cultural, biochemical and physiological features of the pathogen as per standard microbiological procedures (7,10,20).

Purification and maintenance of bacterial culture: The typical bacterial colonies were picked up with the help of sterilized inoculation loop and streaked on the Petri plates containing Nutrient Agar media. The Petri plates were kept for

incubation at $27 \pm 1^{\circ}\text{C}$ for 48 hours. Observations were made for the development of well separated typical yellow, mucoid colonies, such pure colonies were further streaked into the Yeast Extract Calcium Carbonate Glucose Agar (YDC) slants and the culture were maintained in the refrigerator at 5°C which served as a stock culture for further studies.

Morphological characters: The morphological characteristics of the pathogen such as cell shape, gram reaction, staining characters were studied as per the standard procedures described by (3,35). The gram staining was reconfirmed by potassium hydroxide (KOH) solubility test.

Shape of bacterial cell: The shape of bacterial cell was studied by negative staining using 10 percent Nigrosin solution. A loopful of bacterial suspension was mixed with an equal amount of the Nigrosin solution on the glass slide and spread evenly on a microscope slide, allow to air dry and examined under the microscope.

Clonial morphology on differential media: The most frequently used media for isolation are standard non selective (Nutrient Agar), Phosphatase production was tested on bacterial isolates which precultured on plates with King's B

medium plates at 28°C for 24 hrs and semi-selective media generally used for *Xanthomonas* spp. (YDC) (23,35). The media were prepared and sterilized by autoclaving at 121°C for 15 minutes (15 lbs pressure). The media were poured into the sterilized Petri plates and kept at room temperature to eliminate the surface moisture before use. A loopful of 48 hours old bacterial culture was used. The bacterial suspension was uniformly spread over the surface of the medium to obtain well separated bacterial colonies. The inoculated plates were incubated at 28°C for 72 hours. Observations were write for colony characters of the pathogen (3).

Biochemical and physiological tests: The biochemical and physiological characters of the isolates of the bacterium were studied for hydrolysis of starch, catalase test, gelatin liquefaction, hydrogen sulphide production, oxidase test, casein hydrolysis, xanthan gum production test, motility test, acid from different sugars viz. glucose, fructose, sucrose and requirement of oxygen. The tests were conducted as per the methods described by (1,14,35).

Potato rot test: Fresh potato tubers were washed and peeled, alcohol was flamed, and sliced into approximately 7 mm - 1 cm width (1slice for each isolate and 1slice for

control). The slices were dipped in alcohol and flamed, placed in Petri-dishes, then sterile distilled water was added to a depth of half of the slice. Nicks were formed in the center of the potato with a sterile tool and inoculate with 100 µl of bacterial suspension.

**Results **

Isolation of bacterial spot disease: The causal organism *Xanthomonas arboricola* pv. *pruni* was isolated from infected leaves showing typical symptoms of bacterial spot. Figure (1), the resulting bacterial suspensions per plate were spread on YDC and NA incubated from 2 to 6 days at 28°C. The isolated colonies were sub-cultured three times for purification and then transferred to nutrient agar medium with glucose before performing biochemical and physiological tests. Isolation was made from the infected tissue by streaking technique on Nutrient Agar produced typical *Xanthomonas* colonies in 48 hours. The colonies were yellow, convex, smooth, flat, glistening, shiny and round in shape Figure (2).



Figure(1). leaves of almond showing typical symptoms on field.



Figure(2). Isolation and purification of *Xanthomonas arboricola* pv. *pruni*.

Hypersensitivity reaction: *Nicotiana tobaccum* var. *xanthi* was used for the hypersensitive reaction. Rapid collapse and water soaking of infiltrated tissue within 24 h or at the most 48 h followed by

a dry, light-brown localized necrosis within 3 days was observed, which indicates that the test isolates were positive to hypersensitive response Figure (3).

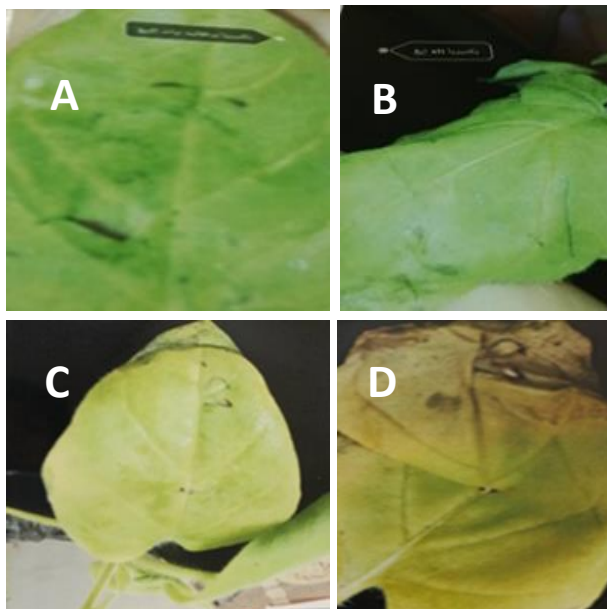


Figure (3). Hypersensitivity reaction of tobacco; (b) = pathogenicity tests by immersing; (c) = spread of small spots and dry leaves; (d) = plant death.

Pathogenicity test: For proving pathogenicity, the bacterial cell suspension (10^6 cfu/ml) of the isolates was sprayed on

to the leaves of almonds. The characteristics symptoms were observed on almond leaves after ten days of

inoculation as small, water-soaked lesions, which became circular to irregularly shaped, dark brown spots on the leaves Figure (4). Re-isolation were carried out

from lesions and comparisons were made with the original culture to confirm the identity of the pathogen.



Figure (4). Pathogenicity test of leaf almond in water agar media.

Identification of the spots bacteria on almonds leaves :

Morphological, physiological and biochemical test, Clonial morphology on differential media: Well separated out colonies isolated from the infected leaves were purified by streaking on the surface of Nutrient Agar Medium. The culture was maintained on Yeast Extract Calcium

Carbonate Glucose Agar (YDC) slants at 5⁰C. These were kept as the stock cultures for further studies. The bacterial colonies on YDC medium were yellow, mucoid, convex, glistening and irregular to round in ,but colonies on King's B media were slightly raised, shiny, mucoid, smooth-domed Figure (5).



Figure (5). Colonies of *Xanthomonas arboricola* pv. *purni* on: A (Nutrient gar media) B:YDC media, C: King B

Shape of bacterial cell: The bacterium was rod shaped with rounded ends, cells appeared single, Gram negative. The suspension becomes viscous and form thread like slime when picked up with a

toothpick during KOH test, aerobic, having single polar flagellum. The cells readily stained with common strain such as crystal violet (Table 1).

Table (1). Morphological characteristics of *Xanthomonas arboricola* pv. *pruni*

Characters	Isolates
Shape	Rod
Occurrence/ arrangement	In Single
Flagellation	Monotrichous
Gram reaction	Negative
%3KOH	Viscous (+ ve test)

Physiological and biochemical tests: The results obtained on various biochemical and physiological characteristics of the pathogen are presented in (Table 9) which revealed that, the bacterium was found to be oxidase negative. It was positive for starch hydrolysis, catalase test after covering with few drops of hydrogen peroxide to a slide by a loopful of test bacteria, there was production of gas

bubbles. liquefaction of gelatin, produced hydrogen sulphide motility test, casein hydrolysis, required oxygen and utilized glucose, fructose and sucrose.

Potato rot test: After 3 days of incubation of potato tablets treated with bacteria at a temperature of 28° appeared colonies of yellow bacteria with a large viscosity and became potato tissue with a clear texture and took yellow after 5 days (Figure 6)



Figure (6). Potaro rot test.

Table (2). Biochemical characteristics of *Xanthomonas arboricola* pv. *Purni*.

Biochemical tests	Isolate I	Isolate II
Starch hydrolysis	-	-
Levan production	+	+
Gelatin liquefaction	+	+
Hydrogen sulphide reaction	+	+
Oxidase reaction	-	-
Catalase reaction	+	+
Casein hydrolysis	+	+
Motility test	+	+
Growth at 35°C	+	+
Acid produce from		
1. d-arabinose galactose	+	+
2. d-glucose	+	+
3. Maltose	+	+
4. Mannose	+	+
5. Sorbitol	+	+
6. Sucrose xylose	+	+

(+) positive reaction (-) negative reaction

**Discussion **

The causal organism was isolated from the infected leaves by following standard streak plating technique using Nutrient Agar medium. Repeated isolation of the isolates collected from different areas yielded well separated, typical, yellow, mucoid bacterial colonies on Nutrient

Agar medium after 48 hours of incubation at 27 °C. Culture was purified by streaking suspected single colony on to the Nutrient Agar medium. The isolates produced pale yellow to dark yellow colonies with mucoid and convex appearance on Nutrient Agar medium. Bacterial cultures were maintained on Yeast dextrose

carbonate agar (YDC), King B slants. Similar, observation were also made by (15,16,17,18,22,25,26,27,28,31).

Biochemical and physiological characters, with the results of present study, it was observed that, the bacterium can hydrolyze the starch and casein, liquefied the gelatin, produced xanthan gum, motility test and was positive for H₂S production, catalase, required oxygen and negative for oxidase enzyme activity. The organism utilized various carbon sources viz. glucose, sucrose, fructose and produced mild acid from these carbon sources. These biochemical characteristics identified in the present investigation were in accordance with the results obtained by (4, 20, 22, 31, 32, 37). Studies on morphological characteristics of the pathogen indicated that the bacterium was rod shaped with rounded ends occurred singly or rarely in pairs, gram negative, with single polar flagellum. The results obtained in the present study on

morphological characters were in agreement with the reports of earlier workers (3,28,31), Pathogenicity tests. Approximately 7 days after inoculation the two strains *X. arboricola* pv. *pruni* strains isolated in AL-Gabel AL-Akter from almond leaves and the 2 strains obtained from different collections induced water soaked angular leaf spots. These spots turned into brown lesions during the next 7 days. Symptoms caused by all *X. arboricola* pv. *pruni* strains were similar on detached almond leaves, thus confirming pathogenicity of all isolates of the pathogen previously identified as *X. arboricola* pv. *pruni*

All the biochemical characters under present study were co-related with the characters reported by (21). Where all the isolates of *Xanthomonas* were subjected to different biochemical tests and were found positive for 3 per cent KOH test, gelatin liquefaction, catalase test while negative for starch hydrolysis and oxidase tests (13)

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الصفات المورفولوجية والبيوكيماوية لبكتيريا التبقع

Xanthomonas arboricola pv. *pruni*

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أجريت هذه الدراسة للتحقق في مرض التبقع البكتيري حيث تم التعرف على الأعراض النموذجية و حصلنا على اثنين من العزلات من مواقع مختلفة من الجبل الاخضر (المرج، البيضاء). في هذه الدراسة، تم تحديد البكتيريا المعزولة وفقا استجابتها لتفاعل فرط الحساسية كذلك شكل المستعمرات على الوسائط المختلفة (YDC ، NA ، King's B) وكذلك الاختبارات الفسيولوجية والكيميائية الحيوية. جميع العزلات تتوافق علي انها متحركة، هوائية، عصوية سالبة لصبغه جرام، موجبة لليفيان، ونتيجتها سلبية مع أوكسيديز وأرجنين ديهيدريولاز . وقد تم فحص قدرة البكتيريا المعزولة للتسبب في مرض التبقع على أوراق اللوز ، وقد تم تحديدها النهائي من خلال التحقق من أمراضيتها على مجموعة متنوعة من اللوز. بعد 9 أيام من التلقيح، لوحظ ظهور عدد كبير من البقع الصغيرة على الأوراق الملقحة. فهي متطابقة مع *Xanthomonas arboricola* pv. *pruni*

الكلمات الدالة : *Xanthomonas arboricola* pv. *pruni* , King B, NA , YDC , اختبارات التصنيف البيو كيميائية.