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Biological, serological and molecular identification of *Xathomonas* sp antibiotic resistance infecting fruit and vegetative crops

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ABSTRACT

Antibiotics have been used for the control of plant pathogenic bacteria and it has become a problem in pathosystems. The current study aimed to detection plasmid curing in species of the genus Xathomonas antibiotic resistance isolated from citrus, almonds and beans. ***Methods:*** Common bacteria blight disease in citrus , almond and beans were detected by external symptoms and DAS-ELISA .The casual against was detected and isolated on selected media by morphological and biochemical characteristics of the colonies, Indirect immunofluorescence assay, bioassay of leaf detached leaves and *Hgene* by PCR .The isolated bacteria were tested antibiotic sensitivity.

Results: Typical symptoms of common blight disease *Xanthomonas* sp on citrus, almond and beans that, necrotic spots surrounded by chlorotic haloes, sunken and corky lesions with oozing gum. Bright yellow spots on the underside of leaves, followed by erumpent brownish lesions. Symptoms were recorded at late summer through to autumn 2017. The canker, chlorotic haloes and halo blight spots gave positive serological reaction with polyclonal antibodies specific *Xanthomonas* sp by DAS-ELISA . *Xanthomonas* sp was isolated on YPGA medium and yellow pigment colonies from naturally infected citrus, almond and beans leaves. *Xanthomonas* sp isolates were identified which showed some differences of physiological and biochemical properties. *Xanthomonas* isolates inoculation of intact .immature leaves of *Nicotiana tabacum cv* samson showed 65–75% to fully expanded were preferred and the lesions develop 15–20 days. Three *Xanthomonas* sp isolates gave positive serological reaction with polyclonal antibodies specific *Xanthomonas* sp *by* DAS-ELISA. The *Hgene* sequence in the genome of *Xanthomonas* spp isolates suggested the original assignment of this isolates to the species *X.citri*, *X.arboricola* and *X. campestris* based on *Hgene* sequence *X.campestris* pv. campestris str. ATCC 33913. Phylogenetically sequence comparison confirmed that *Xanthomonas* spp isolates closest *Hgene* genetic homologues all belong to *X. citri* subsp. citri, *X. arboricola* and *X. campestris* pv. phaseoli. Antibiotics sensitivity of *Xanthomonas* sp isolates showed resistance against Penicillin G, Streptomycin and more resistance against, Sulphadiazole and Tetracycline. On the contrary *Xanthomonas* isolates after plasmids curing treatment showed sensitivity where formed inhibition zone with different area to Penicillin G, Streptomycin, Sulphadiazole and Tetracycline.

***Keywords*:** Bacterial spot, *Xanthomonas* spp, Antibiotic sensitivity, *H gene*, plasmid curing

Introduction

Black rot, caused by the bacterium *Xanthomonas sp* is one of the most dangerous diseases of stone fruit and vegetative crops. In warm and wet conditions losses due to black rot may exceed up to 50% due to the rapid spread of the disease (Sain *et al.,* 2005). The disease may quickly black-rot lesions in field and rot after harvest due to secondary softrotting organisms. The control of bacterial diseases of plants are difficulties because of scarcity of known, recommended bactericides in the market and antibiotics are costly and may not be fully effective in all cases. On the other hand resistance bacteria against antibiotics have increased. (Sain *et al.,* 2008).

Antibiotic resistance in plant-pathogenic bacteria has become a problem in pathosystems. Where the genetic basis for resistance has been examined. For example, the strAB streptomycin-resistance genes occur in Erwinia amylovora, Pseudomonas syringae, and Xanthomonas campestris,

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and these genes have presumably been acquired from nonpathogenic epiphytic bacteria colocated on plant hosts under antibiotic selection. The knowledge of antibiotic resistance is critical to the development of robust resistance management strategies to ensure the safe and effective continued use of antibiotics in the management of critically important diseases (Sundin and Wang, 2018). The genetic basis for resistance has been examined, antibiotic resistance in plant pathogens has most often evolved through the acquisition of a resistance determinant via horizontal gene transfer. For example, the strAB streptomycin-resistance genes occur *in Erwinia amylovora, Pseudomonas syringae, and Xanthomonas campestris*, and these genes have presumably been acquired from nonpathogenic epiphytic bacteria colocated on plant hosts under antibiotic selection. The current study aimed to understand the antibiotic resistance in the isolates of genus Xathomonas isolated from citrus, almonds and beans and detection plasmid curing in species of the genus Xathomonas antibiotic resistance.

Materials and Methods

Collecting samples:

Leaves, stems, shoots and fruits were showed symptoms canker on trees and halo blight from late summer through to autumn, were collected at season 2017 from Qalubia governorate. Leaves, stems, shoots and fruits showed symptoms of Almond leave spot disease were collected at season 2017 from Elgable El-Khdar region in Libia (Fawziam, 2000).

Detection in symptomatic plants:

The bacteria infection was detected in naturally infected citrus , Almond and Beans plants based on distincted bacteria symptoms (CABI, 2006), Disease severity also depends on the susceptibility of the host plant species and cultivars (Goto, 1992). Morphological characteristics of the bacteria colonies on nutrient media, serologically kits were obtained from Agric. Research Center (ARC) provided by (LOEWE Biochemica GmbH Germany) by enzyme linked immunosurbant assay (ELISA) according to ISPM 27 (2006) and molecular testing by polymerase chain reaction (PCR) and bioassay of detached leaves.

Serological detection in plant according to ISPM 27 (2006):

Plant tissue is ground in freshly prepared antioxidant maceration buffer (polyvinylpyrrolidone (PVP)-10, 20 g; mannitol, 10 g; ascorbic acid, 1.76 g; reduced glutathione, 3 g; PBS, 10 mM, 1 litre; pH 7.2) .The sap extractions were filter-sterilized using a sterile 0.22 µm membrane. Aliquots of 25 µl of each bacterial preparation or plant tissues were coated a plastic wells by pipetted as well as positive and negative controls as are used for ELISA were allowed to air-dry. Commercially polyclonal antibodies were diluted with PBS (pH 7.2) and 25 µl of appropriate dilutions were added to the plastic wells coated. Plastic wells were incubated in a humid chamber at room temperature for 30 min. The droplets are shaken off The plastic wells were shacked , washed three times for 5 min each in PBS and then allowed to air-dry. 25 µl of the diluted anti-species gamma globulin-fluorescein isothiocyanate conjugate (FITC) was pipetted into each plastic wells. The plastic wells were incubated in the dark at room temperature for 30 min, washed and allowed to air-dry. Finally, 10 µl of 0.1 mmol/litre phosphate-buffered glycerine (pH 7.6) with an anti-fading agent is added to each plastic wells, which is then covered with a coverslip. The OD is measured using a spectrophotometer with a 405 nm filter for plant tissues. The slides are examined under immersion oil with a fluorescence microscope at 600× or 1000× magnification for bacteria.

Isolation of bacteria:

Freshly prepared sample extracts are essential for successful isolation of *Xanthomonas* sp from symptomatic plant material and stored at 4-8^{o}C until processing. *Xanthomonas* sp colonies with citrus, Almond and Beans which is also commonly isolated from canker lesions and halo blight and produces morphologically similar colonies on standard bacteriological media. Lesions and halo blight are macerated in 0.5–1.0 ml saline (distilled sterile water with NaCl to 0.85%, pH 7.0), and when required they may be disinfected before and with 1% NaClO for 1 min, rinsed three times with sterile distilled water, and pulverized. An aliquot of the extract was streaked on nutrient agar supplemented with

0.1% glucose (NGA), yeast peptone glucose agar (YPGA) (yeast extract, 5 g; Bacto Peptone, 5 g; glucose, 10 g; agar, 20 g; distilled water, 1 litre; pH 7.0). Filter-sterilized cycloheximide (100 mg/litre) can be added when necessary as a fungicide after autoclaving the media. Integration of kasugamycin and cephalexin in the medium (semiselective KC or KCB medium) inhibits several saprophytic bacteria and facilitates isolation of the pathogen (Graham *et al.,* 1989; Pruvost *et al.,* 2005).

Purification and identification of bacterial isolates:

Bacterial colonies obtained from all previously mentioned media were chosen and picked up according to variation in culture characteristics and colony formation then purified by streak-plate method on Nutrient agar medium. Pure isolates were maintained on slants of the same medium at 4^{o}C for subsequent identification. Almost all microscopically examinations and biochemical testing used for identification were carried out according to Bergey's manual, (2009), Collins and Lyne (2004) and Cheesbrough (2006). Antibiotics resistance before and after plasmid curing.The sensitivity test for Tetracycline was tested against *Xanthomonas* sp before and after plasmid curing by the disc diffusion method with the Tetracycline and the same concentration as before plasmid curing. Commercially prepared antibiotic discs (6 mm in diameter) belonging to different groups were chosen for investigating their potency against *Xanthomonas* sp isolated from infecting citrus, Almond and Beans plants. The discs were obtained from (Oxoid, UK.) Properties of the target antibiotics are listed in Table (1).

Table 1: Antibiotic sensitivity test for genus *Xathomonas* sp isolated from citrus, almonds and beans.

Scientific Name	Trade Name	Symbol	Disc Potency (mcg)
Chlorominiphcol	Chlorominiphcol	CN	30
Ampicillin	Ampicillin	CN	25
Sulphadiazole	Sulphadiazole	TE	30
Gentamicin	Gentamicin	CN	10
Kanamycin	Kanatrex	CN	30
PenicillinG	Penicillin	CN	10
Streptomycin	Streptomycin	CN	25
Tetracycline	Tetracycline	TE	30

In this test, the standard Kirby-Bauer disk diffusion method (Bauer *et al*., 1966) was performed in which, 8 commercially prepared antibiotic discs (6 mm in diameter) belonging to different groups . Four to five similar colonies from overnight growth plate were transferred aseptically in sterile distilled water and vigorously agitated to give a turbidity that matches the 0.5 McFarland standard (approximately 10^8 cfu/ml) according to D'Amato and Hochstein, (1982). Within 15min, sterile cotton swab dipped into the culture suspension was used for inoculating the surface of solidified Mueller-Hinton agar plates (NCCLS/CLSI, 2007). Antibiotic discs were dispensed onto the inoculated plate surface and gently pressed down using a sterile forceps to insure complete contact with agar. Within 15 minutes of applying discs, the inverted plates were aerobically incubated at 37^{o}C for 24h. The resulted diameters of inhibition zones around the antibiotic discs were measured to nearest whole mm and interpreted according to protocols standardized for the assay of antibiotic compounds as guided by National Committee for Clinical Laboratory Standards "NCCLS". The results were categorized as: R (resistant), I (intermediate sensitive), and S (sensitive) (Hindler, 1998 and NCCLS/CLSI, 2007).

Plasmid curing:

Plasmids can be eliminated (cured) from bacteria by several chemical and physical agents. Plasmid curing by elevated temperature according to Sambrook *et al.,* (1989). Bacterial growth (10^5cell/ml nutrient broth medium at late log phase) at elevated temperature 43^{0}C (5-7^{0}C above the normal growth temperature. The culture was diluted 1:20 by taking 0.5 ml of the Bacterial growth into 10 ml of nutrient broth and the culture was grown at 43^{0}C for 24 h. Two drops of the treated culture were placed on nutrient agar plates to obtain separated colonies. Individual colonies were tested the

loss plasmid –coded functions antibiotic resistance. Antibiotics resistance of after plasmid curing. The sensitivity test for Tetracycline was tested against Xanthomonas sp before and after plasmid curing by the disc diffusion method with the Tetracycline and the same concentration as before plasmid curing.

Detection by bioassays

Detached leaf enrichment.

The highly susceptible hosts, *leaves of N.tabacum cv.samson* were washed for 10 min in running tap water, surface-disinfected in 1% NAClO for 1 min, and aseptically rinsed thoroughly with sterile distilled water. The lower surface of each leaf is aseptically wounded by puncturing it with a needle and the whole leaves are placed onto 1% agar in sterile water in the wells of ELISA plates with their lower surface up. Droplets of 10– 20 µl of macerated from symptomatic leave tissues and added to the wounds. After 4–12 days at 25 ºC in a lighted incubator, pustule development is evaluated and *Xanthomonas sp* was isolated from either the pustules or the symptomless wounded leaf tissue as described above (EPPO, 1998) This bioassay is a very specific and sensitive (10^2 cfu/ml) diagnostic method (Verdier *et al.*, 2008).

Molecular detection

Molecular detection of *Xanthomonas* sp. by Heat Shock Gene :

Genomic DNA extraction.

All DNA isolation procedures were carried out in Biological Safety Cabinet in accordance with the Good Molecular Diagnostic Procedures guidelines of Millar *et al.* (2002). Extracted DNA was stored at - 80 ^{0} C prior to PCR amplification. For each batch of extractions, a negative extraction control was performed that contained all reagents minus any organism, as well as an extraction positive control with *Xanthomonas sp* standard.

Design of oligonucleotide primers (H Gene).

DNA sequence data of the heat shock protein genes (groES and groEL) were obtained from GenBank; conserved and variable regions were subsequently identified by aligning published sequences of Xanthomonas sp isolates by the CLUSTAL alignment method in the DNASTAR sequence alignment software package. A novel primer pair was designed, forward H1511 5' GACGTCGCCGGTGACGGCACCACCAC-3' H1261 5'-CGACGGTCGCCGAAGCCCGGGG CCTT-(26-mer)], which targeted conserved regions of the heat-shock protein gene yielding a fragment of 600 bp.

Thermocycling parameters.

An Eppendorf Mastercyler or a BioRad iCycler with 0.2mL thin-wall strip tubes and the instrument ramp rate set to approximately 2 degrees per second: 50% of maximum on the Eppendorf. 5 min at 94^{0}C 40 cycles of [30 sec at 94, 30 sec at annealing temp**, 45 sec at 72] 10 min at 72C **For H729/H730 our usual annealing temp is 50C. For H1594/H1595 we raise this to 57C. Some users report cleaner results using fewer cycles) (24-30 instead of 40**).**

PCR reaction components.

PCR reaction components (Hill lab generic recipe for amplification of PCR products for direct sequencing with H729/H730 or H1594/H1595). Each 50µL reaction contains: 1x PCR buffer, 2.5 mM MgCl2 *, 400nM each primer, 200µM dNTPs, Taq polymerase*, template DNA (variable), water to 50 µL. *We don't endorse any particular brand of Taq polymerase and have had success with many different types. Magnesium concentrations of 2-2.5 mM have proven the best in our lab.

PCR product purification.

PCR amplicons were purified by using a QIAquick PCR Purification kit (Qiagen) and eluted in Tris/HCl (10 mM, pH 8·5) prior to sequencing to remove dNTPs, polymerases, salts and primers.

Amplicons were sequenced in both directions on an ALFexpress II automated sequencer (Amersham Biosciences) by using both the forward and reverse primers, H1511 5' and H1261 which were labeled with Cy-5 fluorescent dye (Oligosynthesis Unit, The Queen's University of Belfast, UK) and used in conjunction with a Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing kit (Amersham Biosciences). The resulting sequences were aligned by employing MegAlign software (DNASTAR) and compared with those stored in GenBank by using BLAST alignment software.

Results

***Xanthomonas* blight diseases:**

The bacteria blight disease were diagnosed in citrus ,almond and beans plants cultivated in autumn season 2017 based on distinct *Xanthomonas* sp symptoms and DAS-ELISA . The casual against was detected by bioassay of detached leaves, morphological characteristics on nutrient media, Indirect immunofluorescence assay and PCR.

Symptoms and signs:

Symptoms of citrus canker was recorded on young trees from late summer through to autumn 2017. Bright yellow spots are first apparent on the underside of leaves, followed by erumpent brownish lesions on both sides of the leaves, which become rough, cracked and corky .The canker spot is corky or spongy, is raised, and has a ruptured surface branches , Crater-like lesions develop on the surface of the citrus fruit, (Fig.1)

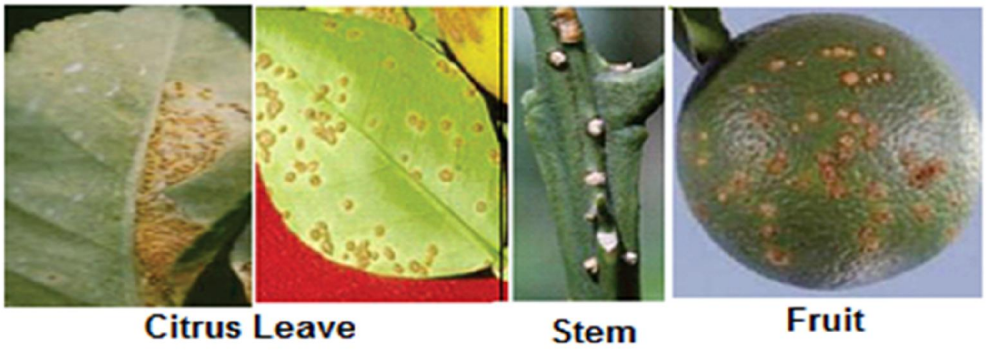

Fig. 1: Leave, Stem and fruits of citrus tree showing typical citrus canker symptoms surrounded by yellow halo , bright yellow spots underside of leaves and fruit.

Typical symptoms of common blight disease *Xanthomonas* sp on almond. Necrotic spots surrounded by chlorotic haloes on leaves, sunken and corky lesions with oozing gum on fruit. Lesions on a nut with dehydrated mesocarp, and dark brown spots on the nut endocarp (fig. 2).

Fig. 2: Leave, branch and fruits of almond tree showing Typical symptoms *Xanthomonas* sp. Necrotic spots surrounded by chlorotic haloes on leaves , sunkenand corky lesions with oozing gum on fruit.

Symptoms of common blight disease appeared similar to halo blight, but halo blight has leaf spots with large, pale yellow margins. Initial water soaked spots on bean pods (Fig. 3).

Fig. 3: Leave , and pods beans plant showing Typical symptoms of *Xanthomonas* sp. Necrotic spots surrounded by chlorotic haloes on leaves and dark brown spots on the nut endocarp.

***2*- Serological detection of Common blight disease.**

The samples should be processed following the general procedure recommended for the specific serological test to be used. Common blight disease in citrus, almond and beans gave positive results by DAS-ELISA consisted of a reference *Xanthomonas* stander (table, 1). Negative results consisted of healthy host plant extract.

Table 1: Detection of *Xanthomonas* sp in diseased citrus, almend and beans plants by DAS-ELISA using specific polyclonal antibodies for *Xanthomonas* in field.

Samples	DAS-ELISA		Samples	DAS-ELISA		Samples	DAS-ELISA	
citrus	*Symptoms	O.D.	almend	*Symptoms	O.D.	beans	*Symptoms	O.D.
Leaves	42/ Leaves	0.421	Leaves	16/ Leaves	0.378	Leaves	21/ Leaves	0.326
Leaves	25/ Leaves	0.374	Leaves	12/ Leaves	0.315	Leaves	25/ Leaves	0.412
Leaves	8/ Leaves	0.262	Leaves	5/ Leaves	0.223	Leaves	16/ Leaves	0.273
Leaves	11/ Leaves	0.245	Leaves	13/ Leaves	0.235	Leaves	11/ Leaves	0.215
Leaves	11/ Leaves	0.236	Leaves	13/ Leaves	0.234	Leaves	11/ Leaves	0.254
Leaves	Healthy	0.075	Leaves	Healthy	0.075	Leaves	Healthy	0.075
Branches	4 / 15 cm	0.191	Branches	4 / 15 cm	0.178	Stems	4 / 15 cm	0.187
Branches	5 / 15 cm	0.179	Branches	5 / 15 cm	0.195	Stems	5 / 15 cm	0.212
Branches	7/ 15 cm	0.202	Branches	7/ 15 cm	0.203	Stems	7/ 15 cm	0.214
Branches	3/ 15 cm	0.181	Branches	3/ 15 cm	0.185	Stems	3/ 15 cm	0.216
Branches	3/ 15 cm	0.176	Branches	3/ 15 cm	0.193	Stems	3/ 15 cm	0.204
Branches	Healthy	0.075	Branches	Healthy	0.075	Stems	Healthy	0.075
Fruits	13 /Fruit	0.301	Fruits	4 /Fruit	0.238	Fruits	5/ Fruit	0.210
Fruits	4 / Fruit/	0.174	Fruits	9 /Fruit	0.285	Fruits	4 /Fruit	0.202
Fruits	5 / Fruit	0.201	Fruits	5/ Fruit	0.211	Fruits	3/ Fruit	0.183
Fruits	9 / Fruit	0.255	Fruits	3/ Fruit	0.179	Fruits	5 /Fruit	0.218
Fruits	9 / Fruit	0.237	Fruits	6 /Fruit	0.184	Fruits	5/ Fruit	0.232
Fruits	Healthy	0.075	Fruits	Healthy	0.075	Fruits	Healthy	0.075

+V= 0. 523 OD, -V= 0.075 OD at 450 nm , *Number of spot lesion

3-Identified *Xanthomonas* sp.

Isolation of the causal organism was performed by streaking lesion extracts onto plates of nutrient agar (NGA), yeast peptone glucose agar (YPGA) and Wakimoto suitable media, on which colonies of *Xanthomonas* sp have a characteristic appearance. The colony morphology on all three media is round, convex and smooth-edged, and the colony is mucoid and creamy yellow. Growth is evaluated after incubation at 25–28 ºC for three to five days.

For serological *Xanthomonas* detection by Indirect immunofluorescence assay, a positive control consisted of a reference *Xanthomonas* resuspended in phosphate-buffered saline (PBS) , negative control consisted of suspension of a non-target bacterial species (for identification of

bacterial cultures) . *Xanthomonas* sp cells was serological precipitate reaction with specific polyclonal antibodies by DAS-ELISA. Freshly prepared substrate solution containing 1 mg/ml p-nitrophenyl phosphate in diethanolamine buffer (pH 9.8) is added (100 µl/well). The plates are incubated for 30–60 min at room temperature. The OD is measured using a spectrophotometer with a 405 nm filter. SX Positive samples are determined as for DAS-ELISA. It was found that the highest percentage of *Xanthomonas* sp cells infection were found in leaves of beans followed by citrus and almond leaves ranged 0.421 to 0.215 OD , followed by branches ranged 0.216 to 0.176 OD and fruit ranged 0.301 to 0.174 OD This method permits detection of approximately 10^3 cfu./ml.

Xanthomonas sp isolates (Citrus, almond and beans) were identified with pathogenicity test on *N.tabacum cv.* Samson. Assayed Leave of susceptible hosts by inoculation with a syringe showed the pathogenicity of bacterial isolates. Immature leaves that are 65–75% to fully expanded are preferred due to *Xanthomonas* sp isolates. On the other hand, Lesions develop 15–20 days after inoculation of intact leaves or detached leaves. Isolated bacteria grown in liquid media or colonies from a freshly streaked agar plate are resuspended in sterile distilled water and the concentration is adjusted to 10^6–10^8 cfu/ml for inoculation into hosts.

Xanthomonas sp isolates were a Gram-negative, straight, rod-shaped bacterium measuring 1.8–2.0 × 0.5– 0.75 µm. It is motile by means of a single polar flagellum. *Xanthomonas* sp isolates were showing some differences of physiological and biochemical properties . It is chemolithotrophic and obligatorily aerobic with an oxidative metabolism of glucose. The yellow pigment is xanthomonadin. Some of the biochemical characteristics that identify *Xanthomonas citri*, *Xanthomonas arboricola* and *Xanthomonas campestris* were listed in Table (2) .

Table 2: Morphological and biochemical characteristics of *Xanthomonas sp* isolated from citrus, almond and beans.

Test	***Xanthomonas* sp isolates**			
	Reference	**Citrus**	**Almond**	**Beans**
Shape	**Rode shaped**	**Rode shaped**	**Rode shaped**	**Rode shaped**
Gram stain	-V Gram	-V Gram	-V Gram	-V Gram
Enzymes				
Catalase	+	+	+	+
Oxidase	+	– or weak	– /+	+
Nitrate reduction	-	– /+	-	– /+
Hydrolysis				
Starch	+	+	+	+
Casein	+	+	+	+
Tween 80	+	+	+	+
Aesculin	+	+	+	+
Gelatin liquefaction	+	+	+	+
Pectate gel liquefaction	+	+	– /+	+
Utilization of asparagines	-	-– /+	-	-
Growth requires:				
Methionine	+	+	+	+
Cysteine	+	-	-	+
0.02% TTC (w/v)	-	– /+	-	-
Incubation Temperature	28°C	28°C	30°C	26°C
Plasmid	+	+	+	+

0.02% triphenyl tetrazolium chloride (TTC) (w/v)

Total *genomic DNA* was extracted from four *Xanthomonas* isolates (*Xanthomonas* Reference, XR , *X. citri*, XC , *X. arboricola* , XA and *X. campestris* XB were separated on agarose 1.5% (fig.4,A).The *yield of genomic DNA* were 4.5,3.2,1.5and 4.2 mg as well as the DNA purity (260/280 OD) were 1.3, 1.4, 2.3 and *1.5* for *Xanthomonas* isolates respectively **.**

Molecular identification for these isolates was done by using Heat Shock Genes. Total DNA were isolated from four selected bacterial isolates using Heat Shock Genes method. The H-Gene primers were H1511 GACGTCGCCGGTGACGGCACCACCAC-3' H12615'-CGACGGTCGCCGA

AGCCCGGGGCCTT-3.PCR product of H DNA gene for four bacteria isolates with expected size 600 bp(Fig. 4,B).

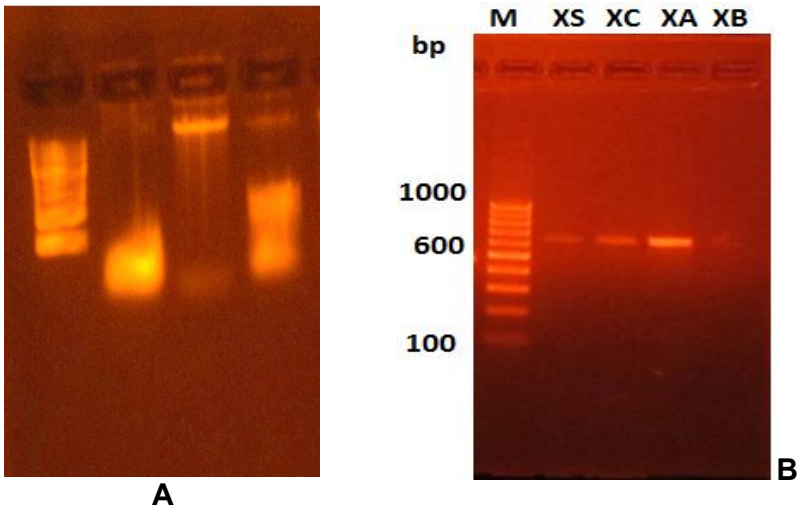

Fig. 4: Gel electrogram showing (A) total *genomic DNA* for *Xanthomonas* four bacteria and (B) PCR product of *H DNA gene* for four bacteria isolates with expected size 600 bp , M = DNA leader , XR = *Xanthomonas* Reference, XC= *X. citri*, XA= *X. arboricola* and XB= *X. campestris*.

A phylogenetic tree of *X. citri* , *X. arboricola* and *X. campestris* isolates and *Xanthomonas* Reference revealed Genetic distance with 0.07 a moderate degree of similarity to 12.3 isolate from Turkey isolated from pepper (Accession no. AJ303095) (Fig.6).

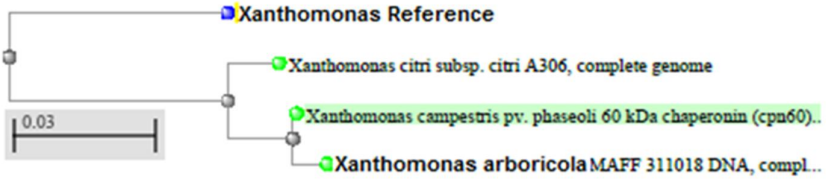

Fig. 6: Neighbour-joining tree of *Hgene* of *X. citri* , *X. arboricola* and *X. campestris* isolates and *Xanthomonas* Reference published in Gen Bank. Numbers represent bootstrap percentage

Antibiotics sensitivity of Xanthomonas sp isolates were showed differences response in Ampicillin Chlorominiphcol Gentamicin and Kanamycin antibiotics sensitivity. As well as three isolates and Xanthomonas References showed resistance against Penicillin G, Streptomycin (1 - 9.5 mm) and more resistance against, Sulphadiazole and Tetracycline (0 mm) (table, 3).

Table 3: Antibiotic sensitivity of different isolates of *Xanthomonas sp*

Antimicrobial Agents	**Disc Potency (mcg)**	***Xanthomonas* isolates**			
		***Xanthomonas.* Reference**	***X. citri***	***X. arboricola***	***X. campestris***
Ampicillin	25	28.5	25.5	25	12.5
Chlorominiphcol	30	4.5	6.5	7.0	5.5
Gentamicin	10	8.5	13.5	12.5	9.5
Kanamycin	30	15	10.5	14	13
PenicillinG	10	1.5	1	9.5	3.5
Streptomycin	25	4.5	1	7.5	2
Sulphadiazole	30	0	0	0	0
Tetracycline	30	0	0	0	0

*- R = resistance (0-10 mm), MS = moderately sensitive (11-25 mm); S-sensitive (more than 26 mm) CD (*P=0.05*) , Treatment (T) 2.78 , Genotype (G) 1.35, T x G. 0.75.

Sensitivity of cured plasmids to antibiotics.

The sensitivity test of *Xanthomonas* isolates after plasmids curing treatment to Chloramphenicol (30 mcg), Gentamicin (10 mcg), PenicillinG (10 mcg) , Streptomycin (25 mcg), Sulphadiazole (30 mcg), Tetracycline (30 mcg), Kanamycin (30 mcg) and Ampicillin (25 mcg) showing formed inhibition zone with different area (table, 4) according to antibiotics compared with non cured plasmids in table, (3).

Table 4: Antibiotic sensitivity of different isolates of *Xanthomonas sp* cured plasmids

Antimicrobial Agents	**Disc Potency (mcg)**	***Xanthomonas*. Reference**	***Xanthomonas* isolates**		
			X. citri	***X. arboricola***	***X. campestris***
Ampicillin	25	22.5	26.5	28	22.5
Chlorominiphcol	30	14.5	16.5	17.0	15.5
Gentamicin	10	8.5	13.5	12.5	9.5
Kanamycin	30	16.5	12.5	15.5	14.5
PenicillinG	10	11.5	14.5	13.5	12.5
Streptomycin	25	6.5	8.5	7.5	8.5
Sulphadiazole	30	13.5	15	4.5	15
Tetracycline	30	10.5	4.5	8.5	4.5

*- R = resistance (0-10 mm), MS = moderately sensitive (11-25 mm) ; S-sensitive (more than 26 mm) CD (*P=0.05*) , Treatment (T) 2.78, Genotype (G) 1.35, T x G. 0.75.

Discussion

Diagnosis of common *Xanthomonas* blight diseases in citrus, almond and beans can be achieved by observing external symptoms. Symptoms of citrus canker was recorded on young trees bright yellow spots of leaves, followed by erumpent brownish lesions , which become rough, cracked and corky (canker spot) and has a ruptured surface branches . Crater-like lesions develop on the surface of the citrus fruit. The disease characteristically causes scabs or crater-like lesions on the rind of fruits and on leaves stems and shoots. Symptoms of citrus canker can occur on seedlings in any season and on young trees from late summer through to autumn, when a flush of abundant growth of angular shoots occurs (CABI, 2006 and IPPC, 2016). The disease becomes sporadic as trees reach full fruiting development, because fewer angular shoots are produced and older leaf tissue and mature fruit are more resistant to citrus canker infection under natural conditions. Disease severity also depends on the susceptibility of the host plant species and cultivars (Goto, 1992 and IPPC, 2016). Typical symptoms of common blight disease *Xanthomonas* sp on almond. Necrotic spots surrounded by chlorotic haloes on leaves, sunken and corky lesions with oozing gum on fruit. Lesions on a nut with dehydrated mesocarp, and dark brown spots on the nut endocarp. Almond, leaf symptoms are similar to those observed on peach. In early spring, infected fruits show sunken corky lesions with oozing gum that streams or clumps. The sunken lesions become raised following dehydration of the meso- carp in summer. Sometimes circular dark spots appear on the endocarp and may affect the nut. Infected fruits either fall prematurely (Fawzia, 2000 and Lamichhane, 2014). Symptoms of common blight disease appeared similar to halo blight, but halo blight has leaf spots with large, pale yellow margins. Initial water soaked spots on bean pods. Several bacterial diseases infect common bean including common bacterial blight (CBB), halo blight, and bacterial brown spot caused by *X.axonopodis* pv. *phaseoli* (*Xap*), *Pseudomonassyringae* pv. *phaseolicola* and *Pseudomonas syringae* pv. *syringae*, respectively (Akhavan *et al.,* 2013) . Diagnosis of common *Xanthomonas* blight diseases in citrus, almond and beans can be achieved by serological testing by DAS-ELISA using polyclonal antibodies (Álvarez *et al.,* 1991 and IPPC, 2016). The casual against was detected via morphological and biochemical identifications of the colonies on nutrient media according to the Bergey's Manual of Systematic Bacteriology according to the Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) . Gram-negative, straight, rod-shaped bacterium measuring 1.5–2.0 × 0.5– 0.75 μm. It is motile by means of a single polar flagellum. It shares many physiological and biochemical properties with other members of the genus *Xanthomonas*. It is chemoorganotrophic and obligatorily aerobic with an oxidative metabolism of glucose. The yellow pigment is xanthomonadin

(Koizumi, 1971 and Francis *et al.*, 2010). *Xanthomonas* sp the casual againt was isolated from naturally infected Citrus, almond and beans plants and identified based on biologically, serologically and molecularly. *Xanthomonas* sp the casual against was detected serological by Indirect immunofluorescence assay using monoclonal antibodies. Indirect ELISA with monoclonal antibodies described by Alvarez *et al.* (1991 and IPPC, 2016) can be used for culture identification. ELISA kits containing all the necessary components for the identification of *X. citri* subsp. *citri* are available commercially (e.g. from Agdia, Inc.). In theory, all *X. citri* subsp. *citri* strains can be identified, but it has been reported that some phenotypically distinct strains isolated in South- West Asia do not react with the available monoclonal antibodies (Vernière *et al.*, 1998). *Xanthomonas sp* was identified by bioassay (pathogenicity test) on a panel of indicator hosts of leaf discs or detached leaves *of N.tabacum cv.* samson. *X. citri* subsp. *citri* should be identified by pathogenicity on a panel of indicator hosts such as *C. paradisi* var. Duncan (grapefruit), *Citrus sinensis* (Valencia sweet orange) or *C. aurantiifolia* (Mexican lime) for confirmation of the diagnosis. Leaf assays by infiltration with a syringe with or without needle on susceptible cultivars of *Citrus* hosts allow demonstration of pathogenicity of bacterial colonies. Immature leaves that are 50–70% to fully expanded are preferred due to their higher level of susceptibility. Lesions develop 7–14 days after inoculation of intact leaves or detached leaves (Francis *et al.*, 2010; Koizumi, 1971) after incubation at 25 ºC in high humidity. Molecular testing (polymerase chain reaction (PCR) using Heat check gene *by specific primers of Hgene* for *Xanthomonas* sp. *Xanthomonas* sp incidence was determined in Citrus, almond and beans plants cultivated in autumn season 2017 based on distinct *Xanthomonas* sp symptoms. Nada Abderatty *et al.,* (2018) applied PCR using Heat check gene *by specific primers of Hgene* for *PGPR bacteria* . *Xanthomonas* sp incidence was determined in Citrus, almond and beans plants cultivated in autumn season 2017 based on distinct *Xanthomonas* Features of citrus-attacking xanthomonads including *X. citri* subsp. *citri* and the genus *Xanthomonas* as a whole have been characterized at the molecular level to develop quick and accurate methods for reclassification and identification. The procedures include DNA–DNA hybridization (Vauterin *et al.*, 1995), genomic fingerprinting (Hartung *et al.,* 1987; Lazo *et al.*, 1987), multilocus sequence analysis (Young *et al.*, 2008) and rep-PCR (Cubero and Graham, 2002, 2004). Fingerprints (band patterns) can be compared and analysed for similarity by eye, but patterns can also be transformed into peak patterns and strains compared using a computer software program such as BioNumerics (Applied Maths). Identification should be based on similarity to patterns of control (reference) strains. Sequence analysis of all *Xanthomonas* (citrus, almond and beans) amplicons demonstrated the presence of a single sequence type and direct sequence analysis showed a highly conserved amplified region. This sequence, which encodes a partial region of the heat-shock protein GroES, has subsequently been deposited in GenBank under *Xanthomonas* Reference accession no. AY150814. This sequence demonstrated 98·2% similarity to that of *Xanthomonas* Reference (Gen Bank accession no. S77424), followed by 76·7% similarity to P. stutzeri (Y13828), 72·7% similarity to P. putida (X78435) and 62.5% similarity to Enterobacter cloacae (M88012) (Clarke *et al.,* 2003). Although there have been several publications that described PCR detection of P. aeruginosa (McIntosh *et al.,* 1992; Karpati & Jonasson, 1996), there have been relatively few descriptions of generic *Pseudomonas* PCR assays that are designed to detect Pseudomonas spp. besides P. aeruginosa. Given that the groES locus is highly conserved in multiple bacterial genera, the current database of available groES sequences is not as comprehensive as those for other highly conserved gene loci, such as the 16S rRNA gene, and hence may not contain sequence data for several commonly isolated, as well as newly emerging, CF organisms. Therefore, the proposed use of these primers in diagnostic screening assays, where Pseudomonas spp. are detected in a molecular manner, should be confirmed phenotypically and antibiotic susceptibility patterns should be obtained.

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