RAPD-PCR technique for detecting the DNA-polymorphism among some *Xanthomonas campestris* isolates causing mango canker disease in Egypt

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Abstract

Studying the virulence of three bacterial isolates of *Xanthomonas campestris i.e.,* Xm4, Xm6 and Xm8 isolated from different parts of cankered mango trees, in addition to, three isolates of *Xanthomonas campestris i.e., X.c.* pv. *vesicatoria* isolate (Xv1) isolated from tomato plants, *X.c.* pv. *campestris* isolate (Xc2) isolated from cabbage plants and *X.c.* pv. *citri* isolate (Xc3) isolated from citrus fruits on some vegetable hosts revealed that no one of the six tested *X. campestris* isolates was able to exhibit any one of their pathogenic effects on the different tested vegetable seedlings at 3 days post inoculation by injection or spraying. On the other hand, the three isolates of *Xanthomonas campestris i.e.,* Xm4, Xm6 and Xm8 were able to infect all tested vegetable cultivars with various extents. Also, Xc2 (cabbage isolate) was the highly effective isolate among the six tested isolates in infecting cabbage seedlings either with injection or spraying methods at 10 days post inoculation while, Xv1 isolate was the highest effective one among the six tested isolates in infecting tomato cv. super strain b seedlings. Studying the virulence of the six aforementioned bacteria on some different fruit hosts revealed that three *X. campestris* isolates (Xm4, Xm6 and Xm8) were the highly pathogenic isolates of mango (cv. Ewasy), peach cv. Florida and pear cv. Lecont transplants among the six tested isolates when inoculated by injection or spraying followed by Xc2, Xc3 and Xv1, respectively. Also, transplants of plum cv. Hollywood and apple cv. Ana were not able to infect with any one of the tested *Xanthomonas* isolates. On the other hand, *X. campestris* p.v *citri* (Xc3) was the only isolate among the six tested isolates had the ability to infect orange and lemon transplants with highly pathogenic reaction. As for the DNA-polymorphism among the six tested *X. campestris* isolates using RAPD-PCR technique with five primers *i.e.*, D3, RI, A1R, D02 and E07, data exhibited that the five tested RAPD primers were good in revealing the DNA-polymorphism among the six tested *Xanthomonas campestris* isolates *i.e.,* Xm4, Xm6, Xm8, Xv1, Xc2 and Xc3 where most of them exhibited RAPD-PCR amplicons with molecular weights recorded with the six tested bacterial isolates to confirm the high similarity among them. Also, the RAPD primer RI was the best one among the five tested primers in revealing RAPD-PCR amplicons with molecular weights confirmed the entirely similarity and relatively within the three tested mango bacterial isolates *i.e.,* Xm4, Xm,6 and Xm8 and confirmed also the similarity of them with the three other tested bacterial isolates.

Keywords: RAPD-PCR, DNA-Polymorphism , Xanthomonas campertris mango, canker

Introduction

Mango (*Mangifera indica* L.) is very important nutritional fruit tree in the world where it has been considered the 'king of fruits' (Purseglove, 1972). Bacterial canker of mango trees caused by *Xanthomonas campestris* (Van Hall) has become a serious problem in many regions of the world. Plessis (1988) determined virulence of five strains of *Xanthomonas campestris* to peach, plum and apricot cultivars after inoculation of detached leaves and leaves on trees in a greenhouse. Virulence of these five strains and 10 additional strains of *X.c* to four peach cultivars was also measured on detached leaves where a highly significant interaction occurred between strains and cultivars. Pruvost *et al*. (2000) concluded that the occurrence of several *xanthomonads* associated with mango and the diversity existing within *Xanthomonas campestris* should be considered for germplasm evaluation. Pruvost and Gagnevin (2002) used the pathovar *mangiferae indicae* mango patho-system as a model to characterize the biological significance of *Xanthomonas campestris* from Brazilian pepper (*Schinus terebinthifolius*) as an inoculum source for mango infections. Ah-You *et al* (2007) described the bacterial canker caused by *Xanthomonas campestris* as an important disease of mango (*Mangifera indica*) as well as, several other plant genera of the family *Anacardiaceae* as host species for xanthomonads. Tamir *et al*. (2007) reported that *X. campestris* is the causal agent of bacterial spot disease of tomato and pepper. The disease process is interactive and very intricate and involves a plethora of genes in the pathogen and in the host. In the pathogen, different genes are activated in response to the changing environment to enable it to survive, adapt, evade host defenses, propagate, and damage the host. Sherif *et al*. (2012) studied the host range of *X. campestris*, the causal agent of bacterial spot disease in peach. They found wide host range of the pathogen including pear, peach, apricot and plum. Rosello *et al*. (2012) observed that *Xanthomomas* caused typical symptoms of bacterial spot disease on stone fruits. During the following years, the pathogen was found affecting different cultivars of Japanes peach.

As for DNA-polymorphism among *X. compestris* isolates, Huang *et al*.(1997) generated a genomic

library of the mango pathogen *Xanthomonas campestris* (X.c) strain, from the nucleotide sequences of insert DNAs in pXCM21, pXCM24 pXCM24-1 (subcloned from pXCM24) and pXCM58-P1 (subcloned from pXCM58), respectively. The 4 primer sets specifically amplify 1.9, 2.2, 0.9 and 1 kb DNA fragments, respectively, using chromosomal DNAs of *X.c* strains as templates in PCR. The rates of detection for the 4 primer sets using 35 strains of *X.c* were 82.9%, 100%, 94.3% and 100%, respectively. In sensitivity test, P24-3/P24-7 and P58-P1-3/P58-P1-7 detected the lowest level of DNA, 10-100 fragments and lowest number of cells, 100-500. It is concluded that 2 primer sets, P24-3/P24-7 and P58-P1-3/P58-P1-7, can be potentially developed to diagnose fruits naturally infected with bacterial canker using PCR. Verdier *et al.* (1998) analyzed strains of *Xanthomonas campestris* for genotypic markers by two methods containing a gene required for pathogenicity from *Xanthomonas campestris*. Ribotyping revealed a unique pattern for all tested strains that corresponded to the previously described ribotype rRNA7. Based on polymorphism detected by pthB among *X.c* strains, nine haplotypes were defined. Said *et al.* (2003) used the Biolog system, fatty acid methyl ester analysis using microbial identification system (MIS), rep-PCR and pathogenicity tests to identify and characterize *Xanthomonas campestris* strains from Tanzania. Great diversity was observed among *X.c* strains in their Biolog and rep-PCR profiles. Specific rep-PCR genomic fingerprints were linked to some geographical areas in the country. Most of the *X.c* strains were clustered in two groups based on their fatty acid profiles and symptom expression in cabbage although some deviant strains were found. Each of the methods allowed a degree of identification from species, pathovar to the strain level. Biolog and MIS identified all *X.c* strains at least to the genus level. Aritua *et al.* (2007) used repetitive sequence based genomic fingerprinting that uses a PCR-mediated amplification of DNA sequences located between specific interspersed sequences of highly conserved elements in prokaryotic genomes to characterize a collection of *Xanthomonas campestris* isolates from banana in Uganda. Fingerprints of bacterial isolates collected from *X. campestris* symptom bearing banana plants grown in production fields from 10 districts in Uganda revealed similar patterns. Cluster analysis of pair wise similarity values performed using un-weighted pair group method with arithmetic averages clustering technique did not generate any differences in the fingerprint patterns. Gama *et al.* (2011) characterized thirty-one pigmented strains of *Xanthomonas* for phenotypic, pathogenic and molecular attributes. These strains were similar to *X. campestris* in phenotypical characteristics, sensitivity to antibiotics and copper compounds used in agriculture, epidemiology and repetitive sequence-based polymerase chain reaction (rep-PCR) profiles. When inoculated into pepper, mango and plum seedlings, the pigmented strains of *X. campestris* produced similar symptoms. Moreover, based on the results of rep-PCR and IS1595-PCR amplification, these strains constitute a variant of *Xanthomonas campestris*. Sabin *et al.* (2012) studied the genetic diversity among seven *Xanthomonas* isolates representing four species using RAPD and ISSR PCR-based techniques. Both techniques revealed high degrees of polymorphisms among the studied isolates. A cluster dendrogram based on the combined data of RAPD and ISSR showed that genetic diversity exists in local isolates of *Xanthomonas*. In terms of percentage similarity values, the genomic variation was found to be in the range of 29% -100% among the isolates. *X. campestris* pv. *mangiferaeindicae* remained un-clustered in cluster dendrogram and revealed a unique genomic profile compared to other isolates used in this study.

This study aimed to throw the light on mango bacterial canker disease caused by *X. compestris* which appeared recently on some mango varieties in Egypt. Also, detecting the DNA-polymorphism among the tested *X. compestris* isolates (3 isolates of mango and 3 other isolates of different hosts).

Materials & Methods

Source of *Xanthomonas compestris* isolates

Three bacterial isolates of *Xanthomonas campestris i.e.*, Xm4, Xm6 and Xm8 which isolated from different parts of cankered mango trees (El-Sisi, 2013), in addition to three isolates of *Xanthomonas campestris i.e.*, X.c. pv. *vesicatoria* isolate (Xv1), isolated from tomato plants, *X.c.* pv. *campestris* isolate (Xc2), isolated from cabbage plants and *X.c.* pv. *citri* isolate (Xc3) which isolated from citrus fruits were tested in this trail to determine their pathogenic effects on different fruit and vegetable hosts in addition to the similarity or diversity among them. The latest three isolates were isolated and identified previously in Plant Pathology Branch, Agric. Botany Dept. Fac. Agric. Moshtohor, Benha Univ.

Virulence of tested canker bacteria on different fruit and vegetable hosts

In this trail, three bacterial isolates of *Xanthomonas campestris i.e.*, Xm4, Xm6 and Xm8 which isolated from different parts of cankered mango trees, in addition to three isolates of *Xanthomonas campestris i.e.*, *X.c.* pv. *vesicatoria* isolate (Xv1) which isolated from tomato plants, *X.c.* pv. *campestris* isolate (Xc2) which isolated from cabbage plants and *X.c.* pv. *citri* isolate (Xc3) which isolated from citrus fruits were tested for their pathogenic reactions on different host plants. All six *X. campestris* were tested for their pathogenic

reactions on five different vegetable cultivars *i.e.,* sweet pepper cv. California wander (*Capsicum annuum*), chili pepper cv. Anaheim (*Capsicum annuum*), cabbage cv. Sabeany (*Brassica oleracea*), eggplant cv. Balady long white (*Solanum melongena*) and tomato cv. super strain b (*Solanum lycopersicum*) as well as seven different fruit varieties i.e., Mango cv. Ewasy (*mangifera indica*), peach cv. Florida (*Prunus persica***)**, plum cv. Hollywood **(***Prunus domestica*), pear cv. Le Conte (*Pyrus communis*), apple cv. Anna (*Malus domestica*), Orange cv. navel (*Citrus sinensis*) and lemon cv. Balady (*Citrus limon*) under greenhouse conditions using two methods of inoculation *i.e.,* injection method and spraying method. The first method was achieved by injecting 0.2 ml of the previously prepared bacterial suspension (10^7 cfu) in the tip of growing shoots of tested fruit transplants or vegetable seedlings using a fine hypodermic syringe. The second method was achieved by spraying the entire canopy of the selected fruit transplants (two year-old) or vegetable seedlings directly without wounding with bacterial suspension (10^7 cfu) using a hand atomizer (each 12 transplants or seedlings were sprayed with about 25 ml of prepared bacterial suspension). The inoculated fruit transplants or vegetable seedlings were covered with plastic sheet for 24 hrs directly. The disease incidence% was determined at 3 and 10 days post inoculation of the tested vegetable seedlings and at 5 weeks of tested fruit transplants.

DNA-polymorphism among the six tested *X. campestris* isolates

The RAPD-PCR technique (Random amplified polymorphic DNA) was used to investigate the similarity and diversity among the tested canker bacterial isolates of those identified as *Xanthomonas campestris* (mango isolates) in addition to three other *Xanthomonas campestris* isolates *i.e,* Xv1 (tomato isolate), Xc2 (cabbage isolate) and Xc3 (citrus isolate) using 5 primers as listed in Table (1).

Table 1. The used primers and their sequences

Primer Name	Nucleotide sequence
(OPERON –A1R)	5-TACGGCAAGGCGACGCTGACG-3-
(OPERON –D02)	5'-GGACCCAACC-3'
(OPERON –D3)	5'GACAGACAGACAGACA3'
(OPERON –E07)	5'-AGATGCAGCC-3'
(OPERON –RI)	5'- TTTCGTCGTCATCTGGC-3'

DNA preparation

Total genomic DNA was extracted from 10 ml of 24-h shake cultures of bacterial cells. After centrifugation at 10,000 × g for 10 min, the bacterial pellet was re-suspended in 1.5 ml of buffer (100 mm Tris-HCl [pH 7.5], 100 mm EDTA [pH 8.0]). The pellet was rinsed twice with cold 70% ethanol, dried in vacuum, and dissolved in 0.5 ml of TE (Tris-HCl + EDTA) buffer. One microliter of ribonuclease at 10 mg/ml was added (final concentration 20µg/ml) and kept at 4°C overnight to completely digest the DNA. The DNA was re-precipitated, rinsed with cold 70% ethanol, dried and dissolved in 40 µl of TE. The DNA was quantified by the mini-gel method. After quantification, the DNA was dissolved in 200 µl of TE and kept at –20°C for later use.

DNA concentration by UV spectroscopy

A dilution of DNA by adding 20 µl of the refrigerated DNA solution to 0.98 ml of distilled water in a micro-centrifuge tube was prepared and mixed well. The UV lamp of the spectrophotometer (SPECTRONIC 20-D) was warmed up for 20 min and wavelength of the spectrophotometer was set to 260 nm. Distilled water was added to one cuvette as a blank and set the absorbance to zero. The absorbance of the diluted DNA was measured. The concentration of DNA was calculated according to Sambrook *et al.* (1989), assuming that DNA at a concentration of 50 µg/ml had an optical density (OD) of 1 at 260 nm as follows:

DNA concentration (µg/µl) =

$$\frac{OD_{260} \text{ x dilution factor x } 50\ \mu g/ml}{100}$$

After quantification, the DNA was dissolved in 200 µl of TE and kept at –20°C for later use.

RAPD-PCR amplification

A working DNA solution was made by diluting the stock DNA solution to about 0.1µg/µl. Each amplification reaction was performed in a 13-µl volume consisting of 0.2 mm. each of dATP, dCTP, dGTP, and TTP (Sigma Chemical Co., St. Louis, MO); 2 mm $MgCl_2$; 0.3 units of Taq DNA Polymerase (Promega, Madison, WI); 4 µM primer, 0.2 µg of DNA template; 1.25 µl of 10x Taq polymerase buffer (Promega) and sterile water added to a final volume of 13 µl. Sterile distilled H_2O was used in place of DNA template as a control to ensure that there was no contamination. The solution was overlaid with mineral oil. Amplification was carried out in a Perkin-Elmer model 480 thermal cycler programmed for 10 min at 94°C for initial denaturation and 30 cycles that consisted of 3 min at 94°C, 1 min at 50°C, and 1 min at 72°C, followed by a final 10 min extention at 72°C. The fastest ramp time was used for temperature transition. After

amplification, 5µl of the solution for each sample was electrophoresed in a 1.2% agarose gel in 1X TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). A 1-kb DNA ladder (0.15 µg) (Gibco BRL, Bethesda, MD) was used to estimate the size of each amplified DNA fragment. The gel was run for 1-2 hours at 100 volts, stained with ethidium bromide (1mg/ml) for 15 min and photographed under ultraviolet light. The test of each primer was repeated at least twice to ensure the consistency of each RAPD band **(Kearns *et al.*, 1998)**.

Results

Virulence of tested *Xanthomonas* isolates on some different vegetable hosts

As clear in **Table (2)**, no one of the six tested *X. campestris* isolates was able to infect the seedlings of tested vegetable hosts at 3 days post inoculation by injection or spraying. However, the results reveal that cabbage seedlings (cv. Sabeany) was highly susceptible one among the four tested vegetable cultivars to infection with the six tested *X. campestris* isolates followed by sweet pepper (cv. California wander) and eggplant (cv. Balady long white) seedlings respectively when inoculated by injection or spraying at 10 days post inoculation. Meanwhile, chili pepper seedlings (cv. Anaheim) were the least infective vegetable cultivar with the six tested *X. campestris* isolates. Also, Xc2 (cabbage isolate) was the highly effective isolate among the six tested isolates in infecting cabbage seedlings followed by Xm8, Xm6 and Xm4 respectively either with injection or spraying methods of inoculation at 10 days post inoculation. While, Xv1 isolate was the highly effective one among the six tested isolates in infecting tomato seedlings (cv. super strain b). On the other hand, the three isolates of *X. campestris i.e.,* Xm4, Xm6 and Xm8 which isolated from different parts of cankered mango trees were able to infect all tested vegetable cultivars with various extents.

Table 2. Virulence of tested *Xanthomonas* isolates on some different vegetable hosts.

Vegetable host (seedlings)	Isolate Code	Injection method		Spraying method	
		Disease incidence %		Disease incidence %	
		3 days	10 days	3 days	10 days
Sweet pepper (cv. California wander)	Xm4	0.0	50.0	0.0	44.0
	Xm6	0.0	48.0	0.0	46.0
	Xm8	0.0	45.0	0.0	45.0
	Xv1	0.0	63.0	0.0	55.0
	Xc2	0.0	44.0	0.0	34.0
	Xc3	0.0	10.0	0.0	8.0
Chili pepper (cv. Anaheim)	Xm4	0.0	10.0	0.0	8.0
	Xm6	0.0	12.0	0.0	8.0
	Xm8	0.0	15.0	0.0	11.0
	Xv1	0.0	15.0	0.0	10.0
	Xc2	0.0	10.0	0.0	7.0
	Xc3	0.0	7.0	0.0	5.0
Cabbage (cv. Sabeany)	Xm4	0.0	70.0	0.0	65.0
	Xm6	0.0	72.0	0.0	61.0
	Xm8	0.0	83.0	0.0	71.0
	Xv1	0.0	69.0	0.0	55.0
	Xc2	0.0	96.0	0.0	88.0
	Xc3	0.0	60.0	0.0	48.0
Eggplant (cv. Balady long white)	Xm4	0.0	11.0	0.0	10.0
	Xm6	0.0	14.0	0.0	10.0
	Xm8	0.0	16.0	0.0	12.0
	Xv1	0.0	88.0	0.0	73.0
	Xc2	0.0	70.0	0.0	64.0
	Xc3	0.0	50.0	0.0	41.0
Tomato (cv. super strain b)	Xm4	0.0	40.0	0.0	30.0
	Xm6	0.0	43.0	0.0	32.0
	Xm8	0.0	43.0	0.0	31.0
	Xv1	0.0	98.0	0.0	89.0
	Xc2	0.0	67.0	0.0	61.0
	Xc3	0.0	55.0	0.0	50.0

Xm = *X. campestris* (4,6,8) isolates of mango
Xv1 = *X.c.* pv. *Vesicatoria* (Tomato isolate)
Xc2 = *X.c.* pv. *Campestris* (cabbage isolate)
Xc3 = *X.c.*pv. *citri* (citrus isolate)

Virulence of tested *Xanthomonas* isolates on some different fruit hosts

Results in Table (3) exhibit that the three *X. campestris* isolates (Xm4, Xm6 and Xm8) were the highly virulent isolates of mango (cv. Ewasy), peach (cv. Florida) and pear (cv. Le Conte) transplants among the six tested isolates when inoculated by injection or spraying followed by Xc2, Xc3 and Xv1 respectively. Also, transplants of plum (cv. Hollywood) and apple (cv. Anna) were not able to infect with any one of the tested *Xanthomonas* isolates. On the other hand, *X. campestris* p.v *citri* (Xc3) was the only infective isolate among the six tested isolates of orange and lemon transplants with highly pathogenic reaction.

Table 3. Virulence of tested *Xanthomonas* isolates on some different fruit hosts.

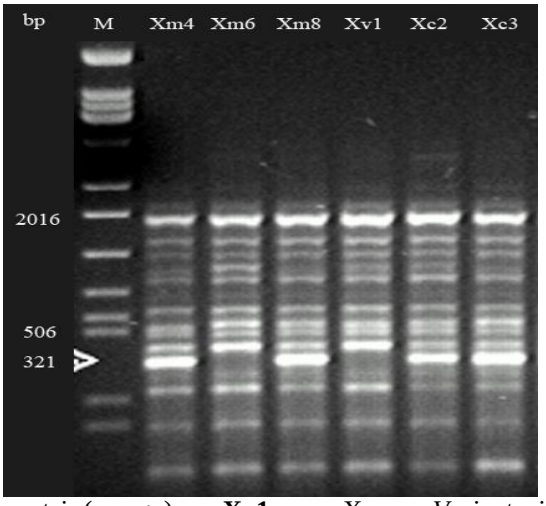
Fruit host (transplants)	Isolate No.	Injection method	Spraying method
		Disease incidence %	Disease incidence %
Mango (cv. Ewasy)	Xm4	96.0	88.0
	Xm6	94.0	83.0
	Xm8	95.0	82.0
	Xv1	25.0	20.0
	Xc2	62.0	51.0
	Xc3	43.0	31.0
Peach (cv. Florida)	Xm4	80.0	55.0
	Xm6	82.0	51.0
	Xm8	94.0	62.0
	Xv1	19.0	12.0
	Xc2	15.0	10.0
	Xc3	33.0	21.0
Plum (cv. Hollywood)	Xm4	0.0	0.0
	Xm6	0.0	0.0
	Xm8	0.0	0.0
	Xv1	0.0	0.0
	Xc2	0.0	0.0
	Xc3	0.0	0.0
Pear (cv. Le Conte)	Xm4	51.0	44.0
	Xm6	47.0	36.0
	Xm8	44.0	33.0
	Xv1	20.0	11.0
	Xc2	51.0	41.0
	Xc3	31.0	28.0
Apple (cv. Anna)	Xm4	0.0	0.0
	Xm6	0.0	0.0
	Xm8	0.0	0.0
	Xv1	0.0	0.0
	Xc2	0.0	0.0
	Xc3	0.0	0.0
Orange (cv. Navel)	Xm4	0.0	0.0
	Xm6	0.0	0.0
	Xm8	0.0	0.0
	Xv1	0.0	0.0
	Xc2	0.0	0.0
	Xc3	96.0	85.0
Lemon (cv. Balady)	Xm4	0.0	0.0
	Xm6	0.0	0.0
	Xm8	0.0	0.0
	Xv1	0.0	0.0
	Xc2	0.0	0.0
	Xc3	88.0	72.0

Xm = *Xanthomonas campestris* (4,6,8) isolates of mango
Xv1 = *X.c.* pv. *Vesicatoria* (Tomato isolate)
Xc2 = *X.c.* pv. *Campestris* (cabbage isolate)
Xc3 = *X.c.*pv. *citri* (citrus isolate)

DNA-polymorphism among the six tested *X. campestris* isolates

In this trial, five RAPD primers *i.e.*, D3, RI, A1R, D02 and E07 were used to investigate the DNA-polymorphism among the six tested bacterial isolates of *Xanthmonas campestris* i.e., Xm4, Xm6, Xm8, Xv1, Xc2 and Xc3 which isolated from cankered mango trees and some other different hosts.

Data in Table (4) and Figs. (1&2) show that using the RAPD primer D3 cleared the DNA-polymorphism among the six tested bacterial isolates which isolated from mango (3 isolates) and other hosts (3 isolates). Data of Figs. (1 & 2) reveal that the highest similarity was recorded among Xm4 and Xm8 isolates (100%) where the two isolates lied in one cluster. Meanwhile, the similarity among cabbage isolate (Xc2) and (Xm4 and Xm8) was 93%. Also, the similarity between citrus isolate (Xc3) and (Xc2, Xm4 and Xm8) was 91%. On the other hand, the similarity between tomato isolate (Xv1) and mango isolate (Xm6) reached 100% where the two isolates lied in one cluster. However, the overall similarity among the Xm6 and Xv1 isolates and the other tested four tested isolates was high where it reached about 83%. Amplification patterns obtained with primers D3 revealed three major amplicons at 2016, 506 and 321bp to be found with all six *Xanthomonas* isolates isolated from mango trees in addition to those isolated from tomato, cabbage and citrus. Also, results in Table (4) clear that the RAPD primer D3 was good in revealing the initiated PCR banding patterns of the fractionated DNA fragments (bp) of the six tested *X. campestris* isolates *i.e.*, Xm4, Xm6, Xm8 Xv1, Xc2 and Xc3. In this respect, the RAPD-PCR amplicons with molecular weights at 92, 161, 247, 413, 490, 542, 650, 941, 1276,1514, 1930 and 2317 bp were recorded with the six tested bacterial isolates to confirm the high similarity among them.



Xm4	*X.campestris* (mango)	**Xv1**	X.c. pv. *Vesicatoria* (tomato)
Xm6	*X.campestris* (mango)	**Xc2**	X.c. pv. Campestris (cabbage)
Xm8	*X.campestris* (mango)	**Xc3**	X.c.pv. citri (citrus)
Primer	5'GACAGACAGACAGACA3'		(D3)

Fig. (1): DNA-polymorphism of six tested *X. campestris* isolates using RAPD-PCR with primer (D3).

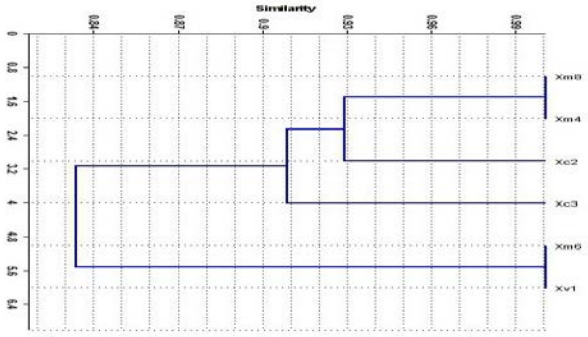
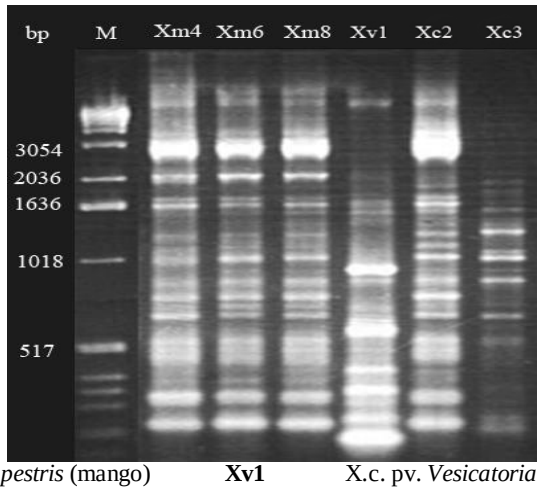


Fig. (2): Dendogram showing DNA polymorphism of six tested *X. campestris* isolates using RAPD-PCR with primer (D3).

Table 4. Analysis of RAPD-PCR products of primer (D3) for six tested *X. campestris* isolates.

MW(bp)	Molecular weights of RAPD-PCR products of tested Xanthomonas isolates					
	Xm4	Xm6	Xm8	Xv1	Xc2	Xc3
2317	1	1	1	1	1	1
1930	1	1	1	1	1	1
1514	1	1	1	1	1	1
1276	1	1	1	1	1	1
1057	0	1	0	1	1	0
941	1	1	1	1	1	1
650	1	1	1	1	1	1
542	1	1	1	1	1	1
490	1	1	1	1	1	1
413	1	1	1	1	1	1
348	1	0	1	0	1	1
289	0	1	0	1	0	1
247	1	1	1	1	1	1
161	1	1	1	1	1	1
92	1	1	1	1	1	1

Data in Table (5) and Figs. (3&4) show that using the RAPD primer RI cleared the DNA-polymorphism among the six tested bacterial isolates which isolated from mango (3 isolates) and other hosts (3 isolates). Data of Figs. (3&4) exhibit that the two isolates Xm4 and Xm6 lied in one cluster with similarity reached 100% while, the similarity among the mango isolate Xm8 and the other two isolates of mango (Xm4 and Xm6) were 96%. On the other hand, the similarity among cabbage isolate (Xc2) and the three isolates of mango (Xm4, Xm6 and Xm8) were 84%. Also, the similarity between tomato isolate (Xv1) and the other isolates *i.e.*, Xc2 (cabbage), Xm4, Xm6 and Xm8 (mango) were 52% while, the similarity among Xc3 (tomato isolate) and the five rest isolates was about 44%. Amplification patterns obtained with primers (RI) revealed two major products at 1636 and 1018 bp to be found with all six tested *Xanthomonas* isolates isolated from mango trees in addition to those isolated from infected samples of tomato, cabbage and citrus. Moreover, data in Table (5) clear that the RAPD primer (RI) was good in revealing the initiated PCR banding patterns of the fractionated DNA fragments (bp) among the six tested *X. campestris* isolates *i.e.*, Xm4, Xm6, Xm8, Xv1, XC2 and Xc3. In this respect, the RAPD-PCR amplicons with molecular weights at 268, 731, 900, 985, 1636, 1809 and 2035 bp were recorded with the six tested bacterial isolates to confirm the similarity among them. While, the RAPD-PCR amplicons with molecular weights at 268, 336, 385, 414, 469, 500, 538, 663, 731, 795, 900, 985, 1090, 1355, 1636, 1809, 2035, 2147, 2818, 3138, 4050 and 4434 bp were recorded with the three tested bacterial mango isolates *i.e.*, Xm4, Xm,6 and Xm8 to confirm entirely similarity among them.



Xm4 *X.campestris* (mango) **Xv1** X.c. pv. *Vesicatoria* (tomato)
Xm6 *X.campestris* (mango) **Xc2** X.c. pv. Campestris (cabbage)
Xm8 *X.campestris* (mango) **Xc3** X.c.pv. citri (citrus)
Primer (5' IIICGICGICATCIGGC 3') (RI)

Fig. (3): DNA polymorphism of six tested *X. campestris* isolates using RAPD-PCR with primer (RI).

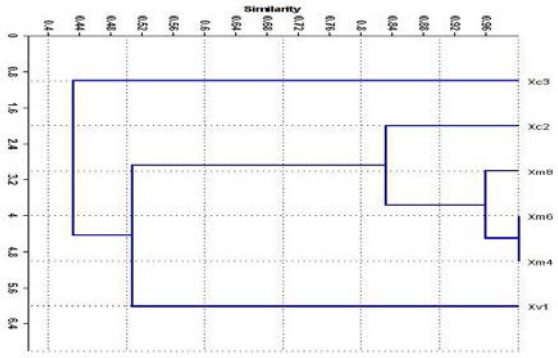
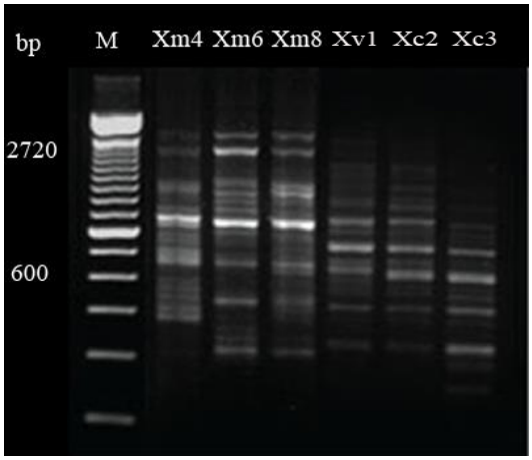


Fig. (4): Dendogram showing DNA-polymorphism of six tested *X. campestris* isolates using RAPD-PCR with primer (RI).

Data in Table (6) and Figs. (5 &6) reveal that the RAPD primer (OPERON–A1R) was good in revealing the initiated PCR banding patterns of the fractionated DNA fragments (bp) among the six tested *Xanthomonas campestris* isolates *i.e.,* Xm4, Xm6, Xm8, Xv1, Xc2 and Xc3. In this respect, data of Figs. (5&6) reveal that the three mango bacterial isolates *i.e,* Xm4, Xm6 and Xm8 lied in one cluster with similarity being 100%. Meanwhile, the similarity between tomato isolate (Xv1) and citrus isolate (Xc3) was also 100% whereas, the similarity between cabbage isolate (Xc2) and the other two isolates (Xv1 and Xc3) of tomato and citrus was 80%. On the other hand, it is clear from the obtained data that the similarity among the cluster containing Xm4, Xm6 and Xm8 and the other three tested isolates was more than 43%. Also, amplification patterns obtained with the RAPD primer (OPERON–A1R) revealed two major condensed amplicons at 2720 and 600 bp to be found with the six tested Xanthomonas isolates isolated from mango trees in addition to those isolated from infected samples of tomato, cabbage and citrus. On the other hand, data of Table (6) indicate to presence of the RAPD-PCR amplicons with molecular weights at 600, 900, 985, 1205, 1355, 1445, 1636 and 1809 bp with the six tested bacterial isolates of *Xanthomonas* to verify the relatively and similarity among them.



Xm4	*X.campestris* (mango)	Xv1	X.c. pv. *vesicatoria* (tomato)
Xm6	*X.campestris* (mango)	Xc2	X.c. pv. campestris (cabbage)
Xm8	*X.campestris* (mango)	Xc3	X.c.pv. citri (citrus)
Primer	(5-CTACGGCAAGGCGACGCTGACG-3)		(OPRON-A1R)

Fig. (5): DNA polymorphism of six tested *X. campestris* isolates using RAPD-PCR with primer (OPERON-A1R).

Table 5. Analysis of RAPD-PCR products of primer (RI) for six tested *X. campestris* isolates.

MW(bp)	Molecular weights of RAPD-PCR products of tested Xanthomonas isolates					
	Xm4	Xm6	Xm8	Xv1	Xc2	Xc3
4434	1	1	1	1	1	0
4050	1	1	1	1	1	0
3565	0	0	1	0	0	0
3379	0	0	0	0	1	0
3138	1	1	1	0	1	0
2818	1	1	1	0	1	0
2147	1	1	1	0	0	0
2035	1	1	1	1	1	1
1809	1	1	1	1	1	1
1636	1	1	1	1	1	1
1445	0	0	0	0	1	1
1355	1	1	1	0	1	1
1205	0	0	0	0	1	0
1162	0	0	0	0	0	1
1090	1	1	1	0	1	1
985	1	1	1	1	1	1
900	1	1	1	1	1	1
795	1	1	1	0	1	0
731	1	1	1	1	1	1
663	1	1	1	0	1	1
589	0	0	0	1	0	0
538	1	1	1	0	1	1
500	1	1	1	1	1	0
469	1	1	1	0	1	0
414	1	1	1	1	1	0
385	1	1	1	1	1	0
361	0	0	0	1	0	0
336	1	1	1	1	1	0
268	1	1	1	1	1	1
234	0	0	0	1	0	0

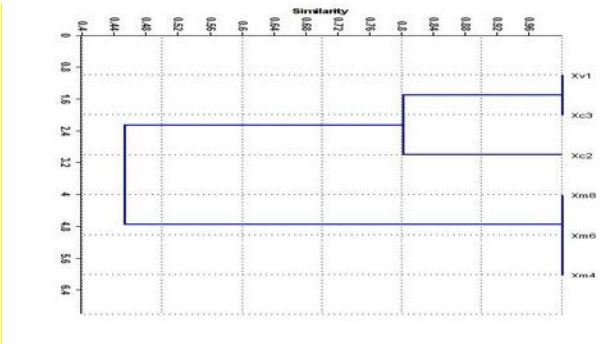
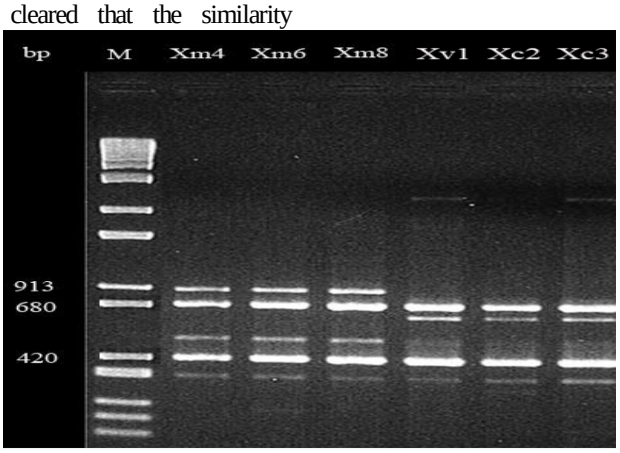


Fig. (6): Dendogram showing DNA polymorphism of six tested *X. campestris* isolates using RAPD-PCR with primer (A1R).

Table 6. Analysis of RAPD-PCR products of primer (A1R) for six tested *X. campestris* isolates.

MW(bp)	Molecular weights of RAPD-PCR products of tested Xanthomonas isolates					
	Xm4	Xm6	Xm8	Xv1	Xc2	Xc3
3138	1	1	1	0	0	0
2818	0	0	0	1	1	0
2720	1	1	1	1	1	0
2035	0	0	0	1	1	0
1809	1	1	1	1	1	1
1636	1	1	1	1	1	1
1445	1	1	1	1	1	1
1355	1	1	1	1	1	1
1205	1	1	1	1	1	1
1162	0	0	0	1	1	1
1090	1	1	1	0	0	0
985	1	1	1	1	1	1
900	1	1	1	1	1	1
795	1	0	0	0	0	0
731	1	0	0	0	0	0
600	1	1	1	1	1	1
589	1	0	0	0	0	0
538	1	0	0	0	0	0
500	0	1	0	0	0	0
469	0	1	1	1	1	1
414	0	1	1	0	0	0
385	0	0	0	0	0	1
361	0	0	0	0	0	1

Data in Table (7) and Figs. (7 &8) reveal the initiated PCR banding patterns of the fractionated DNA fragments (bp) among the six tested *Xanthomonas campestris* isolates i.e.,Xm4, Xm6, Xm8, Xv1, Xc2 and Xc3 in case of using the RAPD primer (OPERON –D-02). In this respect, data of Figs. (7 &8) exhibit that the six tested bacterial isolates were divided to three main clusters where cabbage isolate (Xc2) and citrus isolate (Xc3) lied in one cluster with 100% similarity. Also, the similarity between tomato isolate (Xv1) and mango isolate (Xm6) was 100% (the second cluster). On the other hand, the similarity between mango isolates (Xm4 and Xm8) ranged between 90% and 93% (the third cluster). Also, the results cleared that the similarity between the cluster containing (Xm4 and Xm8 isolates) and the cluster containing (Xv1 and Xm6 isolates) ranged between 78 and 81%. The similarity among the three main clusters i.e., (Xm4 and Xm8), (Xv1 and Xm6) and (Xc2 and Xc3) of the tested bacterial isolates was 69%. Amplification patterns obtained with primer (OPERON–D02) revealed two major condensed amplicons at 680 and 420 bp to be found with all tested bacterial isolates. On the other hand, data Table (7) indicate to presence of the RAPD-PCR amplicons with molecular weights at 344, 416 and 699 bp with the six tested bacterial isolates of *Xanthomonas* to verify the relatively and similarity among them.



Xm4 *X.campestris* (mango) Xv1 X.c. pv. *vesicatoria* (tomato)
Xm6 *X.campestris* (mango) Xc2 X.c. pv. campestris (cabbage)
Xm8 *X.campestris* (mango) Xc3 X.c. pv. citri (citrus)
Primer 5'-GGACCCAACC-3' (OPERON –D02)

Fig. (7): DNA polymorphism of six tested *X. campestris* isolates using RAPD-PCR with primer (OPERON–D02).

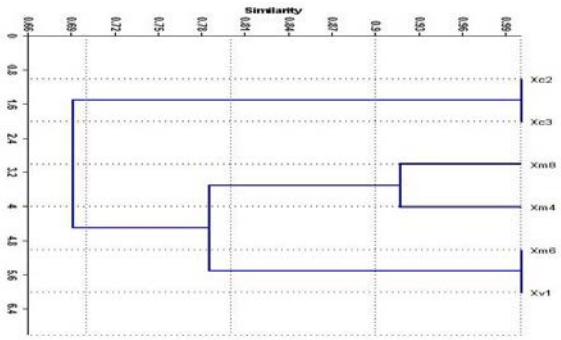
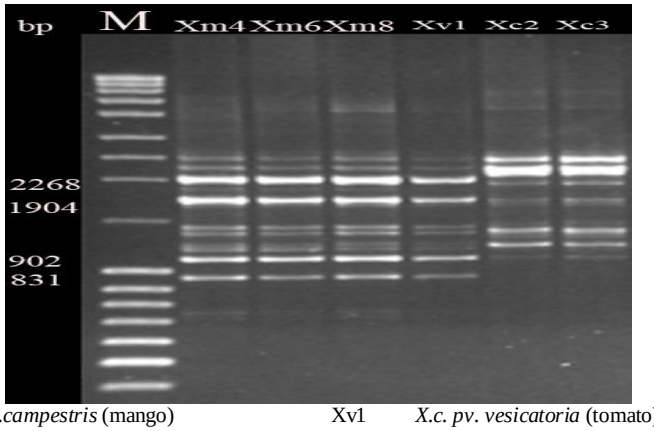


Fig. (8): Dendogram showing DNA polymorphism of six tested *X. campestris* isolates using RAPD-PCR with primer primer (OPERON–D02).

Table 7. Analysis of RAPD-PCR products of primer (OPERON–D02) for six tested *X. campestris* isolates.

MW(bp)	Molecular weights of RAPD-PCR products of tested Xanthomonas isolates					
	Xm4	Xm6	Xm8	Xv1	Xc2	Xc3
895	1	1	1	0	0	0
699	1	1	1	1	1	1
613	0	0	0	1	1	1
503	1	1	1	0	0	0
416	1	1	1	1	1	1
344	1	1	1	1	1	1
204	0	0	0	1	0	1

Data in Table (8) and Figs. (9 &10) reveal the initiated PCR banding patterns of the fractionated DNA fragments (bp) among the six tested *Xanthomonas campestris* isolates i.e., Xm4, Xm6, Xm8, Xv1, Xc2 and Xc3 in case of using the RAPD primer (OPERON–E07). In this respect, data of Figs. (9&10) reveal that tomato isolate (Xv1) and cabbage isolate (Xc2) are closely similar to each other with 100% similarity. Meanwhile, the similarity between citrus isolate (Xc3) and the cluster containing (Xv1 and Xc2 isolates) ranged between 66% and 69%. On the other hand, the two mango bacterial isolates Xm6 and Xm8 are similar to each others with 93% similarity while, the similarity between Xm4 and the cluster containing (Xm6 and Xm8 isolates) was 63%. The results cleared also that the similarity between the main cluster containing Xv1, Xc2 and Xc3 and the other main cluster containing mango isolates (Xm4, Xm6 and Xm8) was 54%. Amplification patterns obtained with primer (OPERON–E07) reveal two major amplicons at 2268 and 902 bp to be found with all tested bacterial isolates. On the other hand, data of Table (8) indicate to presence of the RAPD-PCR amplicons with molecular weights at 1084, 1227, 1385, 1464, 1870, 2173, 2460 and 2740 bp with the six tested bacterial isolates of *Xanthomonas* to verify the relatively and similarity among them.



Xm4	*X.campestris* (mango)	Xv1	*X.c. pv. vesicatoria* (tomato)
Xm6	*X.campestris* (mango)	Xc2	*X.c. pv. campestris* (cabbage)
Xm8	*X.campestris* (mango)	Xc3	*X.c.pv. citri* (citrus)
Primer	5'-AGATGCAGCC-3'	(OPERON-E07)	

Fig. (9): DNA polymorphism of six tested *X. campestris* isolates using RAPD-PCR with primer (OPERON–E07).

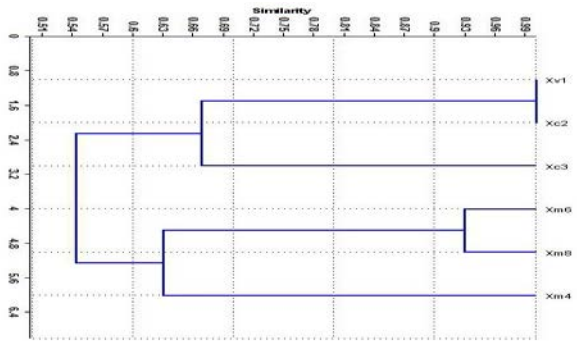


Fig. (10): Dendogram showing polymorphism of DNA of six tested *X. campestris* isolates using RAPD-PCR with primer (E07).

Table 8. Analysis of RAPD-PCR products of primer (OPERON – E-07) for six tested *X. campestris* isolates.

MW(bp)	Molecular weights of RAPD-PCR products of tested Xanthomonas isolates					
	Xm4	Xm6	Xm8	Xv1	Xc2	Xc3
5080	0	0	0	0	1	1
4287	0	0	1	0	1	1
2740	1	1	1	1	1	1
2460	1	1	1	1	1	1
2173	1	1	1	1	1	1
1870	1	1	1	1	1	1
1464	1	1	1	1	1	1
1385	1	1	1	1	1	1
1227	1	1	1	1	1	1
1084	1	1	1	1	1	1
900	1	1	1	1	0	0
638	1	0	1	0	0	0
610	1	0	1	0	0	0

DISCUSSION

Bacterial canker disease on mango trees caused by *Xanthomonas campestris* (Van Hall) has become a serious problem in many parts of the world. Studying the virulence of three bacterial isolates of *Xanthomonas campestris i.e.*, Xm4, Xm6 and Xm8 isolated from different parts of cankered mango trees, in addition to, three isolates of *X. campestris i.e., X.c.* pv. *vesicatoria* isolate (Xv1) isolated from tomato plants, *X.c.* pv. *campestris* isolate (Xc2) isolated from cabbage plants and *X.c.* pv. *citri* isolate (Xc3) isolated from citrus fruits on some vegetable hosts revealed that no one of the six tested isolates was able to exhibit any one of their pathogenic effects on the different tested vegetable seedlings at 3 days post inoculation by injection or spraying. On the other hand, the three isolates of *X. campestris i.e.*, Xm4, Xm6 and Xm8 were able to infect all tested vegetable cultivars with various extents. Also, Xc2 (cabbage isolate) was the highly effective isolate among the six tested isolates in infecting cabbage seedlings either with injection or spraying methods at 10 days post inoculation while, Xv1 isolate was the highly effective one among the six tested isolates in infecting tomato cv. super strain b seedlings. Studying the virulence of the six aforementioned bacteria on some different fruit hosts revealed that three *X. campestris* isolates (Xm4, Xm6 and Xm8) were the highly pathogenic isolates of mango, peach cv. Florida and pear cv. Lecont transplants among the six tested isolates when inoculated by injection or spraying followed by Xc2, Xc3 and Xv1 respectively. Also, no one of the tested *Xanthomonas* isolates was able to infect transplants of plum cv. Hollywood and apple cv. Ana. On the other hand, *X. campestris* p.v *citri* (Xc3) was the only isolate among the six tested isolates had the ability to infect orange and lemon transplants with highly pathogenic reaction. These obtained results are in harmony with the findings of Plessis (1988) who determined the virulence of five strains of *Xanthomonas campestris* to peach, plum, and apricot cultivars after inoculation of detached leaves and leaves on trees in a greenhouse confirming highly significant interaction occurred between strains and cultivars. Also, the results of Ah-You *et al.* (2007), on several other plant genera of the family *Anacardiaceae* as host species for xanthomonads supported our obtained results. Moreover, the virulence results of *X. campestris* on different host plants could be interpreting in light the findings of Tamir *et al.* (2007) who reported that *X. campestris* is the causal agent of bacterial spot disease of tomato and pepper where, the disease process is interactive and very intricate and involves a plethora of genes in the pathogen and in the host. In the pathogen, different genes are activated in

response to the changing environment to enable it to survive, adapt, evade host defenses, propagate, and damage the host. To understand the disease process, it is imperative to broaden our understanding of the gene machinery that participates in it and the most reliable way is to identify these genes *in vivo*. Also, Sherif *et al*. (2012) studied the host range of *X. campestris*, the causal agent of bacterial spot disease on peach. They found wide host range of the pathogen including pear, peach, apricot and plum. While, Rosello *et al*. (2012) observed that *Xanthomomas* caused typical symptoms of bacterial spot disease on stone fruits. During the following years, the pathogen was found affecting different cultivars of Japanes peach. As for the DNA-polymorphism among the six tested *X. campestris* isolates using RAPD-PCR technique of five RAPD primers *i.e.*, D3, RI, A1R, D02 and E07, data exhibited that the five tested RAPD primers were good in revealing the DNA-polymorphism among the six tested *X. campestris* isolates *i.e.,* Xm4, Xm6, Xm8, Xv1, Xc2 and Xc3 where most of them exhibited RAPD-PCR amplicons with molecular weights recorded with the six tested bacterial isolates to confirm the high similarity among them. Also, the RAPD primer RI was the best one among the five tested primers in revealing RAPD-PCR amplicons with molecular weights confirmed the entirely similarity and relatively within the three tested mango bacterial isolates *i.e.,* Xm4, Xm,6 and Xm8 and confirmed also the similarity of them with the three other tested bacterial isolates. The obtained results are in agreement with those of Huang *et al* .(1997) who used 4 primer sets to differentiate among 35 strains of *Xanthomonas campestris* (X.c) the causal of mango canker disease. The rates of detection for the 4 primer sets using 35 strains of X.c were 82.9%, 100%, 94.3% and 100%, respectively. They concluded that 2 primer sets, P24-3/P24-7 and P58-P1-3/P58-P1-7, can be potentially developed to diagnose fruits naturally infected with bacterial canker using PCR. Also, the documented results of Verdier *et al*. (1998) supported our obtained results where they reported that there was clear variation among the isolated strains of *Xanthomonas campestris* from cowpea leaves with blight which collected from various geographic areas. The observed genetic variation was independent of the geographic origin of the strains and of pathogenic variation. In addition, our obtained results could be interpreting in light the findings of Ignatov *et al*. (2007), Gama *et al*. (2011) and Sabin *et al*. (2012) who studied the genetic diversity among seven *Xanthomonas* isolates representing four species using RAPD and ISSR PCR-based techniques. Both techniques revealed high degrees of polymorphisms among the studied isolates. A cluster dendrogram based on the combined data of RAPD and ISSR showed that genetic diversity exists in local isolates of *Xanthomonas*. In terms of percentage similarity values, the genomic variation was found to be in the range of 29%-100 % among the isolates. *X. campestris* pv. *mangiferaeindicae* remained un-clustered in cluster dendrogram and revealed a unique genomic profile compared to other isolates used in this study.

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استخدام تقنية RAPD–PCR لكشف التعدد الشكلي في DNA فيما بين بعض عزلات بكتيريا زانسوموناس كمبسترس المسببة لمرض تقرح المانجو في مصر

عبده مهدي محمد مهدي، نوال عبد المنعم عيسي ، جهاد محمد دسوقي الهباء، أحمد عبدالهادي السيسي

*** قسم النبات الزراعى – كلية الزراعة بمشتهر – جامعة بنها – مصر.**

الملخص العربي

درست الضراوة المرضية لثلاث عزلات من بكتيريا زانسوموناس كمبسترس وهي Xm4 Xm6, Xm8, المعزولة من أجزاء مختلفة من أشجار المانجو المصابة بالتقرح ، بالإضافة الي ثلاث عزلات اخري من زانسوموناس كمبسترس هي *X.c.* pv. *vesicatoria* (Xv1) *X.c.* pv. *campestris* (Xc2) المعزولة من نباتات الطماطم و *X.c.* pv. *citri* (Xc3) المعزولة من نباتات الكرنب هذا بالإضافة الي المعزولة من اشجار الموالح علي بعض عوائل الخضر وقد أظهرت الدراسة عدم قدرة أي من الست عزلات علي إظهار أي تأثيرات مرضية علي شتلات الخضر المختبرة عند ثلاثة أيام بعد العدوي بطريقتى الحقن أو الرش. وعلي الجانب الآخر كانت عزلات زانسوموناس كمبسترس الثلاثة وهيXm4 ، Xm6 ، Xm8 المعزولة من المانجو قادرة علي إصابة كل عوائل الخضر المختبرة بدرجات متفاوتة. كما وجد ايضا ان العزلة Xc2 (عزلة الكرنب) هي أكثر العزلات الست المختبرة فاعلية في اصابة شتلات الكرنب ، بينما كانت العزلة Xv1 (عزلة الطماطم) هي الأكثر فاعلية في اصابة شتلات الطماطم (سوبر استرين بي) عند 10 أيام من العدوي بطريقتي الحقن أو الرش. كما أظهرت دراسة الضراوة المرضية للعزلات الست السابقة الذكر علي عوائل مختلفة من الفاكهة ان الثلاث العزلات Xm4 ، Xm6 ، Xm8 هي الأكثر مرضية علي شتلات المانجو (صنف عويسي) والخوخ (صنف فلوريدا) والكمثري (صنف ليكونت) من بين العزلات الستة المختبرة باستخدام طريقتي العدوي بالرش أوالحقن كما تبعها في الإصابة الثلاث عزلات الأخرى وهيXc2, Xv1, Xc3 علي التوالي. كما لم يكن أي من العزلات الست المختبرة قادرا علي إصابة شتلات البرقوق (صنف هوليود) والتفاح (صنف آنا). وعلي الجانب الآخر كانت عزلة Xc3 (عزلة الموالح) هي العزلة الوحيدة من بين الست عزلات المختبرة القادرة علي إصابة شتلات البرتقال واليمون وبدرجة كبيرة. أما بالنسبة للتعدد الشكلي في DNA فيما بين الست عزلات المختبرة لبكتيريا زانسوموناس كمبسترس باستخدام تقنية RAPD–PCR لخمس بادئات هي RI, A1R, D02, E07, D3 فقد أظهرت النتائج أن الخمس بادئات المستخدمة كانت جيدة في إظهار التعدد الشكلي في DNA للست عزلات المختبرة حيث ظهر مع معظم الست عزلات البكتيرية المختبرة روابط واضحة من DNA بأوزان جزيئية متباينة لتؤكد درجة التشابه العالية بين الست عزلات المختبرة. أيضا كان البادئ RI هو الأفضل بين الخمس بادئات المختبرة في إظهار التعدد الشكلي في DNA حيث اكد استخدام هذا البادئ درجة التشابه والقرابة العالية بين عزلات المانجو الثلاثة المختبرة (Xm8, Xm4, Xm6) ومدي التشابه والقرابة العالي لتلك العزلات مع الثلاث عزلات الأخري.