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Research Article

FIRST REPORT OF BACTERIAL BLACK SPOT OF MANGO IN IRAN

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ABSTRACT

During 2012, mango's orchards in different regions of Hormozgan province of Iran were evaluated. Plants with bacterial black spot symptoms were collected and transferred to the laboratory. Samples were washed with running water for 3 minutes, split to small pieces in sterile distilled water and were maintained in the laboratory condition for an hour. The resulting suspension was cultured in NA medium and incubated at 28˚C. Colonies that appeared at 2-5 days were purified and tested. All isolates were gram negative, obligate aerobic and were able to grow on SPA medium. Strains were positive in hypersensitive reaction on geranium, catalase, levan production, starch hydrolysis, hydrolysis of tween 80, H_2s production from pepton and Simmon citrate agar. All isolates showed alkali reaction on litmus milk. Isolates were negative in oxidase test, arginin hydrolysis, nitrate reduction and acetoin production. Strains were able to use arabinos, inositol, methionin, raffinose, melibiose, mannitol, mannose, sucrose, erythritol, adonitol, trihalose, salicine, dulcitol, inolin and galactose; whereas weren't able to use sorbitol. Pathogenicity test on mango' leaf, were evaluated as positive. Symptom on treated leaf was very similar to mango black spot disease. Polymerase chain reaction with two *Xanthomonas* specific primers (RS21, RS22) led to amplify 1Kbp fragment. Furthermore, the results of PCR product sequencing and blast search showed that these strains have 98 percent similarity to *Xanthomonas citri* pv. *citri.* On the basis of phenotypic features, pathogenicity tests and molecular results, strains identified as *Xanthomonas citri* pv. *mangiferaeindicae*. This is the first report of mango bacterial black spot caused by *X.citri* pv. *mangiferaeindicae* in Iran.

Keywords: *Mango, Bacterial Black Spot, PCR, X. Citri Pv. Mangiferaeindicae, Iran*

INTRODUCTION

Bacterial black spot is one of the most destructive bacterial diseases of mango (Fam: Anacardiaceae) in worldwide (Pruvost and Gagnevin, 2001). It does'nt induce decline in infected trees, but leads to substantial crop losses, change quality and decrease market value (Pruvost and Manicom, 1993).

Bacterial black spot initially described in South Africa in 1915 (Doidge, 1915), then it was reported from many major mango producing region (Gagnevin *et al.*, 1997). Until 2009, pathogen was known as a distinct pathovar of *X. campestris*, but further studies showed that this pathogen is a pathovar of *Xanthomonas citri*, so it must identified as *X. citri* pv. *mangiferaeindicae* (pathotype strain CFBP 1716) (Ah-you *et al.*, 2009).

The major strains of *X. c*. pv.*mangiferaeindicae* show typic characteristics associated with *Xanthomonas* genus, except for Xanthmonadin production. Many strains produce non pigmented colonies when cultured on Nutrient agar medium (Pruvost and Gagnevin, 2001), but in recently years, a few yellow pigmented producing strains have been isolated from mango in Brazil, Florida, South Africa and Reunion (Pruvost and Gagnevin, 2001).

In Iran, mango's cultivation is very valuable and economic in Hormozgan province. Minab and Roudan have more than 92 percent mango cultivation in mentioned province (Anonymous, 2011). Despite the wide distribution symptom of mango black spot in Hormozgan province, any information about existance of disease does not exist. The aim of this study was detection of mango bacterial black spot in Hormozgan province of Iran.

MATERIALS AND METHODS

Isolation

From April to September 2012, different mango orchards in Minab and Roudan County (Hormozgan province, Iran) were investigated. Leaves showing browny to dark, flat and angular spots were collected and transferred to the laboratory. Samples were washed with tap water, renised twice with sterile distilled water, finally ground in a small amount of distilled water. A loopful of suspension was cultured on sucrose nutrient agar and incubated at 28°c. Two to seven days after culturing, different colonies appeared on medium. Small white and yellow, slightly mucoid or non-mucoid, gram negative and obligate aerobe colonies were selected, purified and stored at 4°C for complementary tests (Schaad *et al.*, 2001).

Determination of Phenotypic Features

Biochemical, nutritional and physiological tests were performed according to standard methods (Schaad *et al.*, 2001; Suslow *et al.*, 1982; Fahy and Hayward, 1983).

Phenotypic tests consist of gram, levan production, oxidase, potato rot, argenin dehydrolase, hydrolysis of gelatin, ascolin hydrolysis, tyrosinase and tartarat, carbohydrates utilization test and the other standard tests (Schaad *et al.*, 2001; Fahy& Parsley, 1983).

Pathogenicity Test

Twelve strains identified as *X. citri* based on phenotypic and biochemical tests, were injected on local seedling of mango. The seedlings had 3 years old related to Minab tropical fruites production nursery. A bacterial suspension with a concentration 10^8 cfu/ml (OD_{600}= 1) prepared from bacterial fresh culture and using a strile needle injected to young leaves of mango's seedling through artificial wounds. Seedlings were maintained in mist nursery Jehad-agriculture management of Minab County. For moisture retention, inoculation leaves were covered with hyaline plastic bags for 3-4 days. Strile distilled water was used as negative control. Treated plants were monitored for symptom develoption for 3 weeks. Progressive necrosis symptoms in treated leaves and the absence of these symptoms in control were evaluated as positive bacterial pathogenicity.

DNA Preparation

Bacterial isolates were grown on nutrient agar medium at 28°C for 3 days. A loopful bacteria was suspended in sterile distilled water to a concentration of 10^8 cfu. The suspention was boiled for 10 minutes, cooled at room temprature and directly used as template in PCR (Yaish, 2006).

Detection of Pathogen with Specific Primers

Two primers, RS21 (5'-gCA-CgC-TCC-AgA-TCA-gCA-TCg-Ag-<g>-3') and RS22 (5'-ggC-ATC-TgC-ATg-CgT-gCT-CTC-Cg-<A>-3') (Sinagen Co., Iran) were used. These primers located into the hrp gene cluster in *X.citri* and amplify a DNA fragment approximatly1075 bp lengh in size. The PCR reaction was performed in BioRAD-I thermocycler (USA) in 25µl PCR mixture consist of 12.5 µl of Master mix 2x, 1 µM of each primer and 2 µl of boiled bacterial suspension. PCR reaction was carried out for 1 cycle as a primary denaturation at 94°C for 3 min, followed by 36 cycles as the following condition: 94°C for 1min, 60°C for 45s and 72°C for 45s and final extension 72°C for 5 min (Leite *et al.*, 1994).

The PCR products were electrophoresed on 1.5 % TBE agarose at 80V/cm for 1h. The gel was stained with ethidium bromide, viewed and photographed under UV illumination (Bio Rad, USA). In order to sequencing, PCR product was sent to South Korea. Similarity sequence of our isolates with sequences in gene bank was evaluated with blast search in NCBI site.

RESULTS AND DISCUSSION

All strains were oxidase negative, but catalase and hypersensitive reaction on geranium were positive. Strains were obligating aerobic and abled to grow on SPA. The other results are recorded in tables 1 and 2.

Table 1: Phenotypic characteristic of bacterial strains isolated from mango's trees in Hormozgan province

X. citri	Mango strains in this study	Test Name
-	-	Gram reaction
-	-	Potasreaction
+	+	Levan Production
-	-	Oxidase
+	+	Catalase
O	o	Oxidative / Fermentative test (o/f)
-	-	Urease
-	-	Tween 20
+	+	Hydrolysis of gelatin
+	+	Hydrolysis of esculin
+	+	Hydrolysis of starch
+	+	Tween 80
+	+	Litmus milk
-	-	Tyrosinase
+	+	Hypersensitive reaction on geranium
-	-	Urease
-	-	Reducing compound from sucrose
-	-	Indole formation
-	-	Acetoin production
-	-	Nitrate reduction
+	+	H_2S from pepton
-	-	Lecitinase
-	-	Methyl red

Table 2: Nutritionalcharacteristicsof bacterialstrains isolated from mango in Hormozgan province

X. citri	Mango strain in this study	test name
+	+	Sucrose
+	+	Xylose
+	+	Mannose
_	_	Raffinose
+	+	Arabinose
_	_	Rhamnose
_	_	Maltose
_	_	Melibiose
_	_	Trehalose
+	+	Cellobiose
+	+	Arabitol
+	+	Mannitol
+	+	Sorbitol
_	_	Inulin
+	+	Galactose
+	+	Fructose
+	+	Glucose
-	-	Dulcitol
+	+	Inositol
+	+	Erythritol
-	-	Adonitol
-	-	Salicin
-	-	Methionine

Pathogenicity Test

One week after inoculation, disease symptoms consist of progressive necrotic leaf spot and angular leaf spot were appeared in treated seedlings (Figure1). On the other hand, in control plant, these symptoms were absent.

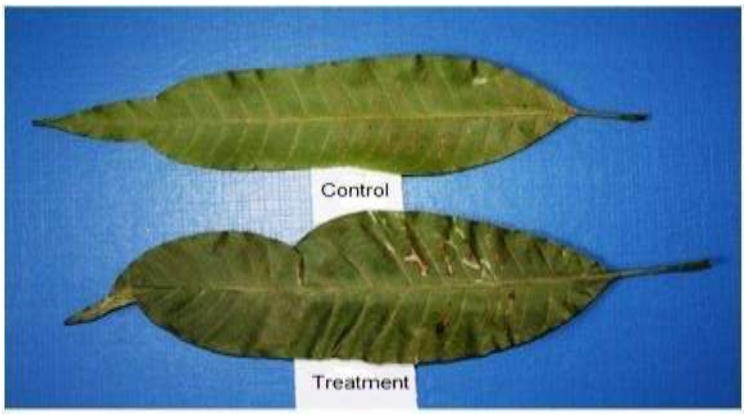

Figure 1: Necrotic symptoms on mango's leaf inoculated with bacterial strains, isolated in this study

On the basis of phenotypic, biochemical and physiological results obtained from isolated bacteria, these isolates identified as *Xanthomonas citri*. Some differntial features of *Xanthomonas* spp. are recorded in table 3.

Table 3: Some differntial features of *Xanthomonas* spp. (Schaad *et al.*, 2001)

Isolates used in this study	*X.fragaria*	*X.citri*	*X.campestris*	*X. axonopodis*	*X.albilineans*	Species/feature
-	-	-	_	_	-	Oxidase
+	-	+	_	_	_	Starch hydrolyse
+	_	+	_	_	_	H_2S proruction of peptone
38	33	38	35-39	35-37	37	Max. growth temp.
+	_	+	_	_	_	Catalase
Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	O/F
						Acid production from
+	-	+	+	-	-	Arabinose
+	+	+	+	-	+	Mannose
+	-	+	+	-	-	Galactose
+	-	+	+	+	-	Trehalose
+	-	+	+	-	-	Cellobiose
+	+	+	-	-	-	Fructose

Genotypic Features

Detection of Pathogen with Specific Primers

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In twelve strains, using specific primers (RS21, RS22), a DNA fragment approximately 1000 bp in lengh was amplified (Figure 2).

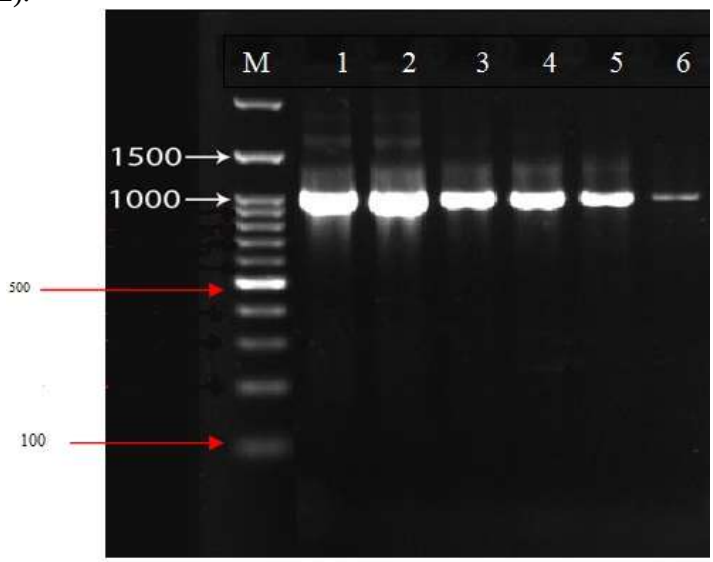

Figure 2: Gel electrophoresis of PCR products with specific primers (RS21, RS22): M, marker 100 bp.1-6 *X.c.m* strains

Sequence Allignment with Gene Bank

The results of the sequences were alligned with sequences in gene bank at NCBI site. Results revealed that our strains have up to 98% similarity to *X. Citr i*pv. *citri* (Figure 3).

Alignments

Description	Max score	Total score	Query cover	E value	Max ident	Accession
Xanthomonas citri subsp. citri Aw12879, complete genome	1847	1847	99%	0.0	98%	CP003778.1
Xanthomonas axonopodis Xac29-1, complete genome	1847	1847	99%	0.0	98%	CP004299.1
Xanthomonas axonopodis pv. citri str. 306, complete genome	1847	1847	99%	0.0	98%	AE008923.1
Xanthomonas axonopodis pv. glycines strain 8ra hrp pathogenicity island, complete sequence	1781	1781	99%	0.0	97%	AF499777.1
Xanthomonas campestris pv. glycines HrcU homolog, HrcV homolog, HpaP homolog, HrcQ homolog, HrcR homolog, and HrcS homolog gene	1757	1757	99%	0.0	97%	AF160874.1
Xanthomonas campestris pathogenicity-related ORF1 and ORF2, complete cds	1729	1729	99%	0.0	96%	M64094.1
Xanthomonas fuscans subsp. fuscans strain CFBP4834-R hrp type III secretion system gene cluster, partial sequence	1609	1609	99%	0.0	94%	EU215367.1
Xanthomonas axonopodis pv. citrumelo F1, complete genome	1365	1365	99%	0.0	90%	CP002914.1
Xanthomonas campestris pv. vesicatoria complete genome	1360	1360	99%	0.0	90%	AM039952.1
Xanthomonas campestris pv. vesicatoria hrp gene cluster, partial sequence	1360	1360	99%	0.0	90%	AF056246.2

Figure 3: Blast search results of isolated bacteria in this study

Discussion

During 2012, mango's orchards showing bacterial black spot symptoms were observed in different regions of Hormozgan province (Iran). Leaf spots were brown, flat and angular. These symptoms were very similar to those were reported by Ah-you and collagues in Brazil (Ah-you *et al.*, 2007). Moreovere, Ploetz reported similar symptoms in Egypt (Ploetz, 2003).

Twellve strains were isolated from infected tissues. All isolates were gram negative, obligate aerobic and were able to grow on SPA medium. Strains were positive in hypersensitive reaction on geranium, catalase test and levan production. Other results were recorded in table 1. According to our findings, studied bacteria were identified as *X.citri*.

In many cases *X. citri* pv. *mangiferaeindicae* produce light creamy colonies (Pruvost and Gagnevin, 2001; Manicom, 1984; Pruvost *et al.*, 2011; Pawar, 2012). Versus many reports, in this research, studied isolates

produced yellow colonies. This result corresponds with the findings of a few researchers. For example Alwarez and collagues showed that causal agent of mango black spot is *X.citri* pv. *mangiferaeindicae* and unlike to the other Xanthomonads they did not produce yellow colonies. Also, there are several reports of yellow colonies in some regions such as Brazil (Robbs *et al.*, 1978), Reunion (Pruvost and Luisetti, 1989), South Africa (Pruvost and Manicom, 1989) and Malaysia (Lim and Sijam, 1991). Lim and Sijam (1991) have reported that the causal agent of mango bacterial black spot produces yellow colony on SPA and YDC these results led us to conclude that Iranian strains are different from strains reported in other countries such as Ghana and Burkinafaso (Pruvost *et al.*, 2011), Pakistan (Steyn *et al.*, 1974), United Arab Emarates and Australia (Gagnevin and Pruvost, 2001). This finding is unlike to primary description of this pathovar (Pruvost and Gagnevin, 2001). All strains were able to produce acid from Arabinose, Cellbiose, Mannose and Glucose that accommodate to others studies (Lim and Sijam, 1991; Manicom and Wallis, 1984; Steyn *et al.*, 1974). All strains were catalase positive and urease negative. Nitrate reduction was negative in all strains. Furthermore all isolates were able to produce H_2S from Cystein and showed alkali reaction on litmus milk. These results corresponds to the Lim and Sijam's findings (Lim and Sijam, 1991).

In pathogenicity test, one week after inoculation, disease symptoms was appeared on treated plants. All 12 strains were pathogenic to mango leaves and produced angular necrotic flat symptoms. On the basis of phenotypic and genotypic features, strains were identified as *X. citri* pv. *mangiferaeindicae*. It must remind that, based on Dye difinition of pathovar, a distincte pathovar can be differentiate from the others by pathgenicity test on the main host (Dye *et al.*,, 1980). All isolates that identified as *X.citri* pv. *mangiferaeindicae* based on segregational properties and bacteriological tests, were used in PCR test with two specific primers (RS21, RS22). All strains were able to amplify a 1000 bpDNA fragment in size. These results confirm that all strains harbor hrp gene in their chromosome.

It must note that hrp genes are necessary for pathogenicity in susceptible plants and hypersensitive reaction in resistant ones. Moreover in plant pathogenic bacteria this gene cluster is protected in individual genus (Leite *et al.*, 1994). RS21 and RS22 are specific primers for hrp gene cluster in many Xanthomonads and synthesize a part of hrpC-hrpD loci in *X.citri* (figure 4).

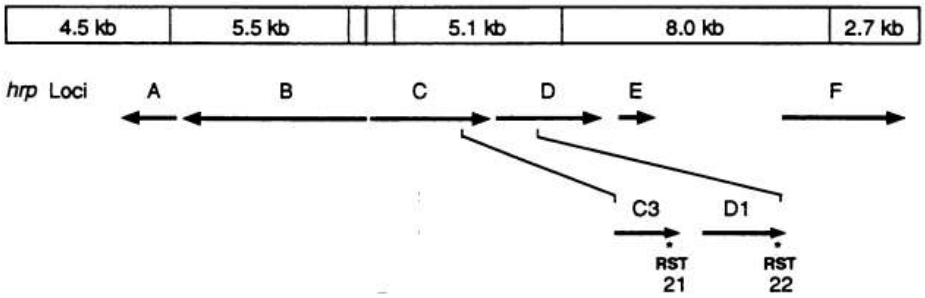

Figure 4: Genetic map of hrp loci showing RS21- RS22 site in hrp gene cluster

The major advantage of these primers is that they don't have any homology with DNA of non pathogenic Xanthomonads or opportunist ones (Leite *et al.*, 1994). Trindade and collagues used specific primers of hrp region for detection of *X. citri* pv. *mangiferaeindicae* in Brazil (Trindade *et al.*, 2007).

According to what mentioned above, reveals that bacterial isolates in this study are pathogenic and belonging to *X. citri*.

In this study, sequencing of PCR product and alligning them with gene bank data showed that our strains have similarity up to 98% to *X.citri* pv. *citri* (Figure 3). Ah-you and collagues, using polyphasic taxonomy of several Xanthomonads which were pathogenic on Anacardiaceae family, showed that the causal agent of mango bacterial disease must be consider as a member of *X. citri* pathovars (Ah-you *et al.*, 2009).

On the basis of our results, it was confirm that bacterial black spot of mango, caused by *X. citri* pv. *mangiferaindicae,* exists in Iran (Hormozgan province) This is the first report of mango black spot caused by *X. citri* pv. *mangiferaindicae* in Iran.

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