J. Soils and Crops 29 (1) 38-43, June 2019

ISSN 0971-2836
NAAS Rating - 4.46
Scientific Journal Impact Factor - 6.736

ISOLATION AND MOLECULAR CHARACTERIZATION OF *Xanthomonas euvesicatoria* CAUSING BACTERIAL LEAF SPOT OF TOMATO IN TAMIL NADU

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ABSTRACT

An experiment entitled "Isolation and molecular characterization of *Xanthomonas euvesicatoria* causing bacterial leaf spot of tomato in Tamil Nadu" was undertaken at Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore during the year 2017 – 2019. Twenty locations in Tamil Nadu were surveyed and bacterial leaf spot infected tomato fruit samples were collected and 24 strains were isolated on Nutrient Agar medium. Morphological and biochemical characters *viz.,* Gram's reaction, colony characters, pigment production, KOH test, catalase test, starch hydrolysis, acid production and gelatin liquefication test were carried out. All the strains showed positive results for all the tests performed. For further confirmation, molecular characterization using the bacterial universal primer fd1 rp2 targeting the 16SrRNA region, *Xanthomonas campestris* pv*vesicatori a*s pecific primer and *Xanthomonas* race specific primers were used.It is observed from this study that all the bacterial strains isolated from the 20 different locations were identified as *Xanthomonas euvesicatoria.* Morphological and biochemical characterization confirmed the strains to be *Xanthomonas* whereas the molecular confirmation with three different primers confirmed the isolated strains to be *Xanthomonas euvesicatoria.* This study lead to the conclusion that the bacterial leaf spot strains infecting tomato plants in Tamil Nadu was *Xanthomonas euvesicatoria.*

(Key words: Tomato, bacterial leaf spot, *Xanthomonas campestris* pv *vesicatoria, X. euvesicatoria*)

INTRODUCTION

Tomato is one of the highly consumed vegetable after potato. It is consumed worldwide and is one of the most widely cultivated vegetables. Tomato is often vulnerable to attack by several pests and pathogens. One such problem in tomato cultivation is the infection of *Xanthomonas euvesicatoria* causing bacterial leaf spot of tomato. The disease is characterized by the production of small, brown angular, water soaked lesions on leaves, stems and fruits and also results in defoliation and direct fruit damage. Significant yield losses can occur due to severe infection (Pernezny *et al.,* 2003). The disease is considered as a quarantine pest and causes economic losses. The disease is often confused with bacterial speck, bacterial canker and bacterial leaf spot all of which are prevalent throughout India. Therefore, this study was aimed in isolation and molecular characterization of *Xanthomonas euvesicatoria* causing bacterial leaf spot of tomato in Tamil Nadu and thereby reporting the prevalence of bacterial leaf spot in Tamil Nadu state.

MATERIALS AND METHODS

Survey and sample collection: Bacterial leaf spot infected tomato samples were collected from different locations of Hosur, Krishnagiri and Coimbatore (Table 1). Fruits showing typical symptoms of bacterial leaf spot were collected and brought to laboratory in sample collection bags. The samples were wiped to remove excess moisture and stored at 4°C for future use.

Isolation: Fruits with brown to black sunken spots were selected and washed with sterile water and moisture was removed with tissue paper. The fruits were then surface sterilized by wiping them with 0.1% sodium hypochloride. The infected portion was cut with a clean and sterilized blade and was dipped in 0.1% sodium hypochloride solution followed by three washes of sterile distilled water. The infected pieces were placed on tissue paper after the final rinse with sterile water to remove the excess moisture. The infected pieces were then dropped in an Eppendorf tube containing 500µl of water and was crushed vigorously with the help of an micro pestle. This ensured breaking of plant

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cells and release of the bacteria. The water turned turbid indicating the release of bacteria. Bacterial loop or inoculation loop was inserted in the Eppendorf tube containing the turbid water a loopful of water was used to streak on the plates amended with nutrient agar medium. The plates were kept inverted at 37° C for 48 hrs.

Morphological and biochemical characters: The isolated culture was preliminarily confirmed to be *Xanthomonas euvesicatoria* by scoring for various morphological characters based on the colony's characters like colour, texture, appearance, etc. The positively scored cultures were then proceeded for various biochemical tests like Gram staining, KOH test, Catalase test, Methyl Red (Acid production) test (Schaad*et al.*, 2001), Gelatinliquification test(Patil*et al.*,2017), Starch hydrolysis(Jadhav*et al.*, 2018).

Pathogenicity test: Pathogenicity on the host of isolation was proved by making slight modifications in the method given by Keyon *et al.* (2016).The isolated strains were inoculated in Nutrient broth and maintained in shaker at 37°C and 200 rpm for 24 hours. The broth was centrifuged at 12000 rpm and the pellet was suspended in sterile water and the absorbance was checked in spectrophotometer to be 1 x 10^8cfu/ ml at OD_{600}. This suspension was sprayed on the 25 days old tomato seedlings that were pin pricked with a sterile needle. The plants were maintained in humid environment for 48 hours. Symptoms were observed two to three weeks post inoculation.

DNA Extraction: DNA was extracted for the isolated bacterial strains by the method described by Gomes *et al.*(2000).

Molecular confirmation: Molecular confirmation for the isolated samples was done by performing PCR using universal primers targeting the 16Sr RNA of the bacteria. The primer used were fd1 (5'AGAGTTTGATCCTGGCTCAG3') and rp2 (5'ACGGCTACCTTGTTACCACTT3') with amplification at 1500 bp (Weisburg *et al.,* 1991). Total volume of the PCR mixture was 10µl (5 µl ready to use Smart Prime Mastermix; 1 µl Forward Primer; 1 µl Reverse Primer; 2 µl DNA and 1 µl water). Conventional PCR was performed in Eppendorf thermo cycler (Eppendorf master cycler) with following temperature profile: Initial denaturation :94°C for 5mins; denaturation: 94°C for 1 min; annealing: 58°C for 1 min; extension: 72°C for 5 mins; and final extension: 72°C for 10 mins with 35 cycles. The PCR amplicons were electrophorized with on 1% agarose gel stained with EtBr and amplification was observed at 1500 bp.

Sequencing: The samples were further sent for sequencing to a private company (J.K. Scientific Company). The sequences were confirmed by BLAST Analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGETYPE+BlastSearch) and the confirmed sequences were submitted in NCBI GenBank database.

Confirmation with *Xanthomonas campestris pv vesicatoria* specific primer: *Xanthomonas campestris pv vesicatoria* specific primers XCVF (5' AGAAGCAGTCCTTGAAGGCA3') and XCVR (5'AATGACCTCGCCAGTTGAGT3') designed from *Rhs*family protein of *X. campestrispv. vesicatoria* str.85–10 (GenBankAccessionNo. AM039952, GI78048271), with a predicted PCR product of 517 bp was used (Park *et al.,* 2009). PCR was performed in Eppendorf thermo cycler (Eppendorf master cycler). PCR mixture consisted of 10 µl reaction (5 µl ready to use Smart Prime Mastermix; 1 µl Forward Primer; 1 µl Reverse Primer; 2 µl DNA and 1 µl water). PCR with a temperature profile of initial denaturation at 94°C for 5 mins, denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 60 sec and final extension at 72°C for 10 mins was performed. The PCR amplicons were electroporesed on 1% agarose gel stained with EtBr.

Confirmation with race specific primer: In order to identify the race to which the isolated strains belonged, the strains were tested against four race specific primers of *Xanthomonas* as given in table 2 (Song *et al.,* 2018*;* Koenraadt *et al.,* 2009).PCR was performed in Eppendorf thermo cycler (Eppendorf master cycler). PCR mixture consisted of 10 µl reaction (5 µl ready to use Smart Prime (Red) Mastermix; 1 µl Forward Primer; 1 µl Reverse Primer; 2 µl DNA and 1 µl water). PCR temperature profile for each primer is given in table 2**.** The PCR amplicons were separated on 1% agarose gel stained with EtBr and 100 bp Ladder (Nexgen) was used for comparing the size of amplicons.

RESULTS AND DISCUSSION

Survey and sample collection

Two tomato growing districts (Coimbatore and Krishnagiri) were surveyed for the occurance of disease. The study was performed to identify the race of bacterial leaf spot causing *Xanthomonas* prevalent in tomato growing areas of Tamil Nadu. In accordance to this aim the tomato growing hot spot areas of Tamil Nadu were surveyed and infected samples were collected. Since it is difficult to differentiate between the spots developed on leaves due to infection by bacterial leaf spot causing *Xanthomonas,* bacterial speck causing *Pseudomonas* syringae pv *tomato* and bacterial canker causing *Clavibacter michigensis* subsp *michigeninsis* the fruits were taken during survey. The fruits with typical small brown angular water soaked lesions were collected.The list of places surveyed for sample collection are mentioned in table1.Twenty four strains were isolated from the samples collected from the above mentioned areas. All the strains were bright yellow in colour and mucoid on NA medium(Fig. 1).The strains were labelled as S1 to S24.

Pernezny *et al.*(2003) reported the presence of brown angular water soaked spots on leaves, stem and fruits which results in defoliation and direct fruit damage. Severe infection can result in significant yield loss. Hassan and Zyton(2017) reported *Xanthomonas campestris* pv

vesicatoria causing bacterial leaf spot on pepper produces water soaked spots that change from initial green colour to dark brown colour and the spots were reported to be surrounded by yellow halos. Agarwal *et al.*(2012) reported *X. campestris* pv. *vesicatoria* produces irregular yellow to brown necrotic lesions on leaves. The target bacteria was isolated on Nutrient agar medium and bright yellow coloured muccoid colonies were observed. This feature is considered as the characteristic feature of *Xanthomonas* genus. *X. campestris* pv. *vesicatoria* isolates on Nutrient Agar medium appeared to be circular, raised and yellow coloured surrounded by a clear ring (Sijam *et al.,* 1991). In accordance to the colonies observed, Chand and Kishun (1991) reported the isolated *Xanthomonas campestris* pv *punicae* colonies also displayed mucoid, circular, convex, yellow, rounded, glistening and raised colonies. Ravikumar (2007) also had similar observations of yellow, mucoid, glistening, slimy, convex and round colonies on the NA medium for *Xanthomonas campestris* pv *vignicola.*

Morphological, biochemical and molecular characters

Morphological, biochemical and molecular characters for all the 24 strains were tested and all the strains shared similar characters.Biochemical tests were performed for all the 24 strains isolated. All the strains responded in a similar pattern to the tests performed (Table 3). In accordance to the results obtained, Roch *et al.* (2018) reported *Xanthomonas* genus to be gram negative, oxidase negative, catalase positive rods producing yellow colonies. Based on the morphological and biochemical characters the isolates bacteria were confirmed to be *Xanthomonos* species and to further confirm this molecular approach was trailed.

DNA was extracted for all the 24 strains and were subjected to molecular confirmation with the bacterial universal primer (FD1 and RP2) targeting the 16S r RNA conserved region of bacteria and development of amplicon of 1500 bp size confirmed all the strains to belong to the bactreial kingdom.

Confirmation with 16s r RNA primers: DNA of all the 24 strains were tested with the FD1 RP2 16 Sr RNA universal primers to confirm the starins belong to the bacteria Kingdom and also identify the organism through sequence similarity using NCBI BLAST database. All the 24 strains showed amplification at 1500 bp (Fig. 2) confirming the strains to belong to the bacterial kingdom. The PCR products were sent for sequencing at a private company and the sequences in FASTA files were Blasted on NCBI Search engine. All the strains showed 99-100% similarity with *Xanthomonas euvesicatoria* and *X. campestris* pv *vesicatoria*, confirming the isolated bacteria to be *Xanthomonas campestris pv vesicatoria or Xanthomonas euvesicatoria.*Although both refer the same organism, for a further clarification the isolated strains were subjected to PCR analysis with *Xanthomonas campestris* pv *vesicatoria* specific primers and also with *Xanthomonas* race specific primer.

Confirmation with XCV specific primer : DNA of the 24 strains were tested with XCV specific primer or species specific primer and all the strains showed amplification at ~517 bp confirming the strains to the *Xanthomonas campestris* pv *vesicatoria*(Fig. 3).Now that the strains were identified to be *X. campestris pv vesicatoria,* the next step was to identify the race of the Bacetrial leaf spot causing *Xanthomonas* for which the race specific primer was used.

Weisburg *et al.*(1991) reported that this set of primer (fd1 rp2) can be used for identifying a wide range of bacteria. The authours claimed that each of the three set of primers they designed can amplify full length 16 S ribosomal DNA from a wide range of bacterial genera. Wood *et al.*(1998) used the same set of primer for identifying *Bacteroides* and *Prevotella* gut bacteria.Song *et al*. (2004) also have used the fd1 rp2 universal primer to identify the 16S rRNA sequence of the 34 strains identified to be the potato scab causing *Streptomyce* spp. In a recent study conducted in France, Togo *et al*.(2018) used the fd1- rp2 16S rRNA primers to identify a new baeria from the milk of a lactating women (Togo *et al.,* 2018).With the universal primer it was confimed that all the isolated strains were bacteria and sequence analysis showed similarity with both *X. campestris* pv *vesicatoria* and with *X. euvesicatoria,* although both the later is the new nomenclature or to be precise it *X. euvesicatoria* is one of the races of the bactreial leaf spot causing *X. campestris* pv *vesicatoria.*

Race identification

In order to confirm the pathovar, the strains were subjected to PCR analysis with *X. campestris* pv *vesicatoria* pathovar specific primer targeting the *rhs* gene family. All the 24 strains showed amplification at ~170 bp when they were combined with *X. euvesicatoria* specific primer for PCR analysis (Fig. 4).The strains did not show any bands for the other three primers confirming the strains to be *X. euvesicatoria.* Finally all the 24 strain sequences were submitted as *X. euvesicatoria* in the NCBI gene bank and accession number was derived (Table 4).Separation of the PCR products at 517 bp confirmed the isolated strains to be *X. campestris* pv *vesicatoria.* Park *et al.*(2009) designed a primer that would very accurately and precisely identify the *X. campestris* pv *vesicatoria* .They tested 31 different bacteria species of *Xanthomonas* and other pathovars of *X. campestris.* Similar to our results only the *X. campestris* pv *vesicatoria* strains yielded bands at 517 bp whereas no other *Xanthomonas* speciesor *X. campestris* pathovars displayed bands indicating the accuracy of the primers. Koenraadt *et al.(*2007) developed four set of primers for specific detection and identification of the four races of bacterial leaf spot causing *Xanthomonas* species (*X. euvesicatoria, X. vesicatoria, X. gardeneri* and *X. perforans*). The primers helped in accurate identification of the races. The accuracy of these primers was further authenticated by Song *et al.* (2018). They used the same primers to identify the species or race of *Xanthomonas* causing bacetrial spot in *Physalis pubescens* in Northeast China. The authours used the same primers as in our experiment to identify the race. The primers aided in identification of the bacteria as *Xanthomonas euvesicatoria*

pv *euvesicatoria.* The same primers were used in our study which developed bands at 170 bp when combined with primer specific for *X. euvesicatoria* but yielded no bands when combined with the other three race specific primers.

It is cocnluded from this study that all the bacterial strains isolated from the 20 different locations were identified as *Xanthomonas euvesicatoria.* Morphological and biochemical characterization confirmed the strains to be *Xanthomonas* whereas the molecular confirmation with three different primers confirmed the isolated strains to be *Xanthomonas euvesicatoria.* The study concluded the bacterial leaf spot strains infecting tomato plants in Tamil Nadu to be *Xanthomonas euvesicatoria.*

Table 1. List of places surveyed in Tamil Nadu

Sr. No.	Place	District	Latitude	Longitude
1	Malayandhali	Krishnagiri	12.6257°N	78.0195°E
2	Manimadikottai	Krishnagiri		
3	Guddur	Krishnagiri		
4	Puddur	Krishnagiri		
5	Koorampatti	Krishnagiri		
6	Neekunthi	Krishnagiri		
7	Velampatti	Krishnagiri		
8	Karimangalam	Krishnagiri		
9	Bagalur	Hosur	12.7409°N	77.8253°E
10	Athimugam	Hosur		
11	Bedapalli	Hosur		
12	Checkpost	Hosur		
13	Andhivadi	Hosur		
14	Advanapalli	Hosur		
15	Deverayapuram	Coimbatore	11°00’35" N	76°49’41"E
16	Kulathuppalayam	Coimbatore		
17	Narasipuram	Coimbatore		
18	Dhaliyur	Coimbatore		
19	Theethipalayam	Coimbatore		
20	Deenapalayam	Coimbatore		

Table 2. List of race specific primers for identifying race of *Xanthomonas*

Sr. No.	Primer	Sequence (5’- 3’)	Amplicon size	Target	Temperature
1	BS- XeF BS - XeR	CATGAAGAACTCGGCGTATCG GTCGGACATAGTGGACACATAC	173 bp	X *euvesicatoria*pv. *vesicatoria*	**Initial denaturation**: 94°C for 30s
2	BS- XvF BS- XvR	CCATGTGCCGTTGAAATACTTG ACAAGAGATGTTGCTATGATTTGC	138 bp	*X. vesicatoria*	**Denaturation**: 94°C for 30s
3	BS- XgF BS-XgR	TCAGTGCTTAGTTCCTCATTGTC TGACCGATAAAGACTGCGAAAG	154 bp	*X. gardneri*	**Annealing**: 56°C for 30s **Extension**: 72°C for 30s
4	BS-XpF BS-XpR	GTCGTGTTGATGGAGCGTTC GTGCGAGTCAATTATCAGAATGTGG	197 bp	*X. euvesicatoria*pv. *perforans*	**Final extension**: 72°C for 10 mins **Cycles**: 30

Table 3. Morphological and biochemical characters studied for the 24 isolates

Sr No	Characters	XCV1	XCV2	XCV3	XCV4	XCV5	XCV6	XCV7	XCV8	XCV9	XCV10	XCV11	XCV12	XCV13	XCV14	XCV15	XCV16	XCV17	XCV18	XCV19	XCV20	XCV21	XCV22	XCV23	XCV24
	Morphological characters																								
1	Mucoid colonies	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Xanthomonadin pigment	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Biochemical characters																								
3	Gram's staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	KOH test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Catalase test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	Acid production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	Gelatinliquification	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 4. Accession numbers of all the 24 strains of *X. euvesicatoria*

Sr. No	Strain Name	Accession No
1	S1	MK680127
2	S2	MK680113
3	S3	MK680124
4	S4	MK680125
5	S5	MK681859
6	S6	MK680128
7	S7	MK681860
8	S8	MK720047
9	S9	MK720049
10	S10	MK720050
11	S11	MK720051
12	S12	MK720052

Sr. No	Strain Name	Accession No
13	S13	MK720061
14	S14	MK720062
15	S15	MK720063
16	S16	MK720064
17	S17	MK722385
18	S18	MK720065
19	S19	MK720066
20	S20	MK722360
21	S21	MK720067
22	S22	MK720071
23	S23	MK720072
24	S24	MK757606

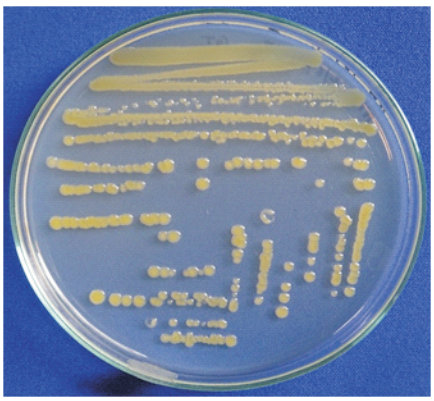

Fig. 1 Bacterial leaf spot causing *Xanthomonas* culture on nutrient agar medium isolated from tomato fruits

Healthy plant **Inoculated plant**

Fig. 2.Pathogenicity on tomato plant. The figure depicts the development of typical bacterial leaf spot symptoms upon inoculation with the isolated strains of *X. euvesicatoria*

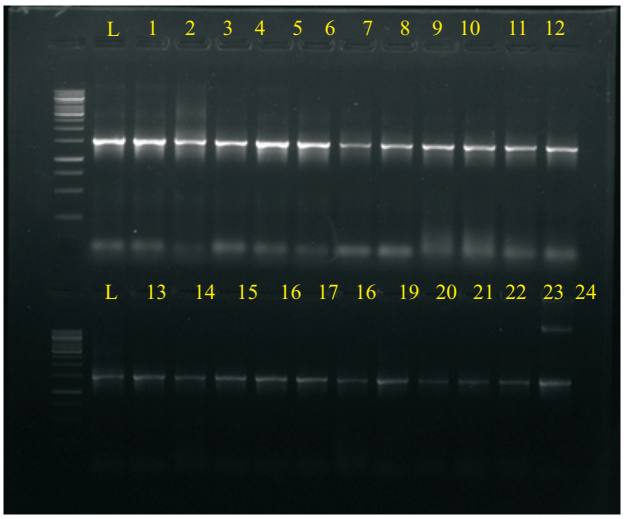

Fig. 3.Agarose gel electrophoresis showing amplification of the isolated strains with Universal primer targeting 16s r RNA region (FD1 RP2) at 1500 bp.
L : 1 Kb ladder (splitting at intervals of 250). 1-12: strains 1- 12. 13 – 14 : strains 13- 14.

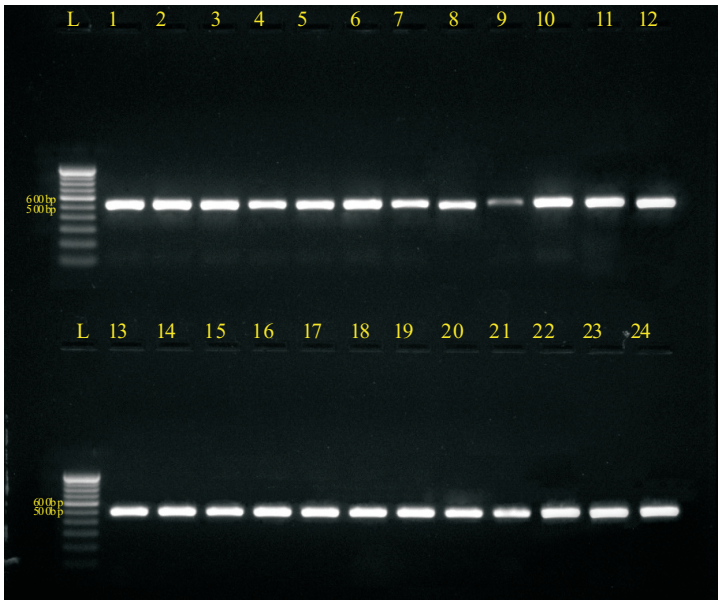

Fig. 4. Amplification of isolated bactreia with*Xanthomonas campestris* pv *vesicatoria* specific primer (XCVF - XCVR).
L : 100 bp ladder. 1-12: strains 1- 12. 13 – 14 : strains 13- 14.

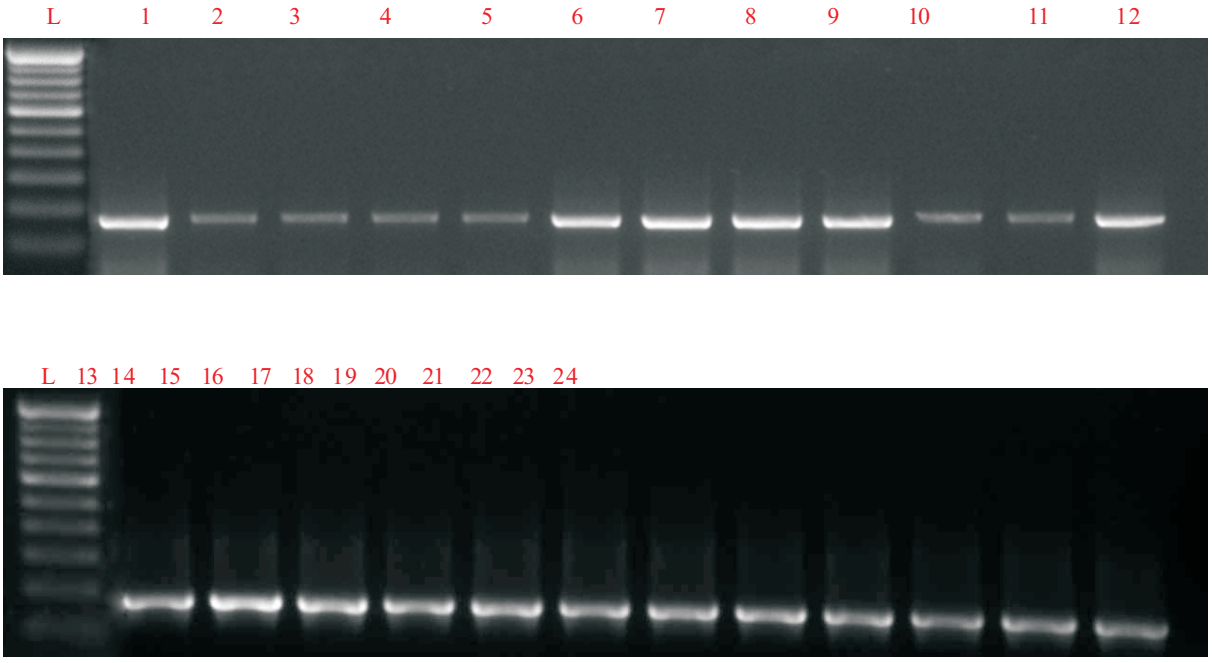

Fig. 5.Agarose gel electrophoresis for *X. euvesicatoria*specific primer, with amplification at 170 bp confirming the strains to be *X. euvesicatoria.*
L : 100 bp ladder. 1-12: strains 1- 12. 13 – 14 : strains 13- 14.

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Rec. on 01.05.2019 & Acc. on 07.05.2019