JOURNAL OF PURE AND APPLIED MICROBIOLOGY, December 2012. Vol. **6**(4), p. 1699-1706

Microbial, Biochemical, Pathogenicity and Molecular Characterization of *Xanthomonas axonopodis* pv. *punicae* from Pomegranate

P.B. Kale[1], V.P. Chimote[1], K.S. Raghuvanshi[2], A.A. Kale[1], A.S. Jadhav[1] and S.G. Borkar[2]

[1]State Level Biotechnology Centre, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India
[2]Department of Plant Pathology and Agricultural Microbiology, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra, India.

(Received: 29 April 2012; accepted: 03 June 2012)

In the present study, an isolate of *Xanthomonas axonopodis* pv. *punicae* (Xap) from oily spot infested pomegranate orchard of Nashik was subjected to characterization by microbial, biochemical as well as plasmid profiling methods. Microbial studies confirmed that, it is Gram-negative and rod-shaped bacteria with smooth, slightly raised, round, pale yellow coloured colonies. On biochemical analysis the bacterium was positive for Potassium Hydroxide (KOH) strings (Gram-negative), catalase (aerobe), weakly positive in oxidase test (non enteric Gram-negative), liquefied gelatin, hydrolyzed starch and casein. However it was unable reduce nitrate or to produce hydrogen sulphide and indole. Observations of typical characters for genus *Xanthomonas* includes: inability to utilize Asparagine as a carbon and nitrogen source and inhibition on 0.1% triphenyltetrazolium chloride (TTZ) were also recorded. This *Xanthomonas* isolate was further used for pathogenicity test to confirm virulence, and re-isolated to confirm Koch's postulate. Plasmid profiling was carried out to confirm the presence of megaplasmids typical of *Xanthomonas*. Restriction digestion of these plasmids with two restriction enzymes, *viz. Hind III* and *Sac I* revealed its size 93.64 kb and 96.18 kb, respectively.

Key words: Pomegranate, Bacterial blight, *Xanthomonas axonopodis* pv. *punicae*, Microbial characterization, Biochemical characterization, Plasmid profiling.

Pomegranate is medicinally and economically important fruit crop from the arid zones of India. The pomegranate tree is native from Iran traveled to the Himalayas in northern India and has been cultivated since ancient times throughout the Mediterranean region of Asia, Africa and Europe (Morton 1987). Earlier pomegranate cultivation was relatively free of most pests and diseases. With increase in intensive cultivation the reports of losses by diseases such as bacterial blight, anthracnose and wilt are becoming frequent.

Oily spot or bacterial blight of pomegranate is a serious disease in many pomegranate producing areas especially in peninsular India. The major symptoms include water soaked lesions on leaves, fruits, and stems at early stage and corky, dark oily spots at later stages of infection. Damages due to the bacterial blight in pomegranate range from 30 to100% (Anonymous,

* To whom all correspondence should be addressed.
E-mail: kalepb_btl@rediffmail.com

2006). Presently decline in orchards, particularly in pomegranate belt of peninsular India i.e. Maharashtra and Karnataka is being observed due to this disease. Oily spot disease lowers the yield as well as market value of produce and makes it unsuitable for export. The impact is worsened because the presence of infested samples of an area triggers restrictions, disrupting the movement of fresh fruit.

The causal organism of bacterial blight was first identified as *Xanthomonas campesrtis* by Hingorani *et al*., (1952). Afterwards it has reclassified and named as *Xanthomonas axonopodis* pv. *punicae* (*Xap*) (Hingorani 1959, Chand and Kishun, 1991, Akhtar, and Bhatti 1992, Upasana Rani and Verma, 2002). *Xanthomonas* is a large genus of Gram-negative, yellow-pigmented, exclusively plant-associated or pathogenic bacteria that collectively cause disease in many plant species. The broad host range of the genus contrasts with stringent host and tissue specificity for individual species and pathovars (Bogdanove *et al*., 2012). Now pathogen is considered as a target organism for management and phytosanitary regulations in many countries.

Morphological and physiological uniformity within *Xanthomonas* has hampered the establishment of a stable taxonomy (Hayward, 1993, Starr, 1981). Many a times it is difficult to detect *Xanthomonas* just on the basis of microbial and biochemical characterization. Other yellow-pigmented bacteria may be isolated from the infected tissues instead of those belonging to the genus *Xanthomonas* (Kale, 2009). As the conventional pathological characterization by pathogenicity test on susceptible seedlings are time-consuming (~2 weeks) and not well suited for rapid screening of samples. Molecular methods in combination with conventional methods have a great potential in rapid plant pathogen diagnosis. Nucleic acid based methods are most widely applied now days in diversity and diagnosis studies. Plasmid profiling also has proved their role in the following studies like: diversity, epidemiology, differentiation, host-pathogen interaction, virulence and antibiotic resistance / susceptibility. Accordingly plasmid profiling was also done in the present study to check for presence of characteristic megaplasmids of *Xanthomonas axonopodis* pv. *punicae*.

MATERIALS AND METHODS

Collection of samples, isolation and preservation of bacterium

Oily spot disease infected samples (leaves and fruits at early infection stage) were collected from pomegranate growing area (*Tehsil:* Deola) of Nashik district (Fig. 1-A). Pure culture was obtained and maintained on nutrient agar (NA) plates after incubation at 28°C for 48-72 hours (Schaad, 1988). Pure culture were further sub-cultured and used for subsequent microbial, pathological, biochemical and plasmid profiling. Glycerol stocks were prepared and stored for long time at -80°C for further work.

Microbial- microscopic and biochemical tests

To know the basic information and properties of the target pathogen, basic microbiological and biochemical tests are important. All the tests were performed as per the standard methods described here.

- Gram-iodine (Schaad,1988)
- KOH strings test
- TTZ i.e. Ttriphenyltetrazolium chloride (TTZ; at 0.05%, 0.1%, 1.5% concentrations) (Singleton and Sainsbury, 1988);
- Asparagine medium test (Bradbury, 1984);
- Casein hydrolysis test (Aneja, 2001);
- Gelatin liquefaction test on gelatin medium (Dickey and Kelman, 1988);.
- Starch hydrolysis (Aneja, 2001);
- Nitrate reduction test (Dickey and Kelman, 1988);
- H_2S and Indole production test (Aneja, 2001);
- Oxidase test (Dickey and Kelman, 1988) and
- NaCl tolerance test (nutrient agar supplemented with 0.5%, 1%, 1.5%, 2%, 3%, 4% and 5% NaCl) as described by Singleton and Sainsbury, 1988 for differential salt tolerance ability in Xanthomonads itself, whereas inoculated 0.5% salt-NA (Nutrient Agar) plate was used as positive control and un-inoculated NA plate were used as negative control and growth response was recorded.

Pathogenicity test, approval of Koch's postulate and hypersensitivity tests

Fully expanded young leaves of one year old pomegranate plants were used for pathogenicity tests and also for approval of Koch's postulate. Pellets from fresh suspension culture

were re-suspended in appropriate amount of sterilized water so that to adjust its OD to 0.3 at 460nm (i.e. 10^7-10^8 cfu/ml bacterial cell concentration). Equal aliquot of the bacterial suspension were inoculated to leaves (Bobosha, 2003), both by infiltration using syringe and surface spreading the inoculums with a brush. The reaction of the plant was observed at 5, 10, 15 and 20 days after artificial infection for symptom development. Characteristic pin headed oily spots at early stage leading to the angular black spot surrounded by oily ring at late stage were recognized as a positive pathogenicity test. Re-isolation of bacterium was made after preliminary symptom development on NA medium at 28°C for 48-72 hours and further growth was observed for colony characteristics (OEPP/EPPO, 2005). An aliquot of same bacterial suspension in sterile distilled water (Prepared in earlier experiment) was used and infiltrated into expanded leaves of a month old tobacco plant (*Nicotiana tabacum*) for hypersensitivity test. Sterilized distilled water was used as negative control treatment and all plants were kept in controlled condition until hypersensitive response symptom develops. Yellowing of tissues occurred around the spot of inoculation was taken as positive for the hypersensitivity test (Bobosha, 2003).

***Xanthomonas* Plasmid DNA isolation and restriction analysis**

Plasmid DNA was isolated from 48hrs old nutrient broth of *Xanthomonas* using QIAGEN Mega Plasmid Purification kit and used for further studies. Due to large size of plasmids it was difficult to characterize them by simple agarose gel electrophoresis. Therefore, restriction analysis was carried out using two restriction enzymes (MBI Fermentas) i.e. *Hind* III and *Sac* I separately at 37°C for 1 hr and then subjected to agarose gel (0.4%) electrophoreses in 1X TBE buffer. Fragments of restricted plasmid DNA compared with 1kb DNA marker.

RESULTS AND DISCUSSION

Present investigation was undertaken to provide in-depth information of the pathogen causing bacterial blight/oily spot disease in pomegranate (*Punica granatum* L.). Pathogen identity was determined using a bacterial isolate. For this purpose, microbial-biochemical tests and molecular analysis were undertaken.

Microbial characterization

Xanthomonas are slow growing and difficult to isolate usually due to masking effect of other fast growing, yellow pigmenting bacteria (Canteros *et al*., 1985). The bacterial isolate studied was able to form smooth, slightly raised, round, pale yellow coloured colonies on 48-72 hr incubation at 28°C. Microscopic observation revealed that it is rod shaped and Gram-negative bacterium. In KOH-string test, viscous mixture and strings formation confirmed that, it is Gram-negative (Fig. 1-B). Cell walls lyses due to 3% KOH in case of Gram negative; while those are not disrupted in Gram-positive bacteria.

The bacterium was confirmed to be *Xanthomonas* on the basis of TTZ and Asparagine test (Fig. 1-C). The bacterial growth was not observed on Asparagine as an only carbon and nitrogen source (Bradbury, 1984). This test is used as a diagnostic test for *Xanthomonas* as a typical character of the genus. Others like yellow Enterobacteriaceae and many Pseudomonads can grow on aspergine. Similarly it was inhibited on NA medium containing 0.1% TTZ, typical for genus

Table1. Symptom development studies for two different methods of artificial infection to fully expanded young leaves

S. No.	Method of Artificial Infection	Days after artificial infection (DAAI)			
		5	10	15	20
1	Infiltration by syringe	+	+++	++++	+++++
2	Surface spreading by painting brush	-	+	+++	++++

Note: + Single pin headed water soaked spot; +++ Brown and oily angular spot surrounded by water soaked ring; ++++ Black water soaked spot surrounded by yellow ring on leaves;
+++++ Yellow leaves with black oily spot ready to senescence; - No symptoms observed.

Xanthomonas in Bergey's Manual of Determinative Bacteriology (Bradbury, 1984). Even at 0.05% TTZ concentration exceptional single colony was detected.

In the other biochemical studies, the bacterium was found able to liquefy gelatin, hydrolyse starch, hydrolyze casein indicating proteolysis, and was found not able to produce hydrogen sulphide and indole; nitrate was not reduced in nitrate reductase test (Fig. 1-D). Positive catalase test confirms that the bacterium studied is an aerobe; as aerobic microbes produce enzyme catalase that detoxifies hydrogen peroxide into water and oxygen (Fig. 1-E). Oxidase test was weakly positive indicating that it is a non enteric Gram-negative bacterium. Many aerobic bacteria, including *Xanthomonas*, *Pseudomonas* species have Cytochrome oxidase. On the other hand, members of the family Enterobacteriaceae (Gram-negative facultative anaerobes) are oxidase negative.

In the present investigation collected bacterium showed growth on 0.5 % to 1.5% NaCl concentrations (Fig. 1-F). There was growth at 1.0 % and 1.5 % NaCl after increased incubation period as compared to 0.5 % and 1.0 % NaCl, respectively. As per Singleton and Sainsbury (1988) *Xanthomonas campestris* tolerates 2-5 % NaCl and *Xanthomonas axonopodis* tolerates 1% NaCl, while other uncommon species fail to tolerate more than 1% NaCl.

The authentication of an organism in the current investigation in respect of symptomatic, microscopic, biochemical and pathological study matches with the previous description given by Hingorani and Singh (1959) and Ramaswami (1962). These characters studied have important role in identification of the organism as *Xanthomonas punicae* (Jamdade; 2007).

Pathogenicity and Hypersensitivity

In pathogenicity test, typical symptoms were observed within a week on the leaves (Fig. 1-G). The development of symptoms in case of infiltrated leaves was earlier than uninjured surface inoculated leaves (Table 1). Bacteria were re-isolated from the infected tissue and it showed the similar microbial characteristics (Fig. 1-H). In general the pattern of development of symptoms in artificial inoculation experiment was identical with the symptoms observed in naturally infected plants.

Positive reaction to the non-host, tobacco (*Nicotiana tabacum*) is a confirmation for the bacterium to be pathogen (Fig. 1-I). The infiltrated tobacco leaves showed development of yellow to light yellow symptoms around the area of injection in 5-7 days. Jamdade (2007) and Hingorani and Singh (1959), described similar type of symptoms on leaves and fruit of pomegranate, which confirmed the result of present investigation.

Plasmid profiling of *Xanthomonas*

Most plant pathogenic bacteria are known to harbor plasmids carrying genes responsible for virulence, antibiotics resistance and exopolysaccharide production (Sundin, 2007). Plasmid profiling procedure can simultaneously targets pathogenicity associated loci therefore this method is sensitive in detecting pathogen. Algeria *et al*., (2005) observed that *Xanthomonas axonopodis* pv. *citri* contains two *virB* gene clusters, one on the chromosome and another one on a 64-kb plasmid, each of which codes for a previously uncharacterized type IV secretion system.

Indigenous mega plasmids have been reported from various species of *Xanthomonas* (Pruvost *et al*., 1992) with size ranging from 7 to 100kb. Satyanarayana and Verma (1993) found that highly virulent races of *X. axonopodis* pv. *malvacearum* harbour at least five plasmids among which two large plasmids of 60 kb and 40kb size were common. However there was greater variability

Table 2. Profile of restriction endonuclease digested plasmid DNA fragments

S. No.	*Hind* III restriction profile	*Sac* I restriction profile
1.	28759 bp	27591 bp
2.	12819 bp	12299 bp
3.	11800 bp	11558bp
4.	10421 bp	10421 bp
5.	9421 bp	8471 bp
6.	8340 bp	7631 bp
7.	7030 bp	5597 bp
8.	5046 bp	2405 bp
9.	2547 bp	1808 bp
10.	-1648 bp	
11.	-1097 bp	

Approximate Total (Minor bands excluded)
96183 bp HIND (III) 93648 bp (Sac I)

Fig. 1(a-i): Various steps involved in characterization of *Xanthomonas axonopodis* pv. *punicae.*

in smaller plasmids that were likely involved in extra aggressiveness. Continuous subculture resulted in loss of these smaller plasmids (Naara *et al*., 2004). *X. axonopodis* pv. *citri* 306 has two plasmids (64.92 kb and 33.7 kb) in addition to a circular chromosome of 5.17Mb (da Silva *et al*., 2002). *X. axonopodis* pv. *vesicatoria* 85–10 that causes bacterial spot of pepper and tomato have a total of 5.42 Mb genome size with main circular chromosome (5.17 Mb); in addition to 4 plasmids (182.572 kb; 38.116 kb; 19.146 kb and 1.852 kb). However in *Xanthomonas oryzae* pv. *oryzae* no autonomous plasmids were observed (Lee *et al*., 2005).

The plasmid DNA fingerprinting reveals higher polymorphism and is quicker than RFLP analyses of genomic DNA. In the present study, total plasmid with purity and integrity was used for digestion of with *Hind* III and *Sac* I separately. Restriction pattern resulted in 9 fragments by *Hind* III and 11 fragments by *Sac* I. Fragments generated by restriction enzyme *Hind* III were of size from 2.547kb to 28.759kb and by *Sac* I were of 1.648kb to 27.591kb (Table 2). Plasmid profiling of *Xanthomonas* revealed that the approximate composite size of these plasmids is in between 94kb (*Sac* I) to 96kb (*Hind* III) Fig. 2. The sum total size of the plasmids coincide with that of two plasmids i.e. ~100kb (60-65 kb and 34-40kb) of other *Xanthomonas axonopodis* pathovars (Satyanarayana and Verma, 1993; da Silva *et al*., 2002; BioMed, 2002). Morever Sharma *et al*., (2012) reported that genome sequences of *X. axonopodis* pv. *punicae* are 99% identical to those of *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus canker.

An ideal molecular target for diagnostic purposes must be genetically stable, and specific for the species/pathovar of interest (Zaccardelli *et al*., 2007). Our plasmid profiling protocol is suitable for rapid and specific identification of *Xap* from pure cultures during the confirmatory phase of diagnosis. Based on the study of the pathotypic diversity of *Xap*, an overall strategy for the identification and further characterization of pathotypes in different regions at a country level is proposed.

The authentication of an organism in the current investigation matches with the previous description given by studies viz. the cultural, morphological characters, biochemical reactions and identified the organism as *Xanthomonas axonopodis* pv. *punicae.* Mega plasmid profiling may provide a more sensitive measure of diversity and provide a convenient technique for tracking strains in epidemiological studies or in quarantine applications. The experimental results will be helpful for better understanding of new pathogen, its virulence establishing evolutionary relationship and to study spread of pathogen.

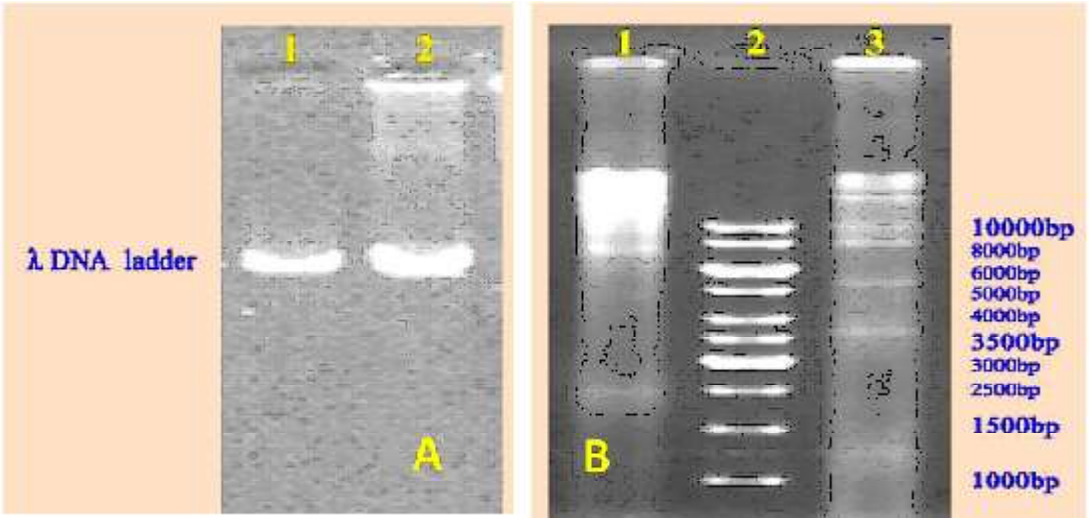

Fig. 2. Plasmid isolation and restriction profiling from *Xanthomonas axonopodis* pv. *punicae* (A) Lane 1: Lambda DNA, Lane 2: Plasmid (B) Lane 1: Digestion with *Hind* III, Lane 2: marker 1kb, Lane 3: Digestion with *Sac* I.

REFERENCES

1. Akhtar, M.A., Rahber Bhatti, M.H. Occurrence of bacterial spot of pomegranate in Pakistan. *Pak. J. Agri. Res.*, 1992; **13**(1): 95-97.
2. Alegria, M., Souza, D., Andrade, M., Docena, C., Khater, L. Identification of New Protein-Protein Interactions involving the products of the chromosome and plasmid-encoded type IV secretion loci of the phytopathogen *Xanthomonas axonopdis* pv. *citri*. *J. Bacteriol.*, 2005; **187**: 2315-25.
3. Aneja, K.R. *Experiments in Microbiology, Plant Pathology, Tissue Culture and Mushroom Cultivation*. 3rd edn. New Delhi: New Age International (P) Limited, 2001. p. 607.
4. Anonymous. Diagnosis of bacterial blight of pomegranate and its management- Training Manual, National Research Centre on Pomegranate, Solapur, 2006.
5. BioMed Central, Comparing *Xanthomonas*-The Scientist, 28 May, 2002.
6. Bobosha, K.: Characterization of *Xanthomonas campestris* pv. *musacearum* isolates: causal agent of enset bacterial wilt disease. Thesis submitted to School of Graduate Studies (M.Sc. Applied Microbiology) at Addis Ababa University. 2003.
7. Bogdanove, A.J., Koebnik, R., Lu, H., Furutani, A., Angiuoli, S.V., Patil, P.B., Van Sluys, M.A., Ryan, R.P., Meyer, D.F., Han, S.W., Aparna, G., Rajaram, M., Delcher, A.L., Phillippy, A.M., Puiu D., Schatz, M.C., Shumway, M., Sommer, D.D., Trapnell, C., Benahmed, F., Dimitrov, G., Madupu, R., Radune, D., Sullivan, S., Jha, G., Ishihara, H., Lee, S.W., Pandey, A., Sharma, V., Sriariyanun, M., Szurek, B., Vera-Cruz, C.M., Dorman, K.S., Ronald, P.C., Verdier, V., Dow, J.M., Sonti, R.V., Tsuge, S., Brendel, V.P., Rabinowicz, P.D., Leach, J.E., White, F.F., Salzberg, S.L. Two new complete genome sequences offer insight into host and tissue specificity of plant pathogenic *Xanthomonas* spp. *J. Bacteriol.*, 2011; **193**: 5450 –64.
8. Bradbury, J.F. *Xanthomonas*, In: *Bergey's Manual of Systematic Bacteriology,* Vol. 1 (Krieg NR, Holt, JG eds). Baltimore: Williams and Wilkins, 1984; pp 199-210.
9. Lee, B.M., Park, Y.J., Park, D.S., Kang, H.W., Kim, J.G., Song, E.S., Park, I.C., Yoon, U.H., Hahn, J.H., Koo, B.S., Lee, G.B., Kim, H., Park, H.S., Yoon, K.O., Kim, J.H., Jung, C., Koh, N.H., Seo, J.S., Go, S.J. The genome sequence of *Xanthomonas oryzae* pathovar *oryzae* KACC10331, the bacterial blight pathogen of rice. *Nucleic Acids Res*., 2005; **33**(2): 577–86.
10. Canteros, B.J.I., Zagory, D., Stall, R.E. A medium for cultivation of the B strain of *Xanthomonas campestris* pv. *citri* causes cancrosis B in Argentina and Uruguay. *Plant Dis*., 1985; **69**: 122-3.
11. Chand, R., Kishun, R. Studies on bacterial blight of pomegranate. *Indian Phytopatol*., 1991; **44**(3): 370-372.
12. da Silva, A.C.R., Ferro, J.A., Reinach, F.C., Farah, C.S., Furlan, L.R., Quaggio, R.B., Monteiro-Vitorello, C.B., Van Sluys, M.A., Almeida, N.F. Jr, Alves, L.M.C. Comparison of genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature*, 2002; **417**: 459–63.
13. Dickey, R.S., Kelman, A. 'Caratovora' or soft rot group. 2nd edn. In: *Laboratory guide for identification of plant pathogenic bacteria* (Schaad NW ed).; St. Paul: APS Press, 1988; pp 81-84.
14. Hayward, A.C. The hosts of *Xanthomonas*. In: *Xanthomonas* (Swings JG. and Civerolo EL eds). London: Chapman and Hall, 1993; pp 1–119
15. Hingorani, M.K., Mehta, P.P. Bacterial leaf spot of pomegranate. *Indian Phytopathol.,* 1952; **5**: 55-56.
16. Hingorani, M.K., Singh, N.J. *Xanthomonas punicae* sp. on *Punica granatum* L. *Indian J. Agric. Sci.,* 1959; **25**: 45-48.
17. Jamdade, K.D. Studies on Oily Spot Disease of Pomegranate, Thesis submitted for M.Sc. (Agriculture) at Mahatma Phule Krishi Vidyapeeth, Rahuri, 2007.
18. Kale, P.B.: Molecular characterization of oily spot disease of pomegranate. Thesis submitted for M.Sc. (Agril. Biotech.) at Mahatma Phule Krishi Vidyapeeth, Rahuri, 2009.
19. Morton, J.: Pomegranate. In: *Fruits of warm climates*. (Julia FM, Miami FL, eds) Miami: Julia F. Morton, 1987; pp 352–355.
20. Narral, H.P., Jayaraman, J., Verma, J.P., Fox, R.T.V. Stability of plasmid profiles of highly virulent races of *Xanthomonas campestris* pv. *malvacearum* during storage and subculturing. *J. Plant Pathol.,* 2004; **86**(2): 171-175.
21. OEPP/EPPO PM 7/44 (1). *"Xanthomonas axonopodis* pv. *citri". OEPP/EPPO Bulletin* 2005; **35**: 289-294.
22. Pruvost, O., Hartung, J.S., Civerolo, E.L., Dubois, C., Perrier, X. Plasmid DNA fingerprints distinguish pathotypes of *Xanthomonas campestris* pv. *citri*, the causal agent of Citrus bacterial canker disease. *Mol. Plant Pathol.,* 1992; **82**(4): 485.
23. Ramaswami, G. (ed): Bacterial plant diseases in

India. Bombay: Asia Publishing House, 1962.

24. Sathyanarayana, N., Verma, J.P. Possible role of plasmids in the virulence of *Xanthomonas campestris* pv. *malvacearum*. *Indian Phytopathol,,* 1993; **46**: 165-166.
25. Schaad, N.W.: Initial Identification of Common Genera, 2nd edn. In: *Laboratory guide for identification of plant pathogenic bacteria,* (Schaad NW ed). St. Paul: APS Press, 1988; pp 81-84.
26. Sundin, G.W. Genomic insights into the contribution of phytopathogenic bacterial plasmids to the evolutionary history of their hosts. *Annu. Rev. Phytopathol.*, 2007; **45**: 129-151.
27. Sharma, V., Midha, S., Ranjan, M., Pinnaka, A.K., Patil, P.B. Genome of *Xanthomonas axonopodis* pv. *punicae* strain LMG 859. *J. Bacteriol.*, 2012; **194**(9): 2395.
28. Singleton, P., Sainsbury, D. (eds.): Dictionary of Microbiology and Molecular Biology, 2nd edn. Singapore: John Wiley and Sons, 1988; p 660.
29. Starr, M.P.: The genus *Xanthomonas*. In: *The prokaryotes, Volume 1*. (Starr MP, Stolp H, Truper HG, Balows A and Schlegel HG, eds), Berlin: Springer-Verlag, 1981; pp 742–763.
30. Upasana, Rani, Verma, K.S. Perpetuation and spread of *Xanthomonas axonopodis* pv. *punicae* causing black spot of pomegranate. *Plant Dis. Res.,* 2002; **7**(1): 46-50.
31. Zaccardelli, M., Campanile, F., Spasiano, A., Merighi, M. Detection and identification of the crucifer pathogen, *Xanthomonas campestris* pv. *campestris*, by PCR amplification of the conserved Hrp/type III secretion system gene *hrcC*. *Eur. J. Plant Pathol.*, 2007; **118**: 299-306.