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Characterization of *Xanthomonas campestris* pv. *viticola* causing bacterial leaf spot of grapes in Maharashtra, India

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Authors Info

A.K. Kamble^{1,2}, S.D. Sawant¹,
I.S. Sawant¹, S.B. Ghule¹,
A.C. Patil¹ and S. Saha^{1*}

¹Department of Plant Pathology,
ICAR- National Research Centre
for Grapes, Pune-412 307, India

²Department of Agrochemicals
and Pest Management,
Shivaji University,
Kolhapur-416 004, India

*Corresponding Author Email :
sujoyta@gmail.com

Edited by

Dr. Prabhat Shukla

Reviewed by

Dr. R. Selva Kumar
Dr. Ravindra Kumar

Abstract

Aim : The aim of the present study was to isolate, characterize and identify pathogenic bacteria from bacterial leaf spot infected grape vineyards of Maharashtra.

Methodology : Collected diseased samples were subjected to isolation. All isolates were further subjected to different morphological and biochemical characterization. These isolates were also used to standardize inoculation methods to study host range and test pathogenicity to confirm infectivity. Genomic DNA was isolated from bacteria for phylogenetic analysis using 16S rDNA.

Results : The disease is characterized by water soaked, angular leaf spot which later turns to irregular, dark brown to black necrotic region on the leaf surface. Microbial studies confirmed that it is a Gram negative, rod shaped bacterium with white, mucoid, glistening and convex circular colonies on artificial medium. Pathogenicity test was performed to confirm virulence. Phylogenetic analysis of 16S rDNA gene exhibited more than 99% similarity with other *Xanthomonas campestris* pv. *viticola*. The bacterium was found resistant to vancomycin, penicillin and oxacillin antibiotics. The bacterium is also capable of infecting *Mangifera indica* and *Citrus limon* along with eight weed species.

Interpretation : Based on morphological, biochemical and molecular characteristics, it was identified as *Xanthomonas campestris* pv. *viticola*.

Key words: 16S rDNA, Bacterial leaf spot, Grape vineyards, *Xanthomonas campestris* pv. *viticola*

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Introduction

Grapes are grown in India in about 138.0 lakh ha land with annual production of 2980 lakh tones (NHB, 2017-18). Maharashtra is a leading producer of grapes occupying 90,000 ha followed by Karnataka, Telengana, Andhra Pradesh and Tamil Nadu (NHB, 2017-18). During the year 2017-18, 2,14,440.57 MT of grapes was exported from India worth Rs. 2,14,621.94 lacs (APEDA 2017-18). This crop is of high commercial interest but suffers from many diseases like downy mildew, powdery mildew, rust, bacterial leaf spot and anthracnose. With the changing climate bacterial leaf spot of grape is caused by *X. campestris* pv. *viticola* (Nayadu) Dye is gradually gaining importance. The disease is characterized by water soaked, angular spots on leaves which later becomes necrotic (Chand *et al.*, 1990; Halfeld-Vieira *et al.*, 2006). In 1984, the disease appeared as destructive outbreak in September pruned vineyards of cultivar Thompson Seedless in Sangli and Solapur districts of Maharashtra (Patil, 1988) and a crop loss of 60-70% was estimated by Chand *et al.* (1990). The disease has cause considerable damage to table grapes in Brazil (Marques *et al.*, 2009). Moreover, it prevents the global transfer of grapevine propagating material as *X. campestris* pv. *viticola*, is a quarantine pathogen in many countries (Naue *et al.*, 2014). Preliminary investigation about the causal agent have been carried out and this bacterium forms typical smooth, round, mucoid convex colonies with entire margins (Trindade *et al.*, 2005).

Due to its relatively recent emergence as an important economic pathogen, little is known about the biology of *X. campestris* pv. *viticola*. Essential prerequisites for understanding and controlling plant diseases are detection and identification of causal agents and systems for early detection of pests might prevent spread of diseases and food losses (Scala *et al.*, 2018). Fang and Ramasamy (2015) opined advanced disease detection methods to minimize and prevent damages caused due to diseases during growth, harvest and postharvest processing, are imperative and grapes are not an exception. In light of the objective of this study was to characterize field strains of bacterium collected from 2015 to 2017, for proper identification of pathogens.

Materials and Methods

Sample collection : A total of eight samples were collected from Solapur (3), Sangli (2), Nashik (1), Osmanabad (1) and Pune (1) grape growing regions of Maharashtra during July to November in 2015 to 2017. The samples were collected on the basis of typical symptoms observed on leaves, petioles and shoots.

Isolation: Infected tissue segments were cut along with little healthy portion with sterilized blade. The cut segments were surface sterilized with 70% alcohol for 60 sec, washed with sterilized distilled water and macerated in 5 ml sterile distilled water. After 2 min, a loop full suspension was streaked on nutrient agar (HiMedia M001) medium plates and incubated at $28 \pm 1^\circ\text{C}$

for 48 hr. Single isolated colonies were purified on nutrient agar slants and were stored at 4°C for further studies.

Pathogenicity and standardization of inoculation techniques: Different inoculation methods viz., injection infiltration, silicon carborundum abrasion, pinprick and scissor clipping (Mazi *et al.*, 2015) were applied on 3 to 4 month old healthy Thompson Seedless potted plants. For all methods, bacterial suspension was adjusted to a density of 10^8 cfu ml⁻¹ with the help of sterile distilled water by using McFarland's standards (McFarland, 1907). The different techniques of inoculation were studied by completely randomized block design (CRD) method. For pathogenicity test, fourth to sixth leaf from the apical side of plant (Chand *et al.*, 1991) were selected for inoculation. Ten plants for each inoculation method were maintained and from each plant single leaf was considered as a replicate. After inoculation, the plants were kept in glass house and observations were taken on alternate days.

Morphological and biochemical characterization : Different morphological and biochemical tests were conducted for identification of bacterium. All the tests were performed by using single isolated colony. The physiological and bio chemical characters of the bacterium was studied for Gram staining, hydrolysis of starch, 3% KOH test, gelatin liquefaction, indole production, urease production, casein hydrolysis, utilization of Asparagine as sole source of carbon and nitrogen and oxidase reaction. The tests were conducted as per methods described by Brad Bury (1970) and Schaad and Stall (1988) with minor modifications.

Host range studies : Perennial fruit plants viz. *Mangifera indica*, *Citrus limon*, *Punica granatum*, *Psidium guajava* and other sixteen different weed species normally present in vineyards viz., *Cyanotis axillaris*, *Portulaca oleracea*, *Amaranthus viridis*, *Euphorbia hirta*, *Lantana camara*, *Oxalis corniculata*, *Cyperus rotundus*, *Alternanthera sessilis*, *Euphorbia heterophylla*, *Brachiaria plantaginea*, *Solanum nigrum*, *Taraxacum officinale*, *Sida acuta*, *Coccinia indica* and *Conyza Canadensis* were selected to as certain alternative host of pathogenic bacteria. Ninety days old susceptible Thompson Seedless plants were used as positive control while each host species inoculated only with sterile distilled water represented negative controls. The artificial inoculation was carried out using injection infiltration method. Five leaves of each species were inoculated, each representing a replication.

The inoculated plants were kept in glass house where temperature ($30 \pm 2^\circ\text{C}$) and humidity (90%) were maintained. Observations were taken on alternate days up to 30 days after inoculation. The plants were categorized on the basis of occurrence of water soaked, chlorotic and necrotic symptoms on leaves. Incidence of symptoms was recorded as positive (+) and no incidence as negative (-).

Antibiotic sensitivity test: Nine different antibiotics namely, penicillin, ampicillin, clindamycin, chloramphenicol, vancomycin,

erythromycin, kanamycin, gentamycin and oxacillin were evaluated to study the sensitivity of isolates under *in vitro* conditions using antibiotics strips of varying concentration ranging from 0.16–256 mcg ml⁻¹. A sterile cotton swab was dipped into freshly prepared bacterial suspension and bacterial lawn was prepared on nutrient agar media. After drying of the lawn, antibiotic strips were placed on the inoculated plates. Plates with bacterial lawn without any antibiotic strip served as control. Inoculated plates were incubated at 28 ± 1°C for 72 hr. Lysis of bacterial lawn around the strip was recorded and the results of sensitivity were reported as minimum inhibitory concentration (MIC).

Molecular characterization : The pathogenic bacteria were grown in nutrient broth (HiMedia MM244) at 150 rpm for 24 hr at 28 ± 1°C in dark and DNA was extracted using the QIAamp DNA mini Kit (Qiagen). DNA obtained was quantified using Nano photometer (IMPLEN ver. 2008). 16S rDNA was amplified by PCR using a combination of universal primers, 27F (5'-AGAGTTTGATCCTGGTCAGAA CGCT-3') and 1492R (5'-TACGGCTACCTTGTACGACTTCAC CCC-3') which amplify approximately 1.4 kb fragment from bacteria (Heuer et al., 1997). PCR amplifications were performed in 25 µl reaction mixture containing 1 U Taq polymerase (Merck Bioscience), 2.5 µl of 10X Taq buffer, dNTP at 200 µM, 10 pmol of each primer and 25 ng of DNA. PCR System programmed was initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec annealing at 55°C for 30 sec primer extension at 72°C for 1.5 min and a final extension at 72°C for 10 min. The obtained PCR products were checked on 1.2% agarose gel (1X TAE) embedded with ethidium bromide. The resulting PCR products were purified and used directly for sequencing (Sci genome, Kochin, India). The resulting forward and reverse sequences were aligned and edited to remove ambiguous base calls using BioEdit Sequence Alignment Editor.

16S rDNA gene comparison and phylogenetic analysis: The 16S rDNA sequences were compared with the sequences of different *Xanthomonas* species available at the National Centre for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) using BLAST search. The multiple sequence analysis and construction of phylogenetic tree based on 16S rDNA sequences was performed using CLUSTAL and MEGA 6 program, respectively (Tamura et al., 2013). The pairwise evolutionary distances were computed using Kimura-2-parameter distance model and phylogenetic tree was constructed using Neighbor Joining (NJ) method. For phylogenetic analysis, isolate sequences were compared with authentic sequences of 16S rDNA sequences of *Xanthomonas campestris* pv. *campestris*, *Xanthomonas albilineans*, *Xanthomonas oryzae*, *Xanthomonas theicola*, *Xanthomonas sacchari*, *Xanthomonas hyacinthi* and *Xanthomonas populi* which were retrieved from GenBank. Relative support for particular clades in the neighbor joining tree was estimated using 1,000 replicates of bootstrap procedure. *Stenotrophomonas maltophilia* was used as out group reference strain.

Statistical analysis: The collected data were statistically analysed and subjected to analysis of variance (ANOVA) by using the statistical software package (SAS, 2002).

Results and Discussion

All eight isolates produced white colored, circular, mucoid, glistening, convex colonies on nutrient agar medium. Yellow pigment namely xanthomonadin is a specific characteristic of genus *Xanthomonas* which is found in majority species of *Xanthomonas* (Bradbury, 1984; Swing et al., 1993). Yellow pigments namely xanthomonadins comprise a unique group of halogenated aryl polyene pigments but their function is poorly understood (Andrews et al., 1973). In the present study, all isolates produced white colonies instead of typical yellow colonies, suggesting possibility of mutation in xanthomonadin biosynthetic pathway. A pigmented, white colored colony has also been reported in other species of *Xanthomonas* (Pruvost et al., 1998; Samanta et al., 2013 and Sabet et al., 1959). Morphologically, similar types of colony characteristics were reported by Nascimento et al. (2004) and Halfeld-Vieira et al. (2006) but yellow colored colony of bacterial leaf spot pathogen was also reported by Jambenal et al. (2011) hinting a phenotypic diversity which needs to be investigated further. Upon inoculation by different methods, shiny, water soaked, angular small spots were observed on lower side of leaves. Later these spots became necrotic, dark brown to black in colour, angular to irregular in shape, measuring about 3–4 mm in size and spread all over the leaves. Similar type of symptoms of bacterial leaf spot in grapes were described by Araujo, (2000); Nascimento et al. (2004) and Chand et al. (1990). Water soaked-angular spots on leaves which later become necrotic have also been reported in other crops eg., bacterial blight of cotton caused by *Xanthomonas axonopodis* pv. *malvacearum* (Saha et al., 2001), which supports the present findings that the symptoms are typical ones caused by *Xanthomonas* sp.

Observations were recorded on alternate days up to 30 days after inoculation (DAI) to evaluate the best method of inoculation in order to study symptoms expression and its subsequent development. In injection infiltration method, small water soaked spots were observed 3 DAI which increased within 12 DAI and became necrotic. Among four inoculation methods, injection infiltration method showed early symptom expression and maximum number of spots development on leaf surface. Similar observations were reported by Halfeld-Vieira et al. (2006) and Maji et al. (2015) hence injection infiltration method was found best for rapid test of pathogenicity with phytopathogenic *Xanthomonas*. All eight isolates were found to be Gram negative, rod shaped and motile. All isolates gave negative response to oxidase test, indole test, urease test and utilization of asparagine as sole source of carbon and nitrogen tests and positive for 3% KOH solubility test, starch hydrolysis, casein hydrolysis and gelatin liquefaction (Table 1) (Malavolta, 1999; Nascimento et al., 2004). Additional tests, i.e., acid production from sugar (sucrose,

Fig. 1: Phylogenetic position of *X. campestris* pv. *viticola* isolates from Maharashtra inferred by analysis of 16 rDNA sequences.

xylose, glucose and galactose), NaCl tolerance, inhibition by triphenyl-tetrazolium chloride (TTC), H_2S and levan production and nitrate reduction were conducted by Burr *et al.* (1980) and Chand *et al.* (1990) for identification of *Xanthomonas* sp. Gram negative bacteria which were oxidase negative, catalase positive and were unable to use asparagines as sole carbon and nitrogen source are primarily characteristics of *Xanthomonas* sp. (Bradbury *et al.*, 1984) and present study conform to all the above criteria, thereby confirming that the identified the isolates belong to genus *Xanthomonas*.

All the isolates showed >99% to 100% similarity with authentic sequences of *Xanthomonas campestris* pv. *viticola* isolates showing >99% similarity could be classified as same species (Bosshard *et al.*, 2003). The results obtained from direct

sequencing of purified PCR products showed that all the isolates belong to *Xanthomonas campestris* pv. *viticola*. The 16S rDNA gene sequences of eight isolates have been deposited in GenBank database under the accession numbers KX257172 - KX257183. The phylogenetic tree obtained by sequence analysis of 16S rDNA of pathogenic isolates and the sequences of *Xanthomonas* spp. obtained from databanks is shown in Fig. 1. 16S rRNA coding genes are universally found among the bacteria and widely used as genomic marker for bacterial identification. A substantial part of 16S rDNA gene is conserved in all bacteria which enable to construct a universal primer and a smaller variable region enables to estimate evolutionary distances for species identification (Nossa *et al.*, 2010). In phylogenetic tree, pathogenic isolates form monophyletic clade with reference isolates of *X. campestris* pv. *viticola* and it is supported by

Table 1 : Biochemical tests for *Xanthomonas campestris* pv. *viticola*

Test		Results
Oxidase test	-ve	No colour produced
Catalase test	+ve	Produced O ₂ bubbles
Starch Hydrolysis test	+ve	Clear zone produced around bacterial colony in medium when stained with iodine
Casein Hydrolysis test	+ve	Clear zone produced around bacterial colony in medium
Gelatin liquefaction test	+ve	Liquefied the gelatin medium
L Asparagines test for Sole source of C and N	-ve	No turbidity produced in medium
Indole test	-ve	No pink colour ring produced
Urease test	-ve	No colour change

-ve = Negative; +ve = Positive

Table 2 : Sensitivity of *Xanthomonas campestris* pv. *viticola* to different antibiotics

Sr. No	Name of antibiotic	MIC (mcg ml ⁻¹)	Sr. No	Name of antibiotic	MIC (mcg ml ⁻¹)
1	Ampicillin	Sensitive (8 mcg)	6	Clindamycin	Sensitive (4 mcg)
2	Gentamicin	Sensitive (75 mcg)	7	Kanamycin	Sensitive (8 mcg)
3	Vancomycin	Resistant	8	Oxacillin	Resistant
4	Penicillin	Resistant	9	Erythromycin	Sensitive (48 mcg)
5	Chloramphenicol	Sensitive (4 mcg)			

bootstrap value of 75%. Thus, molecular analysis confirmed that the isolates investigated in the present study was *X. campestris* pv. *viticola* and is the incitant of BLS. Popovic *et al.* (2014) identified and characterized, *Xanthomonas campestris* pv. *campestris* as causal agent of black rot in oil seed rape crop in Serbia, based on pathogenicity, physiological, biochemical and their 16S rDNA sequence analysis.

The host range study revealed that *Xanthomonas campestris* pv. *viticola* produced almost identical water soaked, chlorotic symptoms on leaf surface within 3-5 days after inoculation, on *Mangifera indica* and *Citrus limon* and on eight weed species viz., *Euphorbia hirta*, *Lantana camara*, *Oxalis corniculata*, *Euphorbia heterophylla*, *Solanum nigrum*, *Taraxacum officinale*, *Sida acuta* and *Coccinia indica*. Chand and Kishun (1990) found that *Mangifera indica* was an alternate, host of *Xanthomonas campestris* pv. *viticola*, but they did not find any response of bacteria on *Citrus limon* and *Lantana camara* as alternative hosts. Santos *et al.* (2014) found that *Xanthomonas campestris* pv. *viticola* had *Azadirachta indica* as potential alternative host. The ability of bacteria to survive in other plant systems, except the major host, is not only key for survival and reoccurrence of pathogen but also leads to danger of epiphytotic. As chemical control is inefficient and eradication is not feasible, preventive measures like elimination of alternative hosts of the pathogen is of paramount importance (Lima, 2000). The occurrence of *Xanthomonas campestris* pv. *viticola* in different alternative hosts gives an insight that these should not be allowed to grow near the vine with an aim to provide a wholistic management of disease. *Xanthomonas campestris* pv. *viticola*

exhibited sensitivity to most of the antibiotics like, ampicillin, chloramphenicol, gentamicin, kanamycin and clindamycin but was resistant to vancomycin, penicillin and oxacillin (Table 2).

McManus *et al.* (2002) reported that ascomycin showed selective toxicity against *Xanthomonas citri* and *Xanthomonas oryzae*. Ravikumar *et al.* (2003) found streptomycin sulphate and streptocyclin effective at 500 ppm against leaf spot of grape caused by *X. campestris* pv. *viticola* *in vitro*. Recently, Kamble *et al.* (2017) reported that *Xanthomonas campestris* pv. *viticola* was sensitive to streptocyclin at 50 ppm, but it did not show any response to validamycin and kasugamycin. Sensitivity and resistance to antibiotics can be used as a marker for correct identification of bacteria. The study has suggested that a proper identification of *Xanthomonas campestris* pv. *viticola* in bacterial leaf spot infected grape vines will make way for the growers to adopt control measures for managing bacterial pathogen. In an advanced research endeavour, specific gene based primers should be deployed for rapid identification of *Xanthomonas* sp. (Kiran *et al.*, 2019) so as to enable quick implementation of effective control strategies. Proper identification of *X. campestris* pv. *viticola* will be the cornerstone for understanding the genetic diversity and origin along with an effective diagnosis of BLS.

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