Diversity of *Xanthomonas citri* pv. *viticola* Populations on Grapevines from Different Locations in India

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The bacterial leaf spot (BLS) disease caused by *Xanthomonas citri* pv. *viticola* (*Xcv*) is a menace to the production of grapes all over India. The maximum incidence of BLS has been reported in the Maharashtra, Karnataka, and Andhra Pradesh states of India. The symptoms are characterised by water-soaked, angular spots which later become necrotic on leaves. The present study was conducted to investigate the variability of 23 *Xcv* strains isolated from infected samples collected from Maharashtra, Karnataka, and Tamil Nadu in India. Samples were collected on the basis of the characteristic symptoms. The diversity of all the isolates was assessed phenotypically and genetically. Phenotypic characters included the morphology of colonies, pathogenicity, hypersensitivity, and biochemical tests. Genetic characterisation was assessed using 16S rRNA sequencing. The results exhibited diversity with respect to various phenotypic aspects, like colony size, colour and opacity, the methyl red reduction test, enzyme production, pathogenicity, and a hypersensitivity reaction. The 16S rRNA sequencing showed a distribution of pathogens into two main clusters, which were further divided into nine sub-clusters. The salient findings on *Xcv* diversity in India will be useful to identify and characterise resistant germplasms against the disease. The non-uniform variability obtained among the isolates suggests that geographical location, climatic factors and the varieties were the driving forces for the evolution of the phytopathogens.

INTRODUCTION

The grape industry is one of the major fruit industries in India, and the total output of grape production reached up to 3 489 000 metric ton from an average area of 162 000 ha in the 2021/2022 production year (Anonymous, 2022). The major grape-growing states are Maharashtra and Karnataka, accounting for about 95% of India's total grape production. Other table grape-growing states include Tamil Nadu, Telangana, Andhra Pradesh, Mizoram, Punjab, Madhya Pradesh, Jammu and Kashmir, Nagaland, Haryana and Rajasthan (Anonymous, 2022). India is one of the biggest grape exporters in the world, with reported earnings of 313.69 million USD in 2022/2023 (Anonymous, 2022). However, grape-related diseases like powdery mildew, downy mildew, anthracnose, bacterial leaf spot and rust have affected the growth of the grape industry and caused huge economic losses. According to a report, India loses 8.23%, i.e. about 223 000 tons, of its grapes every year (Agricultural and Processed Food Products Export Development Authority [APEDA], 2021).

Bacterial leaf spot (BLS) is a disease of grapevine that occurs worldwide, especially during the months of August to September in warm and humid climates (Jones *et al*., 2014). *Xanthomonas citri* pv. *viticola* was reported to cause BLS of grapevine in Maharashtra, characterised by small, brown, angular, water-soaked lesions on the leaves, stems and fruit, leading to defoliation and direct fruit damage (Kamble *et al*., 2019). Severe infection may cause substantial damage to crops, with significant yield losses (Pernezny *et al*., 2003).

The causal bacteria are disseminated via various routes, like contaminated soil, leaves, berries, irrigation water and infected plant debris, which act as potential sources of inoculum (Gitaitis & Walcott, 2007). Initially, bacteria grow epiphytically and then enter the host through either stomata, hydathodes or wounds, spreading systemically to colonise the mesophyll parenchyma (Ryan *et al*., 2011). The distribution and prevalence of BLS-causing *Xanthomonas* species in India is relatively unknown. BLS was first reported in India by Nayudu (1972), but its causal agent was mentioned as *Pseudomonas viticola*. Later, Dye (1978) redefined its taxonomic position and named it *X. campestris*

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pv. *viticola*. Chand and Kishun (1990) also reported the epidemic occurrence of the disease in India and its extensive influence on yield loss. In 2018, Da Gama *et al*. suggested reclassification of *X. campestris* pv. *viticola* as *X. citri* pv. *viticola*.

BLS is primarily controlled by resistant lines, antibiotic treatment, and copper sprays (Roach *et al*., 2018). Due to reliance on the limited range of chemicals, resistance against copper and antibiotics was reported in the *Xanthomonas* population (Martin *et al*., 2004; Griffin *et al*., 2017). Hence, resistant lines, cultural interventions and biological control could be integrated for the successful management of the disease. The evolution of new *Xanthomonas* spp. and pathogenic strains over time has hindered the development and deployment of host resistance to manage bacterial spot disease in grape crops (Timilsina *et al*., 2016). Variation and variability are natural phenomena in plant pathogenic bacteria, and their identification is always a challenge. 16S rDNA sequencing is one of the most rapid and accurate methods for identifying disease-causing bacteria and understanding their epidemiology (Clarridge *et al*., 2004; Faniyan *et al*., 2023). The rRNA genes, such as 16S, 23S and 5S, are highly conserved at the genus and species levels, and thus are believed to be useful tools for grouping bacteria at the taxonomic level.

Although outbreaks of BLS have occurred in most commercial vine-growing regions of India, scanty information is available regarding the genetic diversity and distribution of the causal *Xanthomonas* species. The development of effective management approaches, particularly the selection of resistant plant material, relies upon the accurate identification of the pathogens and a thorough understanding of pathogen diversity and pathogenicity. This study describes the phenotypic and genetic variability of pathogenic *Xanthomonas* spp. associated with BLS in India.

MATERIALS AND METHODS

Sample collection

Infected leaf samples were collected from various grapevine varieties in 23 different locations across Maharashtra, Karnataka and Tamil Nadu (Table 1), according to the symptoms shown in Fig. 1. The leaves were collected in labelled polythene bags and stored at 4°C prior to use.

Isolation of *Xanthomonas*

The collected leaf samples were washed thoroughly under tap water and blot dried. Each leaf was segmented into pieces, 1 cm to 2 cm long, using a sterile scalpel. The pieces were dipped in sodium hypochlorite (1:100) (NaOCl) solution, followed by incubation for 30 sec, and subsequently treated

a) Infected leaves - red circle indicates symptoms

b) Healthy leaf

FIGURE 1
Symptomology of bacterial leafspot in greenhouse trials

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TABLE 1
Samples collected from different locations

Sr no.	State	District	Village			Strains	Variety
			Name	Position			
				Latitude (N)	Longitude (E)		
1	Maharashtra	Nashik	Bhuvan	20.300	73.490	*Xcv* 1	Clone 2A
2	Maharashtra	Nashik	Sompur	20.590	74.198	*Xcv* 2	Crimson Seedless
3	Maharashtra	Nashik	Thengoda	20.590	74.198	*Xcv* 3	SSN
4	Maharashtra	Nashik	Thengoda	20.590	74.198	*Xcv* 4	Manikchaman
5	Maharashtra	Nashik	Palkhed	20.370	73.769	*Xcv* 5	Clone 2A
6	Maharashtra	Nashik	Verkheda	20.234	73.893	*Xcv* 6	Thompson Seedless
7	Maharashtra	Nashik	Verkheda	20.234	73.893	*Xcv* 7	Nanasaheb Purple
8	Maharashtra	Nashik	Satana	20.597	74.200	*Xcv* 8	Thompson Seedless
9	Maharashtra	Nashik	Karsul	20.370	73.769	*Xcv* 9	Thompson Seedless
10	Maharashtra	Nashik	Sarole	18.083	75.850	*Xcv* 10	Clone 2A
11	Maharashtra	Sangli	Belanki	16.817	74.642	*Xcv* 11	Sonaka
12	Maharashtra	Sangli	Walwa	16.494	74.230	*Xcv* 12	Sarita
13	Maharashtra	Sangli	Kuchi	17.021	74.863	*Xcv* 13	Manikchaman
14	Maharashtra	Sangli	Karoli	17.047	74.872	*Xcv* 14	Clone 2A
15	Maharashtra	Pune	Narayangaon	19.162	73.914	*Xcv* 15	Dhanaka
16	Maharashtra	Pune	Narayangaon	19.162	73.914	*Xcv* 16	Manikchaman
17	Maharashtra	Solapur	Mohal	17.809	75.637	*Xcv* 17	Red Globe
18	Maharashtra	Solapur	Pandharpur	17.673	75.294	*Xcv* 18	Thompson Seedless
19	Karnataka	Vijayapura	Vijayapura	16.827	75.725	*Xcv* 19	Crimson Seedless
20	Karnataka	Vijayapura	Vijayapura	16.827	75.725	*Xcv* 20	Thompson Seedless
21	Tamil Nadu	Theni	Theni	10.011	77.478	*Xcv* 21	Manikchaman
22	Tamil Nadu	Theni	Theni	10.011	77.478	*Xcv* 22	Crimson Seedless
23	Tamil Nadu	Theni	Theni	10.011	77.478	*Xcv* 23	Thompson Seedless

with a 70% ethanol solution for another 30 sec. Finally, the leaf segments were washed twice with sterile distilled water, blot dried and placed on a sterile nutrient agar (NA) plate to be incubated at 37°C for 24 h. After 24 h, the observed colonies were selected and characterised morphologically (Costa *et al*., 2012). The selected colonies were sub-cultured and purified, following the method of Shah (2021) with minor modifications. Macroscopic features of the isolated bacterial colonies were assessed for the following criteria: colour, elevation, margin, opacity, consistency and surface of the colony. Microscopic features were determined through gram staining (Bartholomew & Mittwer, 1952).

Biochemical characterisation of purified bacterial isolates

The isolates were subjected to several biochemical tests – the potassium hydroxide (KOH) test, sugar utilisation test, catalase test, urease test, gelatine liquefaction test, indole production test, hydrogen cyanide (HCN) production test, ammonia production, starch hydrolysis test, lipase production test, methylene red reduction, nitrate reduction, oxidase test, citrate reduction, hydrogen sulphide (H_2S) production, cellulase production and protease production (Schaad *et al*., 2001; Vashist *et al*., 2013).

Abiotic stress-tolerance assay

Estimation of salt tolerance

Fresh bacterial culture was inoculated in test tubes containing nutrient broth with different concentrations of sodium chloride (NaCl), viz. 2%, 4%, 6% and 8% (Ullah *et al*., 2018). Growth was observed after 24 h of incubation at 37°C, after which salt tolerance could be evaluated.

Estimation of temperature tolerance

Fresh bacterial culture was inoculated in test tubes containing nutrient broth. Growth was observed after 24 h of incubation at different temperatures, viz. 0°C, 4°C, 26°C and 30°C,

after which temperature tolerance could be evaluated (Ullah *et al*., 2018).

Virulence test

For the assessment of the virulence of *Xcv* on a grape leaf, an inoculum suspension was prepared in sterile water. Pinprick and injection infiltration methods were used for inoculation. In the pin prick method, paper pins were used to bruise the leaves, and the inoculum (10^6 cfu/ml) of *Xcv* was applied. In the injection infiltration method, leaves of young seedlings were inoculated with 1 ml of inoculum using a hypodermic syringe. Control leaves (C) were inoculated with sterilised distilled water (Mazi *et al*., 2015).

Effect of different cultural conditions on the virulence of *Xcv*

Effect of age on the bacterium

Twenty-four hours of growth of the bacterial isolates on NA slants was used as a stock culture, and sub-culturing was done at one-day intervals in order to get five-, four-, three-, two- and one-day-old bacterial growth (Klement, 1963). Optical density of the bacterial suspension of each age level was adjusted to 0.5 and inoculated at the lower surface of the leaf using the syringe infiltration method. Symptoms were observed after 24 h of inoculation.

Effect of temperature

To study the effect of incubation temperature on virulence, each bacterial isolate was plated separately on NA and the plates were incubated at 25°C, 30°C and 35°C respectively. When the colonies emerged, one colony from each temperature treatment was sub-cultured, and the bacterial growth was suspended in sterile distilled water, centrifuged, and the optical density (OD) was adjusted to 0.5 (Klement, 1963). Isolates were inoculated at the lower surface of the leaf and symptoms were observed after 24 h.

Effect of pH

Nutrient agar plates were prepared with varying levels of pH, from 5.0 to 8.0 at intervals of 0.5. Fifty ml of the broth was dispensed in each of the 250 ml Erlenmeyer flasks and autoclaved at 121°C and 15 psi for 20 min. A loopful of 24 h growth of each isolate was suspended in 10 ml of sterile water. The flasks were then inoculated with 0.1 ml of the bacterial suspension prepared from the respective pH levels and incubated (Klement, 1963). Isolates were inoculated at the lower surface of the leaf and observed for lesions after 24 h of inoculation.

Exopolysaccharide (EPS) production

Cultures were grown in basal medium prepared by mixing dipotassium phosphate (K_2HP0_4, 0.12%), potassium dihydrogen phosphate (KH_2PO_4, 0.08%), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$, 0.02 %) and ammonium nitrate (NH_4NO_3, 0.5%) on a rotary shaker for seven days at 28°C to 30°C (Nayudu, 1972). Bacterial cells were removed by centrifugation at 15 000 rpm for 15 min. N-cetylpyridinium chloride (2.0 g per litre) was added to the supernatant for the precipitation of polysaccharide, which was quickened by the addition of one to two pellets of KOH. The brownish-white granular precipitate was removed by centrifugation and dissolved in 10% NaCI, and the polysaccharide were reprecipitated with two volumes of ethanol. This process was repeated thrice. The EPS produced was finally washed with ethanol, and dried before taking the final weight (Chowdhury *et al*., 1980).

Molecular identification

A single pure colony of the bacteria was inoculated in 100 ml of sterile nutrient broth (NB) and cultured on a rotatory incubator shaker at 120 rpm for 24 h at 28 ± 1°C. The genomic deoxyribonucleic acid (DNA) was extracted using the HiMedia Bacterial Genomic Kit according to the manufacturer's instructions, and quantified with a Nanophotometer® NP120 (IMPLEN ver. 2008). The universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used to amplify the 16S rDNA gene. Polymerase chain reaction amplifications were performed in 50 µl reaction mixture including 2U Taq polymerase (Bangalore Genei, Bangalore, India), 5 µl of 10X Taq buffer, dNTP at 200 µM, 10 pmol of each primer (IDT, USA) and 25 ng of DNA template. Initial denaturation was carried out at 94°C for four minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, primer extension at 72°C for 1.5 minutes, and final extension at 72°C for 10 minutes. The sequencing of the resultant polymerase chain reaction (PCR) amplicons was outsourced (GeneMatrix, Pune, India). Using BioEdit Sequence Alignment Editor, the sequences were aligned to obtain consensus sequences, which were then compared to three sequences of *Xcv* species available at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using a BLAST search. MEGA 6.0 software was used to create the phylogenetic tree.

RESULTS

Isolation

Twenty-three isolates were obtained from the samples collected from various locations across India (Table 1). These isolates differed in morphological appearance, viz. colour, shape, size, margin, elevation, consistency and opacity.

Morphological characterisation of *Xcv* isolates

It was observed that all the *Xcv* colonies had the characteristic features, namely round and white in colour, convex, smooth and butyrous in texture. The size of all the isolates ranged from 1 mm to 3 mm in diameter. Eight isolates from the Palkhed (*Xcv*5), Karsul (*Xcv*9), Sarole (*Xcv*10), Belanki (*Xcv*11), Walwa (*Xcv*12), Kuchi (*Xcv*13), Narayangaon (*Xcv*16) and Mohal (*Xcv*17) locations were opaque in nature, while the rest were translucent (see Fig. 2 and Table 2).

Biochemical characterisation of *Xcv* isolates

All the isolates responded positively to the loop test by forming a thread when lifted gently. The loop formation provides confirmation of the Gram-negative bacteria to which all the isolates conformed. The isolates marked as Gram-negative from the loop test were further tested using Gram staining. Similar results were obtained twice, as all isolates retained a pinkish colour, thus confirming that they

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FIGURE 2
Morphological characterisation of 23 isolated *Xcv* from different locations

were Gram-negative.

All the isolates tested negative for H_2S production, giving no black discoloration on lead acetate paper strips. It was observed that all the isolates had negative results for oxidase, urease and nitrate-reduction reactions, but were positive for the catalase test. All the isolates were able to ferment the tested sugars, viz. glucose, galactose, fructose, sucrose, dextrose and lactose. Isolates were neither able to utilise citrate as a sole source of carbon, nor did they show positive indole production. Isolates from eight locations, viz. Palkhed (*Xcv*5), Sarole (*Xcv*10), Walwa (*Xcv*12), Kuchi (*Xcv*13), Karoli (*Xcv*14), Pandharpur (*Xcv*18), Vijayapura (*Xcv*20) and Theni (*Xcv*21), were able to reduce methyl red reagent. It was observed that none of the isolates from Pune district were positive for the methyl red reduction test (Table 3a).

It was noted that eight isolates, from the Bhuvana (*Xcv*1), Satana (*Xcv*8), Walwa (*Xcv*12), Kuchi (*Xcv*13), Vijayapura (*Xcv*19 and *Xcv*20) and Theni (*Xcv*21 and *Xcv*21) regions, were unable to produce protease, lipase or gelatinase enzymes. Protease enzyme was produced by the isolates from Bhuvana (*Xcv*2), Verkheda (*Xcv*6), Balanki (*Xcv*11), Karoli (*Xcv*14), Narayangaon (*Xcv*16), Mohal (*Xcv*17), Pandharpur (*Xcv*18) and Theni (*Xcv*22), whereas lipase was secreted by isolates from Verkheda (*Xcv*6), Sarole (*Xcv*10), Pandharpur (*Xcv*18) and Theni (*Xcv*22). Ten isolates – from Sompur (*Xcv*2), Thengoda (*Xcv*4), Palkhed (*Xcv*5), Verkheda (*Xcv*7), Karsul (*Xcv*9), Sarole (*Xcv*10), Belanki (*Xcv*11), Narayangaon (*Xcv*15), Pandharpur (*Xcv*18) and Theni (*Xcv*22) – were able to hydrolyse gelatine. Amylase and cellulase enzymes were produced by all the isolates from the different locations (Table 3b).

Abiotic stresses

None of the isolates were able to tolerate temperature stress. The maximum growth of *Xcv* took place at 26°C, and no growth was observed at 0°C, 4°C and -30°C. Tolerance to different salt concentrations were tested, and all the isolates were able to tolerate salt concentration of up to 4%, as no growth was observed in broth with a higher salt concentration (Table 4).

Virulence test

The infected grape leaves reacted positively to all isolates within 24 h to 72 h. Mild chlorosis to brown necrosis was observed around the injection point (Fig. 2). As per virulence reaction, the pathogen was categorised as strongly virulent, moderately virulent and mildly virulent. Isolates from eight locations, viz. Verkheda (*Xcv*7), Satana (*Xcv*8), Sarole (*Xcv*10), Walwa (*Xcv*12), Narayangaon (*Xcv*15, *Xcv*16), Vijayapura (*Xcv*20) and Theni (*Xcv*23), were considered to be highly virulent, as they manifested symptoms within 24 h of inoculation, whereas 12 isolates, from Bhuvana (*Xcv*1), Thengoda (*Xcv*3, *Xcv*4), Palkhed (*Xcv*5), Karsul (*Xcv*9), Belanki (*Xcv*11), Kuchi (*Xcv*13), Karoli (*Xcv*14), Mohal (*Xcv*17), Pandharpur (*Xcv*18) and Theni (*Xcv*21 and *Xcv*22), were regarded as moderately virulent, as the symptoms were seen within 48 h. After 72 h of inoculation, symptoms appeared in three of the isolates from Sompur (*Xcv*2), Verkheda (*Xcv*6) and Vijayapura (*Xcv*19) locations,

TABLE 2
Morphological characterisation of purified cultures of *Xcv*

Sr no.	Strain	Shape	Size * (mm)	Colour	Consistency	Elevation	Texture	Opacity	Gram reaction
1	*Xcv*1	C	1.4	W	M	CX	S	T	N
2	*Xcv*2	C	2.1	W	M	CX	S	T	N
3	*Xcv*3	C	3.0	W	M	CX	S	T	N
4	*Xcv*4	C	2.1	W	M	CX	S	T	N
5	*Xcv*5	C	2.9	W	M	CX	S	O	N
6	*Xcv*6	C	1.1	W	M	CX	S	T	N
7	*Xcv*7	C	1.6	W	M	CX	S	T	N
8	*Xcv*8	C	2.8	W	M	CX	S	T	N
9	*Xcv*9	C	2.2	W	M	CX	S	O	N
10	*Xcv*10	C	3.1	W	M	CX	S	O	N
11	*Xcv*11	C	2.9	W	M	CX	S	O	N
12	*Xcv*12	C	2.4	W	M	CX	S	O	N
13	*Xcv*13	C	2.1	W	M	CX	S	O	N
14	*Xcv*14	C	1.9	W	M	CX	S	T	N
15	*Xcv*15	C	2.0	W	M	CX	S	T	N
16	*Xcv*16	C	2.4	W	M	CX	S	O	N
17	*Xcv*17	C	0.9	W	M	CX	S	O	N
18	*Xcv*18	C	1.5	W	M	CX	S	T	N
19	*Xcv*19	C	1.9	W	M	CX	S	T	N
20	*Xcv*20	C	2.3	W	M	CX	S	T	N
21	*Xcv*21	C	0.9	W	M	CX	S	T	N
22	*Xcv*22	C	1.7	W	M	CX	S	T	N
23	*Xcv*23	C	1.9	W	M	CX	S	T	N

*Average of 50 colonies were observed
Key: C - circular; W - white; M - mucoid; CX - convex; S - smooth; N - negative; T - translucent; O - opaque

and these can be categorised as slightly virulent. It is clear from the data that Nashik and Sangli had the highest number of highly virulent strains, whereas strains from Pune, Theni and Solapur were of moderate virulence. No symptoms were recorded in the controls inoculated with distilled water (Table 5).

Two inoculation methods, viz. pin prick and syringe infiltration, were used, and they gave positive responses on the host plants. However, the syringe infiltration method gave more prominent symptoms compared to the pin prick method. The syringe infiltration method therefore was used for the further studies.

Effect of different cultural conditions on the virulence of *Xcv*

Effect of age of the bacterium

The virulence of the bacterial isolates causing BLS was influenced by the age and origin of the bacterial strains. Isolates from Sompur (*Xcv*2), Thengoda (*Xcv*3), Palkhed (*Xcv*5), Verkheda (*Xcv*7), Satana (*Xcv*8), Walwa (*Xcv*12), Karoli (*Xcv*14), Narayangaon (*Xcv*15, *Xcv*16), Mohal (*Xcv*17), Vijayapura (*Xcv*20) and Theni (*Xcv*22, *Xcv*23) manifested the highest virulence in three- and four-day-old cultures, whereas isolates from Bhuvana (*Xcv*1), Thengoda (*Xcv*4), Verkheda (*Xcv*6), Karsul (*Xcv*9), Sarole (*Xcv*10), Belanki (*Xcv*11), Kuchi (*Xcv*13), Pandharpur (*Xcv*18), Vijayapura (*Xcv*19) and Theni (*Xcv*21) showed the highest virulence in two- and three-day-old cultures. Three-day-old cultures of all isolates manifested the maximum virulence compared to the other age levels. Five- and one-day-old cultures were the least virulent. The interaction between the bacterial isolates and their age in relation to virulence was significant.

Effect of temperature

Incubation temperature also influenced the virulence of the bacterial isolates. The pathogen was more virulent when it was incubated at 30°C. However, the virulence of the

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TABLE 3a
Biochemical characterisation of purified sample of *Xcv*

Sr no.	Strain	Sugar fermentation						Oxidase	Catalase	Indole production	Methyl red reduction	Nitrate utilisation	Citrate utilisation
		Glucose	Galactose	Fructose	Lactose	Sucrose	Dextrose						
1	*Xcv*1	+	+	+	+	+	+	-	+	-	-	-	-
2	*Xcv*2	+	+	+	+	+	+	-	+	-	-	-	-
3	*Xcv*3	+	+	+	+	+	+	-	+	-	-	-	-
4	*Xcv*4	+	+	+	+	+	+	-	+	-	-	-	-
5	*Xcv*5	+	+	+	+	+	+	-	+	-	+	-	-
6	*Xcv*6	+	+	+	+	+	+	-	+	-	-	-	-
7	*Xcv*7	+	+	+	+	+	+	-	+	-	-	-	-
8	*Xcv*8	+	+	+	+	+	+	-	+	-	-	-	-
9	*Xcv*9	+	+	+	+	+	+	-	+	-	-	-	-
10	*Xcv*10	+	+	+	+	+	+	-	+	-	+	-	-
11	*Xcv*11	+	+	+	+	+	+	-	+	-	-	-	-
12	*Xcv*12	+	+	+	+	+	+	-	+	-	+	-	-
13	*Xcv*13	+	+	+	+	+	+	-	+	-	+	-	-
14	*Xcv*14	+	+	+	+	+	+	-	+	-	+	-	-
15	*Xcv*15	+	+	+	+	+	+	-	+	-	-	-	-
16	*Xcv*16	+	+	+	+	+	+	-	+	-	-	-	-
17	*Xcv*17	+	+	+	+	+	+	-	+	-	-	-	-
18	*Xcv*18	+	+	+	+	+	+	-	+	-	+	-	-
19	*Xcv*19	+	+	+	+	+	+	-	+	-	-	-	-
20	*Xcv*20	+	+	+	+	+	+	-	+	-	+	-	-
21	*Xcv*21	+	+	+	+	+	+	-	+	-	+	-	-
22	*Xcv*22	+	+	+	+	+	+	-	+	-	-	-	-
23	*Xcv*23	+	+	+	+	+	+	-	+	-	-	-	-

Key: + = positive; - = negative

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TABLE 3b
Biochemical characterisation of purified sample of *Xcv*

Sr no.	Strains	Gelatine hydrolysis	H_2S production	Urease test	KOH hydrolysis	Starch hydrolysis	Casein hydrolysis	Lipase production	Cellulose production
1	*Xcv*1	-	-	-	+	+	-	-	+
2	*Xcv*2	+	-	-	+	+	-	-	+
3	*Xcv*3	-	-	-	+	+	+	-	+
4	*Xcv*4	+	-	-	+	+	-	-	+
5	*Xcv*5	+	-	-	+	+	-	-	+
6	*Xcv*6	-	-	-	+	+	+	+	+
7	*Xcv*7	+	-	-	+	+	-	-	+
8	*Xcv*8	-	-	-	+	+	-	-	+
9	*Xcv*9	+	-	-	+	+	-	-	+
10	*Xcv*10	+	-	-	+	+	-	+	+
11	*Xcv*11	+	-	-	+	+	+	-	+
12	*Xcv*12	-	-	-	+	+	-	-	+
13	*Xcv*13	-	-	-	+	+	-	-	+
14	*Xcv*14	-	-	-	+	+	+	-	+
15	*Xcv*15	+	-	-	+	+	-	-	+
16	*Xcv*16	-	-	-	+	+	+	-	+
17	*Xcv*17	-	-	-	+	+	+	-	+
18	*Xcv*18	+	-	-	+	+	+	+	+
19	*Xcv*19	-	-	-	+	+	-	-	+
20	*Xcv*20	-	-	-	+	+	-	-	+
21	*Xcv*21	-	-	-	+	+	-	-	+
22	*Xcv*22	+	-	-	+	+	+	+	+
23	*Xcv*23	-	-	-	+	+	-	-	+

Key: + = positive; - = negative

pathogen was reduced when incubated at 25°C and 35°˚C, although these did not show a significant difference.

Effect of pH

The pH levels, ranging from 5.0 to 8.0, influenced the virulence of the causal bacterial isolates. All the isolates were significantly virulent at all the tested pH levels, with no direct relationship manifested between the pH levels and the virulence of the bacterial isolates.

Exopolysaccharide production

All the strains were able to produce a significant amount of exopolysaccharides (EPS), ranging from 0.3 mg/ml to 2.2 mg/ml (Table 5). Highly virulent strains of *Xcv* were able to produce larger amounts of EPS compared to the others. Isolates *Xcv*7 (1.8 mg/ml), *Xcv*8 (2.0 mg/ml), *Xcv*10 (1.9 mg/ml), *Xcv*12 (2.2 mg/ml), *Xcv*15 (1.9 mg/ml), *Xcv*16 (2.1 mg/ml), *Xcv*20 (1.8 mg/ml) and *Xcv*23 (2.1 mg.ml), from Verkheda, Satana, Sarole, Walwa, Narayangaon, Vijayapura and Theni respectively, produced the maximum amount of EPS, ranging from 1.8 mg/ml to 2.2 mg/ml. Isolates from Bhuvana (*Xcv*1), Thengoda (*Xcv*3, *Xcv*4), Palkhed (*Xcv*5), Karsul (*Xcv*9), Belanki (*Xcv*11), Kuchi (*Xcv*13), Karoli (*Xcv*14), Mohal (*Xcv*17), Pandharpur (*Xcv*18) and Theni (*Xcv*21 and *Xcv*22) produced moderate amounts of EPS, ranging from 0.8 mg/ml to 1.3 mg/ml. The smallest amounts of EPS were produced by *Xcv*2 (0.5 mg/ml), *Xcv*6 (0.3 mg/ml) and *Xcv*19 (0.4 mg/ml) from Sompur, Verkheda and Vijayapura respectively (Table 5).

Molecular characterisation and cluster analysis

Polymerase chain reaction products produced a single band of approximately 1 500 base pairs (bp). 16S rDNA sequencing of these PCR-amplified products showed diversity in the *Xanthomonas* community. All the isolates had more than 97% sequence similarity with the *Xanthomonas citri* pv. *viticola* strain in a BLAST search. Sequences from seven *Xanthomonas* isolates were obtained from NCBI and

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TABLE 4
Stress tolerance, i.e. temperature and pH of purified sample of *Xcv*

Sr no.	Strains	Growth at different temperatures (°C)				Growth at different salt concentration (%)							
		0	4	26	-30	0	0.5	1	2	3	4	5	7
1	*Xcv*1	-	-	+	-	+	+	+	+	+	+	-	-
2	*Xcv*2	-	-	+	-	+	+	+	+	+	+	-	-
3	*Xcv*3	-	-	+	-	+	+	+	+	+	+	-	-
4	*Xcv*4	-	-	+	-	+	+	+	+	+	+	-	-
5	*Xcv*5	-	-	+	-	+	+	+	+	+	+	-	-
6	*Xcv*6	-	-	+	-	+	+	+	+	+	+	-	-
7	*Xcv*7	-	-	+	-	+	+	+	+	+	+	-	-
8	*Xcv*8	-	-	+	-	+	+	+	+	+	+	-	-
9	*Xcv*9	-	-	+	-	+	+	+	+	+	+	-	-
10	*Xcv*10	-	-	+	-	+	+	+	+	+	+	-	-
11	*Xcv*11	-	-	+	-	+	+	+	+	+	+	-	-
12	*Xcv*12	-	-	+	-	+	+	+	+	+	+	-	-
13	*Xcv*13	-	-	+	-	+	+	+	+	+	+	-	-
14	*Xcv*14	-	-	+	-	+	+	+	+	+	+	-	-
15	*Xcv*15	-	-	+	-	+	+	+	+	+	+	-	-
16	*Xcv*16	-	-	+	-	+	+	+	+	+	+	-	-
17	*Xcv*17	-	-	+	-	+	+	+	+	+	+	-	-
18	*Xcv*18	-	-	+	-	+	+	+	+	+	+	-	-
19	*Xcv*19	-	-	+	-	+	+	+	+	+	+	-	-
20	*Xcv*20	-	-	+	-	+	+	+	+	+	+	-	-
21	*Xcv*21	-	-	+	-	+	+	+	+	+	+	-	-
22	*Xcv*22	-	-	+	-	+	+	+	+	+	+	-	-
23	*Xcv*23	-	-	+	-	+	+	+	+	+	+	-	-

Key: + = positive; - = negative

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TABLE 5
Virulence and exopolysaccharide (mg/ml) produced by purified sample of *Xcv*

Strains	Virulence	Exopolysaccharide (mg/ml)
Xcv1	++	0.9
Xcv2	+	0.5
Xcv3	++	1.1
Xcv4	++	1.0
Xcv5	++	0.8
Xcv6	+	0.3
Xcv7	+++	1.8
Xcv8	+++	2.0
Xcv9	++	1.1
Xcv10	+++	1.9
Xcv11	++	1.2
Xcv12	+++	2.2
Xcv13	++	0.9
Xcv14	++	1.0
Xcv15	+++	1.9
Xcv16	+++	2.1
Xcv17	++	1.3
Xcv18	++	1.0
Xcv19	+	0.4
Xcv20	+++	1.8
Xcv21	++	0.9
Xcv22	++	1.3
Xcv23	+++	2.1
C	-	-

Key: + = slightly virulent; ++ = moderately virulent; +++ = highly virulent; - = negative

subjected to multiple alignments. A dendrogram depicting the estimated phylogenetic relationships was constructed by the neighbour-joining clustering method (Fig. 3). This dendrogram was based on comparisons of all the available 16S rDNA sequence data for the genus *Xanthomonas citri* pv. *viticola* species. The sequence obtained was submitted to GeneBank and an accession number was procured (Table 6). The cluster analysis also clearly discriminated *Xanthomonas citri* pv. *viticola* causing bacterial leafspot of grapes from other *Xanthomonas* species infecting different hosts (Fig. 3).

The dendrogram constructed from the pooled data had two main clusters (Fig. 3) – cluster I and cluster II. A clear distinction could be observed among the isolated *Xcv* and other *Xanthomonas* strains, as all the *Xcv* were grouped under cluster I, whereas cluster II consisted of *Xanthomonas* strains, viz. *Xanthomonas albilineans* (MH491194.1), *Xanthomonas sacchari* (KY486222.1), *Xanthomonas campestris* (MH256551.1) and *Xanthomonas translucens* pv. *graminis* (AY855873.1). Cluster I was further divided into two sub-clusters. Isolates from Karoli (*Xcv*14), Pandharpur (*Xcv*18) and Vijayapur (*Xcv*19) were found to be closely related to each other. Isolates from the Walwa (*Xcv*12), Narayangaon (*Xcv*15 and *Xcv*16) and Mohal (*Xcv*17) regions were grouped in one sub-cluster, whereas isolates from Theni (*Xcv*21 and *Xcv*23) fell into another. All three *Xcv* isolates retrieved from the NCBI were closely related to the remaining isolates, as all were grouped in the same sub-cluster.

DISCUSSION

The pathogenic and genetic diversity of the *Xcv* strains associated with BLS disease in India were analysed through pathogenicity, standard bacteriological tests and 16S rRNA sequence analysis. The results suggest that the *Xcv* populations investigated in the present study were composed of pathologically and genetically diverse strains.

The present study supports the previous findings relating to the occurrence of bacterial leaf spot in India and the characterisation of the causal bacterium, *Xcv* (Kamble *et al*., 2019), and confirms that the disease is widespread in the grape-growing regions of India. Symptoms of the BLS, e.g. water-soaked, angular small spots on the lower surface of the leaves, were observed in all the visited fields. Similar symptoms of BLS have been described by Araujo and Robbs (2000) and Nascimento and Mariano (2004). Necrosis, yellow spots and cankerous lesions on the leaves and shoots are characteristic symptoms caused by any pathogen belonging to the genus *Xanthomonas* (Bradbury, 1970). The symptoms observed on the grapevines were homologous with the previous studies (Bradbury, 1970; Kamble *et al*., 2019).

Bacteria of the genus *Xanthomonas* are straight, Gram-negative rods, typically with yellow pigmentation and a polar flagellum, strictly aerobic chemoorganotrophs, and mostly phytopathogenic. As observed, the colony morphology of *Xcv* revealed white-coloured colonies. In contrast to the present finding, a yellow-coloured colony of bacterial leaf spot pathogen of grapes was reported by Jambenal *et al*. (2011), which hints at phenotypic diversity. Hence, yellow colonies produce the pigment Xanthomonadin (Goel *et al*., 2001), while white or albino colonies do not produce this pigment due to acquired mutations in the Xanthomonadin biosynthesis gene cluster, such as frameshift mutation, deletion and insertion, cause them to lose their pigmentation (Midha & Patil, 2014). Hence there is phenotypic diversity. Similar findings have been made in the case of other albino strains, *Xanthomonas campestris* pv. *mangiferaeindicae* and *Xanthomonas axonopodis* pv. *ricini* (Gama *et al*., 2011). The size and shape of colonies were small to medium, convex and mucoid. The staining reaction was observed under microscope, and the bacteria were found to be Gram negative. The bacterial colonies identified were 1 mm to 3 mm in diameter, and Gram-negative cells were observed under the microscope. These observations regarding colony morphology in the present study correlate with reports by Breed *et al*. (1989) and Kamble *et al*. (2019). The results support those of a previous study (Arshiya *et al*., 2014) in which morphological, biochemical and pathogenicity tests

were performed to identify and characterise the strains of *Xanthomonas* causing bacterial disease. The biochemical test results analysed in the present study confirm the pathogen.

In the present study, variations were observed with respect to the morphological and biochemical characterisation. All *Xcv* were able to utilise carbon in all the tested forms. This finding is in contrast with previous results reported by Young and Triggs (1994), who found that pathovars of *P. syringae* pv. *maculicola* were heterogeneous in their carbon source utilisation. Giri *et al*. (2011) reported that, amongst 16 strains of *Xanthomonas axonopodis* pv. *punicae*, only five could hydrolyse the starch and almost all the strains produced acid from different carbon sources. They further added that 13 strains had produced H_2S gas. The results reflect the biochemical variation exhibited by the strains of *X. axonopodis* pv. *punicae* from different geographical locations (Tables 3a and 3b). Several researchers have reported variations in biochemical tests within the different pathovars (Wiebe *et al*., 1993; Clerc *et al*. 1998; Zhao *et al*., 2008).

Exopolysaccharide production is an important virulence determinant in numerous plant pathogenic bacteria, including *Xanthomonas* spp., *Erwinia amylovora* and *Pseudomonas syringae*, during infection by pathogens (Kemp *et al*., 2004). The present study shows that all the pathogenic *Xcv* were able to produce a significant amount of EPS. It was also seen that EPS production was directly proportional to the virulence, which supports the previous findings of Guo *et al*. (2015) and Nguyen *et al*. (2016). These authors reported that *X. oryzae* pv. *oryzae* and *X. citri* subsp. *citri* employed multiple virulence factors to promote their pathogenicity on rice and citrus respectively, including EPS production. Exopolysaccharide production helps the pathogen to grow and spread in plants by protecting the organism against toxic compounds like superoxide radicals, hydrogen peroxide (H_2O_2), and high or low pH produced by the hosts in defence reactions (Yu *et al*., 2016; Bae *et al*., 2018).

Here, we present a systematic approach to generating and validating diversity among species/supraspecies levels using the 16S rRNA gene, which was also proved by Escapa *et al*. (2020). Previous studies on membrane protein analysis, fatty acid profiling and 16s rRNA analysis showed a high similarity among several strains or pathovars of *Xanthomonas* (Ndongo *et al*., 2018). Comparisons of sequence variation in the 16S rRNA region among 23 strains of *Xcv* from different locations showed more than 97% similarity. In addition, partial 16S rRNA sequences were identical among the strains of *Xcv* studied, and the current findings support the study of Kamble *et al*. (2019). There is limited information describing the genetic diversity of *Xcv*, compared to the closely related pathovars of *Xcc*.

There was a correlation between the groups and the

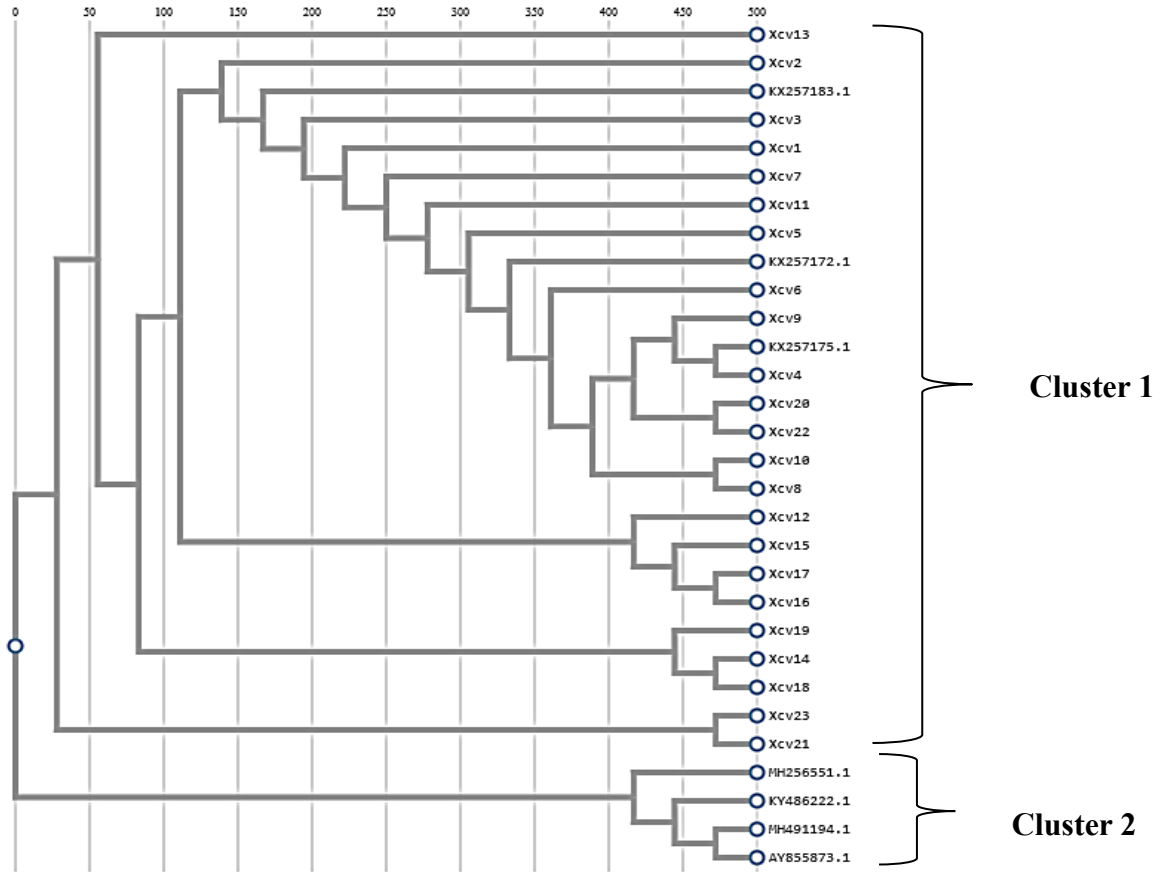

FIGURE 3
Phylogenetic tree of *Xcv* inferred by analysis of 16S rDNA sequences

TABLE 6
Accession number of isolated *Xcv* submitted to the NCBI

Strain	Accession number
Xcv1	OR485223
Xcv2	OR485224
Xcv3	OR485225
Xcv4	OR485226
Xcv5	OR485227
Xcv6	OR485228
Xcv7	OR485229
Xcv8	OR485230
Xcv9	OR485231
Xcv10	OR485232
Xcv11	OR485233
Xcv12	OR485234
Xcv13	OR485235
Xcv14	OR485236
Xcv15	OR485237
Xcv16	OR485238
Xcv17	OR485239
Xcv18	OR485240
Xcv19	OR485241
Xcv20	OR485242
Xcv21	OR485243
Xcv22	OR485244
Xcv23	OR485245

geographical origin of the strains, e.g. from Maharashtra, Karnataka and Tamil Nadu (Fig. 3). Alberts *et al*. (2002) state that it is plausible that the two groups may signify distinct, recent introductions of the pathogen, even though the origin of the distribution is not clear. Previous reports about *Xanthomonas* pv. *manihotis* have shown that there is divergence among the strains that were recently introduced to Africa (Verdier *et al*., 1993). Linkages between groups, indicated by rep-PCR, and geographical origin have been reported for *Xanthomonas* strains pathogenic to other plants. Scortichini *et al*. (2001) reported the existence of genetic diversity in a worldwide collection of *X. axonopodis* pv. *juglandis* isolated from walnuts, and showed that the genetics of the pathogen was unique to each walnut cultivation area. Massomo *et al*. (2003) observed variation among *X. campestris* pv. *campestris* strains isolated from *Brassica* sp. collected in fields in Tanzania, and linked fingerprint patterns to specific geographical areas. They noted that adaptability might be the basis for this phenomenon. Likewise, Mkandawire *et al*. (2004) revealed that *X. campestris* pv. *phaseoli* strains isolated from the common bean of Africa comprised three genotypes – two unique to East Africa and the other associated with strains collected from the New World. Selection for a specialised niche can affect genome organisation and the distribution of repetitive sequences in the bacterium genome, resulting in fingerprints unique to a specific pathovar or strain (Louws *et al*., 1994). Yap *et al*. (2004) reported genetic diversity in *E. carotovora* subsp. *carotovora* strains isolated from the same region in the same season from the same host species. It would be interesting to study the *Xanthomonas citri* pv. *viticola* adaptation when different areas or climatic factors are involved.

The genetic diversity of *Xcv* was found to be based on the different geographical locations in India. The reasons for significant differences are yet to be investigated. However, several factors, such as the migration of individuals, sampling strategies and lumping of strains from different locations, could be possible mechanisms for such variations. Although *Xcv* populations appeared to be highly diverse, one possible explanation for the current observations might be horizontal gene transfer (HGT) and recombination, which probably occurred within the vines (Filip & Skuza, 2021; Ghaly *et al*., 2024). It has been reported that HGT is the predominant force leading to similarities between the genomes of *X. citri* pv. *fuscans* and *X. phaseoli* pv. *phaseoli*. HGT was the main reason for the evolution of *Xanthomonas* strains with the common bean as host (Chen *et al*., 2018). Further studies on *Xanthomonas citri* pv. *viticola* are necessary to clarify the transfer mechanism. Timilsina *et al*. (2019) say that the core genes reveal the extent, source and mechanisms of recombination events that shaped the current population and genomic structure of *X. perforans* in Florida.

CONCLUSION

This study provides new insight into the phenotypic and genotypic diversity and evolution of *Xanthomonas citri* pv *viticola* strains – the causal agent of bacterial leaf spot in grapevines – from Maharashtra, Karnataka and Tamil Nadu in India. Although the sample size was limited, the results indicate diversity in the population structure. Future studies are necessary to identify the factors responsible for the resistance in *Xcv*. Finally, this work provides an excellent basis for further exploration of the specific interaction between the *Xcv* strain and grapevines that will help to implement various preventive strategies. Furthermore, it can help to minimise the losses due to BLS through the development of a PCR-based diagnostic method for early detection of the pathogen.

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