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Study on Plasmid-borne Traits in *Xanthomonas campestris* pv *viticola* Causing Bacterial Leaf Spot in Grapes

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ABSTRACT

Plasmid has been proved an invaluable tool to microbiologist and molecular biologist as they confer a selective and important advantage to the organisms that possess them. This study present the first report on isolation and identification of plasmid from bacteria *Xanthomonas campestris* pv *viticola* which is known to cause bacterial leaf spot disease on grape. Plasmid was isolated from bacteria followed by its curing using different curing agents. Pathogenicity, exopolysacchride production and antibiotic resistance tests were performed with cured and uncured bacteria to check virulence and antibiotic sensitivity. Approximately, 23.1 kb size of plasmid was isolated from bacteria followed by curing it with three different curing agents viz., sodium dodecyl sulphate, ethidium bromide and acridine orange. Plasmid curing exhibited negative impact on colony morphology and exopolysacchride production. Cured bacteria was also found sensitive to the tested antibiotics. It also lost its virulence upon artificial inoculation on different varieties of grapes. Plasmid curing work indicate that colony morphology, exopolysacchride production, antibiotic sensitivity and virulence are plasmid-borne traits.

Keywords Plasmid, Curing, Antibiotics, Pathogenicity, *Xanthomonas campestris* pv *viticola.*

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INTRODUCTION

Bacterial leaf spot caused by *Xanthomonas campestris* pv *viticola* (*Xcv*) was noticed for the first time in grapes (*Vitis vinifera*) cv Anab-e-shahi at Tirupati (Andhra Pradesh) during 1960 (Naydu 1972). Later, it was found causing substantial damage in Maharashtra and Karnataka.

Plasmids are extra chromosomal elements of definite size, which are usually inherited stably within a bacterial cell line and are capable of being transferred between strains, species or genera. There is a growing interest in the role of plasmids in pathogenicity and host specificity as well as the possible advantages of plasmid-borne locations for the genes involved. Different plasmid-borne phenotypes, such a colony morphology, exopolysaccharide, toxin, hormone production and resistance to bactericides have evoked a lot of interest in the recent times. Plasmids are reported to code for phenotypes (Ulaganathan *et al.* 1989) and often carry genes that may benefit

the survival of the host organism, such as antibiotic resistance, heavy metal tolerance and toxin production (Kado 1998). The genus *Xanthomonas* cause important diseases to several plant species of horticultural importance such as citrus canker (*Xanthomonas axonopodis* pv *citri*), bacterial blight of common beans (*Xanthomonas axonopodis* pv *phaseoli*), bacterial spot of tomatoes (*Xanthomonas axonopodis* pv *vesicatoria*), black rot of crucifers (*Xanthomonas campestris* pv *campestris*) and blight of pomegranate (*Xanthomonas axonopodis* pv *punicae*).

Plasmids have been reported from many *Xanthomonads* viz. *X. albilineans*, *X. arboricola* pv *pruni*, *X. axonopodis* pathovars cyamopsidis, *dieffenbachiae*, *glycines, manihotis*, *phaseoli*, *vignicola*, *vitians* and *X. citri* pv. *citri*, *X. campestris* pathovars *campetris*, *malvacearum*, *vesicatoria* and *X. hortorum* pathovars *hederae* and *pelargonii* and *X. fuscans* subsp. fuscans and *X. oryzae* pv *oryzae* and complete DNA sequences of some plasmids have been published (Niu *et al.* 2015). Plasmids from *Xanthomonas* are significantly diverse in size and gene composition. Some of them carry genes encoding macro-molecule secretion systems, effectors, heavy metal exporters, plasmid stability factors and DNA mobile elements. Although *Xcv* has been characterized in detail (Kamble *et al*. 2019) but information regarding its plasmid is scanty. The purpose of this study was to isolate the indigenous plasmid and have a preliminary idea on the plasmid borne determinants of *Xanthomonas campestris* pv *viticola.*

MATERIALS AND METHODS

Isolation, maintenance and preservation of *Xanthomonas campestris* pv *viticola*

Xcv was isolated from grape variety, Thompson seedless from Maharashtra state, India. A single colony isolate was purified by repeated sub culturing on nutrient agar (HiMedia MM012) slants. For long term storage the cultures were preserved in 15% glycerol at -20°C.

Plasmid DNA isolation

For extraction of indigenous plasmid, three bacterial strains viz. XCV-A_1, XCV-B_1 and XCV-D_1 were inoculated in sterile nutrient broth (HiMedia MM244) and incubated at 28 ± 1°C in orbital shaker at 150 rpm for 16–18 h. Plasmid DNA was isolated by using modified protocol (Chakrabarty *et al.* 2010). The obtained plasmid was detected on 0.8% agarose gel (1X TAE) embedded with ethidium bromide.

Plasmid curing

Plasmid curing was carried out using three different reagents, namely ethidium bromide (EtBr) (Crameri *et al.* 1986), acridine orange (AO) (Brown 2000) and sodium dodecyl sulphate (SDS) (Saha *et al.* 2000). Three different concentrations of ethidium bromide (75 µg mL^{-1}, 100 µg mL^{-1} and 125 µg mL^{-1}), acridine orange (50 µg mL^{-1}, 75 µg mL^{-1}, 100 µg mL^{-1}) and sodium dodecyl sulphate (8%, 10%, 12%) were used to cure the plasmids. Nutrient broth was prepared and supplemented with the curing agents in aforementioned concentrations. Freshly inoculated overnight grown culture of the bacteria was sub-cultured into 10 ml of the nutrient broth containing curing agent. The samples were then incubated at 30°C for 48 h. All the treatments along with control i.e. without any curing agent were serially diluted with sterile nutrient broth up to 10^{-8}. All dilutions were plated out on nutrient agar plates and incubated at 30°C. Single colonies were restreaked on fresh plates. Colonies were randomly picked and screened for plasmids.

Colony morphology

Macroscopic observation of the different colonies was taken as they appeared on nutrient agar after incubation. The color, shape, appearance, size, elevation, edges and degree of growth, production of pigment, surface and margin were noted. Pure cultures were examined microscopically using trinocular stereo microscope (LeicaDFC425).

Exopolysaccharide production

Exopolysaccharide (EPS) production in synthetic medium was determined as described by Chowdhury and Verma (1980). Cultures were grown for 7 days at 31°C on a rotary shaker. The cells were removed by centrifugation at 15,000 rpm. EPS was precipitated

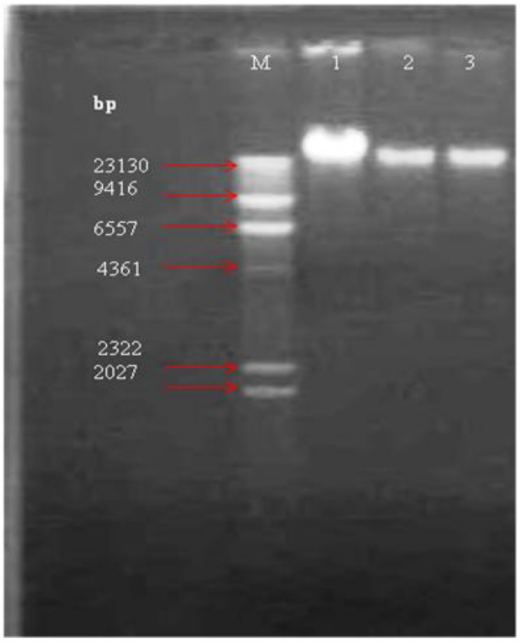


Fig. 1. Plasmid DNA of *X. campestris* pv *viticola*.

from the cell-free supernatant with N-cetylpyridinium chloride at a final concentration of 0.2%, redissolved in 10% aqueous sodium chloride and reprecipitated with two volumes of ethanol.

Pathogenicity test

For pathogenicity test, inoculum was prepared by adding 10 mL of sterile distilled water to 24 h old culture plate and bacterial growth was gently scrubbed with the help of sterile nichrome wire loop. A bacterial suspension for inoculation was adjusted to a density of 10^8 cfu mL^{-1} with the help of sterile distilled water by using McFarland's standards (Kamble *et al.* 2017). For pathogenicity test, four to sixth leaf from the apical side of healthy potted plants (Maji *et al.* 2015) were selected for inoculation. Five susceptible varieties (Kamble *et al.* 2017), Thompson seedless, Manik Chaman, TAS-A-Ganesh, Sonaka and Arkavati were chosen for the study with Manjari Medika as a resistant check variety. The disease intensity was recorded using a 0 to 5 points rating scale (0 = No disease ; 1 = 1–5% of leaf area infected ; 2 = 6–20% of leaf area infection ; 3 = 21–50% of leaf area infection; 4 = 51–80% of leaf area infection ; 5 = > 80% infection) (Chand 1992). Five plants for each treatment were maintained. From each plant single leaf was considered as a replicate and three replicates were used for inoculation. After inoculating by injection infiltration method (Klement 1963), the plants were kept in glass house where absolute temperature and relative humidity was maintained and observations were taken on alternate days.

Table 1. Plasmids isolated from *X. campestris* pv *viticola.*

Pathovar	Identification of strain	No. plasmids detected	Estimated size (kb)
Viticola	NRCG-XCV-A1 NRCG-XCV-B1 NRCG-XCV-D1	1	23.1

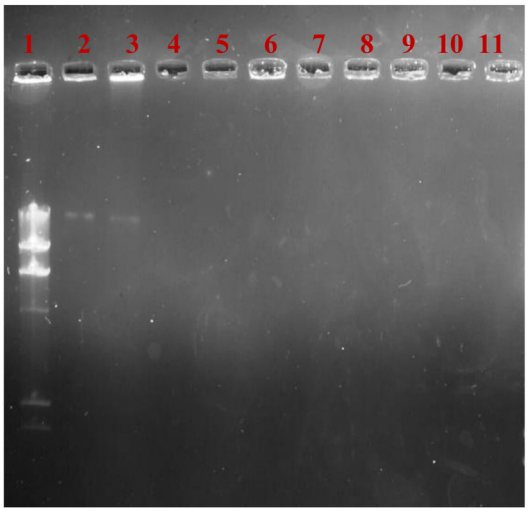
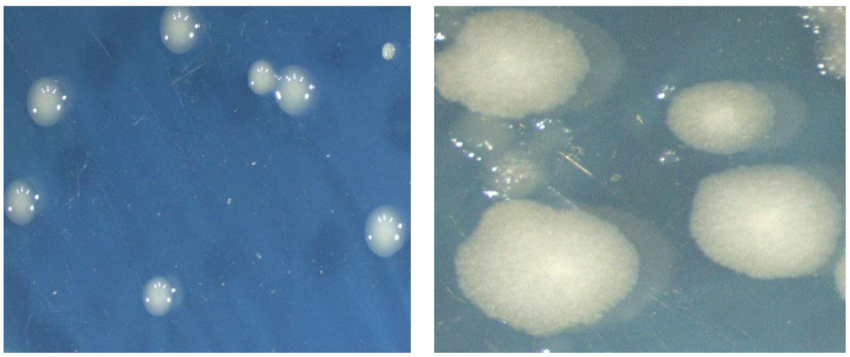


Fig. 2. Plasmid profile of uncured and cured bacterial strain of *X. campestris* pv *viticola.*

Table 2. Effect of curing on colony morphology of *X. campestris* pv *viticola.*

Colony morphology	Uncured *Xcv*	Cured *Xcv*
Shape	Circular	Irregular
Size	Small	Big
Appearance	Shiny and glistening	Dull
Elevation	Raised	Flat
Margin	Regular	Irregular
Edges	Smooth	Rough



a.Uncured **b. cured**

Fig. 3. Effect of plasmid curing on colony morphology of *X. campestris* pv *viticola.*

Antibiotic sensitivity assay

Antibiotic sensitivity was conducted with three antibiotics viz. penicillin, oxacillin and vancomycin, as the wild *Xcv* is reported to be resistant to them (Kamble *et al.* 2019). *In vitro* bacterial strain susceptibility to the antibiotics was tested after curing. Antibiotics strips of varying concentration ranging from 0.16–256 mcg mL^{-1} (HiMedia) were used to test sensitivity against bacteria. The inoculum was prepared by pouring 10 mL of sterile distilled water over a 24 h old culture plate and bacterial growths was gently scraped with the help of sterile nichrome wire loop into a sterile tube. A sterile cotton swab was dipped into the bacterial suspension and bacterial lawn was prepared on nutrient agar media. The inoculated plates were kept aside for drying lawn. The respective antibiotic strips were placed onto the lawn with the help of sterilized forceps. Plates with bacterial lawn without any antibiotic strip served as control. Inoculated plates were incubated at 28 ±1°C for 72 h. Lysis of the bacterial lawn around the strip was recorded to check for sensitivity.

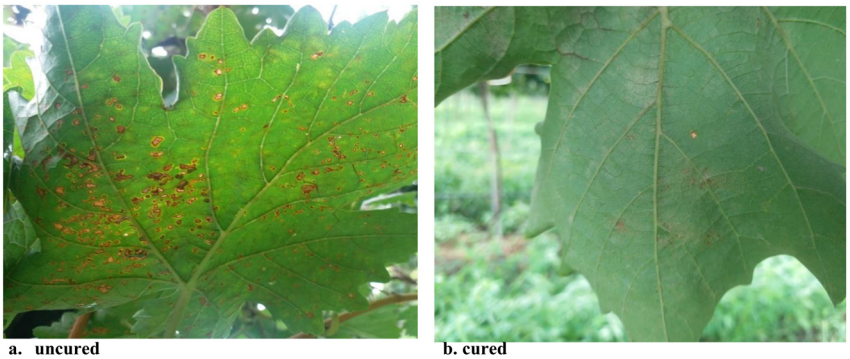
RESULTS AND DISCUSSION

Plasmid DNA analysis

Indigenous plasmid DNA was isolated from three strains XCV-A_1, XCV-B_1 and XCV-D_1 *Xcv*. All isolates showed presence of only single plasmid of size approximately 23.1 kb (Table 1, Fig. 1) which is in concurrence with the finding of (Trindale *et al.* 2007) who reported the presence of a single plasmid band of approximately 23.6 kb in size in *Xanthomonas campestris* pv *viticola.* Variability in the number and size of plasmids in *Xanthomonas* sp. is high. Single plasmid in *Xanthomonas* has been reported in *Xanthomonas axonopodis* pv *citri* (Carvalho *et al.* 2005) and *Xanthomonas campestris* pv *oryzae* (Xu *et al.* 1991) with an approx size of 72.6 kb and 31.7 kb, respectively. Satyanarayana and Verma (1993) reported a strain of *X. campestris* pv *malvacearum* belonging to race 18 containing five plasmids while Chakrabarty *et al.* (1992) in a separate study revealed two other strains of *Xanthomonas campestris* pv *citri* of different origin, but also belonging to race 18, harboured three plasmids each. Ninety per cent isolates of *Xanthomonas campestris* pv *phaseoli* carry one to four plasmids with a size varying between 1–22 kb (Zapata *et al.* 2002). Some variability in plasmid profile amongst different strains within the same race of *X. campestris* pv *malvacearum* has been reported (Chakrabarty *et al.* 1995). However, plasmid of Indian isolates causing bacterial leaf spot of grapes is hereby reported for the first time.

Plasmid curing

While working with plasmids it is often desirable to obtain plasmid cured derivatives for a comparative analysis between plasmid-containing uncured cells and plasmid-cured cells so as to have an insight regarding plasmid-borne traits of the bacteria. In the current study an effort was made to cure plasmid from *Xanthomonas campestris* by using different commonly used curing agents. Plasmid profile of cured cells was achieved with a range of concen-



a. uncured b. cured

Fig. 4. Symptoms developed on grape leaves after inoculation with *X. campestris* pv *viticola.*

tration of ethidium bromide (75–125 µg mL^{-1}) and acridine orange (50–100 µg mL^{-1}). Sodium dodecyl sulphate at 1% and 1.5% were able to cure the cells, but at lower concentration i.e. 0.5%, it was unable to denature the plasmid completely (Fig. 2). Sodium dodecyl sulphate have been successfully used to cure bacterial plasmids in *Pseudomonas aeruginosa* (Raja *et al.* 2009) which supported the findings of Saha *et al.* (2000) that curing by using SDS (40%) was more efficient than elevated temp (28%). Unlike the present study, single concentration of SDS i.e. 0.5% was unable to cure the plasmid, similarly Zaman *et al.* (2010) reported, that out of the three curing agents, acridine orange was unable to cure the plasmid at all concentrations. Plasmid curing experiment have been reported previously on various bacterial groups like, *E. coli, Lactobacillus, Pseudomonas* (Saha *et al.* 2000, Zaman *et al.* 2010, Gohar *et al.* 2015) but there are no standard protocols available and applicable to all plasmids. In fact, the usefulness of curing agents is unpredictable in many bacterial strains (Treveros 1986). Success of curing methods depends on nature of curing agents, its concentration and bacterial host where some may work better than others (Gosh *et al.* 2000). Some curing agents work in a non-specific way, while some seem to act much selectively (Hohn *et al.* 1969). This Plasmid curing experiment in *Xcv* have not been reported previously and is hereby reported for the first time but it demands further investigation.

Comparative analysis of colony morphology

Plasmid cured colonies of *Xcv* showed considerable changes in colony morphology than its wild counterpart. The colonies of cured strain were irregular shaped with irregular margin with a dull non shiny, non mucoid appearance (Fig. 3). Colonies of cured strain were appressed and bigger than the uncured colonies with no change in pigmentation as *Xcv* reported in this case was an albino strain (Kamble *et al.* 2019) (Table 2). Colony morphology of cured strain was similar irrespective of the curing agents used. The non mucoid nature of the cured bacterial colony did not revert on repeated sub culturing and replica plating. Similar results were also reported in *Pseudomonas* sp. (Saha *et al.* 2000), clearly hinting that colony morphology/characteristics is a plasmid-borne trait. Similar change in colony morphology was also observed in heat cured *X. campestris* pv *malvacearum* (Chakrabarty *et al.* 1995) and they further reported that the color of colonies were restored when the cured cells were transformed with the wild plasmid. In another study plasmid curing with acridine orange exhibited a significant negative effect on growth, physiological characteristics and colony morphology of the *Lactobacillus plantarum* when cured and uncured cells subjected to different conditions (Adeyemo *et al.* 2015).

Exopolysacchride production

Cured strain of *Xcv* was also defective in EPS production. Quantitative estimation in synthetic medium clearly exhibits that the cured strain produced smaller amount of EPS viz. 0.05 mg/ml after 7 days (Table 3). However, the EPS production of uncured strain, during the same time span was 1.68 mg/ml. Chen *et*

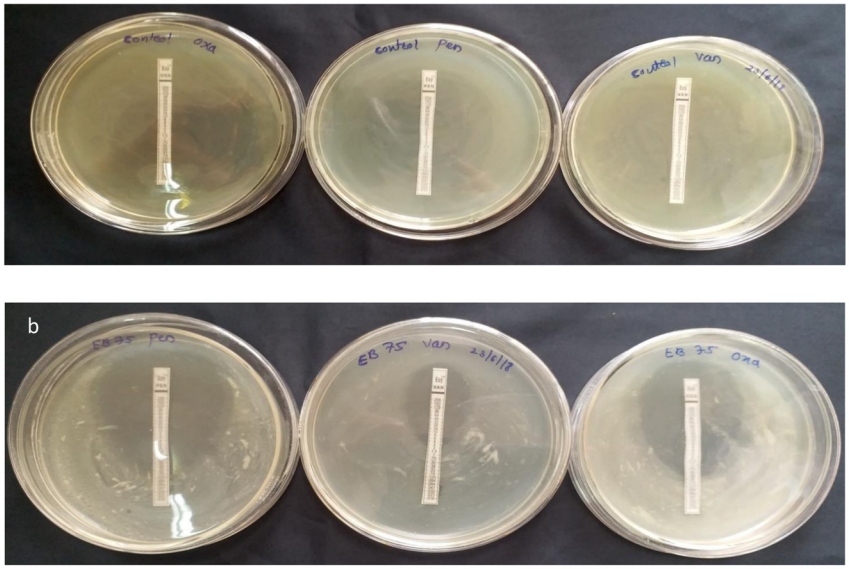


Fig. 5. Sensitivity of uncured (a) and cured (b) bacterial strain of *X. campestris* pv *viticola* to different antibiotics.

al. (1988) reported plasmid encoded EPS production in three strains of *Xanthomonas campestris* pv *oryzae*. The virulence of *Xanthomonas campestris* pv *malvacearum* had been reported to be correlated with EPS production (Borkar 1982, Chowdhury *et al.* (1980), El-Banoby *et al.* (1979). Verma (1986) suggested that the EPS of *Xanthomonas campestris* pv *malvacearum* was needed for permanent water soaking, favourable for the multiplication and spread of the pathogen. As there was an occurrence of defective EPS production in cured strains, less polysaccharide may be available for water soaking, thereby reducing their pathogenicity. A cluster of 12 genes affecting EPS biosynthesis in *Rhizobium meliloti* was present on a mega plasmid (Barbour *et al.* 1989). Udupia *et al.* (1991) also reported that a high molecular mass plasmid of 70–80 MDa encoded the production of EPS in *Rhizobium* sp. isolated from *Cajanus cajan*. Yang *et al.* (1994) demonstrated that *avrb 6*, a plasmid-borne pathogenicity (host recognition) gene is absolutely required for water soaking symptom in cotton and also for increase of bacterium on leaf surface.

Pathogenicity test

The virulence of *Xcv* was adversely affected by plasmid curing and the cured strain was very weakly pathogenic. Disease severity on all the tested varieties ranged from 4–5 when inoculated with wild strain while the corresponding range of 0–1 was observed

Table 3. Exopolysaccharide production and disease severity of *X. campestris* pv *viticola* in different grape varieties. 'a' - Average of five replications.

Bacterial strain (*Xcv*)	Exopoly-sacch-aride [a](mg/ml)	Incuba-tion period [a](days)	Thomp-son seed-less	Disease severity			
				Sonaka	Manik Chaman	TAS A Ganesh	Arkavati	Manjari Medika
Uncured	1.68	3.6	5	5	4	5	4	1
Cured	0.05	23.4	1	0	0	1	0	0
CD	0.23							
SE	0.016							

in case of inoculation with cured strain. The typical water-soaking symptoms of disease were discernible after 72 h on all grape varieties inoculated with uncured strain of *Xcv* (Fig. 4, Table 3). The cured strain, under similar conditions, had prolonged incubation period and very faint water soaking symptoms were visible after 23 days in all the tested varieties. Moreover these water soaked symptoms were localized only at the point of inoculation which signifies its weak localised infection. The cells recovered from the leaves inoculated with the uncured strain possessed the same plasmid profile and colony morphology as the original cultures, whereas the cells isolated from the leaves inoculated with the cured strain were plasmid less and their colonies remained flat, dull and non mucoid. The drastic prolongation of the incubation period of the cured strain in the present study may be due to a defect in the host recognition system of the bacterium as a result of plasmid curing. Current study, clearly suggests that the pathogenicity and virulence genes of *Xcv* was perhaps extra chromosomal and hence the variability in infection by the wild and cured cells.

Antibiotic sensitivity

Antibiotic (vancomycin, penicillin and oxacillin) strips of known range of concentration have been used to test against both cured and uncured bacterial strain. As plasmids frequently carry genes for antibiotic resistance, cured bacteria was tested for their sensitivity against these three antibiotics. At post curing stage, the *Xcv* was found sensitive to vancomycin, penicillin and oxacillin antibiotics (Fig. 5). This might be due to the fact that the plasmid borne antibiotic resistance gene had been denatured or removed in the process of curing. Sundin and Bender (1996) reported that the plasmid pP23TA of *Pseudomonas syringae* pv. *syringae* carried the genes of streptomycin resistance. It was also reported that wild *Pseudomonas aeruginosa* BC 15 was resistant to tetracycline, chloramphenicol, streptomycin, kanamycin and erythromycin, but the cured strain being sensitive to all, clearly indicated the possible plasmid borne nature of gene encoding resistance to these antibiotics (Raja *et al.* 2009). Minsavage *et al.* (1990) concluded that the streptomycin resistance locus in *X. axonopodis* pv *vesicatoria* was located on 68 kb plasmid. On the contrary, Mashaly *et al.* (2013) reported that there was no correlation between plasmid and antibiotic resistance in case of *X. axonopodis* pv *malvacearum.* The probable reason could be unsuccessful curing of the bacteria or as reported by Mills (1990), majority of the plasmids occurring in phytopathogenic bacteria are cryptic. Nevertheless, this study also brings to the fore that antibiotic resistance markers are encoded by the plasmids.

CONCLUSION

Present study reported the isolation and analysis of first indigenous plasmid from *Xanthomonas campestris* pv *viticola* from Maharashtra, India. Approximately, 23.1 kb size of plasmid was isolated. All the studied traits was affected by plasmid curing which clearly state that genes, responsible for colony morphology, antibiotic resistance, exopolysaccharide production and virulence, are present on plasmid. However, for an unequivocal conclusion, transformation studies needs to be carried out. As antibiotics are widely used in bacterial disease control, the management practices should be employed in such a way that acquisition of resistant plasmids is minimum. Furthermore, information regarding the plasmid character and its mobility is a must.

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