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Antibiotic resilience in *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight of pomegranate

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Bacterial blight caused by *Xanthomonas axonopodis* pv. *punicae* (Xap) is one of the economically important diseases of pomegranate in India. Under field conditions, the disease is being managed using antibiotics and copper-based compounds but with limited success due to their poor bio-efficacy. The reduced efficacy of antibiotics and copper compounds against field populations of Xap might be due to the development of bactericide resistance through acquired genes. In the present study, ten bacterial blight-infected pomegranate samples were collected from different geographic locations of Karnataka, India, and causal agent Xap was isolated and identified through 16S rRNA sequencing. Streptomycin resistance genes such as *rps*L, *str*A, *str*B and copper resistance genes *cop*L, *cop*B were detected using gene-specific primers in PCR. All ten isolates were positive for streptomycin resistance genes whereas copper resistance gene *cop*B was absent in three isolates (Xap1, Xap4, Xap6) while *cop*L was absent in Xap4 and Xap6 isolates. Further, *in vitro* experiments using different concentrations of streptomycin on culture media showed lowest growth inhibition up to 1500 µg/ml concentration, supporting the molecular evidence of antibiotic resistance. The present study provides preliminary information on the presence of antibiotic resistance genes in the field populations of Xap.

Keywords: Antibiotics, bacterial blight, copper compounds, disc diffusion, pomegranate, resistant genes.

IN recent years, bacterial blight or oily leaf spot disease has emerged as a major concern for pomegranate cultivation in all pomegranate-growing states of India. The disease is responsible for huge economic losses up to INR

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Table 1. Place of origin and 16S rDNA accession numbers of *Xanthomonas axonopodis* pv. *punicae* isolates used in the study

Isolate ID	Place of origin in Karnataka, India	Geographical co-ordinates	Accession number
Xap1	Chikkanayakanahalli	N13,35′55.4″, E76,18′23″	KY692197
Xap2	Madhugiri	N13,40,37″, E77,25,4.9	KY795999
Xap3	Sira	N13,51,3.8052, E76,54,52.7004	KY796000
Xap4	Hosadurga	N13,49′17.8″, E76,18′35.1″	KY796001
Xap5	Hosadurga	N13,56′1.8″, E76,29′12.31″	KY796002
Xap6	Hiriyur	N13,57′41.6″, E76,33′37.2″	KY796003
Xap7	Arsikere	N13,34′28.5″, E76,27′52.4″	KY796004
Xap8	Arsikere	N13,43′1.7″, E76,19′36.5″	KY796005
Xap9	Chintamani	N13,31,39.0216, E76,3,50.1624	KY796006
Xap10	Madaksira	N13,59,33.9, E77,16,58.7	KY796007

10,000 million to farmers of Karnataka and Maharashtra[1]. Under field conditions, bacterial blight of pomegranate was successfully managed using streptocycline (streptomycin sulphate 90% and tetracycline hydrochloride 10%) antibiotics in combination with copper compound but, in recent times, its effectiveness has reduced due to indiscriminate usage. One of the prime reasons for reduced efficacy of an antibiotic against a bacterial species is the acquired antibiotic resistance by the exposed population due to selection pressure[2]. Previously, presence of antibiotic resistance genes in phytopathogenic bacteria such as *Xanthomonas oryzae* pv. *oryzae*, *Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas syringae*, *Erwinia amylovora*, *Xanthomonas alfalfae* subsp. *citrumelonis* and *Xanthomonas axonopodis* pv. *citri* has been reported[2–5].

Phylogenomic analysis using whole genome sequence of *X. axonopodis* pv. *punicae* (Xap) has confirmed its close relationship with *X. axonopodis* pv. *citri* (Xac)[6]. Reports of antibiotic-resistant strains in Xac were a proof of their close relative Xap possessing all potentialities to acquire resistance to the antibiotic. Till date, no information is available for the existence of antibiotic resistance strains of Xap from any part of the world. Therefore, the present study was conducted to understand the genetic basis for reduced sensitivity against antibacterial compounds in the Xap populations of Karnataka pomegranate orchards. We identified streptocycline and copper compound resistance strains of Xap from the pomegranate orchards of Karnataka, which is one of the major pomegranate-growing regions of India. This information is useful for monitoring the antibiotic-resistant strains in pomegranate orchards and also provides leads on the need for an alternative strategy for the management of bacterial blight disease of pomegranate.

Pomegranate leaves showing symptoms of bacterial blight were collected from different geographical locations such as Hosdurga, Hiryur (Chitradurga district), Arsikere (Hassan district), Sira, Madhugiri (Tumkur districts) and Chintamani (Kolar district) of Karnataka during the August–September 2016 (Table 1). Further, the pathogen was isolated on yeast dextrose calcium carbonate agar (YDCA) medium, and identified based on morphological and cultural characters of the pathogen according to standard microbiological procedures[7].

Five-week-old seedlings of bacterial blight susceptible pomegranate variety cv. Bhagwa were kept in an environmentally controlled polyhouse for two days for conditioning. The temperature and relative humidity in the polyhouse were maintained at 25° ± 5°C and 70–80%, respectively. Pure cultures of all ten isolates were used for pathogenicity assay. The bacterial cell suspension (2.5×10^8 cfu/ml) was sprayed on the superficially pricked leaves using sterilized needle following a previously reported procedure[6]. Reisolation of the pathogen and identification was carried out from the symptomatic diseased leaves for confirming the pathogenicity of the Xap pathogen.

Genomic DNA from the bacterium was extracted as described previously[8]. Pure cultures of all ten isolates were mass-multiplied on YDCA medium after confirming their pathogenicity. The genomic DNA was further used for PCR amplification with universal 16S rRNA primers (F-5′-GAGTTTGATCCTGGCTCA-3′; R-5′-AGAAAGGAGGTGATCCAG-3′). PCR was performed in a thermal cycler (Eppendorf, Vapo protect, Germany), with 100 ng of genomic DNA, 0.1 μM of each primer, 12.5 μl of 2xPCR mastermix (Takara Bio, Inc., Japan) and sterile distilled water to make a final volume of 25 μl. The thermal cycler was programmed with an initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 45 sec, 60°C for 45 sec and 72°C for 2 min, with the final extension at 72°C for 10 min. The amplified products were purified and directly sequenced from both ends using a commercial facility (AgriGenome Labs, Kerala, India). DNA sequences obtained were analysed and consensus sequences thus obtained were compared with the available NCBI database using BLAST analysis[6]. Further, these sequences were deposited in the NCBI GeneBank.

To identify resistance for streptomycin and copper compounds, presence of streptomycin resistance genes (*rps*L, *str*A and *str*B) and copper compound resistance genes (*cop*B and *cop*L) was assayed using gene-specific primers. Primers specific for *str*A and *str*B were designed from streptomycin 3″-kinase of *X. campestris* pv.

Table 2. Oligonucleotide primer sequences used in the present study

Gene	Forward primer sequence	Reverse primer sequence	Expected amplicon size (bp)
*cop*B	TAGACGCGATACCACCCTGA	CGATGTAGGGAGCGAACTTT	1151
*cop*L	TCTACTGCGACTGCTCCTGA	GTCGGATCAGATGAGGCAAG	380
*rps*L	CAAGCGACCACCTACAAGAGT	GTACTTGGAACGGCCTTGAC	315
*str*A	CCAAGTCAGAGGGTCCAATC	TGACTGGTTGCCTGTCAGAG	760
*str*B	TAGATCGCGTTGCTCCTCTT	ACGTTTCGCAACCTGTTCTC	758

vesicatoria strain 85–10 deposited in the NCBI database (Gene IDs CAJ24003.1 and CAJ24002.1). The specific primers of *rps*L were designed from 30S ribosomal protein S12 gene (Gene ID AE008923.1, NCBI) of *X. axonopodis* pv. *citri*. Primers for copper resistance genes such as *cop*B and *cop*L were designed using gene sequence of *X. citri* subsp. *citri* (Gene ID HM362782, protein ID ADR70747.1, ADR70748.1 and ADR70746.1)[3]. All primers were designed using Primer3 software[9]. Table 2 shows the nucleotide sequences of all primers used in this study.

The PCR reaction was carried out in a 25 µl volume containing 100 ng of genomic DNA, 0.1 µM each primer, 12.5 µl of PCR 2X mastermix (Takara Bio, Inc, Japan) and sterile distilled water to make a final volume of 25 µl. The thermal cycler (Eppendorf, Germany) was programmed with initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 1 min for product size of less than 1 kb and 1.5 min for product size of more than 1 kb, followed by final extension at 72°C for 10 min. After PCR amplification, 10 µl of the PCR product was electrophoresed in a 1.5% agarose gel in 1X TAE buffer and visualized by ethidium bromide staining. A 1 kb DNA ladder (Takara Bio, Inc., Japan) was used as molecular weight markers.

All ten isolates were assayed for streptomycin sulphate and copper oxychloride sensitivity at different concentrations. Streptomycin sulphate was used at 100, 200, 300, 400, 500, 750, 1000, 1250 and 1500 µg/ml concentration on nutrient sucrose medium, whereas copper oxychloride was used at concentrations of 500, 1000, 2000, 2500, 3000, 3500, 4000, 4500 and 5000 µg/ml on casitone yeast extract (CYE) medium, following a previously described procedure[10]. Sterile 5 mm Whatman No. 42 filter paper discs were immersed and soaked in the antibiotic solutions for 30 min. Sterilized and nearly cooled medium was inoculated with broth culture of Xap (1×10^9 cfu/ml), then poured into petri plates and allowed to solidify as described in previous studies[11,12]. After solidification, three discs of the same concentration were placed at equal distance in every plate. The plates were refrigerated at 5°C for 1 h and then incubated at 27°C.

After 24 h of incubation, the inhibition zone was measured and values were converted to per cent inhibition using the formulae given below and further statistically analysed.

$$\text{Inhibition (\%)} = 100 - \left[\left(\frac{9(\text{Standard diameter of petri plate}) - \text{Inhibition zone of treatment}}{9(\text{Standard diameter of petri plate})} \right) \times 100 \right].$$

Pure cultures for ten Xap isolates (Xap1–Xap10) were isolated from the diseased samples. Bacterial colonies appeared as well-separated and typically bright yellow in colour on the YDCA medium. The pathogenicity assay revealed the characteristic symptoms on the leaves of susceptible pomegranate cultivar Bhagwa after 10 days post inoculation as small, water-soaked, brown to black-coloured circular to irregular lesions. The taxonomic identity of the pathogen isolates was further confirmed by PCR amplification followed by sequencing of the 16S rDNA fragment using universal primers (Figure 1). Amplicon sequencing and its analysis using BLAST confirmed that the pathogen isolates taxonomically belonged to *X. axonopodis* pv. *punicae* (Xap). The 16S rDNA sequences were deposited in the NCBI database and Table 1 lists their accession numbers.

PCR amplification technique using gene-specific primers was used for assaying antibacterial resistance genes such as *rps*L, *str*A and *str*B (imparts streptomycin resistant), and *cop*B and *cop*L (imparts copper resistance) across the ten isolates. The primer sets used were efficient in reproducing amplification of the expected size (Figure 2). Results revealed that *rps*L, *str*A and *str*B resistant genes were found in all the ten isolates tested by giving positive amplification in the PCR (Figure 2 *c–e*). The *cop*B copper-resistant gene was positive in seven out of ten isolates (Xap2, Xap3, Xap5, Xap7, Xap8, Xap9 and Xap10), whereas, PCR amplification was negative for *cop*B in three isolates, viz. Xap1, Xap4 and Xap6 (Figure 2 *a*). Another copper-resistant gene *cop*L was PCR amplified in eight isolates (Xap1, Xap2, Xap3, Xap5, Xap7, Xap8, Xap9 and Xap10), whereas no amplification was obtained in Xap4 and Xap6 isolates (Figure 2 *b*). The PCR results suggested considerable variation in the *cop*B and *cop*L alleles in Xap populations of Karnataka.

In vitro assay for streptomycin sensitivity of all ten isolates revealed lower per cent inhibition in all isolates at all concentrations tested. At the highest concentration of 1500 µg/ml tested, the inhibition was 35.33% (Table 3).

Table 3. *In vitro* evaluation of streptomycin sensitivity in Xap isolates at different concentrations

Treatment	Percentage of growth inhibition of different isolates									
	Xap1	Xap2	Xap3	Xap4	Xap5	Xap6	Xap7	Xap8	Xap9	Xap10
T1	33.33	21.33	18.67	24.87	28.00	22.67	27.33	36.00	32.67	24.67
	(25.41)	(20.12)	(25.59)	(21.82)	(23.22)	(20.73)	(22.91)	(26.52)	(25.17)	(21.66)
T2	34.67	23.33	32.67	25.33	34.67	36.00	34.00	38.00	35.33	26.67
	(25.99)	(21.08)	(34.86)	(22.01)	(26.02)	(26.54)	(25.75)	(27.35)	(26.29)	(22.58)
T3	36.00	24.00	41.33	26.00	34.67	36.67	35.33	40.00	35.33	29.00
	(26.51)	(21.42)	(40.01)	(22.34)	(26.02)	(26.81)	(26.29)	(28.11)	(26.29)	(23.65)
T4	37.33	25.33	43.67	28.67	37.33	36.67	40.00	42.67	34.67	29.33
	(27.08)	(22.03)	(41.34)	(23.48)	(27.08)	(26.82)	(28.12)	(29.13)	(26.02)	(23.76)
T5	37.33	26.00	46.00	29.33	38.00	38.00	44.00	46.00	35.33	31.33
	(27.04)	(22.33)	(42.70)	(23.79)	(27.35)	(27.33)	(29.60)	(30.35)	(26.26)	(24.64)
T6	35.33	26.00	47.33	30.27	38.67	40.00	50.67	50.00	35.67	31.60
	(26.26)	(22.33)	(43.47)	(24.21)	(27.60)	(28.12)	(32.02)	(31.79)	(26.43)	(24.72)
T7	44.00	28.00	47.33	34.60	40.00	51.33	50.67	51.33	36.80	34.53
	(29.62)	(23.22)	(43.47)	(25.99)	(28.11)	(32.23)	(32.04)	(32.02)	(26.87)	(25.97)
T8	45.33	32.00	63.33	38.00	41.33	52.67	55.33	51.33	38.07	35.33
	(30.11)	(24.93)	(52.76)	(27.35)	(28.61)	(32.71)	(33.67)	(32.26)	(27.38)	(26.29)
T9	49.33	33.33	67.33	44.00	43.33	56.67	56.67	55.33	40.00	35.33
	(31.55)	(25.47)	(55.14)	(29.63)	(29.37)	(34.10)	(34.13)	(33.66)	(28.12)	(26.29)
C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)
S.Em (±)	1.16	0.56	1.08	0.60	0.73	1.14	0.72	0.91	0.75	1.00
CD at 0.01	4.71	2.26	4.39	2.42	2.97	4.62	2.92	3.69	3.06	4.08
CV (%)	4.18	2.47	2.56	2.43	2.70	4.01	2.45	3.02	2.84	4.12

Figures in parentheses represent the arcsine values.

T1, Streptomycin sulphate at 100 µg/ml; T2, Streptomycin sulphate at 200 µg/ml; T3, Streptomycin sulphate at 300 µg/ml; T4, Streptomycin sulphate at 400 µg/ml; T5, Streptomycin sulphate at 500 µg/ml; T6, Streptomycin sulphate at 750 µg/ml; T7, Streptomycin sulphate at 1000 µg/ml; T8, Streptomycin sulphate at 1250 µg/ml; T9, Streptomycin sulphate at 1500 µg/ml; C, Control.

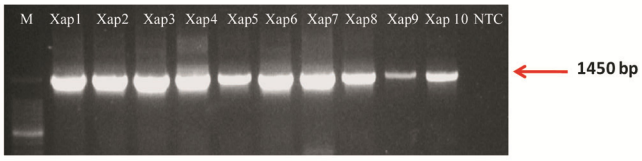

Figure 1. Gel electrophoresis image showing 16S rDNA amplification in the ten isolates of *Xanthomonas axonopodis* pv. punicae (Xap). Lane M, Marker; lanes Xap1–Xap10, isolates and lane NTC, no template control.

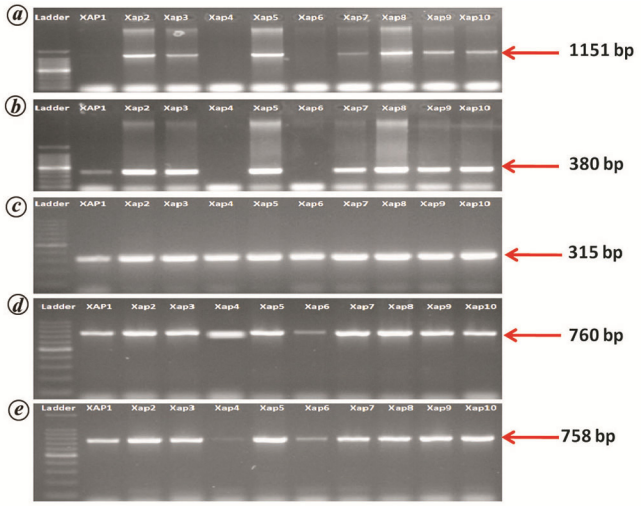

Figure 2. Gel electrophoresis image showing PCR amplification of antibiotic resistant genes in the ten isolates of Xap1–Xap10. (***a***–***e***) Gene amplification of (***a***) *cop*B, (***b***) *cop*L, (***c***) *rps*L, (***d***) *str*A and (***e***) *str*B.

PCR results revealed the presence of three streptomycin resistance genes (*rps*L, *str*A and *str*B) in all Xap strains. Thus, the lower inhibitory effect of streptomycin *in vitro* could be due to the presence such streptomycin resistance genes in all the ten isolates tested.

Similarly, *in vitro* assay for copper sensitivity across the isolates revealed higher inhibition of colony growth by copper oxychloride in Xap4 (48.89%) which did not possess the copper resistance genes (*cop*B and *cop*L; Table 4). Whereas Xap6 with per cent inhibition (44.44) lower than Xap3, Xap7 and Xap8 (45.19), did not possess any copper-resistant genes. Xap1 exhibited inhibition of 44.07% even though it possessed only one of the copper-resistant genes, i.e. *cop*L.

In the present era of high input-based agri-production, antibacterial compounds such as antibiotics and copper-based compounds are trending towards lower bio-efficacy under field conditions, which otherwise were highly effective in combating bacterial plant pathogens in the past. This could be due to acquired bactericide resistance genes by the exposed field populations of a phytopathogenic bacterial species under high bactericidal pressure. Many

Table 4. *In vitro* evaluation of copper oxychloride (COC) sensitivity in Xap isolates at different concentrations

Treatment	Percentage of growth inhibition of different isolates									
	Xap1	Xap2	Xap3	Xap4	Xap5	Xap6	Xap7	Xap8	Xap9	Xap10
T1	20.37	21.11	19.63	23.33	24.44	20.74	10.74	12.59	12.59	14.07
	(26.76)	(27.33)	(26.26)	(28.83)	(29.60)	(27.03)	(19.03)	(20.67)	(20.76)	(21.78)
T2	26.48	22.96	25.81	31.85	27.41	23.33	22.96	13.70	28.52	20.74
	(30.96)	(28.61)	(30.51)	(34.34)	(31.51)	(28.87)	(28.56)	(21.66)	(32.27)	(27.01)
T3	28.93	24.81	28.89	36.85	31.48	30.74	30.00	24.81	30.37	27.04
	(32.53)	(29.84)	(32.49)	(37.37)	(34.11)	(33.66)	(33.16)	(29.81)	(33.40)	(31.28)
T4	35.37	30.00	31.85	40.74	34.44	32.59	34.44	26.67	35.56	32.96
	(36.46)	(33.19)	(34.34)	(39.65)	(35.92)	(34.79)	(35.92)	(31.05)	(36.60)	(35.00)
T5	38.52	34.63	35.33	42.59	35.19	35.56	41.00	35.19	37.04	38.52
	(38.36)	(36.03)	(36.45)	(40.73)	(36.37)	(36.59)	(39.81)	(36.35)	(37.47)	(38.35)
T6	40.81	36.30	38.52	44.81	36.67	40.37	43.33	38.89	38.89	36.30
	(39.70)	(37.04)	(38.35)	(42.02)	(37.25)	(39.44)	(41.16)	(38.56)	(38.58)	(37.04)
T7	42.37	38.89	41.85	45.56	37.78	41.85	44.81	40.74	42.96	37.41
	(40.61)	(38.57)	(40.31)	(42.45)	(37.91)	(40.31)	(42.02)	(39.65)	(40.95)	(37.67)
T8	42.96	42.59	43.33	46.67	40.00	44.07	44.44	44.07	44.07	41.85
	(40.95)	(40.74)	(41.16)	(43.09)	(39.22)	(41.60)	(41.81)	(41.59)	(41.60)	(40.31)
T9	44.07	44.44	45.19	48.89	44.07	44.44	45.19	45.19	44.07	44.07
	(41.60)	(41.81)	(42.24)	(44.36)	(41.59)	(41.81)	(42.24)	(42.24)	(41.81)	(41.59)
C	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)
S.Em ±	0.99	1.01	1.11	1.05	1.24	1.06	1.26	1.42	0.82	1.59
CD at 0.01	4.04	4.10	4.50	4.28	5.02	4.32	5.13	5.76	3.32	6.48
CV (%)	2.73	2.90	3.10	2.69	3.44	2.96	3.52	4.23	2.28	4.60

Figures in parentheses represent the arcsine values.

T1, Copper oxychloride (COC) 500 µg/ml; T2, COC 1000 µg/ml; T3, COC 2000 µg/ml; T4, COC 2500 µg/ml; T5, COC 3000 µg/ml; T6, COC 3500 µg/ml; T7, COC 4000 µg/ml; T8, COC 4500 µg/ml; T9, COC 5000 µg/ml; C, Control.

phytopathogenic bacteria such as *X. oryzae* pv. *oryzae*, *X. campestris* pv. *vesicatoria*, *P. syringae*, *E. amylovora*, *X. alfalfae* subsp. *citrumelonis* and *X. axonopodis* pv. *citri* have been reported to have developed antibiotic resistance by acquiring resistance genes[2–5]. Xap is one of the devastating pathogens of pomegranate in India[13], which has also becoming less sensitive against streptomycin antibiotics and copper compounds (unpublished, pers. commun.).

Streptomycin, an aminoglycoside group of antibiotics, has been extensively used against many phytopathogenic bacteria in agriculture[14]. It has been demonstrated through induced mutation that the bacteria can acquire resistance to streptomycin by modification in the ribosomal S12 protein, i.e. *rps*L[15] or through transposon, i.e. *str*A–*str*B[16]. The genes *str*A and *str*B usually reside on plasmid-borne transposons and were previously reported in streptomycin-resistant strains of *Erwinia*, *Pseudomonas* and *Xanthomonas*[17]. In the present study, ten isolates of Xap collected from different pomegranate orchards in Karnataka were found positive for the presence of three streptomycin-resistant genes, i.e. *rps*L, *str*A and *str*B. These PCR results are well supported by the *in vitro* sensitivity assay for streptomycin antibiotics at different concentrations, confirming the genetic basis for reduced bio-efficacy of the antibiotics. In this study all the isolates exhibited reduced sensitivity towards streptomycin *in vitro* up to 1500 µg/ml, indicating the development of antibiotic resistance in all strains. Presence of three antibiotic resistance genes in all strains indicates the wider distribution of streptomycin resistance strains in majority of the pomegranate orchards in Karnataka.

Copper compounds are widely used to manage bacterial disease of crop plants either alone or in combination with antibiotics. Like antibiotics, resistance to copper compounds has also been reported in many phytopathogenic bacteria such as *X. citri* subsp. *citri*, *X. alfalfa* subsp. *citrumelonis* and *Pseudomonas* sp.[3]. Copper resistance genes are known to be located on the chromosome, and copper resistance is mediated by plasmid borne *cop* operon that sequesters the excess copper ions and reduces toxic levels in the bacterial periplasmic membrane[4,18]. In the present study, we observed that seven out of ten isolates were positive for the presence of *cop*B gene and eight isolates are positive for *cop*L gene, indicating the copper resistance population distributed in pomegranate orchards of Karnataka. This molecular evidence is well supported by the copper sensitivity assay, where *cop*B (Xap1, Xap4 and Xap6) and *cop*L (Xap4 and Xap6) gene-positive strains showed reduced sensitivity towards copper oxychloride on culture medium.

The Indian subcontinent is a centre of diversity of pomegranate that provides an excellent host diversity and climatic conditions for the emergence and establishment of bactericide resistance strains. The situation will be worse if such acquired resistance genes get transferred to

other related phytopathogenic bacteria through horizontal gene transfer. There is an urgent need to monitor and develop strategies to manage bactericide resistance strains as well to avoid the development of such strains in future. Further, development of alternative management strategies such as host plant resistance, biocontrol, botanicals, etc. to complement and protect the bio-efficacy of antibacterial chemicals is essential.

Conflict of interest. The authors declare no conflict of interest.

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