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Research Article

Genetic Basis and Phenotypic Characterization Of *Xanthomonas citri* Resistance to Copper in Citrus Orchards of Pakistan

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ABSTRACT

Copper resistance in citrus orchards is an emerging concern, requiring effective strategies to manage the spread of bacterial citrus canker. During a two-year surveillance (2020-2021), 5,490 citrus canker lesion samples were collected from major citrus-growing regions of Pakistan, including Punjab, Khyber Pakhtunkhwa, and the Federal area. Of these, 3,160 isolates were successfully cultured, giving an overall recovery rate of 58%, with variation across regions, districts, and cultivation zones. Sargodha district, the primary citrus-producing area of Punjab, was the main focus for copper-resistance assessment. A total of 120 bacterial isolates, along with a copper-resistant reference strain (LH-201), were evaluated on nutrient glucose agar amended with copper sulfate at 0.5 g, 1 g, and 2 g concentrations. Phenotypic characterization showed that most isolates tolerated 0.5 g CuSO₄ (60% recovery), fewer survived at 1 g (24%), and none grew at 2 g. In contrast, the reference strain (LH-201) exhibited complete resistance at all tested levels. Copper-tolerance patterns also varied among Sargodha sub-locations: isolates from Chak 48 and Choranga (2020) and Chak 82 (2021) showed higher recovery at 1 g CuSO₄. Overall, resistance frequency was greater in 2020 than in 2021. Molecular screening using primers targeting copper-resistance genes (CopLAB) detected no such genes in any isolates, despite their phenotypic tolerance at lower copper concentrations. Overall, Pakistani citrus canker populations were largely copper-sensitive, particularly at higher concentrations, with no genetic evidence of stable copper-resistance mechanisms. These findings highlight spatial and temporal variation in pathogen recovery and provide important understandings for copper-based management practices in Pakistan.

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Introduction

Citrus canker, a destructive plant disease caused by *Xanthomonas citri* subsp. *citri*, adversely affects various citrus species, resulting in significant fruit loss and a

consequent decline in overall production. Implementing effective management strategies, particularly the use of chemical agents such as copper-based compounds, is crucial to mitigate the impact of the disease (Arif et al.,

2022). Copper sulphate, a widely used bactericide, can suppress *X. citri* infections and help control citrus canker; however, its efficacy varies depending on factors such as concentration, mode of application, environmental conditions, and the presence of copper-resistant bacterial populations. Ongoing research is focused on evaluating the effectiveness of copper sulphate against citrus canker, particularly its bactericidal activity, effects on *X. citri*, and potential phytotoxicity. Nevertheless, the long-term efficacy of copper-based treatments is threatened by the emergence of copper-resistant strains (Behlau et al., 2011; Marin et al., 2019).

Management of citrus canker is critically important because the disease poses a major threat to the economically significant citrus industry, especially in citrus-producing countries. Current control strategies are costly and largely aimed at eliminating the pathogen, with millions of dollars spent annually on eradication, prevention, and control efforts. As no curative treatment is available, disease management primarily depends on the removal and destruction of infected plants and trees (Cubero et al., 2011). Several approaches may be adopted to manage the disease, including the application of nanoparticles, botanical extracts with antimicrobial properties, and chemical bactericides such as copper-based sprays. Among these, silver nanoparticles have emerged as promising biocontrol agents against *X. citri*, offering advantages such as ease of synthesis, safety, and high efficacy at low concentrations (Arif et al., 2022).

Several medicinal plant species have traditionally been used to treat various ailments. The genus *Euphorbia*, one of the largest angiosperm genera, is widely distributed across the Himalayan region extending from Pakistan to China. This genus contains numerous secondary metabolites commonly used to treat skin disorders and edema in humans. Methanolic and ethanolic extracts of *Euphorbia* have also been employed in the synthesis of silver nanoparticles, which have shown significant inhibitory effects against plant-pathogenic microorganisms (Thorley and Tetley, 2013; Phull et al., 2020).

Understanding the molecular mechanisms underlying the bactericidal effects of copper sulphate is essential for developing effective and sustainable management strategies. These mechanisms involve identifying specific bacterial proteins and cellular pathways disrupted by copper, ultimately leading to disease

suppression. In *X. citri*, resistance to copper is largely attributed to the *copABCD* and *copL* gene clusters, which are involved in copper homeostasis and detoxification. The *copA* and *copB* genes function in exporting excess copper ions from the cell, while *copC* and *copD* participate in copper binding and sequestration. The *copL* gene acts as a regulatory component, contributing to enhanced resistance observed in highly tolerant strains (Phull et al., 2020).

Copper sulphate (CuSO_4) has been widely used worldwide, including in Pakistan, for managing citrus canker. However, the effectiveness of this strategy diminishes when pathogen populations develop resistance to copper-based compounds. Genetic variability within *X. citri* populations allows the emergence of resistant strains, reducing the overall efficacy of copper-based treatments (Ali et al., 2017). Copper compounds such as cuprous oxide, copper sulphate, and copper hydroxide remain widely used bactericides for citrus canker management, but their intensive use has raised environmental concerns and contributed to the selection of copper-resistant *X. citri* strains (Marin et al., 2019). Copper resistance was first reported in Argentina in the 1990s due to prolonged exposure of citrus nurseries to copper-based sprays (Behlau et al., 2011). Furthermore, excessive copper accumulation can cause phytotoxicity, damaging fruit rinds and root systems (Graham et al., 2016).

Zinc oxide has been investigated as an alternative management option, reducing citrus canker lesions by up to 42% under greenhouse conditions, although its field efficacy remains limited (Graham et al., 2016). Other approaches, such as the application of antibiotics (e.g., streptomycin), are restricted due to concerns about resistance development and legal prohibitions in several countries, including Brazil and parts of Europe (Martins et al., 2020). Consequently, there is an increasing need for eco-friendly and sustainable management strategies. Emerging alternatives include genetic engineering, the development of resistant cultivars, and biological control techniques. Advances in genome editing have enabled the modification of citrus defense-related genes to enhance tolerance to *X. citri* and strengthen the plant's overall immune response (Martins et al., 2020).

Therefore, the present study was designed to isolate and characterize *X. citri* strains from different locations in the Sargodha district of Pakistan and to evaluate their resistance to increasing concentrations of CuSO_4 .

Material and Methods

Study area and sample collection

This study was conducted at the Institute of Biotechnology & Genetic Engineering, The University of Agriculture, Peshawar, Pakistan. Disease surveillance and sample collection were carried out in different citrus orchards over a two-year period (2020-2021). A total of 5,490 canker lesion samples (3,400 in 2020 and 2,090 in 2021) were collected from citrus-growing regions across Pakistan.

In Khyber Pakhtunkhwa (KP), sampling was conducted in Bajaur, Dargai, Dir, Haripur, Khyber, Mansehra, Nowshera, Peshawar, Shangla, and Swat, with the highest number of samples collected from Peshawar District (1,040 in 2020 and 240 in 2021). In Punjab, samples were collected from the districts of Khushab, Lahore, Mianwali, Sahiwal, Sargodha, and Toba Tek Singh. Sargodha contributed the largest number of samples (520 in 2020 and 270 in 2021), followed by Toba Tek Singh (330 in 2021). Overall, the greatest number of samples originated from Khyber Pakhtunkhwa, comprising 1,290 samples in 2020 and 1,980 samples in 2021. Of the total collected samples, 3,160 were cultured on growth media to assess the

recovery of bacterial isolates.

Bacterial culturing and sub-culturing

Citrus canker-infected leaf samples were collected from major citrus-growing regions of Pakistan. Leaves showing typical canker symptoms were excised and rinsed with tap water (Figure 1). Lesions were carefully removed and surface-sterilized using 0.9% mercuric chloride. The sterilized lesions were air-dried and transferred to Eppendorf tubes containing phosphate-buffered saline (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl; pH 7.4). The lesions were macerated to obtain a bacterial suspension.

The suspension was streaked onto nutrient agar medium supplemented with antibiotics and incubated at 28 °C for 72 h. Three replicate plates were used for each isolate, and all plates were sealed with parafilm. Bacterial colonies obtained from each plate were inoculated into nutrient broth and incubated in a shaking incubator at 28°C for 6 h. The resulting suspension was sub-cultured on nutrient agar for an additional 72 h at 28°C (Figure 1). Pure isolates were preserved in sterilized glycerol and stored at 4°C until further use for resistance studies (Pruvost et al., 1992; Ullah et al., 2021).

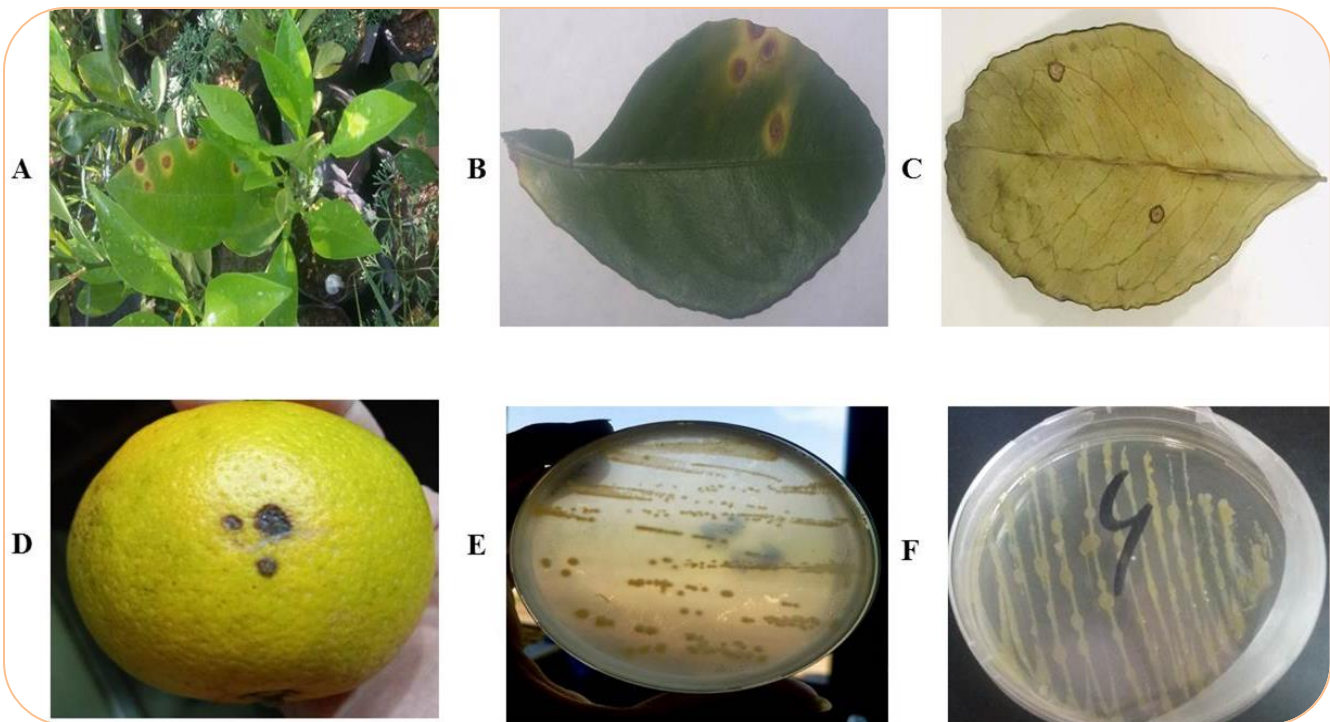


Figure 1. Citrus canker symptoms on plants (A-D) and yellow bacterial colonies on nutrient agar plates (E-F). A) Symptoms on sweet orange; B) Symptoms on young leaves; C) Symptoms on old leaves; D) Symptoms on fruit; E-F) Yellow bacterial colonies on nutrient agar medium.

Morphological characterization

The morphological characteristics of *X. citri* pv. *citri*, including colony color, shape, texture, opacity, and configuration, were recorded to support the identification of the citrus canker pathogen (Table 1).

Table 1. Cultural and morphological characteristics of *X. citri* isolates from different citrus-growing regions of Pakistan.

S. No.	Characteristics	Appearance
01	Color	Yellow or light yellow
02	Shape of colony	Round or Filiform
03	Texture	Mucoid
04	Opacity	Translucent
05	Configuration	Single and small

Characterization of bacterial strains for CuSO₄ resistance

Among various management practices, the application of CuSO₄ is considered one of the most effective strategies for controlling citrus canker. However, excessive and prolonged use has contributed to the emergence of copper-resistant strains of the pathogen *X. citri* (Behlau et al., 2011). In this study, isolates collected from major citrus-growing orchards in Sargodha were characterized for copper resistance. The level of resistance was evaluated on solid media, and resistant strains were further screened for copper resistance genes using PCR (Table 2).

Phenotypic screening for copper resistance in the *X. citri* population

The selected isolates were cultured on nutrient glucose agar (nutrient agar 28 g L⁻¹, glucose 0.1 g L⁻¹) to identify copper-resistant strains from district Sargodha, following

the protocol of Graham & Gottwald (1990). Copper sulfate was incorporated into the medium at concentrations of 0, 0.5, 1, and 2 g L⁻¹. The *X. citri* strain LH-201, known to be copper resistant, served as the reference strain.

Isolates were incubated at 28°C for 24 h, and their copper resistance was assessed by comparing growth on copper-amended media with growth on copper-free media. Strains exhibiting comparable growth on both media after 72 h of incubation at 28°C were classified as copper resistant. Minimum inhibitory concentrations (MICs) were subsequently determined for these resistant isolates.

Strain LH-201 was used as a positive control (CuSO₄-resistant), while nutrient media without CuSO₄ served as the negative control. To ensure standardization across experimental replicates and enable direct comparison among isolates, the response of each isolate was converted into a relative value. Cephalexin was added to the medium to suppress contamination or unwanted fungal growth during phenotypic characterization.

Genotypic screening for CuSO₄ resistance

Genomic DNA from bacterial isolates was extracted using a modified cetyltrimethylammonium bromide (CTAB) method following Ullah et al. (2021). PCR amplification of copper resistance genes (*copL*, *copA*, and *copB*) was performed using specific primers (Table 2). The PCR protocol included an initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 63°C for *copL*, 64°C for *copA*, and 60°C for *copB* for 45 sec; and extension at 72°C for 1 min. A final extension was completed at 72°C for 10 min.

Amplified PCR products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized using a gel documentation system.

Table 2. Details of PCR primers used for the characterization of copper-resistance genes in *X. citri* isolates from citrus-growing regions of Pakistan.

Gene	Forward primer	Reverse primer	Annealing temp	Band size
<i>copL</i>	CCGTGTCAGCCTCCTCACTTCTAC	CAGCGGCATGACATCCAGGCC	63	356
<i>copA</i>	CCTCCATGGCAGGACACTTCCATC	CCAGACATATCCATCGACCCATGATCCA	64	870
<i>copB</i>	CTCAGGATCACTCTGCACATCAG	GCACGTAGCTCTTAATCGAGTTGTC	60	531

Data analyses

Resistance screening, based on both morphological and genotypic traits, was performed on representative isolates collected from the Sargodha district. The experimental data were analysed using a generalized

linear model (GLM), in which location and host were treated as fixed effects, while isolates nested within locations were treated as random effects. Significant differences among means were determined using the least significant difference (LSD) test at the 1% probability

level. All statistical analyses were performed in the R statistical environment. Multivariate analyses were conducted to illustrate the overall distribution of isolates based on their resistance profiles, and the results were presented in the form of tables and bar graphs.

Results

Recovery of citrus canker isolates collected across Pakistan

Out of the total 5,490 samples collected, the number of samples recovered from Khyber Pakhtunkhwa was 1,040 in 2020 and 870 in 2021; from Punjab, 700 in 2020 and 520 in 2021; and from the Federal Region, 20. The distribution of samples and their recovery varied across the major citrus-producing zones of Pakistan. In the KP commercial zone, 770 samples were collected in 2020 and 540 in 2021, with recovery rates ranging from 51% to 67%. In the commercial zone of Punjab, a 50% recovery rate was recorded for the 1,160 samples collected in 2020, whereas a 67% recovery rate was observed for the 780 samples collected in 2021. In 2020, the highest number of samples (1,280) was collected from other Pakistani zones, with a recovery rate of 50%. The recovery rates from the newly merged districts were 64% in 2020 and 67% in 2021, while in the Himalayan zone, the recovery ranged from 67% to 100% across the two years (Table 5).

Growth of *X. citri* and Phenotypic characterization of copper-resistant bacteria

Phenotypic assessment of copper resistance in *X. citri* strains showed that all bacterial isolates, including the reference strain (Reunion), were able to grow on

in 2020 and 10 in 2021 (Table 3).

Considering the samples recovered across different districts, the minimum number of samples was collected from the Federal Region, particularly Islamabad, with 20 samples in both 2020 and 2021. The recovery rates for these samples were 50% and 100%, respectively. In KP, the maximum number of samples was collected from Peshawar district (1,040 in 2020 and 240 in 2021), where the recovery rate exceeded 50%. In Punjab, the highest number of samples was collected from Sargodha district (520 in 2020 and 270 in 2021), followed by Toba Tek Singh (330 in 2021), with recovery rates above 50% for all districts (Table 4).

nutrient glucose agar (NGA) medium amended with varying concentrations of copper sulfate (0 g, 0.5 g, 1 g, and 2 g). All strains (100%) successfully grew on copper-free medium, while most isolates (60%) were recovered at 0.5 g CuSO₄. A smaller proportion (24%) grew on medium containing 1 g CuSO₄. None of the isolates grew on 2 g CuSO₄, except for the reference strain, which was able to grow at all tested copper concentrations (Figure 2).

Overall, the highest proportion of isolates (60%) showed resistance at 0.5 g Cu, followed by 24% at 1 g Cu, while no field isolate survived at 2 g CuSO₄. Considering year-wise variation, isolates collected in 2020 exhibited relatively higher copper resistance than those from 2021. In 2020, 100% of isolates grew on 0 g Cu, followed by 69% on 0.5 g Cu and 25% on 1 g Cu, with no growth at 2 g Cu. In 2021, 53% of isolates were recovered at 0.5 g Cu and 22% at 1 g Cu; again, no isolate survived at 2 g Cu (Table 6; Figure 2 and 3).

Table 3. Recovery rate (%) of citrus canker isolates collected from different provinces during 2020 and 2021.

Year	Region	No. of samples collected	No. of samples cultured	Recovery (%)
2020	Federal Area (Pothohar region)	20	20	100
	Khyber Pakhtunkhwa	1980	1040	53
	Punjab	1400	700	50
	Year total	3400	1760	52
2021	Federal Area (Pothohar region)	20	10	50
	Khyber Pakhtunkhwa	1290	870	67
	Punjab	780	520	67
	Year total	2090	1400	67
Overall population		5490	3160	58

Table 4. Recovery rate (%) of citrus canker isolates collected from different locations in Pakistan during 2020 and 2021.

Year	Region	District	No. of sample collected	No. of sample cultured	Recovery (%)
2020	Federal Area	Islamabad	20	20	100
	Khyber	Bajaur	80	40	50
	Pakhtunkhwa	Dargai	120	60	50
		Dir	40	20	50
		Haripur	160	80	50
		Khyber	30	30	100
		Mansehra	60	60	100
		Nowshera	240	120	50
		Peshawar	1040	520	50
		Shangla	10	10	100
		Swat	200	100	50
	Punjab	Khushab	120	60	50
		Lahore	240	120	50
		Mianwali	240	120	50
		Sahiwal	280	140	50
		Sargodha	520	260	50
2021	Federal Area	Islamabad	20	10	50
	Khyber	Bajaur	210	140	67
	Pakhtunkhwa	Haripur	60	40	67
		Khyber	30	30	100
		Malakand	270	180	67
		Mansehra	30	20	67
		Nowshera	240	160	67
		Peshawar	240	160	67
		Swat	210	140	67
	Punjab	Khushab	90	60	67
		Mianwali	90	60	67
		Sargodha	270	180	67
		Toba Tek Singh	330	220	67

Table 5. Recovery rate (%) of citrus canker isolates collected from different citrus cultivation zones during 2020 and 2021.

Year	Region	No. of collected samples	No. of samples cultured	Recovery (%)
2020	Pothohar region	20	20	100
	Himalayan zone	60	60	100
	KP commercial zone	770	390	51
	NMDs Zone	110	70	64
	Other zone	1280	640	50
	Punjab commercial zone	1160	580	50
2021	Pothohar region	20	10	50
	Himalayan Zone	30	20	67
	KP commercial zone	540	360	67
	NMDs Zone	240	170	67
	Other zone	480	320	67
	Punjab commercial zone	780	520	67

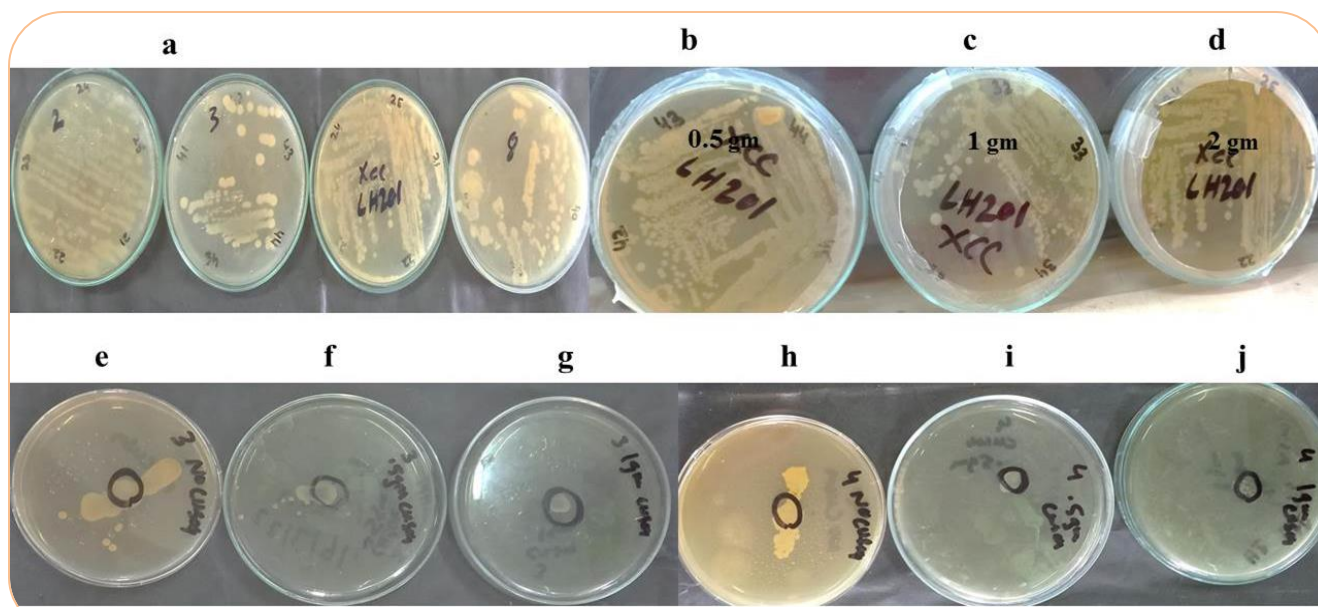


Figure 2. Representative examples of citrus canker isolates grown on nutrient glucose agar amended with different copper concentrations (0.5 g, 1 g, and 2 g). (a) Growth on 0 g copper sulfate (negative control); (b) growth of the reference strain at 0.5 g CuSO_4 ; (c) growth of the reference strain at 1 g CuSO_4 ; (d) growth of the reference strain at 2 g CuSO_4 ; (e-j) growth of various isolates at different copper sulfate concentrations.

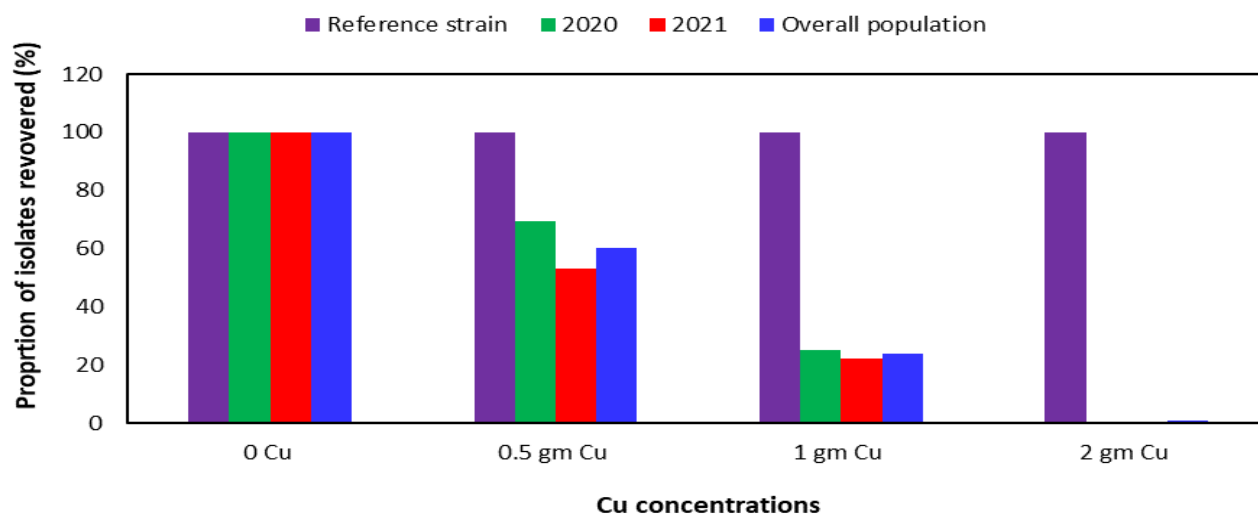


Figure 3. Percentage recovery of citrus canker isolates (%) at different copper concentrations from Sargodha district during 2020-2021.

Table 6. Revival (%) of citrus canker isolates on different copper concentrations, sampled from Sargodha district during 2020-2021.

Year	Sample size	Number of isolates revived			
		0 Cu Con.	0.5 g Cu Con.	1 g Cu Con.	2 g Cu Con.
2020	52	100	69	25	0
2021	68	100	53	22	0
Reference strain	1	100	100	100	100
Overall population	121	100	60	24	1

Recovery of *X. citri* collected from different locations in district Sargodha

The recovery rate of *X. citri* exposed to different concentrations of copper varied across the sampled locations in district Sargodha. At 0.5 g Cu, the maximum recovery (100%) during 2020 was recorded for isolates from Chak 36, Chak 48, Chak 70, Chak 8, Chak 9, Marhe, and Choranga. In 2021, only two locations, Chak 82 and Chak 100, showed 100% recovery at this concentration.

At 1 g Cu, the highest recovery in 2020 was observed for isolates from Chak 48 and Choranga (83% each), whereas no recovery (0%) was recorded from Chak 8, Chak 85, Chak 88, Chak 9, Chak 95, and Marhe. In 2021, the maximum recovery at 1 g Cu was reported for Chak 82 (67%), followed by Chak 100 and Chak 84 (50% each). The minimum recovery (0%) was noted for Chak 18, Chak 71, Chak 81, and Chak 85. However, no isolate was recovered at 2 g Cu during either year (Table 7).

Correlation of the growth response of citrus canker isolates (%) at different copper concentrations

The relationship between the growth responses of citrus canker isolates at varying copper concentrations was further evaluated through correlation analysis. Pairwise comparisons were performed between the responses at two concentrations at a time, specifically 0.5 g vs. 1 g, 0.5 g vs. 2 g, and 1 g vs. 2 g of copper. The results showed varying degrees of association between isolate responses across the different concentrations (Figure 4). In some cases, a positive correlation indicated that isolates exhibiting higher tolerance at a lower copper concentration (e.g., 0.5 g) also tended to maintain tolerance at higher levels. Conversely, weaker or inconsistent correlations suggested that certain isolates responded differently as the copper concentration increased, possibly due to genetic or physiological differences influencing copper tolerance.

Characterization of citrus canker based on copper resistance genes

To assess the presence of copper resistance among *X. citri* isolates collected from district Sargodha, three primer sets targeting the copper resistance genes CopL, CopA, and CopB were used in PCR assays. The well-characterized copper-resistant reference strain LH 201 served as a positive control. As expected, PCR amplification of the reference strain produced distinct products corresponding to the predicted sizes of CopL, CopA, and CopB (Figure 5), confirming the specificity of the primers.

In contrast, none of the field isolates from Sargodha yielded amplification products for any of the three target genes (Figure 5). The absence of PCR bands in these isolates indicates that they likely lack the genetic determinants associated with copper resistance. Therefore, the *X. citri* populations in this region appear to remain sensitive to copper-based compounds.

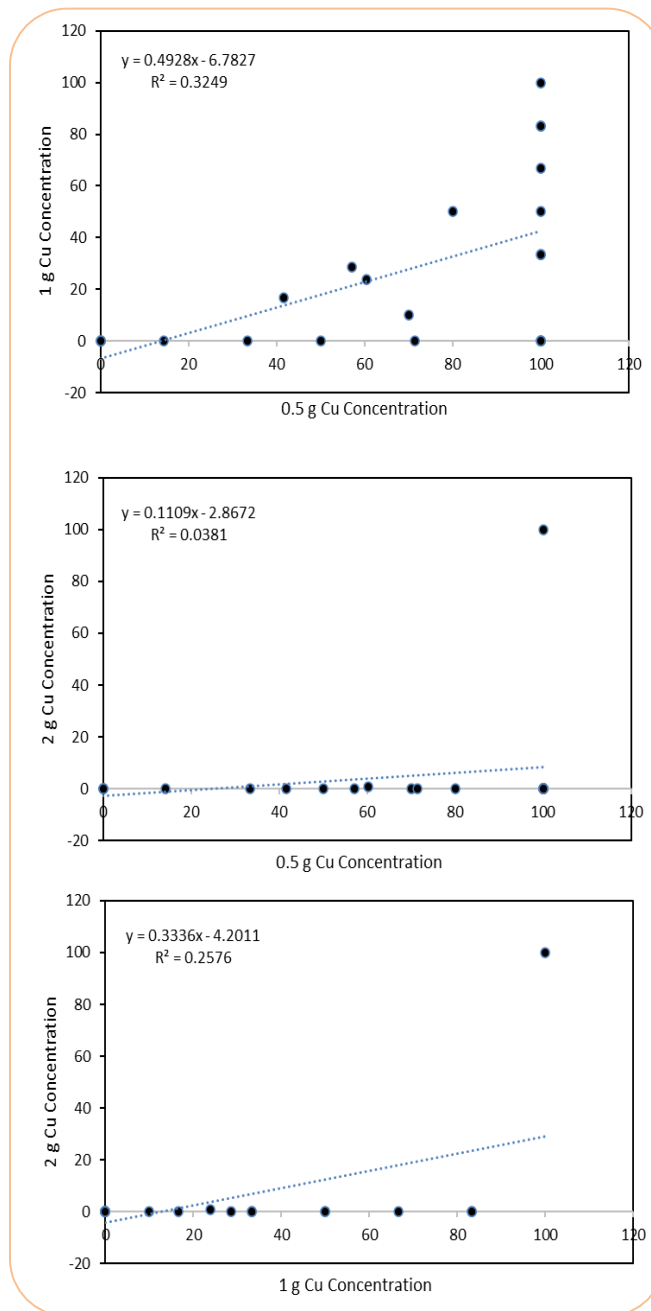


Figure 4. Correlation of the responses of citrus canker isolates (%) to different copper concentrations from Sargodha district over 2020-2021.

Table 7. Recovery (%) of citrus canker isolates on different copper concentrations from various locations in Sargodha district during 2020 and 2021.

Year	Location	Sample size	Proportion of isolates revived (%)			
			0 Cu	0.5 g Cu	1 g Cu	2 g Cu
2020	Chak 36	3	100	100	33	0
	Chak 48	6	100	100	83	0
	Chak 70	3	100	100	33	0
	Chak 8	2	100	100	0	0
	Chak 82	10	100	70	10	0
	Chak 85	6	100	33	0	0
	Chak 88	5	100	0	0	0
	Chak 9	1	100	100	0	0
	Chak 95	4	100	0	0	0
	Choranga	6	100	100	83	0
	Marhe	6	100	100	0	0
2021	Chak-100	4	100	100	50	0
	Chak-15	12	100	42	17	0
	Chak-18	9	100	0	0	0
	Chak-71	6	100	50	0	0
	Chak-81	7	100	71	0	0
	Chak-82	6	100	100	67	0
	Chak-84	10	100	80	50	0
	Chak-85	7	100	14	0	0
	Chak-91	7	100	57	29	0
Reference	Reference strain	1	100	100	100	100
Overall population		121	100	60	24	1

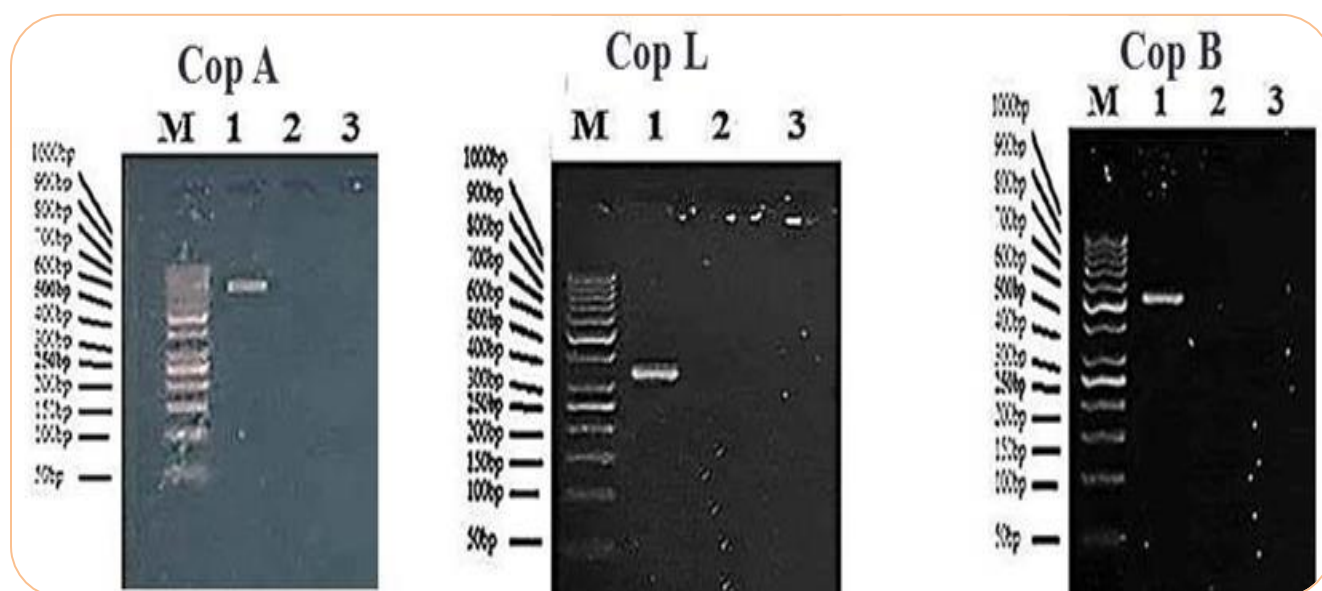


Figure 5. Specific PCR amplification of copper-resistance genes (CopLAB) in *X. citri* isolates from different locations in Sargodha district. Lane M: 50 bp plus DNA ladder; Lane 1: Reference strain LH-201; Lanes 2-3: Test strains; CopA: CopA resistance gene; CopL: CopL resistance gene; CopB: CopB resistance gene.

Discussion

This study investigated the genetic and phenotypic characteristics of *X. citri* strains exhibiting tolerance to copper, providing insights into the molecular mechanisms that support bacterial adaptation under copper stress (Marin et al., 2019). The occurrence of phenotypic tolerance in Pakistani isolates suggests that resistance development may result from the combined effects of selection pressure, genetic variability, and environmental factors (Behlau et al., 2017). Copper-based bactericides remain the primary management strategy for citrus canker worldwide, requiring multiple applications per season (Ibrahim et al., 2016). However, continuous use has led to the emergence of resistant strains in several regions, including Argentina, Reunion, and Martinique (Behlau et al., 2017; Richard et al., 2017). In Pakistan, farmers largely rely on copper-based sprays or tree removal for disease control, highlighting the urgent need for integrated and sustainable management strategies. Novel genome editing tools, such as CRISPR, offer promising avenues for developing resistant citrus varieties (Martin et al., 2020).

Over two years (2020-2021), a total of 3,160 isolates were recovered from 5,490 citrus canker lesions collected across major citrus-growing regions of Pakistan, reflecting a 58% recovery rate. The variation in recovery among years and regions highlights the dynamic nature of disease occurrence and its association with climatic and management conditions (Behlau et al., 2021). The Federal region exhibited a sharp decline in recovery from 100% in 2020 to 50% in 2021, likely due to temporal factors such as rainfall variation, temperature shifts, or changes in orchard hygiene (Marin et al., 2019; Kumar et al., 2020). Similar temporal variability has been reported in Brazil, where bacterial recovery was influenced by climatic fluctuations and pesticide usage (Behlau et al., 2013).

Punjab and Khyber Pakhtunkhwa showed moderate but stable recovery rates (50-67%), indicating relatively steady pathogen populations. Sargodha, recognized as the “citrus capital” of Pakistan, recorded the highest number of isolates, confirming its status as a hotspot for citrus canker. The Himalayan zone exhibited the highest recovery (67-100%), likely due to cool and humid conditions favorable for bacterial survival, whereas the Newly Merged Districts (NMDs) displayed moderate yet consistent recovery (64-67%), possibly reflecting limited exposure to copper sprays (Das, 2003; Dhakal et al., 2009).

The observed variation in copper tolerance among isolates from Sargodha suggests that local environmental and management practices influence the development of resistance. A reduction in tolerance in 2021 compared to 2020 may reflect decreased copper use or environmental changes that reduced selection pressure. Similar trends have been reported in Argentina and Cuba, where frequent copper applications led to higher resistance in *X. citri* populations than in less intensively managed orchards (Behlau et al., 2013). These findings highlight the importance of regional monitoring and adaptive management to delay resistance development.

Although all Sargodha isolates exhibited phenotypic tolerance at lower copper concentrations, PCR analysis confirmed the absence of the *CopA*, *CopB*, and *CopL* genes. This suggests that the observed tolerance is likely due to transient physiological responses, such as efflux pump activation, biofilm formation, or stress-response mechanisms, rather than stable genetic resistance. This finding is noteworthy, as it indicates that Pakistani *X. citri* populations remain in the early stages of resistance development. In contrast, studies from Brazil and Florida have reported plasmid-borne *copLAB* gene clusters responsible for stable copper resistance and bacterial growth under high copper concentrations (Behlau et al., 2012). The absence of these genes in Pakistani isolates presents a valuable opportunity to implement proactive, sustainable management strategies before copper resistance becomes widespread.

Overall, the results indicate that *X. citri* populations in Pakistan remain largely copper-sensitive, although low-level tolerance is emerging. Similar early-stage tolerance has been documented in Brazil and Florida before stable genetic resistance was established (Behlau et al., 2012; Richard et al., 2017). Continued monitoring and judicious copper use are therefore essential to prevent the spread of resistant strains.

The findings have several implications for citrus disease management in Pakistan. First, the moderate recovery rates across provinces highlight the importance of ongoing surveillance to detect shifts in prevalence and resistance trends. Second, while phenotypic tolerance observed at 0.5-1 g CuSO₄ is not genetically fixed, it indicates that reliance solely on copper-based bactericides may promote the emergence of more resistant populations. Consequently, integrated disease management (IDM)

practices should be prioritized. These may include the use of biological control agents such as *Bacillus subtilis* or phage therapy, deployment of resistant citrus cultivars, and cultural practices including pruning, sanitation, and crop rotation. Reducing dependence on copper formulations will not only minimize the risk of resistance but also decrease environmental contamination, a growing concern in intensive citrus production systems. Furthermore, farmer education programs in regions such as Sargodha should emphasize integrated approaches rather than single-strategy reliance.

Conclusions

The isolation and characterization of *X. citri* isolates revealed that, while citrus canker remains widespread across major citrus-growing zones of Pakistan, including Sargodha, the majority of isolates from this district showed only partial tolerance to low copper concentrations and failed to survive at higher doses. No copper resistance genes (*CopA*, *CopB*, *CopL*) were detected. These findings indicate that copper-based bactericides are still effective for controlling citrus canker in the region; however, variability in recovery rates across locations and years emphasizes the importance of continuous monitoring.

Recommendations

It is therefore recommended that integrated disease management practices be implemented, combining judicious copper use with cultural and biological control measures, alongside periodic molecular surveillance to detect early signs of resistance development, thereby ensuring the long-term sustainability of citrus production in Pakistan.

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Authors' Contributions

SA, SU and MA conceived and designed the study. SU and SA collected samples and performed laboratory work, including bacterial culturing, DNA extraction, and PCR. SU, SA, and MA analyzed the data. SU, IM, and SA drafted the manuscript. SA provided funding for the study.

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Conflict of Interest

The authors declare no competing interests.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger

SDG 12: Responsible Consumption and Production

SDG 15: Life on Land

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