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Screening and validation of plant extracts and antagonistic microorganisms against *Xanthomonas* *axonopodis* pv. *citri* in Kagzi lime (*Citrus aurantifolia*)

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Abstract

Citrus canker, caused by *Xanthomonas citri* subsp. *citri*, is a destructive bacterial disease that significantly reduces citrus productivity worldwide. The present study evaluated the antibacterial efficacy of eleven phytoextracts and eleven biological control agents against *X. citri* subsp. *citri* under *in vitro* conditions, followed by validation of the most promising treatments under pot culture conditions. The investigation was conducted during 2022–2023 at the Department of Plant Pathology, College of Agriculture, VNMKV, Parbhani. Among the phytoextracts, garlic (*Allium sativum*) extract exhibited the highest antibacterial activity with a mean inhibition zone of 18.70 mm, followed by eucalyptus (16.30 mm) and onion (14.91 mm), whereas copper oxychloride recorded 20.58 mm. Among the bioagents, *Streptomyces californicus* was the most effective antagonist with a mean inhibition zone of 17.54 mm, followed by *Pseudomonas fluorescens* (14.23 mm) and *Bacillus subtilis* (11.71 mm). Based on laboratory screening, garlic extract and *S. californicus* were selected for greenhouse evaluation. After two foliar sprays, garlic extract recorded the lowest mean PDI (43.18) and highest mean PDC (30.35%), showing efficacy comparable to copper oxychloride (31.37% PDC), while *S. californicus* also significantly reduced disease severity compared with the untreated control. The findings demonstrate that garlic extract is an effective, eco-friendly, and sustainable alternative to conventional bactericides and can be integrated into citrus canker management programs. Further field validation and formulation studies are recommended to facilitate its large-scale adoption.

Keywords: Citrus canker, *Xanthomonas citri* subsp. *citri*, Garlic extract, *Streptomyces californicus*, biological control

1. Introduction

Citrus canker, caused by *Xanthomonas citri* subsp. *citri*, is one of the most destructive bacterial diseases affecting citrus cultivation worldwide. The disease infects almost all commercially important citrus species, with severity varying according to the host genotype, age of plant tissues, and prevailing environmental conditions. Warm temperatures, high humidity, and frequent rainfall favour disease development and spread. The pathogen is believed to have originated in India and Southeast Asia and has since spread to nearly all major citrus-growing regions of the world. Amo citrus species grown in India, acid lime is highly susceptible to citrus canker due to its stomatal architecture, resulting in significant yield losses of 16–23% under severe disease pressure (Naqvi *et al.*, 2022) [5].

Moreover, continuous and indiscriminate use of copper compounds has led to the emergence of copper-resistant bacterial strains, reducing their long-term effectiveness (Sigee, 1993) [8]. These limitations have highlighted the need for safer and more sustainable disease management strategies. Biological control agents and plant-derived phytoextracts have emerged as promising alternatives because they are eco-friendly, biodegradable, and less likely to induce resistance in pathogens. Their ability to suppress bacterial growth while minimizing environmental impacts makes them suitable components of integrated disease management programs for the sustainable control of citrus canker.

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2. Materials and Methods

The experiment was carried out during 2022-2023 at the Department of Plant Pathology, College of Agriculture, Parbhani, VNMKV Parbhani. Pure cultures of biocontrol agents were obtained from the Department of Plant Pathology, College of Agriculture, VNMKV, Parbhani; maintained and multiplied on appropriate culture media and used for further studies. Whereas, the aqueous extracts (10% and 20% concentration) of plant extracts were obtained from the surroundings and gardens of the Department of Plant Pathology, College of Agriculture, VNMKV, Parbhani. Seedlings of Sai Sharbati of acid lime were used for various lab and pot culture experiments from Central Nursery Scheme, VNMKV Parbhani. Pure cultures of biocontrol agents alone were evaluated for their efficacy against the growth of *X. axonopodis* pv. *citri*, by the inhibition zone assay method (Raju *et al.*, 2012) [6]. A heavy suspension (3 days old) of *X. axonopodis* pv. *citri* multiplied in nutrient broth (20 ml) was mixed with lukewarm nutrient agar medium (1000 ml) contained in Erlenmeyer's flask. 15-20ml of NA medium was poured into the sterilised Petri plates and allowed to solidify. Bacterial suspension was uniformly spread over this solidified media using a sterilized cotton swab. A sterilized filter paper disc was dipped in a loopful culture of each of the antagonistic organism and was placed in the centre of Petri plates containing the seeded medium. In case of fungal antagonists, mycelial discs of 5 mm (diameter) size taken from actively growing culture were placed in the centre of the plates. The inoculated plates were then incubated at 30 °C for 72 hours. Observations were recorded for the diameter of the zone of inhibition produced by antagonistic microorganisms around the growth of the pathogen.

Aqueous extracts (20%) of selected plants were evaluated for their efficacy against the growth of *X. axonopodis* pv. *citri*, by the inhibition zone assay method (Raju *et al.*, 2012) [6]. The required plant parts extract was prepared by mixing 20ml of phytoextract in 100ml of water for 20%. A heavy suspension (3 days old) of *X. axonopodis* pv. *citri* multiplied in nutrient broth (20 ml) was mixed with lukewarm nutrient agar medium (1000 ml) contained in Erlenmeyer's flask. 15-20ml of NA medium was poured into the sterilized Petri plates and allowed to solidify. Bacterial suspension was uniformly spread over this solidified media using a spreader or a sterilised cotton swab. A sterilised filter paper disc was dipped in a prepared phytoextracts and was placed in the centre of the Petri plates containing seeded medium. The inoculated plates were then incubated at 30 °C for 72 hours. Observations were recorded for the diameter of the zone of inhibition produced by phytoextracts around the growth of the pathogen.

Those bioagents and phytoextracts found most effective against the *X. axonopodis* pv. *citri* during *in vitro* studies were selected and assessed for integrated management of bacterial canker disease of Kagzi lime. The earthen pots (60 cm dia.) filled with potting mixture of soil: sand: FYM (2:1:1) planted with Kagzi lime seedlings were used. One-year-old seedlings cv. Sai Sarbhati with uniform vigour and a stem diameter of approximately 1 cm was cut back to allow one dominant shoot to develop. Four leaves per plant, as well as four areas per leaf were selected for puncturing (pin prick method). In each area, 20 wounds were made and the upper leaf surfaces immediately sprayed with a mixture of *X. axonopodis* pv. *citri* strains. Inoculated seedlings were immediately covered with plastic bags for 48 hrs and were maintained. The per cent index was recorded before spraying. After that, each of plants was sprayed with 20±5 ml of one of the

treatments. After 14 days, per cent disease index was recorded (Graham and Leite, 2004) [4]. The second spraying was done at 15th day and observations were recorded after 14 days. The experiment was carried out in completely randomized design with 4 treatments and 4 replicates with two spraying. One seedling (4 replicates) was sprayed with copper oxy chloride as positive control. One seedling (4 replicates) was sprayed only with sterile distilled water as negative control. Per cent disease control of each treatment after each spray was also calculated and tabulated.

3. Results and Discussion

1. *In vitro* efficacy of phytoextracts against *X. axonopodis* pv. *citri* (20%)

Antimicrobial activity of total 11 antimicrobial phytoextracts at 20% concentration was evaluated *in vitro* by inhibition zone assay method. The results obtained on inhibition zone of test bacterium are presented.

After the 5th day of incubation, the results (table 1, plate 1) indicated that, the phytoextract tested at 20%, significantly inhibited growth of *X. axonopodis* pv. *citri* over control (untreated). However, mean inhibition zone was ranged from 7.18mm (congress weed) to 18.70mm (garlic). Inhibition zone was significantly highest with garlic (18.70mm), followed by eucalyptus (16.32mm), onion (14.91mm), ginger (13.68mm), neem (13.36mm), turmeric (13.06mm), rui (11.15mm), custard apple (9.94mm), Tulsi (9.70mm), Bougainvillea (8.45mm). Whereas, significantly least inhibition zone was found with parthenium weed (7.18mm). However chemical check (copper oxy chloride) mean inhibition zone was found to be 20.58mm. The superior antibacterial activity of garlic extract is attributed to organosulfur compounds such as allicin, ajoene, and diallyl sulfides, which inhibit bacterial growth by disrupting essential enzymes, protein synthesis, and nucleic acid metabolism (Ankri & Mirelman, 1999; Borlinghaus *et al.*, 2014) [2, 3]. Eucalyptus also exhibited strong antibacterial activity owing to essential oils rich in 1,8-cineole and other phenolic compounds that damage bacterial cell membranes. The antimicrobial effect of onion is mainly associated with thiosulfates and flavonoids, while ginger contains bioactive compounds such as gingerols and shogaols. Neem and turmeric owe their antibacterial activity to azadirachtin, nimbin, and curcumin, respectively, which interfere with microbial growth and metabolism. The comparatively lower activity of the remaining phytoextracts may be due to lower concentrations or reduced effectiveness of their antimicrobial constituents against *Xanthomonas axonopodis* pv. *citri*. Overall, garlic was the most effective botanical extract, indicating its potential as an eco-friendly alternative for managing citrus bacterial canker, although copper oxychloride remained the most effective treatment under *in vitro* conditions.

2. *In vitro* efficacy of bioagents against *X. axonopodis* pv. *citri*

Antibacterial activity of total 11 antagonists were evaluated *in vitro* by inhibition zone assay method. The results obtained on inhibition zone of test bacterium are presented.

After incubation the results (table 2, plate 2) indicated that, the bioagents tested, significantly inhibited growth of *Xanthomonas axonopodis* pv. *citri*, over control (untreated). However, mean inhibition zone was ranged from 5.60mm (*Verticillium lecani*) to 17.54 mm (*Streptomyces californicus*). Inhibition zone was significantly highest with *Streptomyces californicus* (17.54 mm), followed by *Pseudomonas fluorescens* (14.23 mm), *Bacillus subtilis* (11.71 mm), *Paecilomyces lilacinus* (9.42 mm),

Metarhizium anisopliae (8.42 mm), *Trichoderma viride* (8.00 mm), *Trichoderma harzianum* (7.94 mm), *Streptomyces tendre* (7.60), *Pink Pigmented Methylobacterium* (6.81 mm), *Streptomyces coeruleofuscus* (6.15 mm), whereas, significantly least inhibition zone was found with *Verticillium lecani* (5.60 mm). However chemical check (copper oxy chloride) mean inhibition zone was found to be 20.58mm. From the ongoing results it was inferred that the *Streptomyces californicus* was highly efficient in inhibiting the bacterial growth and survival, it may be due to secondary metabolites produced from the actinomycetes which processed antibacterial property. This *in vitro* study demonstrated that all eleven bioagents significantly inhibited *Xanthomonas axonopodis* pv. *citri*, highlighting their potential for citrus canker management. *Streptomyces californicus* exhibited the highest efficacy (17.54 mm inhibition), performing comparably to the commercial standard, copper oxychloride (20.58 mm). The strong antagonism of *Streptomyces* species against phytopathogens is well-documented and primarily attributed to their production of broad-spectrum antibiotics, extracellular lytic enzymes (e.g., chitinases), and siderophores, which disrupt cell wall synthesis and restrict nutrient availability. *Pseudomonas fluorescens* (14.23 mm) and *Bacillus subtilis* (11.71 mm) also demonstrated significant inhibition. Suppression by *P. fluorescens* is likely mediated by antimicrobial compounds (e.g., phenazines, DAPG) and aggressive iron competition via fluorescent siderophores. Similarly, the efficacy of *B. subtilis* is linked to its production of membrane-disrupting lipopeptides, including surfactin, iturin, and fengycin. Conversely, fungal antagonists (*Paecilomyces*, *Metarhizium*, *Trichoderma*, and *Verticillium*) yielded moderate to low inhibition (9.42–5.60 mm). While these organisms are highly successful commercial biocontrols against fungi and insects via mycoparasitism and physical penetration, their bactericidal metabolite production *in vitro* is generally less pronounced than that of bacterial antagonists. Although continuous application of copper-based bactericides provides high inhibition, it leads to environmental toxicity, soil accumulation, and the emergence of copper-resistant *Xanthomonas* strains. Therefore, *S. californicus*, *P. fluorescens*, and *B. subtilis* present highly promising, eco-friendly alternatives. Future *in vivo* studies must validate their field stability, formulation viability, and phyllosphere colonization for integration into practical disease management programs.

Green house disease management (Pot culture)

A total 4 treatments (each in four replication), comprising of bioagent and phytoextract found most effective against the *X. axonopodis* pv. *citri* during present *in vitro* studies were selected and assessed for management of bacterial canker disease of Acid lime (pot culture). The results obtained on per cent disease intensity are presented in Table no.3. Results indicated that the both bioagents and phytoextracts tested (pot culture) against *Xanthomonas axonopodis* pv. *citri* significantly reduced the disease intensity after first spraying over control (untreated). Copper oxy chloride was used as a chemical check and disease intensity after first spray of copper oxy chloride was 45.67. However minimum per cent disease index was recorded with Garlic extract (46.90) and found superior, followed by *Streptomyces californicus* (50.51) which was maximum per cent

disease index. After second spraying disease intensity significantly reduced *Xanthomonas axonopodis* pv. *citri* over control (untreated) (65.00). However, minimum per cent disease index was recorded with Garlic extract (39.47) and was again found superior followed by *Streptomyces californicus* (42.72) which was maximum per cent disease index. Per cent disease index of copper oxy chloride was 39.43. Per cent disease index of garlic extract was at par with per cent disease index of copper oxy chloride. Both bioagents and phytoextract tested (pot culture) against *Xanthomonas axonopodis* pv. *citri* significantly reduced the disease intensity after first and second spraying over control (untreated). Copper oxy chloride was used as a chemical check. However, garlic extract was most effective with lowest PDI mean 43.18. Whereas, maximum mean per cent disease index was recorded with *Streptomyces californicus* (46.61). Percent disease control (PDC) achieved after two spraying in all treatments over respective unsprayed control is presented in Table no.4.16. Similar trend as that of present disease intensity was observed. Copper oxy chloride was used as a chemical check and its control after first and second spray was 22.59% and 39.33% respectively. However maximum disease control was achieved with garlic extract 20.50% and 39.27% after first and second spraying, respectively; whereas minimum disease control was achieved with *Streptomyces californicus*. Mean per cent disease control (PDC) achieved with both the treatments was 30.35% for garlic extract which was highest mean disease control followed by *Streptomyces californicus* 24.82% (lowest mean disease control) and for copper oxy chloride (chemical check) it was found to be 31.37%.

The present findings demonstrated that garlic extract was the most effective botanical treatment in reducing bacterial canker severity under pot culture conditions, producing the lowest mean percent disease index (PDI) and the highest percent disease control (PDC), with efficacy comparable to copper oxychloride after the second spray. The superior performance of garlic may be attributed to organosulfur compounds such as allicin and ajoene, which inhibit bacterial growth by disrupting essential metabolic pathways and enzyme activity (Ankri & Mirelman, 1999; Borlinghaus *et al.*, 2014) [2,3]. Although *Streptomyces californicus* reduced disease severity compared with the untreated control, its efficacy was comparatively lower than garlic extract. This may be because the antagonistic activity of *Streptomyces* depends on successful establishment and metabolite production under plant conditions, which can vary with environmental factors.

The present results are in agreement with those of Sharma and Sharma (2009) [7], who reported that garlic extract, particularly when combined with streptomycin sulfate, effectively suppressed the growth of *Xanthomonas axonopodis* pv. *citri*. Similarly, Tahir *et al.* (2016) [10] observed a significant reduction in citrus canker severity with *Allium sativum* and *Azadirachta indica* extracts under greenhouse conditions. Abdallah *et al.* (2013) [1] also demonstrated the antibacterial potential of actinomycetes against bacterial plant pathogens, while Sugathan *et al.* (2023) [9] highlighted the biocontrol potential of *Streptomyces californicus* through the production of hydrolytic enzymes with antimicrobial activity. These findings support the potential use of garlic extract as an eco-friendly alternative for the integrated management of citrus bacterial canker.

Table 1: *In vitro* efficacy of phytoextracts against *X. axonopodis* pv. *citri* (20%)

Tr. no	Treatments	Botanical names	Mean inhibition zone (mm)*		
			3 rd (DAI)	4 th (DAI)	5 th (DAI)
T ₁	Neem	<i>Azadiracta indica</i>	13.02 (3.74)	13.22 (3.77)	13.37 (3.79)
T ₂	Garlic	<i>Allium sativum</i>	18.33 (4.39)	18.55 (4.42)	18.70 (4.43)
T ₃	Onion	<i>Allium cepa</i>	14.65 (3.95)	14.84 (3.98)	14.91 (3.99)
T ₄	Tulsi	<i>Ocimum basilicum</i>	9.45 (3.23)	9.54 (3.24)	9.76 (3.28)
T ₅	Turmeric	<i>Curcuma longa</i>	13.02 (3.74)	13.04 (3.74)	13.06 (3.75)
T ₆	<i>Bougainvillea</i>	<i>Bougainvillea glabra</i>	8.12 (3.02)	8.40 (3.06)	8.45 (3.07)
T ₇	Rui	<i>Calotropis gigantea</i>	11.01 (3.46)	11.05 (3.47)	11.16 (3.49)
T ₈	Congress weed	<i>Parthenium hysterophorus</i>	7.06 (2.84)	7.15 (2.85)	7.19 (2.86)
T ₉	<i>Eucalyptus</i>	<i>Eucalyptus globulus</i>	16.01 (4.12)	16.30 (4.16)	16.30 (4.16)
T ₁₀	Custard apple	<i>Annona reticulata</i>	9.76 (3.28)	9.91 (3.30)	9.94 (3.30)
T ₁₁	Ginger	<i>Zingiber officinale</i>	13.00 (3.74)	13.37 (3.79)	13.69 (3.83)
T ₁₂	Copper oxy chloride	-----	19.01 (4.47)	19.35 (4.51)	20.58 (4.64)
T ₁₃	Control	-----	0 (1.00)	0 (1.00)	0 (1.00)
		S.E.(m)=	0.001	0.005	0.047
		C.D. (P= 0.01)	0.004	0.016	0.137

*Mean of three replications, (DAI)- days after incubation Figures in parenthesis are square root transformed values

Table 2: *In vitro* efficacy of bioagents against *X. axonopodis* pv. *citri*

Tr. No.	Treatments	Mean inhibition zone (mm)*		
		3 rd (DAI)	4 th (DAI)	5 th (DAI)
T ₁	<i>Trichoderma viride</i>	6.30 (2.70)	7.62 (2.93)	8.00 (3.00)
T ₂	<i>Trichoderma harzianum</i>	6.13 (2.67)	7.14 (2.85)	7.94 (2.99)
T ₃	<i>Paecilomyces lilacinus</i>	8.56 (3.09)	8.92 (3.15)	9.42 (3.22)
T ₄	<i>Metarhizium anisopliae</i>	7.52 (2.91)	8.20 (3.03)	8.48 (3.07)
T ₅	<i>Verticillium lecani</i>	4.08 (2.25)	4.26 (2.29)	5.60 (2.56)
T ₆	<i>Pink pigmented methylobacterium</i>	5.71 (2.59)	6.23 (2.68)	6.81 (2.79)
T ₇	<i>Bacillus subtilis</i>	9.51 (3.24)	10.34 (3.36)	11.71 (3.56)
T ₈	<i>Pseudomonas fluorescens</i>	11.29 (3.50)	13.34 (3.78)	14.23 (3.90)
T ₉	<i>Streptomyces californicus</i>	14.5 (3.94)	16.35 (4.16)	17.54 (4.30)
T ₁₀	<i>Streptomyces coeruleofuscus</i>	4.25 (2.29)	4.37 (2.31)	6.15 (2.67)
T ₁₁	<i>Streptomyces tendae</i>	6.00 (2.64)	6.36 (2.71)	7.60 (2.93)
T ₁₂	Copper oxy chloride	17.95 (4.35)	19.09 (4.53)	20.58 (4.64)
T ₁₃	Control	0 (1.00)	0 (1.00)	0 (1.00)
	S.E. (m)=	0.018	0.010	0.064
	C.D. (P= 0.01)	0.052	0.030	0.187

*Mean of three replications, (DAI)- days after incubation Figures in parenthesis are square root transformed values

Table 3: *In vitro* efficacy of most efficient *in- vitro* bioagents and phytoextracts against bacteria (pot culture)

Tr. no	Treatments	PDI before spraying*	PDI after spray*		Mean PDI	PDC after spray*		Mean PDC
			first	second		first	second	
T ₁	<i>Streptomyces californicus</i>	47.84 (43.75)	50.51 (45.27)	42.72 (40.78)	46.61	14.38	34.27	24.82
T ₂	Garlic	48.90 (44.35)	46.90 (43.20)	39.47 (38.90)	43.18	20.50	39.27	30.35
T ₃	Copper oxy chloride	47.82 (43.734)	45.67 (42.50)	39.43 (38.87)	42.55	22.59	39.33	31.37
T ₄	Control	43.29 (41.12)	59.00 (50.16)	65.00 (53.70)	62	0	0	0
	S.E (m)±	0.385	0.392	0.290				
	C.D. (P= 0.01)	1.199	1.221	0.930				

*Mean of four replications Figures in parenthesis are angular transformed values

PDI: - Percent disease index

**Plate 1:** *In vitro* efficacy of phytoextracts against *X. axonopodis* pv. *citri* (20%)**Plate 2:** *In vitro* efficacy of bioagents against *X. axonopodis* pv. *Citri*



Plate 3: *In-vitro* efficacy of most efficient *in-vitro* bioagents and phytoextracts against bacteria (pot culture).

4. Conclusion

The present investigation was conducted to evaluate the efficacy of selected phytoextracts and antagonistic microorganisms against *Xanthomonas citri* subsp. *citri*, the causal agent of citrus canker, under *in vitro* and pot culture conditions. Among the eleven phytoextracts evaluated, garlic (*Allium sativum*) extract exhibited the highest antibacterial activity, recording a mean inhibition zone of 18.70 mm, followed by eucalyptus (16.30 mm) and onion (14.91 mm), while copper oxychloride produced the maximum inhibition zone (20.58 mm). Among the eleven bioagents tested, *Streptomyces californicus* was the most effective antagonist with a mean inhibition zone of 17.54 mm, followed by *Pseudomonas fluorescens* (14.23 mm) and *Bacillus subtilis* (11.71 mm). Based on the laboratory screening, garlic extract and *Streptomyces californicus* were selected for pot culture evaluation. Both treatments significantly reduced citrus canker severity compared to the untreated control after two sprays. Garlic extract recorded the lowest mean percent disease index (43.18) and the highest mean percent disease control (30.35%), with performance comparable to the chemical check, copper oxychloride (31.37% disease control), whereas *Streptomyces californicus* also reduced disease severity but was comparatively less effective. Overall, the study demonstrated that garlic extract is a highly effective, eco-friendly, and sustainable alternative for the management of citrus canker and has the potential to be incorporated into integrated disease management (IDM) programs, thereby reducing dependence on copper-based bactericides and antibiotics. Further field evaluation and formulation studies are recommended to validate its effectiveness under diverse agro-climatic conditions and facilitate its large-scale adoption in citrus cultivation.

5. Disclaimer (artificial intelligence)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

6. Competing Interests

Authors have declared that no competing interests exist.

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