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Research Article

EVALUATION OF CHEMICAL TREATMENTS FOR MANAGING BACTERIAL LEAF SPOT IN CHILI CAUSED BY *XANTHOMONAS CAMPESTRIS* PV. *VESICATORIA*

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ABSTRACT

Chili, a highly important and valuable crop worldwide, belongs to the *Solanaceae* family. In Pakistan, Sindh is the leading producer of chili. However, bacterial leaf spot (BLS) caused by *Xanthomonas campestris* pv. *vesicatoria* poses a significant threat, leading to yield losses of up to 40% globally. Effective management of BLS has become a critical concern. In the current study, five chemicals, Cabrio Top, Oxyrich, Kocide, Electus Super, and Forum Top, were evaluated for their efficacy in managing BLS. An *in vitro* experiment was conducted using a completely randomized design with three replications per treatment and three concentrations (200, 250, and 300 ppm) of each chemical. The results revealed that Cabrio Top exhibited the largest inhibition zone (18.710 mm), followed by Electus Super (16.730 mm), Forum Top (13.653 mm), and Kocide (11.759 mm), compared to the untreated control. Subsequently, an *in vivo* experiment was designed using the randomized complete block design with three replications and three concentrations (1, 2, and 3 g/L) of the best-performing chemicals. The combined treatment of Cabrio Top + Oxyrich demonstrated the most significant results, showing the lowest disease incidence (23.544%), followed by Oxyrich (31.559%) and Cabrio Top (37.193%). The findings of this study conclude that the combined application of Cabrio Top and Oxyrich is a promising approach for managing BLS in chili under field conditions.

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INTRODUCTION

Chili (*Capsicum annuum* L.) belongs to the Solanaceae family and the *Capsicum* genus (Olaes et al., 2020). It is an excellent source of vitamin C (150-180 mg/100 g) and vitamin A, contributing up to 12% of the total pigment content. Vitamin C (ascorbic acid) has antioxidant properties that help protect against cancer, stroke, cardiovascular diseases, and atherosclerosis (Sharma et al., 2019). Byproducts of chili include chili sauce, chili powder, tomato-chili paste, and chili pickle (Tripathi et al., 2022). The global area under chili cultivation spans 2.06 million hectares, with an annual yield of 37.62 million tons (Saha and Bera, 2019). Pakistan ranks 4th in chili production, with an annual output of 103.7 thousand tons. The total area under chili cultivation in Pakistan is 45.7 thousand hectares, with Sindh province leading in production, followed by Balochistan and Punjab. Chili contributes 1.5% to Pakistan's GDP (Arin, 2019; GOP, 2021).

Chili is affected by various biotic (fungal, viral, nematode, and mycoplasma) and abiotic (temperature, relative humidity, wind speed, and rainfall) stresses (Asghar et al., 2020; Saba et al., 2022; Aslam and Mukhtar, 2023a; Yaseen et al., 2023, 2024). Among the biotic stresses, bacterial leaf spot of chili is a destructive and widespread disease caused by the aerobic, gram-negative bacterium *Xanthomonas campestris* pv. *vesicatoria* (Tahir et al., 2017; Otten and Büttner, 2021). This disease affects chili crops globally, particularly in subtropical and tropical climates. It was first reported in Australia in 1944, initially in tomatoes and later in chili (Roach et al., 2018). The pathogen also affects a variety of other host plants and causes significant yield losses in chili crops (Aziz et al., 2024). Due to favorable weather conditions in chili-growing areas, bacterial leaf spot can lead to yield losses of up to 40% (Aftab et al., 2022). The temperature range of 30-38 °C, relative humidity above 80%, and rain splash are critical epidemiological factors for disease development (Hameed et al., 2022). Characteristic symptoms of bacterial leaf spot in chili include dark brown lesions on the leaves, fruit spots, and canker-like structures on the stem. In less humid conditions, the spots may dry up over time, causing damaged tissues to fall off and resulting in a tattered appearance on the affected leaves (Roach et al., 2018).

Several management practices have been employed to control bacterial leaf spot (BLS) of chili, but the use of resistant varieties remains the most effective method for disease control (Ashfaq et al., 2014; Aslam et al., 2017). The adoption of resistant varieties is particularly appealing, as it poses no environmental risk and allows growers to reduce pesticide use. In some host-pathogen systems, resistance may persist for many years, while in others, it may be temporary (Van Bruggen and Finckh, 2016). Due to the lack of resistant varieties and the sudden onset of the disease, farmers often resort to chemicals, which are considered an effective, quick, and cost-efficient solution for disease management (Iqbal and Mukhtar, 2020). Copper-based bactericides are commonly used to control bacterial diseases, making them a focal point of research (Pereira et al., 2022). The primary objective of this study was to optimize the antibacterial efficacy of chemicals against *X. campestris* pv. *vesicatoria*, the causative agent of BLS in chili.

MATERIALS AND METHODS

Collection of diseased samples

Plant samples showing typical symptoms of leaf spot on chili were collected from various locations, including the Ayub Agricultural Research Institute, Samundri, and Chak No. 281 RB, in district Faisalabad. The samples were placed in brown paper bags (13 × 9.5 cm), properly labeled with the name, date, and collection site, and transported to the Molecular Bacteriology Laboratory, Department of Plant Pathology, University of Agriculture Faisalabad. The samples were stored at 4 °C for further analysis.

Isolation, purification, and identification of *Xanthomonas campestris* pv. *vesicatoria*

The collected chili samples were first washed under running tap water to remove surface debris and dust. Small pieces (approximately 5 mm) were cut from the infected areas, including some healthy portions, using sterilized scissors. The excised tissue pieces were surface-sterilized by immersing them in 1% sodium hypochlorite (NaOCl) solution for 30 sec, followed by three rinses with sterilized distilled water to eliminate residual NaOCl toxicity.

Nutrient agar (NA) medium was prepared and sterilized in an autoclave at 121 °C and 15 psi for 15 min. The sterilized tissue pieces were then placed onto Petri plates containing solidified NA medium using a sterilized inoculating needle. The Petri plates were sealed with wrapping tape, labeled, and incubated at 28 °C for bacterial growth. Bacterial colonies were observed after 24 h of incubation.

The bacteria were purified by isolating a single colony from the initial culture plate using a sterilized cotton swab. The single colony was gently streaked onto a fresh plate containing nutrient media in a zigzag pattern and incubated at 28 °C for 24 h. Pathogen identification was carried out using biochemical tests, including the gram staining technique (Shahbaz et al., 2015; Aslam and Mukhtar, 2023b, 2024).

To prepare the bacterial suspension, a pure culture of *X. campestris* pv. *vesicatoria* was mixed with a drop of sterilized water on a microscope slide. The suspension was stained with crystal violet for 30 sec, rinsed with distilled water, and then treated with ethanol for decolorization. Subsequently, a drop of safranin was added as a counterstain, followed by another rinse with distilled water.

The bacteria were identified based on morphological characteristics such as colony color (creamy yellow), growth pattern (raised and mucoid colonies), and the presence of a clear ring around the colonies (Mincă and Mitrea, 2020). Molecular identification confirmed the pathogen as *X. campestris* pv. *vesicatoria*, with the virulent gene *hrp8* being detected (Otten and Büttner, 2021).

Pathogenicity test

The pathogenicity of the isolated *X. campestris* pv. *vesicatoria* was confirmed by fulfilling Koch's postulates. The pathogen was initially identified based on visual symptoms observed on diseased plants. Samples were collected, and bacteria were isolated from the diseased tissues.

Healthy chili plants of a moderately susceptible variety were grown in pots for the pathogenicity test. A bacterial suspension, adjusted to a concentration of 1×10^6 CFU/ml using a spectrophotometer, was inoculated into the leaves at the seedling stage. Inoculation was performed early in the morning, when stomatal openings were maximal, using syringe infiltration with a 25-gauge needle (Yang et al., 2018; Aziz et al., 2024). Control plants were injected with distilled water.

Symptoms appeared 5-7 days post-inoculation. Re-isolation of the bacteria from the artificially inoculated plants was performed, and the recovered bacteria exhibited identical colony morphology to the original culture. This confirmed the ability of the pathogen to cause disease, fulfilling Koch's postulates. The isolated pathogen was subsequently used for further studies.

Evaluation of chemicals against BLS of chili under field conditions

Five chemicals, namely Cabrio Top, Oxynich, Kocide, Electus Super, and Forum Top, were selected for evaluation against *X. campestris* pv. *vesicatoria* using the inhibition zone technique (Table 1). A stock solution was prepared by dividing the percentage of active ingredients in each chemical by 100 and dissolving the resulting quantity in 100 ml of distilled water. Three concentrations, C1 (200 ppm), C2 (250 ppm), and C3 (300 ppm), were prepared by taking 2, 2.5, and 3 ml of the stock solution, respectively, and diluting each to 100 ml with distilled water.

The inhibition zone technique was employed to assess the efficacy of the chemicals. Fresh bacterial cultures were streaked onto Petri plates containing nutrient agar. Filter paper discs approximately 1 cm in diameter were cut, autoclaved, and soaked in the prepared chemical concentrations using sterilized forceps. The treated discs were then placed at the center of the Petri plates. A control plate, treated only with distilled water, was also maintained.

For each concentration, three replicates along with one control were prepared and incubated at 28 °C. The diameter of the inhibition zones was recorded at 24, 48, and 72 h post-incubation.

Evaluation of chemicals against BLS of chili under field conditions

The field was prepared using a moderately susceptible variety, maintaining row-to-row spacing of 75 cm and plant-to-plant spacing of 45 cm. The most effective chemicals identified during laboratory trials, Cabrio Top and Oxyrich, were further evaluated in the field trial. Three concentrations, 1 g/L, 2 g/L, and 3 g/L, were prepared by dissolving 1 g, 2 g, and 3 g of the respective chemicals in 100 ml of distilled water. These concentrations were applied to plants infected with the pathogen in the field. A control group, inoculated only with distilled water, was maintained for comparison.

Data on disease incidence were recorded under a randomized complete block design at weekly intervals. Disease severity was assessed on a 0-4 scale as per Gambley (2018), where:

0 = No symptoms on inoculated leaves,
1 = 1-10 spots on inoculated leaves,
2 = 11-30 spots on inoculated leaves,
3 = More than 30 spots on inoculated leaves,
4 = Confluent necrotic appearance on inoculated leaves.

Disease incidence was calculated using the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{No. of infected plants}}{\text{Total no. of plants}} \times 100$$

Statistical analysis

Laboratory experiments were conducted using a Completely Randomized Design (CRD), while the field trials were carried out under a Randomized Complete Block Design (RCBD). Mean separation was performed using the Least Significant Difference (LSD) test, as described by Stahle and Wold (1989). The recorded data were analyzed using Analysis of Variance (ANOVA) in Minitab 18.1 software.

Table 1. Chemicals, their active ingredinets and mode of action.

Chemical	Active ingredient	Mode of action	References
Oxyrich (40 % WP)	Copper oxychloride Cymoxanil	Its post-infection activity stops the development of the pathogen during incubation.	Islam et al. (2022)
Kocide (52.4 % WG)	Copper hydroxide	It disrupts the cellular proteins.	Majumdar et al. (2021)
Electus Super (30 % SC)	Azoxystrobin Diphenoconazole	It inhibits mitochondrial respiration.	Kumar et al. (2022)
Cabrio top (60 % WDG)	Pyraclostrobin Metiram	It blocks the energy supply of the pathogen.	Selim and Khalil, (2021)
Forum top (53.0 % WG)	Dimethomorph Metiram	Its mode of action is the inhibition of sterol (ergosterol) synthesis.	Sieiro-Sampedro et al. (2020)

RESULTS

In vitro evaluation of different chemicals against *X. campestris* pv. *vesicatoria*

Among all treatments, Cabrio Top exhibited the largest inhibition zone (20.173 mm), followed by Oxyrich (18.710 mm), Electus Super (16.730 mm), Forum Top (13.653 mm), and Kocide (11.759 mm), compared to the control (Table 2). The interaction between treatments and concentrations (T×C) revealed that Cabrio Top produced the maximum inhibition zones of 17.604 mm, 20.340 mm, and 22.576 mm at 200, 250, and 300 ppm, respectively. This was followed by Oxyrich (16.889 mm, 18.123 mm, 21.117 mm), Electus Super (15.289 mm, 16.778 mm, 18.124 mm), Forum Top (11.350 mm, 13.960 mm, 15.650 mm), and Kocide (9.818 mm, 11.220 mm, 14.237 mm), in comparison to the control (Table 3).

Table 2. Impact of different treatments on growth of *X. campestris* pv. *vesicatoria* under laboratory conditions.

Treatments	Active ingredients	Inhibition zone (mm)
Cabrio top (60% WDG)	Pyraclostrobin Metiram	20.173 a
Oxyrich (40% WP)	Copper oxychloride Cymoxanil	18.710 b
Electus super (30% SC)	Azoxystrobin Diphenoconazole	16.730 c
Forum top (53.0% WG)	Dimethomorph Metiram	13.653 d
Kocide (52.4% WG)	Copper hydroxide	11.759 e
Control (Distilled water)	Distilled water	0.0000 f
LSD	0.3533	

Interpretation: As determined by pairwise comparison LSD tests (P≤ 0.05), mean values in a column sharing same letters reflect non-significant difference.

Table 3. Impact of interaction between treatments and concentrations on inhibition zone of *X. campestris* pv. *vesicatoria* under laboratory conditions.

Treatments	Inhibition zone (mm)		
	Concentrations		
	200 ppm	250 ppm	300 ppm
Cabrio Top	17.604 cd	20.340 b	22.57 6a
Oxyrich	16.889 d	18.123 c	21.117 b
Electus super	15.289 fg	16.778 de	18.12 4 c
Forum top	11.350 i	13.960 h	15.650 ef
Kocide	9.818 j	11.220 i	14.237 gh
Control (Distilled water)	0.0000 k	0.0000 k	0.0000 k
LSD		0.6118	

Interpretation: As determined by pairwise comparison LSD tests (P≤ 0.05), mean values in a column sharing same letters reflect non-significant difference.

The interaction between treatments and time (T×D) showed that Cabrio Top also achieved the highest inhibition zones of 20.113 mm, 18.580 mm, and 21.827 mm after 24, 48, and 72 h, respectively. It was followed by Oxyrich (18.638 mm, 17.073 mm, 20.418 mm), Electus Super (16.629 mm, 15.057 mm, 18.506 mm), Forum Top (13.457 mm, 12.338 mm, 15.166 mm), and Kocide (11.719 mm, 10.226 mm, 13.331 mm), compared to the control (Table 4).

Table 4. Impact of interaction between treatments and days on inhibition zone of *X. campestris* pv. *vesicatoria* under laboratory conditions.

Treatment	Inhibition zone (mm)		
	Duration (D)		
	24 h	48 h	72 h
Cabrio Top	20.113 b	18.580 c	21.827 a
Oxyrich	18.638 c	17.073 d	20.418 b
Electus super	16.629 d	15.057 e	18.506 c
Forum top	13.457 f	12.338 fg	15.166 e
Kocide	11.719 g	10.226 h	13.331f
Control (Distilled water)	0.0000 i	0.0000 i	0.0000 i
LSD		0.6118	

Interpretation: As determined by pairwise comparison LSD tests (P≤ 0.05), mean values in a column sharing same letters reflect non-significant difference.

In vivo evaluation of different chemicals against BLS of chili

The combination of both chemicals, Cabrio Top and Oxyrich, resulted in the lowest disease incidence (23.544%), followed by Oxyrich (31.559%) and Cabrio Top (37.193%), compared to the control (58.011%), as shown in Table 5. The interaction between treatments and concentration (T×C) revealed that the combination of both chemicals exhibited the lowest disease incidence at concentrations of 1 g/L (34.222%), 2 g/L (20.211%), and 3 g/L (16.200%), followed by Oxyrich (35.400%, 33.511%, 25.767%) and Cabrio Top (43.622%, 37.333%, 30.622%), in comparison to the control, as presented in Table 6. The interaction between treatments and days (T×D) demonstrated that the lowest disease incidence was recorded with the combination of both chemicals (30.289%, 22.367%, 17.978%) after one-week intervals, followed by Oxyrich (42.733%, 31.511%, 20.433%) and Cabrio Top (46.067%, 37.078%, 28.433%), compared to the control, as shown in Table 7.

Table 5. Impact of different treatments on disease incidence of BLS of chili.

Treatment	Disease incidence (%)
Cabrio top	37.193 b
Oxyrich	31.559 c
Cabrio top + Oxyrich	23.544 d
Control (Water)	58.011 a
LSD	2.0772

Interpretation: As determined by pairwise comparison LSD tests (P≤ 0.05), mean values in a column sharing same letters reflect non-significant difference.

Table 6. Impact of interaction between treatments and concentrations on disease incidence of BLS of chili under field conditions.

Treatment	Disease incidence (%)		
	Concentrations		
	1 g/ L	2 g/ L	3 g/ L
Cabrio top	43.622 b	37.333 bc	30.622 cd
Oxyrich	35.400 c	33.511 c	25.767 de
Cabrio top + Oxyrich	34.222 c	20.2111 ef	16.200 f
Control (Water)	57.318 a	57.483 a	59.231 a
LSD		3.5979	

Interpretation: As determined by pairwise comparison LSD tests (P≤ 0.05), mean values in a column sharing same letters reflect non-significant difference.

Table 7. Impact of interaction between treatments and days on disease incidence of BLS of chili.

Treatment	Disease incidence (%)		
	Weeks (W)		
	1 week	2 weeks	3 weeks
Cabrio top	46.067 b	37.078 cd	28.433 ef
Oxyrich	42.733 bc	31.511 de	20.433 g
Cabrio top + Oxyrich	30.289 de	22.367 fg	17.978 g
Control (Water)	55.408 a	58.282 a	60.342 a
LSD		3.5979	

Interpretation: As determined by pairwise comparison LSD tests (P≤ 0.05), mean values in a column sharing same letters reflect non-significant difference.

DISCUSSION

Chili is an important perennial, self-pollinated crop belonging to the family *Solanaceae* (Singh et al., 2021). Its production is influenced by various biotic and abiotic factors (Saeed et al., 2023; Saeed and Mukhtar, 2024). Among the most significant biotic stresses affecting chili cultivation is bacterial leaf spot, caused by *Xanthomonas campestris* pv. *vesicatoria* (Roach et al., 2020).

To manage this disease, various strategies have been employed, with chemical control being one of the simplest and most effective approaches. In the present study, different chemical formulations, Cabrio Top (60%), Oxyrich (40%), Electus Super (30%), Forum Top (53%), and Kocide (52.4%), were evaluated for their efficacy against *X. campestris* pv. *vesicatoria* under laboratory conditions. Among these, Cabrio Top demonstrated the largest inhibition zone (20.173 mm), indicating the highest effectiveness against the bacterial leaf spot pathogen under laboratory conditions.

The two most effective chemicals from the laboratory trials, Cabrio Top and Oxyrich, were subsequently tested in a field trial. The combined application of these chemicals resulted in the lowest disease incidence (23.544%), highlighting their potential as an integrated management strategy for bacterial leaf spot in chili.

The results of the ongoing study are supported by the findings of Gambley (2018), who evaluated the combined effect of cymoxanil and famoxadone with mancozeb and copper hydroxide, demonstrating enhanced resistance against pathogens. Similarly, the findings align with those of Hassan and Zyton (2017), who reported that Roxil was the most effective treatment for reducing the severity of bacterial leaf spot in peppers.

Furthermore, the present results are corroborated by Khanal (2020), who concluded that spraying a copper-mancozeb mixture effectively controlled the bacterium *X. campestris* pv. *vesicatoria*. The outcomes of this research are also consistent with Rekanović et al. (2019), who demonstrated the effectiveness of a conventional copper-based bactericide combined with acibenzolar-S-methyl (ASM) for managing bacterial spot in peppers.

Furthermore, the conclusions of the study are supported by Poudel and Neupane (2018), who found that the highest yields were achieved with a combination of Agrimycin and Blitox, followed by Blitox alone. Rajwade et al. (2022) also contribute to these findings, showing that while pyraclostrobin alone did not suppress the *in vitro* growth of *X. vesicatoria*, its combination with streptomycin significantly reduced disease severity.

The present findings are further substantiated by Abrahamian et al. (2019), who tested various application programs involving famoxadone, cymoxanil, and ASM in combination with copper hydroxide and mancozeb for managing bacterial spot. Similarly, Šević et al. (2019) evaluated the efficacy of new formulations of copper compounds against *X. campestris* pv. *vesicatoria* under field conditions using artificial inoculation. Their study assessed the performance of Blauvit (copper-hydroxide), Cuprozin (copper-oxychloride), Fungohem (copper-hydroxide), and Cuproxat (copper-sulphate), demonstrating their effectiveness.

Finally, the findings are consistent with those of Gambley (2018), who investigated the use of the compound 2-aminoimidazole (2AI) for controlling bacterial leaf spot. The compound was shown to inhibit and disrupt bacterial biofilms effectively.

Cabrio Top contains pyraclostrobin and metiram, which disrupt the energy supply of pathogens, preventing their further spread within the plant (Younas et al., 2021). Pyraclostrobin penetrates rapidly into leaf tissues and accumulates in the waxy layer, providing prolonged protection (Roylawar et al., 2021). It inhibits mitochondrial electron transport, thereby blocking the energy supply to the pathogen, ultimately causing its death. In contrast, metiram acts as a contact and protective fungicide with a prophylactic effect (Monteiro et al., 2019; Sharma et al., 2020). Moreover, pyraclostrobin degrades into natural precursors of indole-3-acetic acid (IAA) and increases endogenous abscisic acid (ABA) levels, enhancing the plant's tolerance to stress (Tooley, 2019).

Oxyrich was the second most effective chemical evaluated against *X. campestris* pv. *vesicatoria*. Its post-infection activity halts pathogen development during the incubation period (Islam et al., 2022). Oxyrich contains copper oxychloride and cymoxanil as active ingredients. Copper oxychloride, due to its strong bonding affinity with carboxyl groups and amino acids, reacts with proteins and acts as an enzyme inhibitor in target pathogens (Sharma and Gaur, 2021). Cymoxanil interferes with the synthesis of amino acids, nucleic acids, and other cellular processes essential for the lifecycle of the pathogen. It penetrates quickly into plant tissues and remains effective even after rainfall. Cymoxanil also controls diseases during the incubation period, preventing the onset of visible damage to crops (Bhaik et al., 2022).

The current investigation demonstrated the effectiveness of chemical control, corroborating findings from previous research. However, it is crucial to recognize its limitations for ensuring sustainability in the future. Although chemical control strategies are initially effective, they may lead to the development of resistance in pathogens, diminishing their long-term efficacy. Moreover, the accumulation of chemical residues in the environment can harm beneficial organisms and disrupt ecological balance. These limitations underscore the importance of exploring alternative approaches, such as integrated disease management and the development of resilient disease control strategies.

CONCLUSION

Under controlled *in vitro* conditions, Cabrio Top exhibited the highest inhibition zone against the pathogen. In field trials, the combination of Cabrio Top and Oxyrich resulted in the lowest disease incidence, followed by the individual applications of Oxyrich and Cabrio Top. Based on these findings, the combined application of Cabrio Top and Oxyrich is strongly recommended for farmers as an effective strategy for managing bacterial leaf spot in chili crops.

AUTHORS' CONTRIBUTIONS

TF conducted experiments; MA designed and formulated the study; LA, MMJ, and JS helped in conducting the experiments; AN and MJM collected, arranged and analyzed the data; AS, and NRK provided technical assistance; AI and HR addressed the comments of the reviewers.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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