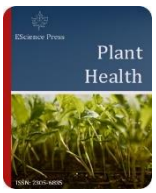
Plant Health 02 (01) 2023. 17-26 DOI: 10.33687/planthealth.02.01.5075



Available Online at EScience Press
Plant Health
ISSN: 2305-6835
https://esciencepress.net/journals/planthealth

Determining the Efficacy of Plant Extracts and Chemicals against Bacterial Leaf Spot of Spinach

[a]Shahbaz Hussain, [a]Muhammad Sajid*, [a]Yasir Mehmood, [b]Muhammad Sheeraz, [c]Muhammad Irfan, [c]Shabir Hussain, [a]Shahid Ameer, [d]Muhammad Mehtab, [a]Tatheer Abbas
[a] Department of Plant Pathology, Bahauddin Zakariya University, Multan, Pakistan.
[b] Department of Agri. Business and Marketing, Bahauddin Zakariya University, Multan, Pakistan.
[c] Department of Agronomy, Bahauddin Zakariya University, Multan, Pakistan.
[d] Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan.

ARTICLE INFO

Article History
Received: June 01, 2023
Revised: July 13, 2023
Accepted: July 30, 2023

Keywords
Bacterial diseases
Spinacia oleracea L.
Pseudomonas syringae
Inhibition zone technique
Disease severity

ABSTRACT

Bacterial leaf spot of spinach is an emerging problem causing significant losses worldwide, particularly in Pakistan. The current study was subjected to mitigate this arising issue by the exploitation of four chemicals and four plant extracts *in vitro* and in green house at three different concentrations. The experiments were performed under CRD through inhibition zone technique and foliar spray method. Laboratory experiments concluded aqueous extract of *Acacia nilotica* with maximum inhibition zone (10.4 mm) followed by *Calotropis gigantea* (5.0 mm), *Citrullus colocynthis* (3.6 mm), and *Moringa oleifera* (1.6 mm) respectively as compared to control (0.00 mm). *In vitro* results of chemicals showed Streptomycin sulphate (13.9 mm) with highest inhibition zone followed by Oxytetracycline (10.2 mm), Kasumin (4.7 mm), and Copper oxychloride (3.2 mm) respectively. Most effective Phytoextracts and chemicals observed *in vitro* were selected for greenhouse evaluation. In greenhouse results Streptomycin sulphate (5.2%) showed as most significant antibacterial agent in managing the disease severity followed by *Acacia nilotica* (10.1%) as compared to control. The current findings would be the valuable insights for researchers and the farming community, assisting in the improved management of bacterial leaf spot of spinach.

Corresponding Author: Muhammad Sajid
Email: sajid1694@bzu.edu.pk

INTRODUCTION

Spinach (*Spinacia oleracea L.*) is a nutrient rich vegetable crop with global production of 32.3 million tons (FAO, 2021). It is a rich source of carotenoids, flavonoids, polyphenols, vitamins (K, E, C and A), oxalic acid, folic acid and mineral elements (Ca, Zn, Mg, P, K, Mn) (Bunea *et al*., 2008; Lasya, 2022). The production of spinach is under several biotic and abiotic factors (Foss and Jones, 2000; Machado and Serralheiro, 2017). Among biotic factors, Fusarium wilt, Downy mildew, Leaf spot complex and damping off are ravaging issues to successful production of spinach (Foss and Jones, 2000). Recently, Bacterial leaf spot of spinach caused by *Pseudomonas syringae* pv. *spinaciae* is found as an emerging problem in Pakistan. This bacterium has been found notorious for 70% crop losses worldwide (Zhao *et al*., 2000). Various management practices including soil fumigation, crop rotations, tillage practices, intercropping, soil disinfestation, solarization, soil steaming, biofumigation and sanitation practices have been evaluated to mitigate these losses (Datnoff *et al*., 2007; Hasna *et al*., 2007; van Bruggen *et al*., 2016; Yogev *et al*., 2009). All of these practices are found somehow successful but have some limitations in the effective

management of Bacterial leaf spot of spinach (Gullino *et al*., 2020). From the last decade, scientists are found involved in utilizing the biological disease management strategies. The concept of biological management encompasses the utilization of microorganisms, as well as their extracts or byproducts derived from animals or plants, for the purpose of mitigating diseases of plants (Johnson, 2010). Plant extracts are extensively acknowledged and consist of diverse phytochemical components (Alrefaee *et al*., 2021). A range of different of botanical species can be employed to obtain organic extracts which are considered as strong antimicrobial agents (Godlewska *et al*., 2021). Various studies found plant extracts as eco-friendly and alternatives of hazardous chemicals to manage plant diseases (Al-Snafi, 2015; Altemimi *et al*., 2017). Based on trends in scientific research, aqueous extracts of *Acacia nilotica, Citrullus colocynthis, Calotropis gigantea* and *Moringa oleifera* were evaluated against *Pseudomonas syringae* pv. *spinaciae* in this study.

Keeping in view of sustainable agriculture and green revolution, farmers and scientists always prioritize the environmentally safe products to manage their crop losses. But in severe cases of disease, farmer is compelled to use chemicals for quick control of dispersing pathogen and to overcome the severe crop losses (Sundin *et al*., 2016). That is why; Streptomycin sulphate, Oxytetracycline, Kasugamycin and Copper sulphate were included in current study.

MATERIALS AND METHODS

Pathogen Identification

During the year 2022-2023, infected samples of spinach leaves were collected Khanewal and Multan District, Punjab, Pakistan. The diseased samples were brought to Phytobacteriology and Biochemical Analysis Lab department of Plant Pathology at Bahauddin Zakariya University, Multan. Nutrient Agar (NA) media was used to isolate *Pseudomonas syringae* pv. *spinaciae* from infected spinach leaves. A screwcap glass bottle was utilized for preparation and thorough mixing of all these ingredients (Sodium chloride 5g, Agar 15g, Peptone 5g, Beef extract 3g, Distilled water 1L). Media was autoclaved for 20 mints at 121^{0}C with 15 psi pressure for sterilization of media. The diseased samples were cut into small pieces precisely with scissors and dipped in distilled water to remove the dust and other impurities. The samples were submerged in 1 percent sodium hypochlorite for disinfection from saprophyte after washing with distilled water the samples were placed on sterilized tissue paper for drying. After pouring and solidification, the infected samples were placed carefully on the media. The plate was sealed with paraffin and was incubated at 27-28^{0}C for 24-72 hours. A minute quantity of bacterial colony was picked with O-loop and was put on the glass slide and examined at 100X lens of compound microscope. Bacteria were straight rods and were motile by means of flagella as described by OZAKI *et al*. (1998).

Pathogenicity Test

The pathogenicity test was carried out on Prickly Seed variety of spinach under greenhouse. After seed germination, leaves were sprayed with distilled water to increase humidity. Inoculum was prepared with serial dilution technique and concentration of 1×10^6 CFU was prepared for inoculation. The pathogen was inoculated directly into the host plant with the help of 10CC syringe and plants were covered with plastic sheets. When symptoms appeared, the leaves were brought to lab for isolation of pathogen.

Biochemical Identification

The biochemical characterization of pathogen was done by Gram Staining, Catalase test, Oxidase test, Starch-hydrolysis test, KOH test, gelatin liquefaction test and Methyl-red test.

In-vitro Evaluation of Plant Extracts

Four different extracts of *Acacia nilotica* (Keeker), *Calotropis procera* (Ak Plant), *Moringa oleifera* (Moringa Plant) and fruit of *Citrullus colocynthis* (Kor Tumma) were obtained for their antibacterial properties. The leaves of these different plants were washed with tap water and dried in shade for two weeks. In case of *C. colocynthis*, the fruits were cut into half and shade dried for 3 weeks. The dried leaves and fruits of plants were grinded into fine powder. The equal weights of powder and distilled water were mixed and placed in incubator shaker at 150 rpm for 24 hours. Then, filtrate of aqueous extracts was obtained through muslin cloth. and Whatman filter paper 44 after 6 hours. Three concentrations 25, 35 and 45% were prepared by adding 25, 35 and 45 mL of stock plant extracts in 75, 65, and 55 mL of distilled water, respectively. In-vitro evaluation of extracts was done by inhibition zone technique. Two methods for the evaluation of plant extracts namely disc diffusion method and well diffusion method were used. In first method, 5 mm discs of Whatman filter paper

No.44 was prepared. NA media was poured in petri plates and on solidification, media was streaked thoroughly with *P. syringae* pv. *spinaciae* by using sterilized streaking loop. After streaking, discs were submerged in plant extracts and placed carefully in the middle of petri plate in order to avoid errors. In second method, after solidification of media, the plate was streaked thoroughly with bacteria and a well was made in the center with the help of cork borer. The extract was poured in well with the help of 1000 µl pipette, allowed to settle down and get absorbed by the media. Then, the plates were wrapped with paraffin tape, incubated at 27±1^{0}C for 24-72 hours and after 72 hours zones were measured with the help of measuring tape. Experiment was carried out in accordance to Complete Randomized Design (CRD) with 3 replications of each treatment in order to mitigate error.

***In-vitro* Evaluation of Different Chemicals**

Four different chemicals including Copper oxychloride, Kasumin, Streptomycin sulphate and oxytetracycline were assessed against *P. syringae* pv. *spinaciae* under Complete Randomized Design (CRD) by using inhibition zone technique (Disc diffusion and Well diffusion methods). Three concentrations 1, 2 and 3% were prepared by adding 1, 2 and 3 mL of stock chemicals in 99, 98, and 97 mL of distilled water, respectively. In Disc diffusion method, 5 mm discs of Whatman filter paper No.44 were prepared. NA media was poured in petri plates and on solidification, media was streaked thoroughly with *P. syringae* pv. *spinaciae* by using sterilized streaking loop. After streaking, discs were submerged in chemicals and placed carefully in the middle of petri plate in order to avoid errors. In Well diffusion method, after solidification of media, the plate was streaked thoroughly with pathogen and a well was made in the center with the help of cork borer. The extract was poured in well with the help of 1000 µl pipette, allowed to settle down and get absorbed by the media. Then, the plates were wrapped with paraffin tape, incubated at 27±1^{0}C for 24-72 hours and after 72 hours, plates were observed for inhibition zone.

Greenhouse Experiments

Most significant plant extract and chemical with most effective concentration observed in laboratory circumstances were exploited in greenhouse under CRD design. Spinach seeds of "Prickly Seed" variety were collected from Vegetable Research Institute, Faisalabad. Spinach seeds were soaked for 2 minutes in 1 percent sodium hypochlorite for disinfection and later, seeds were soaked in distilled water to remove the remnants of disinfectant. Seeds were sown in pots containing sterilized soil to avoid soil-borne pathogens and pots were labelled. A concentration of 1×10^6 CFU was prepared by 24 hours old colony of *Pseudomonas syringae* pv. *spinaciae* through serial dilution technique.

Table 1. Table showing the different treatments under greenhouse conditions.

Treatments	Plant response
T1	Positive Control (Application of distilled water only)
T2	Application of Chemical on inoculated plants
T3	Application of Plant extract on inoculated plants
T4	Negative Control (Inoculated plants)

After germination and growth of 8 weeks, treatments were applied. In positive control, spinach plants were kept un-inoculated and irrigated with distilled water only. In treatment 2 (T2), distilled water was sprayed on the plants to wash off the dust and impurities. The plants were inoculated with *P. syringae* pv. *spinaciae* by help of syringe and covered with plastic sheet to maintain humidity. After 4 days of inoculation, 50 ml foliar spray of 45% aqueous extract of *A. nilotica* was applied on these T2 plants. In treatment 3 (T3), inoculation was done through syringe method same as in T2 plants. Distilled water was sprayed on the plants to wash off the contamination. Plants were inoculated with *P. syringae* pv. *spinaciae* and covered with plastic sheet to maintain humidity. 50 ml foliar spray of 3% aqueous solution of streptomycin sulphate was applied on T3 plants after 4 days of inoculation. In case of treatment 4 (T4), plants were only inoculated with *P. syringae* pv. *spinaciae*. All the experimental units were irrigated on regular basis and after 3 weeks, disease severity was measured by following the method given by Mengist *et al*. (2019).

Table 2. Table showing the disease rating scale for measuring the disease severity.

Disease grade	Description	Percentage infection
0	No spots on leaves	0
1	One spot	<1
3	Few spots on few leaves	1-10
5	Few spots on many leaves	11-25
7	Many large leaf spots	26-50
9	Mostly large leaf spots	>51

$$DS\ (\%) = \frac{\text{Sum of all numerical disease ratings}}{\text{Total number of diseased leaves} \times \text{Maximum disease rating}} \times 100$$

Statistical Analysis

The recorded data was analyzed statistically using statistics 8.1 and means were separated through LSD at α = 0.05.

RESULTS

Pathogenicity Test

The results of pathogenicity test demonstrated that upon inoculation of pathogenic bacterium, spinach leaves produced same symptoms of the formation of small water-soaked spots, which then transitioned into brown to light brown circular spots, the lesions exhibited yellow haloes on their lower surface, lesions were merged together resulting in the formation of large and asymmetrical blotches which exhibited a small protrusion on the upper surface. Bacteria were reisolated on nutrient agar media from artificially infected spinach leaves and were purified which showed the identical physical characteristic features as of the original isolate. The colonies observed on nutrient agar plates exhibited characteristics consistent with a white color, convex shape and translucent appearance displaying smooth surfaces.

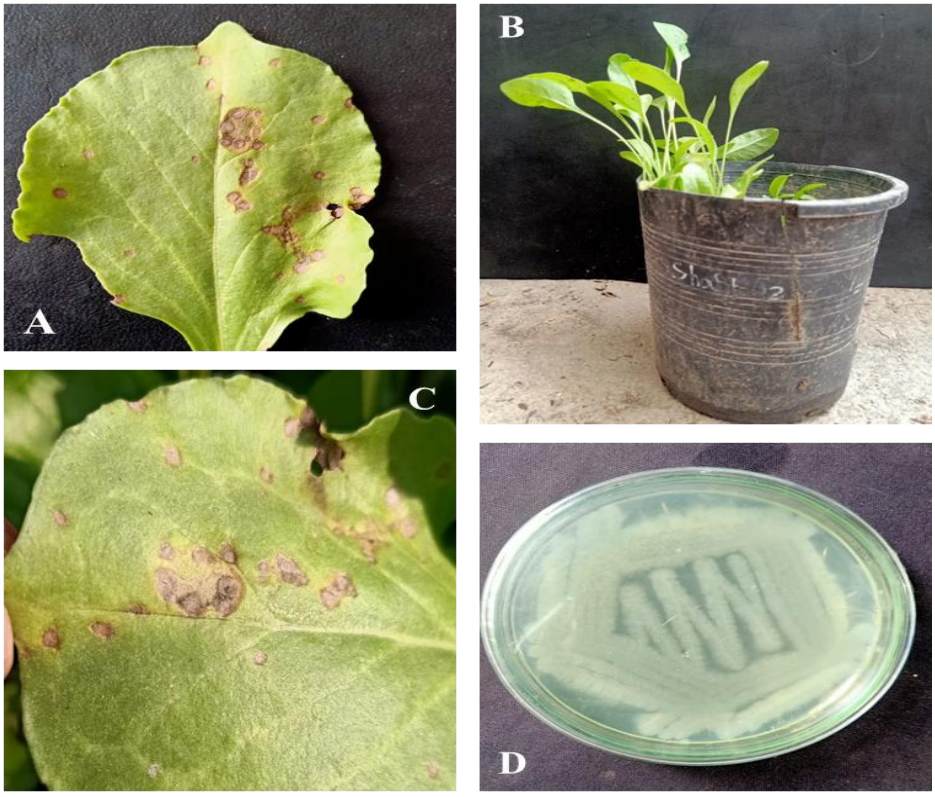


Figure 1. Pathogenicity test results after the application of inoculum. (A) Infected leaf sample, (B) Healthy spinach plants, (C) Diseased leaf of spinach after inoculation and (D) *Pseudomonas syringae* pv. *spinaciae*, pure culture grown on NA media.

Biochemical Characterization

Gram staining

The specimen on our slide was unable to retain the color of crystal violet. When slide was stained with the counterstain safranin, the bacterium was observed pink under microscope.

Starch-hydrolysis test

After incubating at 27-28°C for 7 days, when lugol's iodine was flooded over nutrient starch agar medium plates, a distinct yellow-golden zone was observable surrounding bacterial colonies which indicated that starch was hydrolyzed by the bacterium and isolates of P. syringae pv. spinaciae were tested positive.

Catalase test

When the hydrogen peroxide (H_2O_2) was allowed to interact with the catalase enzyme produced by bacteria, catalase oxidized the H_2O_2 by breaking it into water molecule releasing oxygen gas. The emission of oxygen gas resulted in the formation of bubbles and this formation of bubbles resulted in a positive catalase test which indicated that the bacteria under observation was gram-negative.

Oxidase test

The bacterial isolates gave negative results which proved that the bacteria being examined lack oxidase enzyme. No isolate changed their color from white to purple within one minute.

KOH test

When a drop of 3 percent KOH was added to inoculum on the slide with gentle mixing and the loop was lifted upward along with the inoculum, a stringy viscous appearance was noted. This appearance proved that the bacteria under observation were gram-negative.

Gelatin liquefaction test

In this test, *P. syringae* pv. *spinaciae* changed the solidified media into liquid by hydrolysis of gelatin. The gelatin medium began to flow when the test tubes were kept in a slightly slanting position. The isolate was able to liquefy the gelatin media when they were compared to control.

Methyl-red test

P. syringae pv. *spinaciae* were grown in a broth medium for methyl red test. 5 drops of methyl red indicator were put into the test tubes containing bacteria after 2 to 5 days of incubation at 27 to 28°C. A positive result was shown when the color of methyl red shifted from yellow to red.

In-vitro* Evaluation of Plant Extracts against *P. syringae* pv *spinaciae

In-vitro analysis of plant extracts by disc diffusion method concluded *Acacia nilotica* as the most significant antibacterial agent against *P. syringae* pv *spinaciae* followed by *Calotropis gigantea, Citrullus colocynthis*, and *Moringa oleifera* at 45% concentration as compared to control (Figure 2, Table 3).

Table 3. In-vitro evaluation of plant extracts.

Treatments	Mean Inhibition Zone (mm)
Acacia nilotica	10.4
Calotropis gigantea	5.0
Citrullus colocynthis	3.6
Moringa oleifera	1.6
Control	0.0

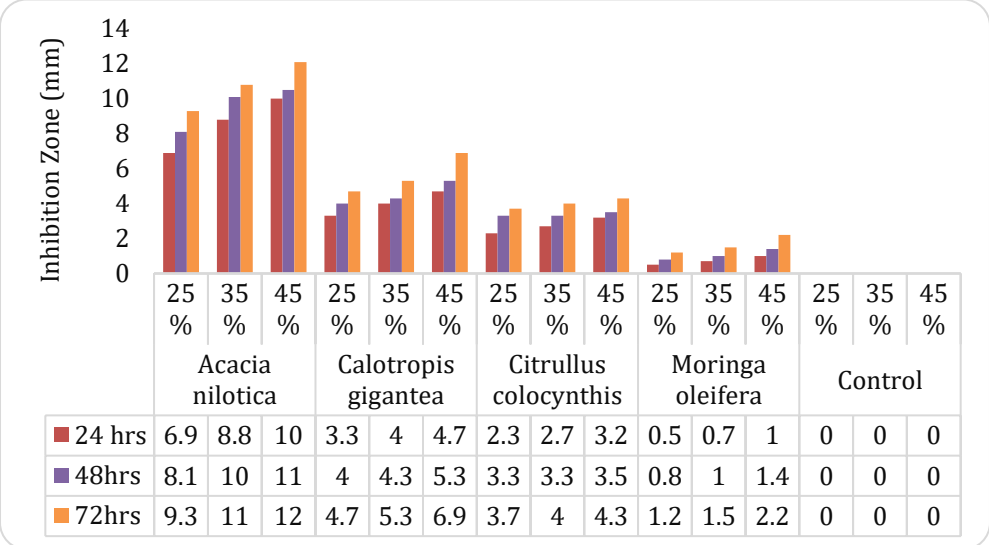
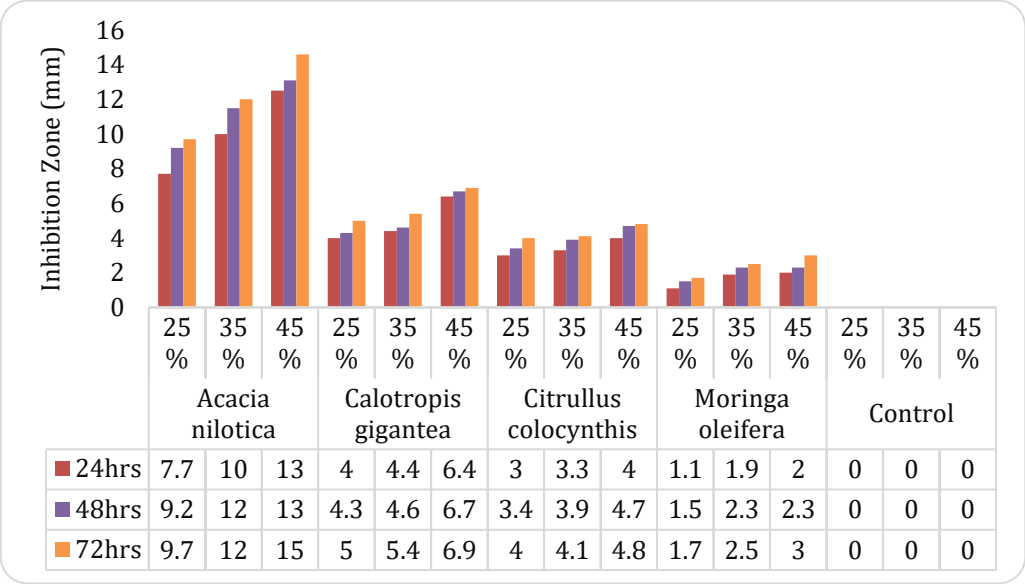


Figure 2. Results of disc diffusion technique for plant extracts.

In vitro evaluation of plant extracts through well diffusion technique represented that *Acacia nilotica* is most effective antibacterial agent against *P. syringae* pv *spinaciae* followed by *Calotropis gigantea, Citrullus colocynthis*, and *Moringa oleifera* at 45% concentration as compared to control (Figure 3, Table.1). It was observed that the results of inhibition zone (mm) in the well diffusion technique were more significant as compared to disc diffusion technique (Figure 2 and 3).

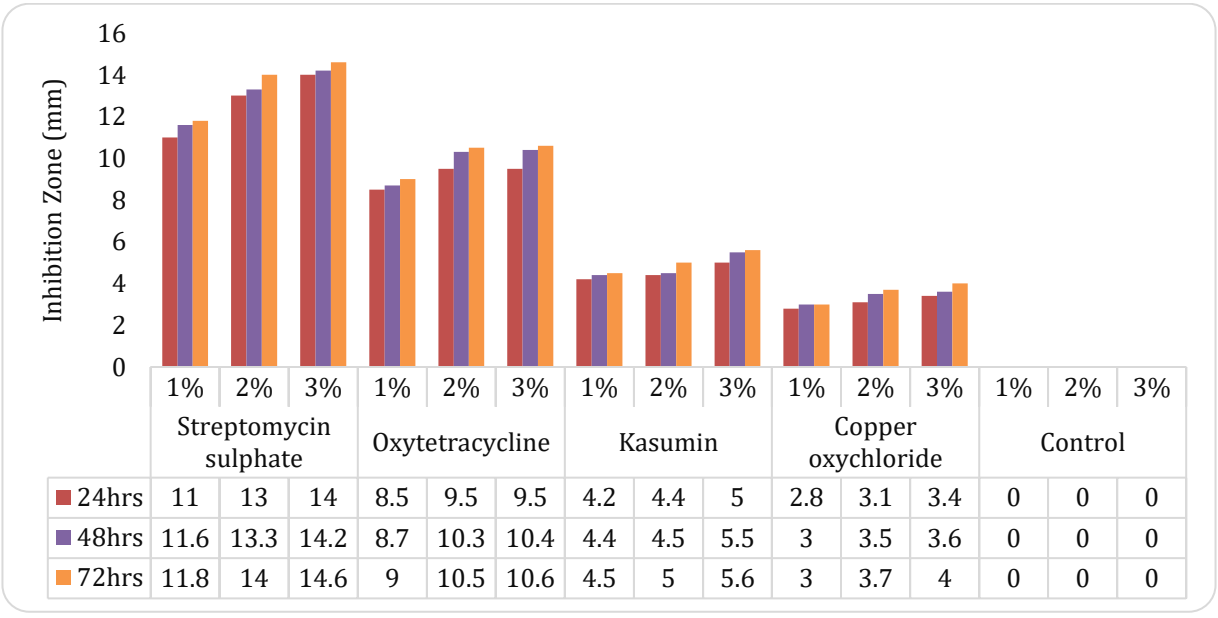


	Acacia nilotica			Calotropis gigantea			Citrullus colocynthis			Moringa oleifera			Control		
	25 %	35 %	45 %	25 %	35 %	45 %	25 %	35 %	45 %	25 %	35 %	45 %	25 %	35 %	45 %
24hrs	7.7	10	13	4	4.4	6.4	3	3.3	4	1.1	1.9	2	0	0	0
48hrs	9.2	12	13	4.3	4.6	6.7	3.4	3.9	4.7	1.5	2.3	2.3	0	0	0
72hrs	9.7	12	15	5	5.4	6.9	4	4.1	4.8	1.7	2.5	3	0	0	0

Figure 3. Results of well diffusion technique for plant extracts.

In-vitro* evaluation of Chemicals against *P. syringae* pv *spinaciae

Laboratory experiment of chemicals by disc diffusion method revealed Streptomycin sulphate as an effective antibacterial agent against *P. syringae* pv *spinaciae* followed by Oxytetracycline, Kasumin, and Copper oxychloride at 3% concentration as compared to control (Figure 4, Table 2).



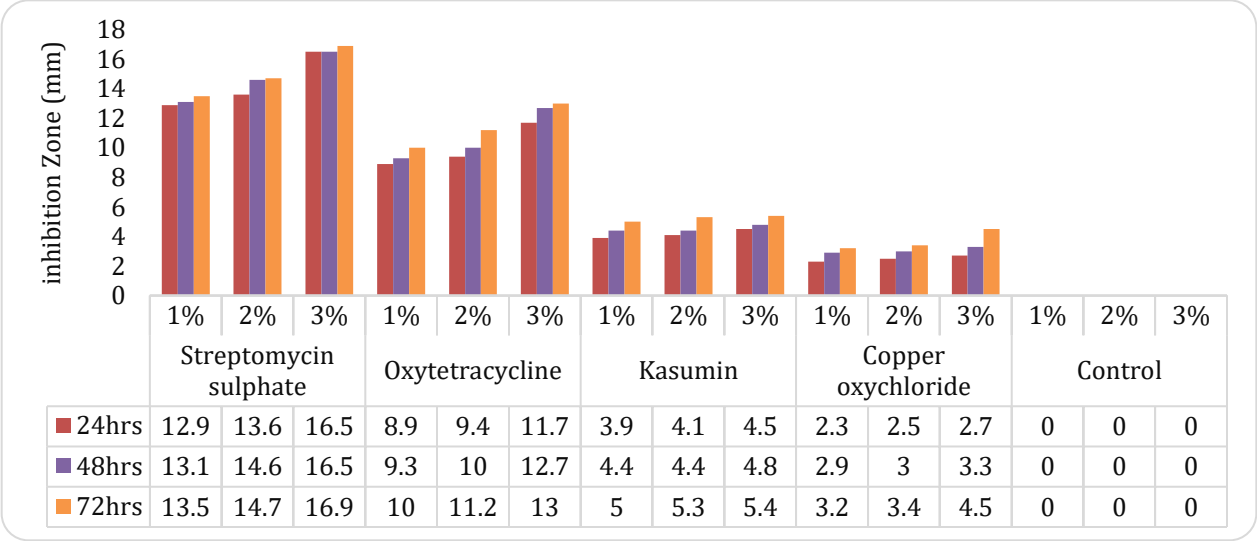
	Streptomycin sulphate			Oxytetracycline			Kasumin			Copper oxychloride			Control		
	1%	2%	3%	1%	2%	3%	1%	2%	3%	1%	2%	3%	1%	2%	3%
24hrs	11	13	14	8.5	9.5	9.5	4.2	4.4	5	2.8	3.1	3.4	0	0	0
48hrs	11.6	13.3	14.2	8.7	10.3	10.4	4.4	4.5	5.5	3	3.5	3.6	0	0	0
72hrs	11.8	14	14.6	9	10.5	10.6	4.5	5	5.6	3	3.7	4	0	0	0

Figure 4. Results of disc diffusion technique for chemicals.

Table 4. *In-vitro* evaluation of chemicals.

Treatments	Mean Inhibition Zone (mm)
Streptomycin sulphate	13.9
Oxytetracycline	10.2
Kasumin	4.7
Copper oxychloride	3.2
Control	0.0

Laboratory experiment of chemicals by well diffusion technique also expressed Streptomycin sulphate as an efficient antibacterial agent against *P. syringae* pv *spinaciae* followed by Oxytetracycline, Kasumin, and Copper oxychloride at 3% concentration as compared to control (Figure 5, Table. 2). Results of inhibition zone (mm) by chemicals observed in well diffusion method were more prominent than expressed by disc diffusion technique (Figure 4 and Figure 5).



	1%	2%	3%	1%	2%	3%	1%	2%	3%	1%	2%	3%	1%	2%	3%
	Streptomycin sulphate			Oxytetracycline			Kasumin			Copper oxychloride			Control		
24hrs	12.9	13.6	16.5	8.9	9.4	11.7	3.9	4.1	4.5	2.3	2.5	2.7	0	0	0
48hrs	13.1	14.6	16.5	9.3	10	12.7	4.4	4.4	4.8	2.9	3	3.3	0	0	0
72hrs	13.5	14.7	16.9	10	11.2	13	5	5.3	5.4	3.2	3.4	4.5	0	0	0

Figure 5. Results of well diffusion technique for chemicals.

Greenhouse Exploitation of Plant Extracts and Chemicals

Most effective chemical and plant extract observed under laboratory conditions were selected for greenhouse evaluation. Greenhouse results indicated that Streptomycin sulphate expressed lowest disease severity followed by *Acacia nilotica* as compared to negative control (Figure 6).

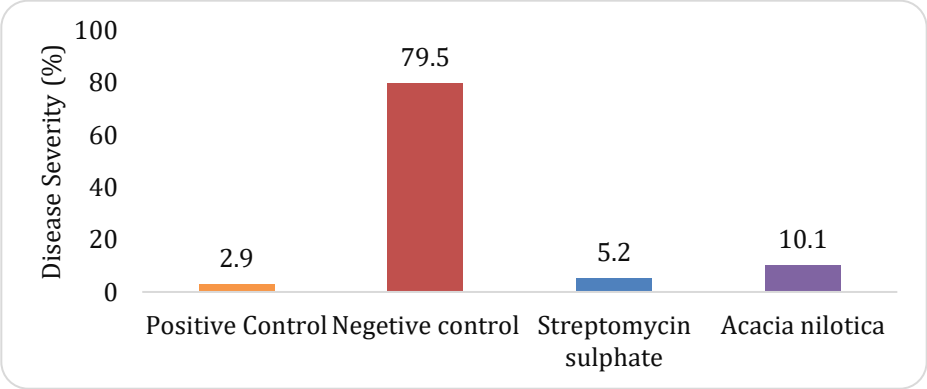


Figure 6. greenhouse evaluation of chemical and plant extract.

DISCUSSION

Spinach is susceptible to various pathogens that are responsible for causing leaf spot diseases. Bacterial blight of spinach induced by *P. syringae* pv. *spinaciae* is globally an emerging problem posing significant economic and yield losses. This economic menace is essential to manage through ecological tactics. Regarding this, current study in laboratory and greenhouse potentially managed the disease through utilizing four chemicals and plant extract. The plant extracts recently got importance due to ecological nature and have been used against various destructive to plant pathogens. The utilization of plant by-products with antimicrobial capabilities against numerous phytopathogenic bacteria and fungi has been described by many researchers (Falcón-Piñeiro *et al*., 2023; Paul and Roychoudhury, 2021). The present research was aimed to determine the efficacy of plant extracts against *P. syringae* pv. *spinaciae*. The results of our experiments were in line with previous reports of Hameed *et al*. (2020), Rasool and Jahanbakhsh (2011), Chawech *et al*. (2015); Kavitha *et al*. (2013), and Sadiq *et al*. (2017). Previous studies have shown that plant extracts have the ability to inhibit the growth of bacteria by destroying the structure and function of cell wall and membrane and induces the leakage of intracellular substances causing the dysfunction of metabolism and energy (Guo *et al*., 2021). Peptide Mop2 in moringa extract has the ability to damage the cellular membrane (Wang *et al*., 2023). *A. nilotica* has the ability to damage the cell membrane of pathogen (Sadiq *et al*., 2017). The rutin compound present in extracts acts as type II topoisomerase inhibitor (Araruna *et al*., 2012). The abundance of polyphenolic compounds in plant extracts is the main reason causing antibacterial activity by altering the bacterial membrane's permeability. This alteration reduces the synthesis of ATP and all abort the functions such as motility and less selectivity towards harmful compounds that depends on ATP. Polyphenolic compounds then enter the cytoplasm and denature the enzymes involved in replication and quorum sensing (Fontana *et al*., 2022). During severe plant disease circumstances, the use of chemicals is the last option to optimize the inputs and yield losses due to high disease management potential. Thus, in current exploitation, the effect of Streptomycin sulphate was particularly significant followed by Oxytetracycline and Kasumin in reducing *P. syringae* pv. *spinaciae* multiplication. The least effective chemical was determined to be copper oxychloride against *P. syringae* pv. *spinaciae*. This revelation is supported by de León *et al*. (2008), Vashist and Jindal (2012), and Zhang *et al*. (2014) regarding the effectiveness of employed chemicals in treating bacterial infections. Streptomycin in streptomycin sulphate interferes with the protein synthesis of bacteria in the ribosome (Singh *et al*., 2020). Tetracycline in oxytetracycline inhibits bacterial growth either by interfering with the synthesis of protein or by destroying the membrane (Schnappinger and Hillen, 1996). Kasugamycin intervenes in the reaction of the 30S subunit of the ribosome and mRNA (Schluenzen *et al*., 2006). Copper oxychloride causes damage to DNA resulting in the death of cell (Iwase *et al*., 2014).

CONCLUSION

This study highlights the potential of Streptomycin sulphate and *A. nilotica* extracts as powerful biocontrol agents against Bacterial leaf spot of spinach. Both treatments actively inhibited the bacterial growth and expressed minimum disease severity under laboratory and greenhouse assessments, respectively, followed by other treatments comparing to control. This revelation suggests Streptomycin sulphate and *A. nilotica* extracts would be the potent treatments to manage *P. syringae* pv *spinaciae* and other bacterial pathogens.

CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

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