OPEN ACCESS

Int. J. Phytopathol. 12 (01) 2023. 63-72

DOI: 10.33687/phytopath.012.01.4306

Check for updates

Available Online at EScience Press

International Journal of Phytopathology

ISSN: 2312-9344 (Online), 2313-1241 (Print)

https://esciencepress.net/journals/phytopath

MORINGA OLEFRA PLANT BASED COPPER AND SILVER NANO PARTICLES AND ITS ANTIBACTERIAL ACTIVITY TOWARDS LEAF SPOT OF CHILLI CAUSED BY *XANTHOMONAS CAMPESTRIS* PV. *VESICATORIA*

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ARTICLE INFO

Article History

Received: February 24, 2023

Revised: March 31, 2023

Accepted: April 18, 2023

Keywords

AgNPs

ZnNPs

Xanthomonas campestris

Inhibition zone technique

Disease incidence

ABSTRACT

Bacterial leaf spot caused by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) is one of the most devastating diseases, causing serious losses in chilli crop yield. The extensive usage of chemicals has increased the toxicity of the environment. Nanotechnology products are indeed being developed as a promising approach for plant disease control with minimal impact on the environment. In the present research, the antibacterial activity of green synthesized nanoparticles (Silver and Zinc nanoparticles) was investigated against *Xcv*. Different concentrations (0.25, 0.6 and 0.75%) of the green synthesized nanoparticles (AgNPs and ZnNPs) along with their combination were evaluated under lab conditions against *Xcv* under Complete Randomized Design (CRD). Results showed that the combination of both nanoparticles (AgNPs+ZnNPs) gave the best results and expressed maximum inhibition zone (25.207 mm), followed by solo applications of AgNPs and ZnNPs (18.458 and 12.253 mm). Whereas, the interaction of treatment and concentration combination (AgNPs+ZnNPs) expressed a maximum inhibition zone (28.459 mm) at 0.75% concentration as compared to other treatments. The concentration (0.75%) at which bacteria showed the maximum inhibition zone was used to control the bacterial leaf spot of chilli under field conditions. The experiment was conducted under a Completely Randomized Block Design (RCBD) where AgNPs+ZnNPs expressed the best results with minimum disease incidence (21.92%) followed by solo treatments of AgNPs and ZnNPs (26.999 and 35.320%).

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INTRODUCTION

Capsicum annum L. is an important spice crop and belongs to the family *Solanaceae* (Pawaskar and Kerkar, 2021). It has many culinary advantages as it comprises numerous chemicals including steam-volatile oils, fatty oils, capsaicinoids, carotenoids, vitamins, protein, fiber and mineral elements (Olatunji and Afolayan, 2018). Many chilli constituents are important for nutritional value, flavor, aroma, texture and color (Ye *et al.*, 2022). Chillies are low in sodium and cholesterol free, rich in vitamins A, C & E, and are a good source of potassium, folic acid (Sonawane and Shinde, 2021). 2.06 million hectares area is under cultivation across the globe yielding 37.62 million tonnes annually (Jamir and Jha, 2020; FAO, 2020). In Pakistan, it share 1.5% in GDP as it is cultivated on an area of 45.7 thousand hectares producing 103.7 thousand tonnes yearly (Arin, 2019; GOP, 2021).

Biotic and abiotic stresses are prime limitations for the escalation in quality and production of chilli crops (Nephali *et al.*, 2020). Among all the other biotic factors, *Xanthomonas campestris* pv. *vesicatoria* is a serious threat and causes bacterial leaf spots of chilli (Aftab *et al.*, 2022). Symptoms appear as small tan to black lesions with an angular appearance. These spot merge and form large necrotic areas on the leaf surface. These symptoms appear on leaves, stems and calyx. Severe infections can result in entire plant death (Roach *et al.*, 2018). The bacteria is gram-negative and aerobic causing up to 45% yield losses when provided with favorable conditions (Petrocelli *et al.*, 2018). Pathogen produced yellow and mucoid colonies (Ogolla and Neema, 2019). The bacterium can only survive for a few weeks in the soil and obtain its food from the debris of plants, seeds, and soil. It spreads through heavy rain, seeds and wind while seeds are the primary source of inoculum. Bacteria can also survive on volunteer plants of pepper along with soil in the presence of residues of the crop (Ahmad and Ahmad, 2022). High relative humidity and warm temperatures (24–30°C) favour the disease development (Reddy, 2016).

Numerous effective management strategies are used to combat the chilli bacterial leaf spot. One of the best strategies is the use of resistant varieties. It is the most cost-effective and environmentally friendly strategic approach (Sandani and Weerahewa, 2018). Leading to a shortage of resistant varieties and the unexpected occurrence of disease, farmers prefer to use chemicals because it is an effective, simple and rapid method for disease control (Shavanov *et al.*, 2022). The prolonged use of chemicals endangers both human and environmental health. Chemical residues devastate the soil ecosystem by negatively impacting non-targeted organisms and lowering soil fertility. Therefore, it needs time to adopt advanced approaches such as nanotechnology. Nanotechnology is a relatively new idea with promising applications for maintaining excessive agricultural inputs and managing environmental balance (Chhipa, 2019).

Nanotechnology has enormous potential for controlling plant disease and it can provide environmentally friendly alternatives to many chemicals (Rajwade *et al.*, 2020). The use of different biological entities in the green synthesis of the NPs can manage many of the negative effects of chemical and physical techniques (Usman *et al.*, 2019). Extraction of the moringa leaves was utilized to evaluate the biosynthesis of the zinc oxide NPs. Many researchers identified the use of the green culinary strategy as it's inexpensive and has no negative environmental impact. Extraction of the moringa leaves is promising for particle size balance (Abel *et al.*, 2021a). An experiment was conducted and concluded that the growth of *Xcv*, was significantly suppressed by copper nanoparticles, which had superior function compared to conventional commercial formulations of copper (Varympopi *et al.*, 2022). A greenhouse pot trial was carried out to evaluate the potential of silica nanoparticles that are green based (SiO2-NPs) against the bacterial leaf spot disease of pepper caused by *Xanthomonas compestris* pv. *vesicatoria* (Awad-Allah *et al.*, 2021). The purpose of the current study was to assess the effect of green-based nanoparticles on the BLS of chilli while considering the seriousness of the situation.

MATERIAL AND METHODS

Isolation, Purification and Identification of *Xanthomonas campestris* pv. *vesicatoria*

Chilli leaf spot samples were collected from the Research Area, Department of Plant Pathology, University of Agriculture Faisalabad, and brought to the laboratory of Phyto-bacteriology. After washing, diseased samples were cut into small pieces of 2-3 mm with sterilized scissors, along with some healthy parts. The samples were sterilized by dipping them in 1 percent sodium hypochlorite (NaOCl) for 30 seconds, followed by three consecutive washes with distilled water to eliminate the residual effects. Nutrient agar (NA) medium was prepared and sterilized in an autoclave for 15 minutes at 121°C and 15 Psi. Three sterilized samples were placed on each Petri plate having solidified NA media using a sterilized inoculating needle, and the plates were wrapped with wrapping tape. These Petri plates were labelled and kept in an incubator at 28°C for bacterial growth. After 24 hours, bacterial growth was observed. Bacteria was purified by gently streaking a single colony from an isolated plate on another plate having media in a zigzag pattern and incubated it at 28°C for 24 hours. The pathogen was identified by performing biochemical tests using gram staining. A bacterial suspension was prepared on a slide by combining pure cultures of *Xanthomonas campestris* pv. *vesicatoria* in a drop of sterilized distilled water. The suspension was stained and left for 30 seconds before being washed with distilled water and ethanol. One drop of safranin was added as a counterstain and washed with distilled water. Bacteria were identified using morphological parameters such as colony color (creamy yellow), growth pattern (raised and mucoid colonies) and the formation of a clear ring around the colonies (Mincă and Mitrea, 2020).

Pathogenicity Test

Confirmation of isolated *Xanthomonas campestris* pv. *vesicatoria* was done by fulfilling the Koch`s postulates. For the pathogenicity test, the pathogen was identified from the diseased plant based on visual symptoms. Samples were collected and bacteria were isolated from these diseased samples. The healthy chilli plants of moderately susceptible variety (Desi) were grown in pots. Bacterial suspension was measured through spectrophotometer (RoHS, UV/VIS Spectrophotometer, UV-1100) (10^5 CFU/mL of H_2O) and inoculated to the plant leaf through syringe infiltration using 25-gauge needle at seedling stage (Yang *et al.*, 2018). Plants which were treated as control, injected with distilled water. Symptoms appeared after 5-7 days of inoculation. Re-isolation of bacteria from artificially inoculated plants/tissues was done and bacteria which showed the same colony pattern as the original culture was noticed as pathogenic fulfilling Koch's postulates and were used for further studies.

In-vitro* Evaluation of Green based Nanoparticles against *Xanthomonas campestris* pv. *vesicatoria

Preparation of Green based Nano Particles

Moringa leaves were taken and shade dried for 1 week. After this, these leaves were sun dried for 2-3 days and then oven dried at 65°C for 3-4 hours. Then the leaves were grinded in fine powder by using pestle and mortar. 20g of obtained powder was added in 100 ml of methanol and placed at a dark place for 24 hours. This mixture was filtered through filter paper then 17g silver nitrate ($AgNO_3$) and 17g zinc oxide (ZnO) were added to the filtrate. After that it was placed on ultrasonic cleaner for 4 hours at 65 ℃ and then on water bath at 65°C for 15 minutes. It was ground in pestle and mortar to obtain fine powder (nanoparticles). Different concentrations were prepared to use it *In-vivo* and *In-vitro*.

Procedure for Evaluation of Green based Nanoparticles under Lab Condition

Four treatments (silver, zinc, combination of silver and zinc, control) were used for evaluation against *Xanthomonas campestris* pv. *vesicatoria* through inhibition zone technique. 3 different concentrations (0.25%, 0.6% and 0.75%) were prepared by adding 0.25g, 0.6g and 0.75g of nanoparticles into 100 mL of distilled water separately. Poured the prepared NA in Petri plates and after solidification of media, transfer the pathogen on Petri plate by streaking method. Sterilized blotter paper was cut and dipped in the prepared concentrations and placed in the center of inoculated plates. For control treatment, blotter paper was dipped in distilled water and placed in the center of inoculated plate. Petri plates were wrapped with parafilm and labelled. All the plates were placed carefully at 28 ℃ for 24 hours. The growth and inhibition zone was checked with the interval of 24 hours. Inhibition zone was measured through scale after every 24 hours till three days. The experiment was conducted under Complete Randomized Design (CRD).

***In-vivo* Evaluation of Green based Nanoparticles against Bacterial Leaf Spot of Chilli**

Field was prepared by growing the moderately susceptible variety maintaining R×R = 75cm and P×P = 45cm. The nanoparticles (Silver and Zinc) which showed the most effective results under lab trials and a combination of both silver and zinc nanoparticles were evaluated in field trial. The concentration (0.75%) at which inhibition zone was maximum in lab was used and prepared by adding 0.75 grams of nanoparticles in 100mL distilled water respectively. One set of plants was inoculated with distilled water and kept as control. Data regarding disease incidence was recorded with one week of interval and disease incidence was measured through the given formula. Disease severity was rated on scale from 0-4, where 0 indicated no symptoms on inoculated leaves, 1 indicated 1-10 spots on inoculated leaves, 2 showed 11-30 spots on inoculated leaves, 3 showed more than 30 spots on the inoculated leaves and 4 indicated the confluent necrotic appearance on the inoculated leaves (Gambley, 2018) while disease incidence was calculated by following formula:

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

Statistical Analysis

Lab experiment was performed under CRD (Completely randomized design) while field trial was conducted under RCBD (Randomized Complete Block Design) design. Data was recorded for each experiment and statistically analyzed by using SAS (Institute, 1990).

RESULTS

In-vitro Evaluation of Different Green based Nanoparticles against *Xanthomonas campestris* pv. *vesicatoria*

Among all the treatments, combination of green based silver and zinc nanoparticles showed maximum inhibition zone (25.207 mm) followed by silver nanoparticles (18.458 mm) and zinc nanoparticles (12.253 mm) as compared to control as shown in Table 1 and Figure 1. The interaction between treatments and concentration (T x C) showed that maximum inhibition zone was expressed by combination of green based silver and zinc nanoparticles (21.94, 25.21, 28.45) mm at 0.25, 0.6 and 0.75% followed by silver (15.19, 18.46, 21.70) mm and zinc nanoparticles (9.86, 11.84, 15.05) mm as compared to control as shown in Table 2 and Figure 2. Interaction between treatments and days (T×D) exhibited that maximum inhibition zone was expressed by combination of green based silver and zinc nanoparticles (21.89, 25.14, 28.57) mm after 24, 48 and 72 hours followed by silver nanoparticles (15.14, 18.39, 21.82) mm, zinc nanoparticles (8.79, 12.20, 15.79) mm as compared to control as shown in Table 3 and Figure 3.

Table 1. Impact of different treatments on growth of *Xanthomonas campestris* pv. *vesicatoria* under lab conditions.

S. No.	Treatments	Inhibition zone (mm)
1	AgNPs	18.458b
2	ZnNPs	12.253c
3	AgNPs+ZnNPs	25.207a
4	Control	0.0000d
	LSD	0.3459

*Mean value in the column sharing similar letter does not differ significantly as determined by LSD test (P≤0.05).

Table 2. Impact of interaction between treatments and concentrations on inhibition zone of *Xanthomonas campestris* pv. *vesicatoria* under lab conditions.

Treatments	Inhibition zone (mm)		
	Concentrations		
	0.25%	0.6%	0.75%
AgNPs	15.197e	18.467d	21.709c
ZnNPs	9.8606g	11.846f	15.052e
AgNPs+ZnNPs	21.947c	25.217b	28.459a
Control	0.0000h	0.0000h	0.0000h
LSD		0.5991	

*Mean value in the column sharing similar letter not differ significantly as determined by LSD test (P≤0.05)

Table 3. Impact of interaction between treatments and days on inhibition zone of *Xanthomonas campestris* pv. *vesicatoria* under lab condition.

Treatments	Inhibition zone (mm)		
	Time (h)		
	24 hours	48 hours	72 hours
AgNPs	15.144e	18.399d	21.829c
ZnNPs	8.7638g	12.201f	15.795e
AgNPs+ZnNPs	21.894c	25.149b	28.579a
Control	0.0000h	0.0000h	0.0000h
LSD	0.5991		

*Mean value in the column sharing similar letter not differ significantly as determined by LSD test (P≤0.05).

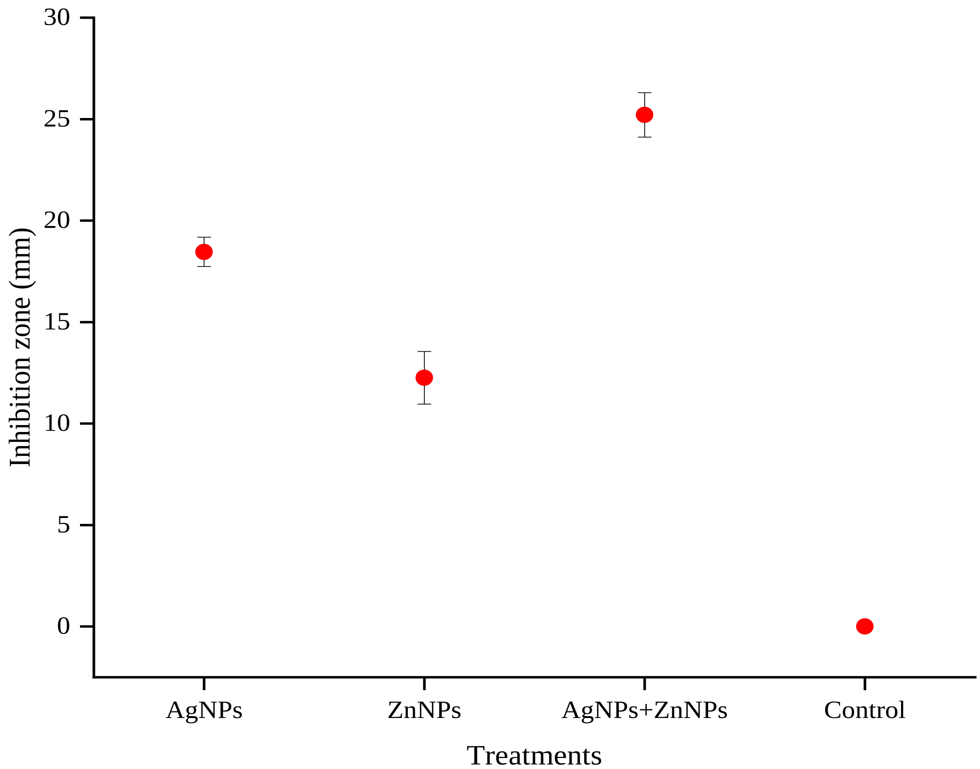

Figure 1. Impact of different treatments on inhibition zone of *Xanthomonas campestris* pv. *vesicatoria* under lab conditions.

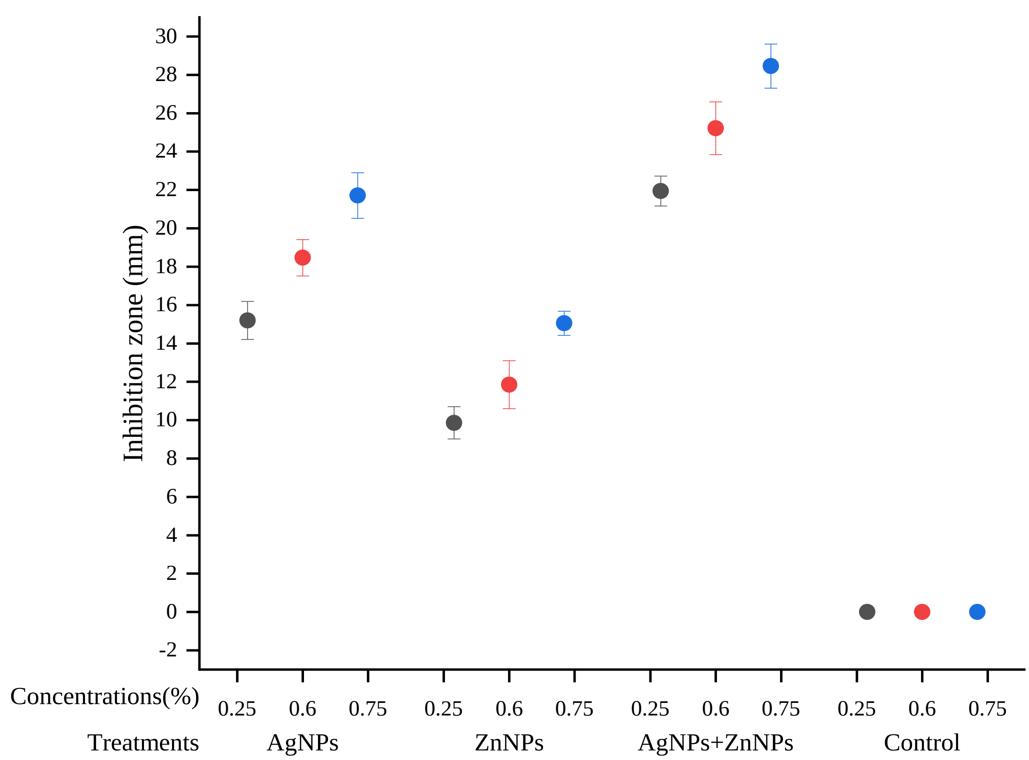

Figure 2. Impact of interaction between treatments and concentration on inhibition zone of *Xanthomonas campestris* pv. *vesicatoria* under lab conditions.

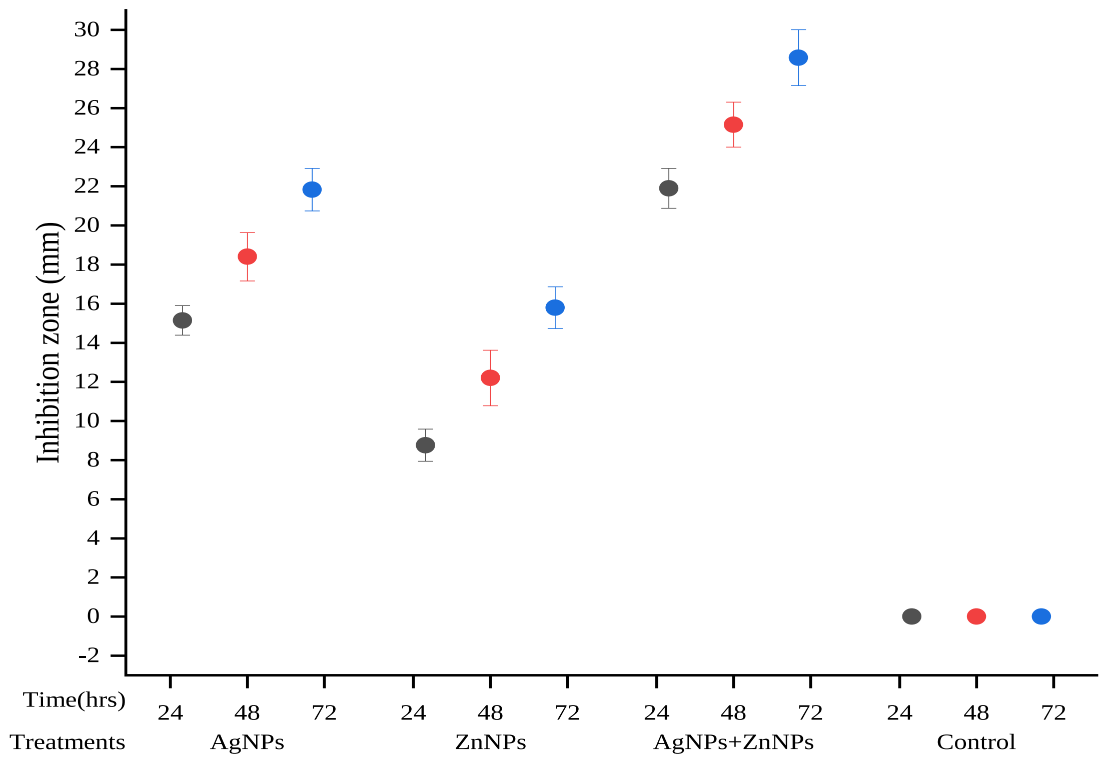

Figure 3. Impact of interaction between treatments and time on inhibition zone of *Xanthomonas campestris* pv. *vesicatoria* under lab conditions.

In-vivo Evaluation of Different Green based Nanoparticles against Bacterial Leaf Spot of Chilli

Combination of green based silver and zinc nanoparticles showed minimum disease incidence (21.92%) followed by silver (26.99) % and zinc nanoparticles (35.32) % as compared to control as shown in Table 4 and Figure 4. Interaction between treatments and days (T×D) showed that minimum disease incidence was shown by combination of green based silver and zinc nanoparticles (27.30, 21.33, 17.13) % after one week interval followed by silver nanoparticles (32.14, 26.08, 22.769) %, zinc nanoparticles (39.26, 35.99, 30.70) % as compared to control as shown in Table 5 and Figure 5.

Table 4. Impact of different treatments on disease incidence of bacterial leaf spot of chilli.

S. No.	Treatments	Disease incidence (%)
1	AgNPs	26.999c
2	ZnNPs	35.320b
3	AgNPs+ZnNPs	21.922d
4	Control	62.240a
	LSD	1.9840

*Mean value in the column sharing similar letter not differ significantly as determined by LSD test (P≤0.05)

Table 5. Impact of interaction between treatments and weeks on disease incidence of bacterial leaf spot of chilli.

Treatments	Disease incidence (%)		
	Weeks		
	After 1 week	After 2 weeks	After 3 weeks
AgNPs	32.146cd	26.083de	22.769ef
ZnNPs	39.260b	35.996bc	30.704cd
AgNPs+ZnNPs	27.300de	21.331ef	17.134f
Control	59.114a	62.154a	65.451a
LSD		3.4363	

*Mean value in the column sharing similar letter not differ significantly as determined by LSD test (P≤0.05)

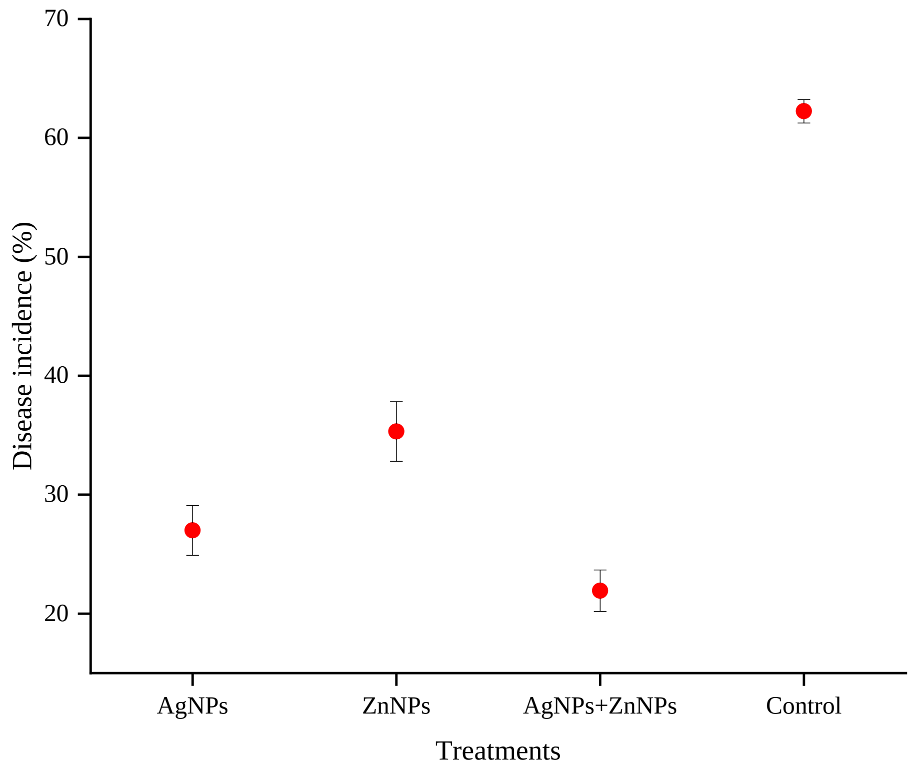

Figure 4. Impact of different treatments on disease incidence of bacterial leaf spot of chilli.

DISCUSSION

Bacterial plant diseases are considered more difficult to control than fungal diseases due to their frequent polycyclic nature and the limitations of systemic and environmentally friendly antibacterial substances (Stefani *et al.*, 2021). Because of major concerns about residues in food and water, as well as the negative environmental impact, the current list of conventional products such as chemicals like fungicides and bactericides should be reduced. Furthermore, due to the overuse of chemicals, *Xanthomonas* species are remarkably successful in developing resistant populations (Kumar *et al.*, 2022). As a result, a longe-term and more effective strategy for controlling disease such as *X. campestris* pv. *vesicatoria* is necessary.

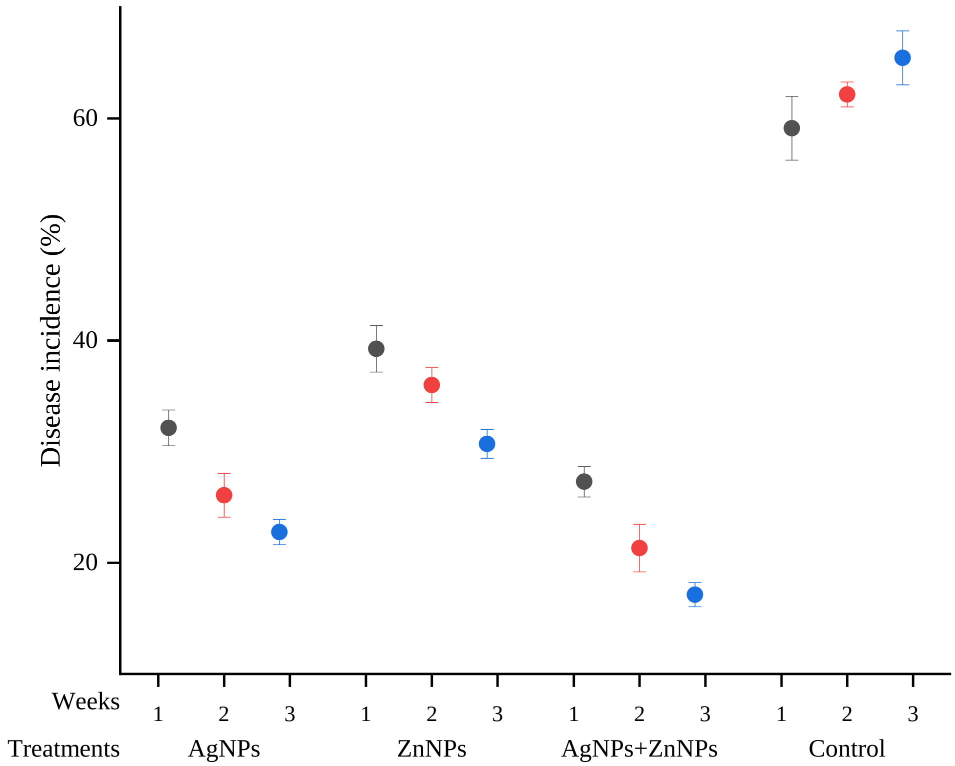

Figure 5. Impact of interaction between treatments and days on disease incidence of Bacterial leaf spot of chilli.

In present research, two green based nanoparticles (Silver and Zinc nanoparticles) were prepared and used at different concentrations and were evaluated *In-vitro* and *In-vivo* conditions alone and with their combination. Leaves of *Moringa olifera* were used to prepare the nanoparticles. *In-vitro* results demonstrated that AgNPs were more effective to inhibit the growth of *Xcv* as compared to ZnNPs and the combination of both nanoparticles showed the maximum inhibition zone against *Xcv*. These nanoparticles were applied under field conditions to control Bacterial leaf spot of chilli at the most effective concentration that was evaluated under lab conditions. The results showed that the combination of both green based nanoparticles gave the best control of the disease. The antibacterial activity of *Moringa olifera* on the bacterium *Xanthomonas campestris* pv. *campestris* looks promising as it has the multiple and synergic mechanisms of action involved with the phytocomplex, the high efficacy, eco-compatibility and sustainability that it would imply, low cost and low risks, make it ideal as a potential means of controlling the disease (Fontana *et al.*, 2021; Goss *et al.*, 2018). Silver nanoparticles showed the best results as compared to zinc nanoparticles as they possess a broad spectrum of antibacterial, antifungal and antiviral properties. Silver nanoparticles have the ability to penetrate bacterial cell walls, changing the structure of cell membranes and even resulting in cell death (Mikhailova, 2020; Prabhu and Poulose, 2012). Their efficacy is not because of their nanoscale size only but also to their large ratio of surface area to volume (Dakal *et al.*, 2016). They can increase the permeability of cell membranes, produce reactive oxygen species, and interrupt the replication of deoxyribonucleic acid by releasing silver ions in pathogen (Huq *et al.*, 2022). The use of silver nanoparticles as an antibacterial agent has received much attention due to their broad spectrum of antibacterial and antifungal activities (Dakal *et al.*, 2016).

The results of the current research is supported by Varympopi *et al.* (2022) who evaluated that growth of *Xanthomonas campestris* pv. *vesicatoria* was significantly suppressed by green based nanoparticles (Varympopi *et al.*, 2022). The outcomes of the present study is also supported by Abel *et al.* (2021b) who investigated the technique of biosynthesis of nanoparticles from the extraction of moringa leaves. Many researchers recognize the use of this method of green culinary technique because it is cost-effective and has no negative impact on the environment. The biosynthesis of nanoparticles from the extraction of Moringa leaves has been demonstrated in several other studies (Virk *et al.*, 2023; Prasad and Elumalai, 2011). The nanoparticles of Moringa leaves are spherical, with a size of 141.6 nm and a polydispersity index of 0.32 (Virk *et al.*, 2023). *Moringa oleifera* leaves extract has been used as a reducing agent in the green biosynthesis of silver nanoparticles due to its strong antioxidant properties (Sathyavathi *et al.*, 2011). Outcomes of the present research is also confirmed by Moradian *et al.* (2018) who evaluated the antibacterial effects of nano-particles (NPs) against a *Xanthomonas campestris*, as well as the study of these nanoparticles effects on expression of the pathogenic gene *hrpE*. The antibacterial effects of nanoparticles against Xanthomonas campestris have been studied in several research articles (Varympopi *et al.*, 2022; Moradian *et al.*, 2018). The effect of nanoparticles on the pathogenic gene expression *hrpE* has also been studied using Real-Time PCR (Moradian *et al.*, 2018). The study of the effects of nanoparticles on the expression of pathogenic genes is important for understanding the mechanisms of action of nanoparticles and their potential applications in the control of bacterial diseases in plants. The growth percentage of bacteria *Xanthomonas campestris* was reduced with increase in the concentration of nanoparticles. The conclusion of this study is also verified by Awad-Allah *et al.* (2021) who carried out a pot experiment under greenhouse conditions to evaluate the potential of green synthesized nanoparticles against pepper bacterial leaf spot disease, caused by *Xanthomonas vesicatoria*. In addition, to assess their efficacy and suppressive effects in reducing disease severity and improving pepper growth, productivity and quality.

CONCLUSION

Present studies reveal that conventional approaches to using synthetic chemicals are being limited in controlling bacterial plant diseases caused by *Xanthomonas* species, which have developed resistant populations due to the overuse of chemicals. Green-based silver and zinc nanoparticles were evaluated in controlling bacterial leaf spot of chilli caused by *Xanthomonas campestris* pv. *vesicatoria*. The *in vitro* results showed that AgNPs were more effective than ZnNPs, and a combination of both nanoparticles showed the maximum inhibition zone against *Xcv*. The field application of both nanoparticles gave the best control of the disease. The study also suggests the antibacterial activity of *Moringa olifera* on the bacterium *Xanthomonas campestris* pv. *campestris* looks promising due to its multiple and synergic mechanisms of action, high efficacy, eco-compatibility, sustainability, low cost, and low risks. Silver nanoparticles showed the best results compared to zinc nanoparticles, possessing a broad spectrum of antibacterial, antifungal, and antiviral properties. The study supports the use of green-based nanoparticles as an effective and environmentally friendly strategy for controlling bacterial plant diseases caused by *Xanthomonas* species.

CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

AUTHORS CONTRIBUTIONS

All the authors have contributed equally to the research and compiling the data as well as editing the manuscript.

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