GREEN SYNTHESIS OF SILVER AND COPPER NANOPARTICLES FROM LEAVES OF *EUCALYPTUS GLOBULUS* AND ASSESSMENT OF ITS ANTIBACTERIAL POTENTIAL TOWARDS *XANTHOMONAS CITRI* PV. *CITRI* CAUSING CITRUS CANKER

ATIQ, M.[1*] – MAZHAR, H. M. R.[1] – RAJPUT, N. A.[1] – AHMAD, U.[1*] – HAMEED, A.[2] – LODHI, A. M.[3] – USMAN, M.[1] – NAWAZ, A.[1] – AMMAR, M.[1] – KHALID, M.[1]

[1]*Department of Plant Pathology, University of Agriculture, Faisalabad, Pakistan*

[2]*Institute of Plant Protection, MNS-Agriculture University, Multan, Pakistan*

[3]*Department of Plant Protection, Faculty of Crop Protection, Sindh Agriculture University, Tandojam, Pakistan*

**Corresponding authors*
e-mail/phone: dratiqpp@gmail.com/+92-33-3439-6981 (M. Atiq); usamasono@gmail.com (U. Ahmad)

(Received 7th Oct 2021; accepted 17th Mar 2022)

Abstract. Green synthesis of nanoparticles has ushered in a new research field known as green or phyto-nanotechnology. Plant based nanotechnology has emerged as an imperative tool for the management of plant diseases. In comparison to other conventional techniques, green synthesis of various nanoparticles was found to be non-toxic, low-cost, and profitable, resulting in more stable synthesized materials. Plant-extract based methods for synthesizing nanoparticles are far more efficient and cost-effective to manage plant diseases. For the management of citrus canker, silver and copper nanoparticles were evaluated under lab conditions. The highest inhibition zone was produced under silver + copper nanoparticles (21.06 mm) follow by silver (18.26 mm) and copper nanoparticles (15.27 mm) respectively. In field, maximum disease incidence was exhibited by copper nanoparticles (41.24%), and minimum disease incidence (35.23%) was observed when silver and copper nanoparticles were used in combination as compared to the control. Minimum disease severity (23.23%) was observed when of silver and copper nanoparticles were applied in combination followed by silver nanoparticles (28.31%) and copper nanoparticles (35.23%) as compared to control under field conditions. Complete Randomized Design (CRD) was used for the laboratory and greenhouse experiments while Randomized Complete Block Design (RCBD) was used for the field experiment.
Keywords: *nanotechnology, canker, AgNPs, Xanthomonas citri pv. citri*

Introduction

Citrus (*Citrus latifolia*) is an important fruit crop of the world. It is a member of the *Rutaceae* family. The extracted pulp and juices of citrus fruit are used for medicinal purposes, as well as for the preparation of various dishes (Alamgir, 2018). In 2020 its production worldwide was 194.4 million tons from an area of 13.9 million ha and in Pakistan citrus production was estimated as 2.29 million tons from an area of 206.6 thousand ha, the worldwide juice industry is utilized to achieve 2.2 million tons (FAOSTAT, 2020). The assessment of production losses has not been fully evaluated. The presence of canker lesions, however, makes the fruit unacceptable for the fresh market. Among all diseases, citrus canker (CC) is the main constraint to the productions of citrus crops in Pakistan as well as the repose of the world (Ware, 2015). Typical symptoms of citrus bacterial canker disease are necrotic lesions on twigs, leaf, stem,

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2003_22052213

fruit, leaves and fruit become corky with a yellow halo and a watery border after a particular time interval tree decline and defoliation (Dewdney and Graham, 2016). In the years 2015-2016, the European Union rejected 144 consignments of Pakistani citrus due to *Xanthomonas citri* pv *citri* (*Xcc*) disease (Pervaiz, 2015). *Xcc* is an obligatory gram-negative rod-shaped bacterium with a single polar flagellum (1.5-2.0 x 0.5-0.75). For successful infection, it needs a temperature of 28-30 °C and its colony color is yellow due to the formation of “*xanthomonadin*” pigment (Afroz et al., 2013).

Different management strategies have been used to control citrus canker like, chemical, biological, cultural control and use of resistant varieties. To stop the disease from spreading pruning of infected trees, copper-based chemicals (Antibiotics and plant extracts) are strongly recommended to reduce the risk of disease (Ullah et al., 2019). The most economical, ecofriendly, and effective management of canker is the use of resistant source but under conducive environmental conditions, resistant varieties become susceptible, and disease appeared in epidemic form. Nanoparticles biosynthesis using different plant parts (i.e. leaves, stem, flower, roots) introduced developing research area referred as phyto-nanotechnology or green nanotechnology. Plant based nanotechnology opens up new door in agricultural field. In this miserable condition farmers have no option except the use of chemicals but due to their health hazard effects and environmental pollution issue, use of nanoparticles is the best option, as they are ecofriendly and have least effect on human health. That is why, in current study, (AgNPs) and (CuNPs) were evaluated against citrus canker (Dipankar and Murugan, 2012). Use of green synthesis of nanoparticles is the most appropriate ecofriendly and with least health hazard effect. Biosynthesis of Nanoparticles using different plant parts (i.e. leaves, stem, flower, roots) introduced developing research area referred as phyto-nanotechnology or green nanotechnology. Plant based nanotechnology opens up new door in agricultural field. Green synthesis of various nanoparticles using this technique found to be non-toxic, low-cost, and profitable which marks as more stable synthesized materials in comparison to other traditional techniques (Parveen et al., 2016). Among other methods the most frequently used approach is the green synthesis of NPs using plant extract. These have some distinctive advantages like less biohazardous, being easily assessable and serve as a source of active constituents (Baker et al., 2013). Copper nanoparticles (CuNPs) can be biologically synthesized using a variety of plant extracts. Copper has great potential in a wide variety of catalytic, biological, and sensor applications and is more commonly used in the form of nanoparticles, leading to the development of different techniques for the formulation of copper nanoparticles. Copper nanoparticles (CuNPs) are important because of their high conductivity, as compared to other metallic nanoparticles such as silver nanoparticles (Kasana et al., 2017). Silver (AgNPs) and copper nanoparticles (CuNPs) are the comprehensively researched nanomaterial, that fascinate scientists because of their broad-spectrum antimicrobial efficacy (Nedelcu et al., 2014). So in current study silver and copper nanoparticles were developed from eucalyptus leaves and were evaluated against *Xcc* causing citrus canker.

Evaluation of nanoparticles in lab for the management of citrus canker

Preparation of nanoparticles

Eucalyptus leaves were collected from the Botanical Garden of UAF. Then, these leaves were washed with distilled water and dried for 10-14 days under shade and then oven dry at 65 °C for 4 hours and then grind to get a fine powder of the leaves. Then

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20 g of eucalyptus powder was mixed with 100 mL of methanol in a beaker that was covered with aluminum foil. For 24 h, solution was kept in a dark room. After that, the solution was stirred for 15 min. at the temperature of 70 °C. Then whatman's filter paper no.41 was used to filter the solution. The extraction was mixed with 0.5 M silver nitrate (17g) and 15.0 g of copper sulfate in a beaker separately. Then the solution was stirred for 5-10 min with the help of a magnetic stirrer. After stirring, kept the beakers in an ultrasonic cleaner (YJ 5120-1) for heating for 60 min and then kept the samples in the furnace oven (Memmert oven 100-800). After 60 min. samples were grinded with the help of pestle and mortar and save them in test tubes for further use and then 0.25, 0.5 and 0.75% concentrations were prepared by adding 0.25 g, 0.55 g and 0.75 g silver and copper nanoparticles in 100 mL bottle separately (*Fig. 1*).

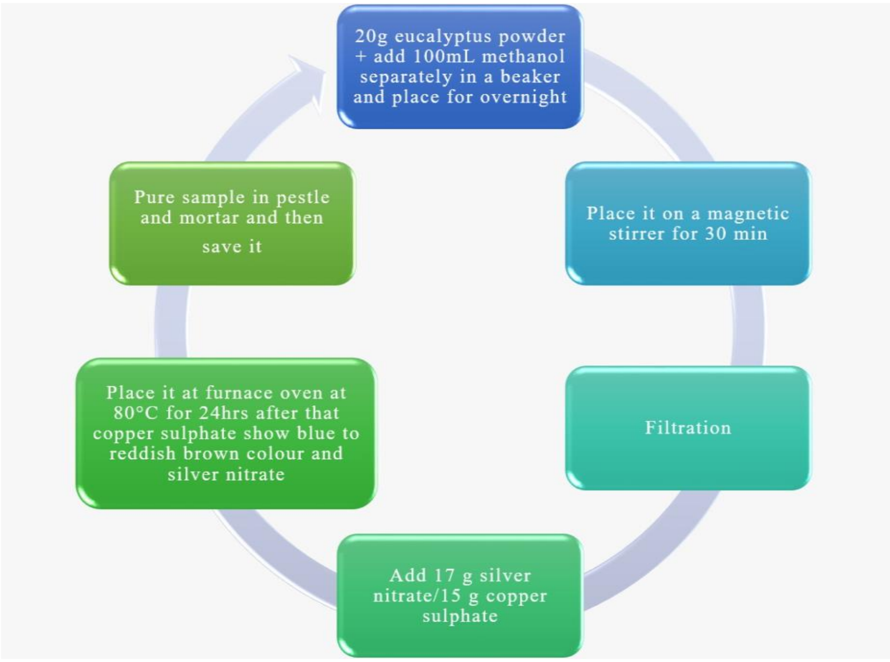

Figure 1. *Summary for preparation of nanoparticles*

Evaluation of (AgNPs) and (CuNPs) nanoparticles in lab condition

Nutrient agar (NA) media was prepared in the molecular Phyto-bacteriology laboratory, Department of plant pathology, University of Agriculture, Faisalabad (UAF). 1 Cm circular pieces of filter paper were cut and autoclaved at 121 °C for 15 Psi for 15 min and placed in NA containing Petri plates. A sterilized cotton swab was used to disperse bacterial culture in the Petri plate (9 cm) in a laminar airflow chamber (RTVL-1312, Robus United Kingdom). Then pieces of sterilized filter paper were dipped into different concentrations of filtered Nanoparticles (0.25, 0.5 and 0.75%) and placed them in the center of the NA plates with *Xcc* culture. These plates were wrapped and incubated at 28 ± 2 °C. The trial was designed by using Completely Randomized Design (CRD) with three replications per treatment. The control plates were treated with distilled water and inhibition zones were measured with the help of a digital Vernier caliper (500-196, Mitutoyo) after 24, 48 and 72 h. Fisher's Least Significant Difference Test (LSD) at 5% probability was used to examine data from inhibition zones (Steel et al., 1997).

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Evaluation of silver and copper nanoparticles in the field for the management of citrus canker

One year old Grapefruit plants were obtained from a nursery, Institute of Horticultural Sciences (IHS), University of Agriculture Faisalabad (UAF) and transplanted in the Research Area, Department of Plant Pathology, University of Agriculture, Faisalabad, using P×P = 1.0 m and R×R = 1.5 m distance in a field trial. These plants were treated with distilled water before being put in the field. Aqueous suspension of the bacteria was prepared from 48-h-old actively growing culture and bacterial concentration was measured with the help of a spectrophotometer @ 1×10^8 CFU/mL (Hitachi U-2001, model 121003UV/Vis). The bacterial suspension was inoculated early in the morning in the plants with the help of a syringe method. Bacterial suspension was injected into the midrib of the leaf as well as veins of the lower surface of the plant leaves. After three days Ag and Cu nanoparticles were assessed under field conditions @ 0.25, 0.5 and 0.75% concentrations while in control treatment only distilled water was sprayed on citrus plants under Randomized Completely Randomized design (RCRD) by maintaining three replications of each treatment. Data regarding canker was recorded after 7, 14 and 21 days intervals.

Analysis of data

Complete Randomized Design (CRD) was used for laboratory and Randomized Complete Block Design (RCBD) was used for field experiment. Least significant difference (LSD) was used with the probability level of 0.05% to observe the difference in treatments impact against *Xcc.*

Results

Maximum inhibition zone was produced by (silver + copper) nanoparticles (21.06) follow by silver (18.26) and copper nanoparticles (15.27) mm respectively as compared to control (*Fig. 2*). Interaction between treatments and concentrations (T×C) showed that maximum inhibition zone (21.50) mm was produced by silver + copper nanoparticles @ 0.75% (21.31 mm) , 0.5% and (20.37) 0.25% respectively while copper nanoparticles exhibited minimum inhibition zones (14.22, 15.13, 16.45) mm @ 0.25, 0.5 and 0.75% concentrations respectively as compared to the control. Treatments and duration of time expressed that copper exhibited minimum inhibition zones (14.07, 15.24, and 16.48) mm followed by silver nanoparticles (17.96, 18.65, and 18.17) mm and silver + copper nanoparticles (20.21, 21.08, and 21.88) respectively (*Fig. 3*). Maximum disease incidence was expressed by copper nanoparticles (41.24) %, and minimum disease incidence (35.23) was observed when silver nanoparticles + copper nanoparticles were applied in combination as compared to control (*Fig. 4*). Treatments and concentration interaction (T×C) of copper nanoparticles showed maximum disease severity 45.450, 42.478 and 35.811% while minimum disease severity was showed by the combination of both silver nanoparticles + copper nanoparticles (42.67, 34.15 and 28.86%) @ 3, 5 and 7% concentrations respectively as compared to control (*Fig. 5*). Treatments and days interaction (T×D) exhibited that copper nanoparticles expressed 42.75, 42.00 and 38.97% disease severity while silver nanoparticles showed (35.46, 35.21 and 35.01) % and silver nanoparticles + copper nanoparticles exhibited (23.25, 22.21 and 21.45) % disease severity when applied @ 3, 5 and 7% after 7, 14 and 21 days respectively as compared to the control (*Fig. 5*).

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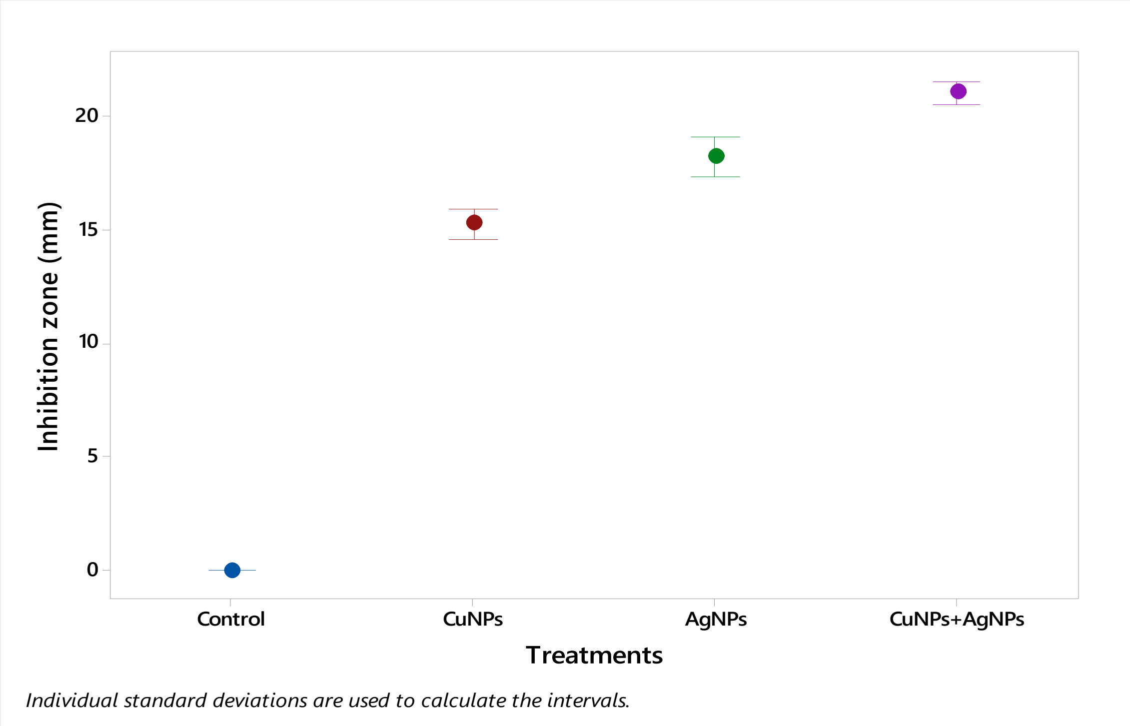

Figure 2. *Evaluation of silver and copper nanoparticles alone and in combination under lab. conditions*

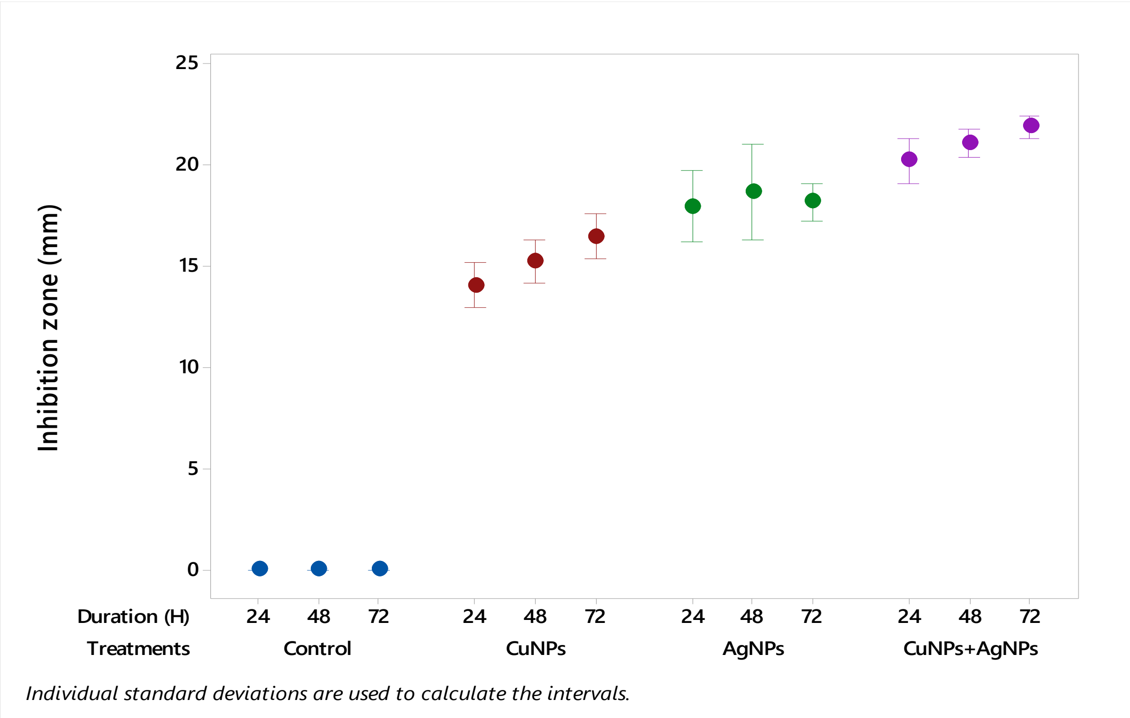

Figure 3. *Impact of interaction b/w treatments and duration of time on growth of Xanthomonas citri pv. citri under lab. conditions*

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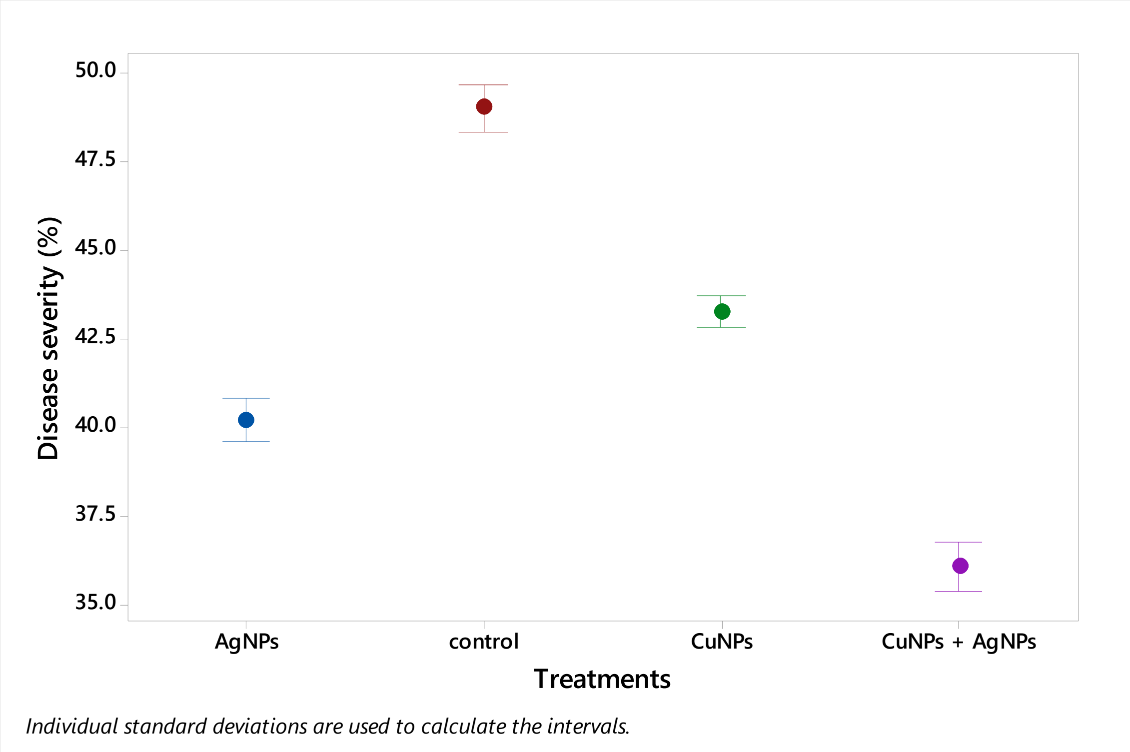

Figure 4. *Evaluation of silver and copper nanoparticles alone and in combination under field conditions*

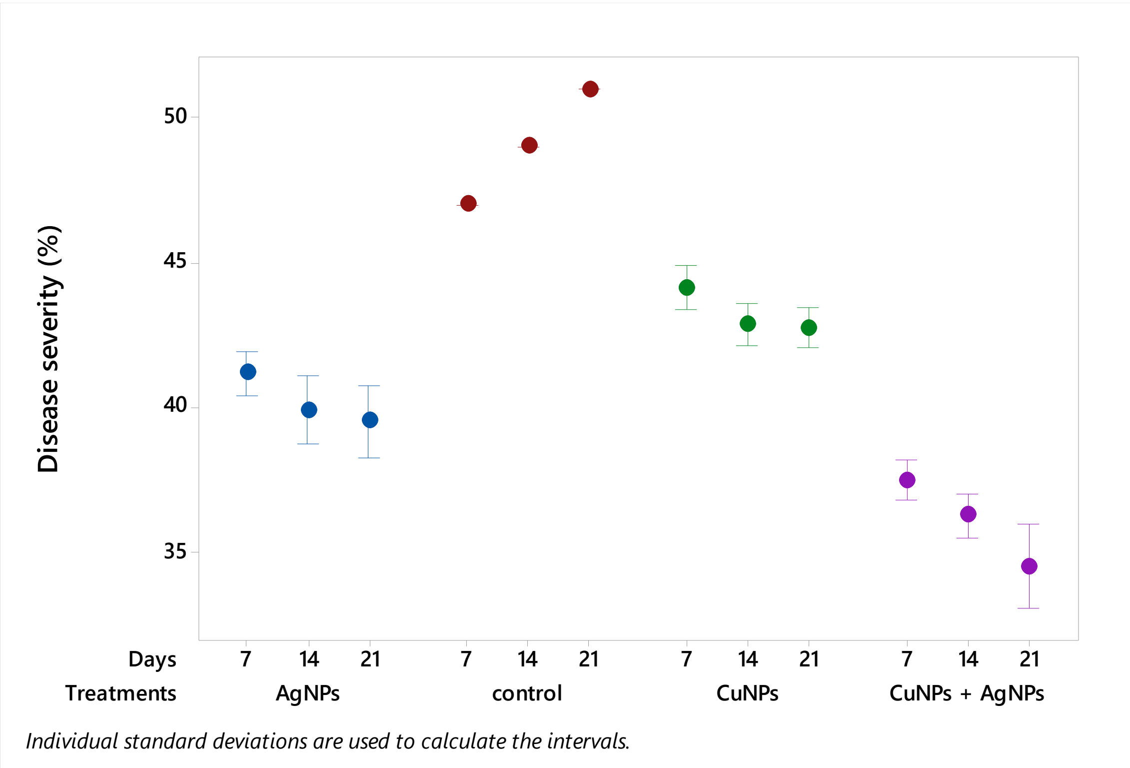

Figure 5. *Impact of interaction b/w treatments and days on the development of citrus canker under field conditions*

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Discussion

Among various control measures, scientist and researchers are striving for environment friendly management of plant diseases. Currently, green nanotechnology draws the attention of scientists to work on nanoparticles for the management of plant diseases. These nanoparticles are of various types including silver, gold, zinc, copper nanoparticles etc. Green nanotechnology offers nano-tools for biosystems transformation to nanomaterial synthesis through green approaches, by inhibiting any chemicals toxicity (Nasrollahzadeh et al., 2019). Green synthesis is well defined as use of environment friendly tools for plant and other microorganism for the biosynthesis of nanoparticles. These efficient green methodologies are free of imperfections related with conventional artificial approaches because nanoparticles are environment friendly. Bio-formulation of plant derived aqueous extracts introduced various benefits which include fast synthesis and more essentially, devoid of expensive processing for nanoparticles formulation (Das et al., 2014). Green synthesis of nanoparticles using plant extracts, provides a broad-scale formation of effective nanoparticles. Phyto-nanotechnology provides targeted distribution to specific sites in a controlled manner, minimizing the use of various plant-protection chemicals, reducing nutrient losses by the use of nano-fertilizers and increasing yield by using proper nutrients (Das et al., 2014). In the contemporary study, eucalyptus based (silver and copper) nanoparticles were evaluated in the lab conditions towards *Xcc* causing citrus canker. Among nanoparticles, silver nanoparticles showed maximum inhibition zone while under greenhouse and field conditions, combination of Ag + CuNPs expressed minimum disease severity. Results of the current study are supported by the finding of Wang et al. (2017), who studied and described that antibacterial efficacy of AgNPs of *Eucalyptus globulus* has inhibitory effect against citrus canker. AgNPs of *E. globulus* plant has great antibacterial properties against citrus canker. Outcomes of the present study were also supported by Vadlapudi and Amanchy (2017) who evaluated silver nanoparticles derived from *Myriostacha wightiana* leaf extract which showed antibacterial activity against *R. solanacearum* On weekly basis, the disease incidence of citrus canker was measured in response to various concentrations of green synthesis of CuNPs and AgNPs at 0.25%, 0.5% and 0.75%. In comparison to other nanoparticles, AgNPs @ 0.75% showed less disease incidence against canker after one week. In an attempt to determine the effect of green synthesized AgNPs in Kinnow, similar results were obtained by Hussain et al. (2018). Same results were also reported by Agnihotri et al. (2014) who tested AgNPs against *Staphylococcus aureus, Vibrio cholera and Pseudomonas aeruginosa* and found effective . Similarly AgNPs were used against citrus canker and observed reduction in disease incidence under green house and field conditions. Because of easy availability and non-toxic nature of plants as well as the stability and antibacterial potential of biosynthesized nanoparticles, green synthesis method of nanoparticle is very simple, speedy, cost-effective and ecofriendly**.** In the field of agriculture, Ag nanoparticles are cost-effective and effective antibacterial agent for large-scale production for the management of plant diseases.

Conclusion

In lab experiments, combination of silver and copper nanoparticles showed best results in reducing microbial growth followed by silver nanoparticles and copper nanoparticles. While in field experiments, maximum disease incidence was expressed by copper nanoparticles while minimum disease incidence was observed when silver

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and copper nanoparticles were used in combination. It was concluded that the combination of silver and copper nanoparticles can be used in future for the management of citrus canker.

Acknowledgements. We are highly thankful to scientists of Molecular Citrus Pathology laboratory, Department of Plant Pathology, University of Agriculture, Faisalabad for providing research facilities under project NRPU-HEC# 6373.

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