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Research Article

DETERMINATION OF ANTIBACTERIAL POTENCY OF DIFFERENT CHEMICALS TOWARDS BACTERIAL CANKER OF TOMATO CAUSED BY *CLAVIBACTER MICHIGANENSIS SUBSP. MICHIGANENSIS.*

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Abstract

Tomato is an imperative fruit/vegetable crop and a treasure trove of active natural metabolites. It is cultivated across the globe but both biotic and abiotic factors have been implicated in decreasing its production. Among all the constraints faced by tomato crop in Pakistan, bacterial canker of tomato caused by *Clavibacter michiganensis subsp. michiganensis (*Cmm is one of the most ravaging biotic stss to tomato crop. Present study was designed to evaluate antibacterial potential of different chemicals (*Cabrio* Top, Oxyrich, Kocide, Electus super, and Forum top) against *Clavibacter michiganensis* subsp. *michiganensis* under vitro and vivo conditions at three different concentrations. In vitro condition, efficacy of these chemicals was determined at (250 ppm, 500 ppm,750 ppm) concentrations out of which Electus super showed maximum inhibition zone at 750ppm concentration. In field condition, Kocide and Electus Super were applied separately and in combination and in combination significant result was expressed with minimum disease incidence. The current investigations pave the way for fruitful disease management through certain chemicals because of their easy availability, fast action and cost effectiveness.

Keywords: Kocide, Electus Super, Cabrio Top, Oxyrich, Concentrations

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1. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the second most valuable fruit/vegetable after potato as it contains vitamins (A, C, E), minerals (Zn, Fe), carotenoids, and phenolic compounds that are vital for human health. Lycopene, a carotenoid in tomato is the main source of prevention from cancer, osteoporosis, and cardiovascular diseases. Phenolic compounds have antioxidants that are effective against several oxidative stress-related diseases such as high blood pressure (Quinet *et al.*, 2019). The worldwide area under cultivation of tomato is 5 million hectares with 186 million tons production. In Pakistan, the area under cultivation is 57 thousand hectares with 0.594 million tons production (FAO, 2020).

Tomato crop is plagued by a variety of diseases, the most devastating of which is bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), which was discovered in 1909 in Michigan, USA (Takishita *et al.*, 2018). It is responsible for 70% of global crop losses in tropical and subtropical regions each year (Siddique *et al*., 2020). *Cmm* is a curved, non-motile, seed-borne, gram positive actinomycete (Nandi, Macdonald, *et al*., 2018). The optimum temperature required

for its growth is 25-30 ºC with high humidity (Singh and Bharat, 2017). *Cmm* uses natural openings (stomata) and wounds to enter the plants (Haggoud *et al.*, 2017). Infected soil, seeds, tomato plant debris, and operating tools play an important role in the pathogen transmission (Takishita *et al*., 2018). The pathogen multiplies in the xylem after getting entry into the plants and blocks the xylem vessels which results in wilting of the plants (Sharabani, Shtienberg *et al*., 2013). Stunting and small water-soaked lesions are observed at the seedling stage. A reddish-brown color discoloration is seen in the stem when it is cut lengthwise. Mature leaves develop necrotic leaf lesions on upper surface of the leaves. Sometimes, circular white spots appear on the leaves as well as on the stem petioles. A white halo surrounds the spots which contain a dark brown center (Peritore-Galve *et al.*, 2020).

Different management strategies are in practice to reduce the pathogen population such as use of resistant varieties, cultural practices, chemical and biological control but the best among them is the use of resistant varieties. When disease appears in the field in epidemic form then farmers have no other option, except the use of chemicals to control the disease because chemicals are fast acting, easily available, less expensive and take less time as compared to the other management strategies. Pereira *et al*. (2021) used 7 chemicals to treat bacterial canker out of which copper sulphate with 8-hydroxy-quinoline was found effective in controlling the disease incidence. Copper based bactericides proved to be effective against bacterial diseases by giving maximal inhibitory action. Abrahamian *et al*. (2019) evaluated different chemicals and out of them copper hydroxide showed the highest reduction of diseases in tomato seedlings. Nandi *et al*. (2018) used hydrochloric acid on the pure culture of *Cmm* to check its effectiveness on the tomato seeds. It was concluded that pathogen did not survive in the acid treated seeds even after 12 months as the plants showed no C*mm* population. Keeping in view the importance of chemicals, the current study was designed to check the most effective chemical against bacterial canker.

1. MATERIALS AND METHODS

1. Isolation, purification, and identification of *Clavibacter michiganensis subsp. michiganensis*

Tomato samples containing typical symptoms of bacterial canker obtained from the research area, Institute of Horticultural Sciences (IHS) University of Agriculture, Faisalabad were brought to the Phyto bacteriology Lab. Disease samples with some healthy portions were isolated in the lab after cutting 2-3mm long pieces and washed to remove the dirt or soil particles. Sterilization of the samples was done after washing with 1% sodium hypochlorite (NaOCl) for 30 seconds followed by three washings with distilled water to disinfect the samples. Nutrient Agar (NA) media was used for bacterial growth and was prepared by using autoclave (RTVL-1312, Robus United Kingdom) at 121 ºC and 15 Psi for 15 minutes. NA media was poured in the Petri Plates and after solidification; three isolated samples were places on each Petri Plate by using sterilized needle. The plates were wrapped with paraffin wax, labeled and placed in an incubator at 30 ºC. The bacterial growth was observed after 24 hours, and bacteria was purified to another plate containing solidified NA media by taking small bacterial colony from the isolated plate with the help of cotton swab and streaked by using zig zag streaking method. It was then placed in incubator at 30 ºC for 24 hours. The bacteria was identified through gram staining technique. Bacterial suspension was prepared on a slide containing pure culture of Cmm in a drop of sterilized water. After staining, the

suspension was left for 30 seconds and washed with distilled water followed by ethanol. A drop of safranin was added on the suspension as counter staining and washed with distilled water. The prepared slide was observed under microscope and bacterial identification was done on the basis of colony color (yellow colony) color and (concave shape) (Tripathi *et al.*, 2022).

2. **PATHOGENICITY TEST**

A bacterial aqueous suspension was made from a 48-hour-old actively growing culture and the concentration was measured by using a spectrophotometer @ 1×10^6 CFU/mL. (Hitachi U-2001, model 121003). The plants were kept in the greenhouse to avoid the effect of environmental factors. The healthy tomato plants in the pots were inoculated by using syringe (18-gauge blunt end needle with 60ml syringe) and spray method with three plants inoculated and one control to confirm the symptoms. The control plant was sprayed with distilled water and all the plants were observed on daily basis. Re isolation and identification of the pathogen was done on morphological characteristics after collecting the symptomatic portions from the leaves of inoculated plants after 7 days, and these symptoms were compared with naturally infected plants.

Evaluation of chemicals against *Clavibacter michiganensis* subsp. *michiganensis* in vitro conditions

Five chemicals such as Cabrio Top, Oxyrich, Kocide, Electus Super, and Forum Top were selected for evaluation against *Clavibacter michiganensis* subsp. *michiganensis* through inhibition zone technique (Cooper, 1963). The percentage of active ingredients of each chemical was divided by 100 and the obtained quantity of chemical was added in 100 ml distilled water to make the stock solution. Three concentrations 150, 250, and 350ppm were prepared from the stock solution by taking 2.5, 5 and 7.5mL stock solution into 100mL distilled water for each concentration. Filter paper, of 1 cm size were cut, autoclaved, and placed at center of the media plate streaked with pure culture of Cmm after dipping in the required concentration of the chemicals. Three replications were made for each concentration with one control and in control plate, the filter paper was dipped in distilled water. The plates were incubated at 30ºC, and bacterial growth was observed after 24, 48 and 72 hours and data was measured through scale or vernier caliper by taking the diameter of the inhibition zone horizontally and vertically.

Evaluation of the most effective chemicals in field conditions for management of bacterial canker of tomato

Two chemicals which showed the best results in the lab were evaluated in field experiment, separately and in combination for the management of bacterial canker. The plants of the moderately resistant variety (Aut-05) were grown in the field by keeping 76cm row to row and 30cm plant to plant distance. The inoculum was applied on three weeks old seedlings. Different concentrations of the chemicals were prepared and applied in the field by using spray method. Four treatments were used, out of which 3 treatments contained chemicals with three replications at three concentrations (1, 2 and 2.5%) and one was treated as control and was sprayed with distilled water.

Data regarding disease incidence was measured after 7 days intervals after symptoms appearance by using a disease rating scale which was given from 0-4. A rating scale of 0 showed no disease symptoms, 1 showed 1-20% wilting, 2 showed 20-40% wilting and 3 showed 40%-60% and 4 showed more than 60%

Table.1. **Summary chemicals along with active ingredients and mode of action towards *Clavibacter michiganensis subsp. michiganensis* causing bacterial canker of tomato**

Trade Name	Active Chemical	Mode of action	Reference
Oxyrich	Copper oxychloride (30%) and Cymoxanil (10%)	It damages the structure of DNA	Liman *et al.* (2021)
Electus Super	Azoxystrobin (22%) and Difenoconazole (8%)	It inhibits the mitochondrial respiration	Ju *et al*. (2019)
Kocide	Copper hydroxide (52.4%)	It disrupts active sites of enzyme and interfere with energy transport system	Lamichhane *et al*. (2018)
Forum Top	Dimethomorph (9.0%) and Metiram (44%)	It inhibits the cellulose synthesis in membranes and affects cell wall architecture	Blum *et al.* (2010)
Cabrio Top	Pyraclostrobin (50%) and Metiram (50%)	It blocks mitochondrial electron transfer reactions	Malhat *et al*. (2019)

symptoms (Umesha, 2005). Disease incidence was measured through the following formula.

Disease incidence (%) =

$$\frac{\text{No. of infected plants}}{\text{Total no. of healthy plants}} \times 100$$

1. DATA ANALYSIS

RCBD was used for field experiment and CRD for lab experiment. Least Significant Difference (LSD) was applied on the recorded data by using statistic 8.1 to find out the significant differences among treatments (Steel *et al.*, 1997).

2. RESULTS

Effect of Chemicals against *Clavibacter michiganensis* subsp. *michiganensis* under lab condition

Among all the treatments, Electus Super showed maximum inhibition zone (21.80 mm) followed by Kocide (17.66 mm), Oxyrich (7.055 mm), Cabrio Top (6.04 mm) and Forum top (4.47 mm) as compared to the control. (Fig 1). The interaction between Treatments and Concentrations (T×C) showed that maximum inhibition zone was expressed by Electus Super (20.41, 20.92, 24.08 mm), at 150ppm, 250ppm and 350ppm concentrations followed by Kocide (15.21, 18.00,19.78 mm), Oxyrich (6.39, 6.94 ,7.83 mm), Cabrio Top (5.47, 6.98,8.78 mm), and Forum Top (3.52, 4.35, 5.54 mm) at 150ppm, 250ppm and 350 ppm

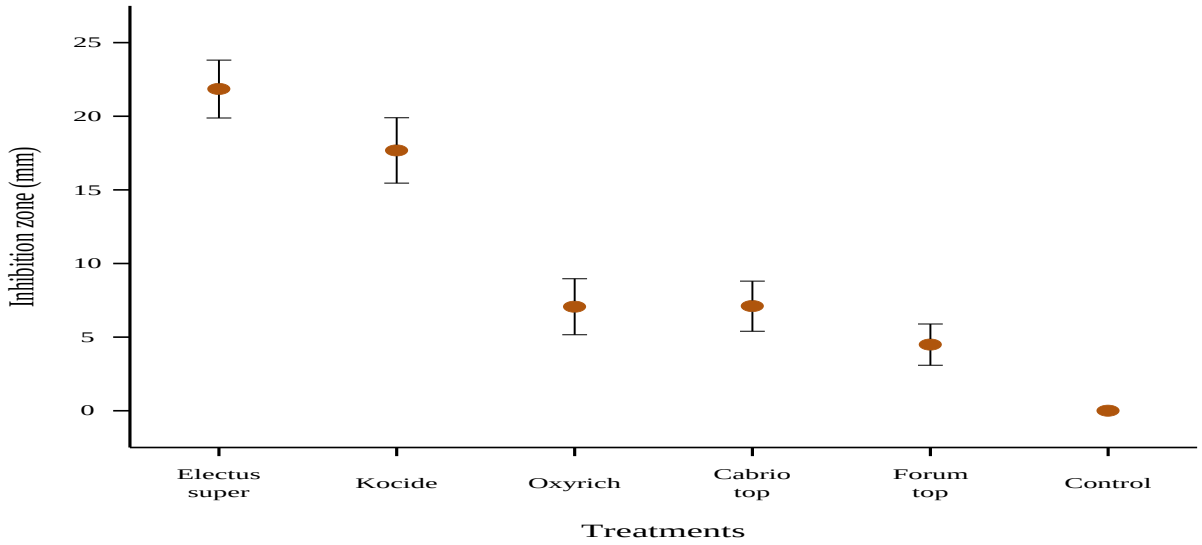

Fig. 1. Effect of different chemicals against Clavibacter michiganensis subsp michiganensis under lab condition.

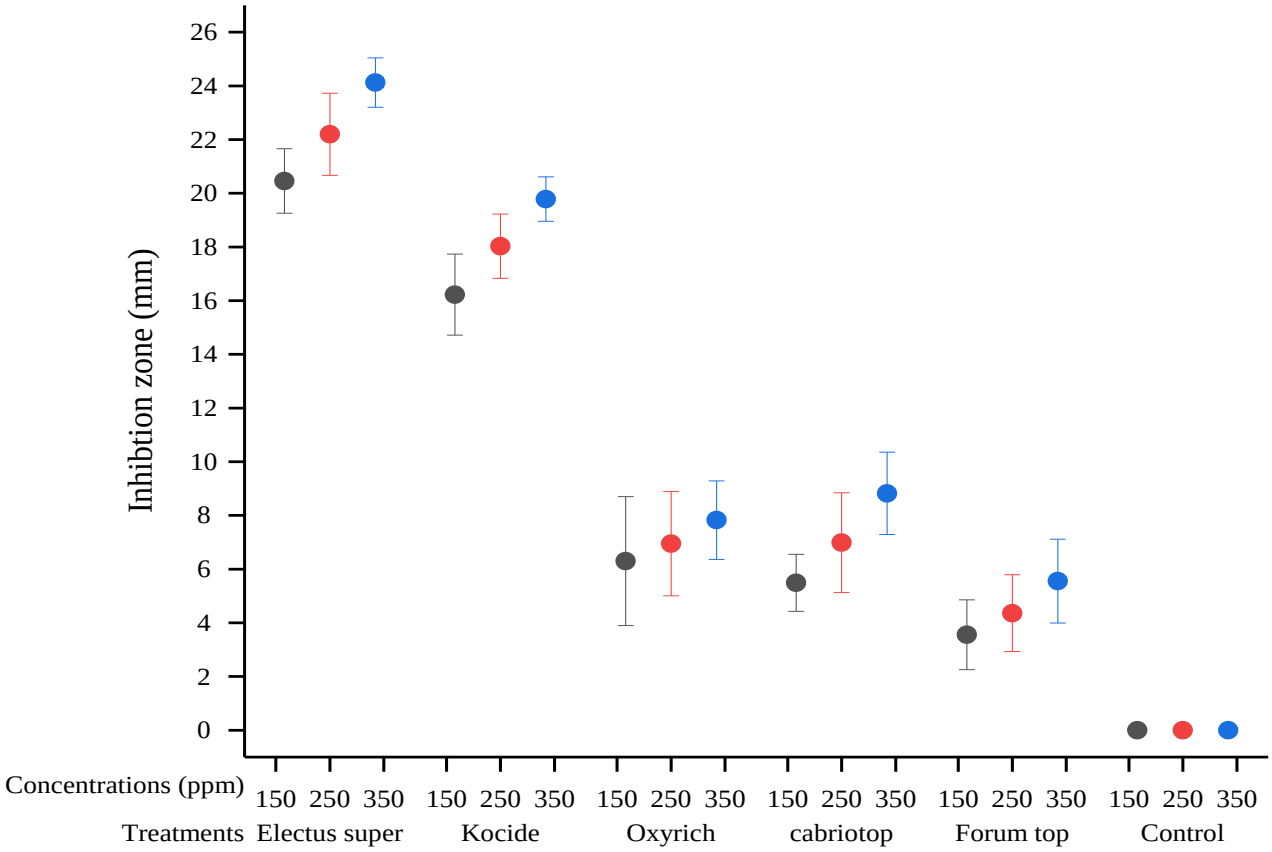

Fig. 2. Evaluation of interaction between treatments and concentrations (T×C) *Clavibacter michiganensis* subsp. *michiganensis* under lab condition.

respectively as compared to the control (Fig 2). The interaction between treatments and duration (T×D) showed that the maximum inhibition zone was expressed by Electus super (21.17, 21.48, 22.77 mm) after 24, 48, and 72 h followed by Kocide (16.85, 17.62, 18.52 mm), Oxyrich (5.36, 7.43, 8.38 mm), Cabrio Top (6.32, 7.06, 7.86 mm) and

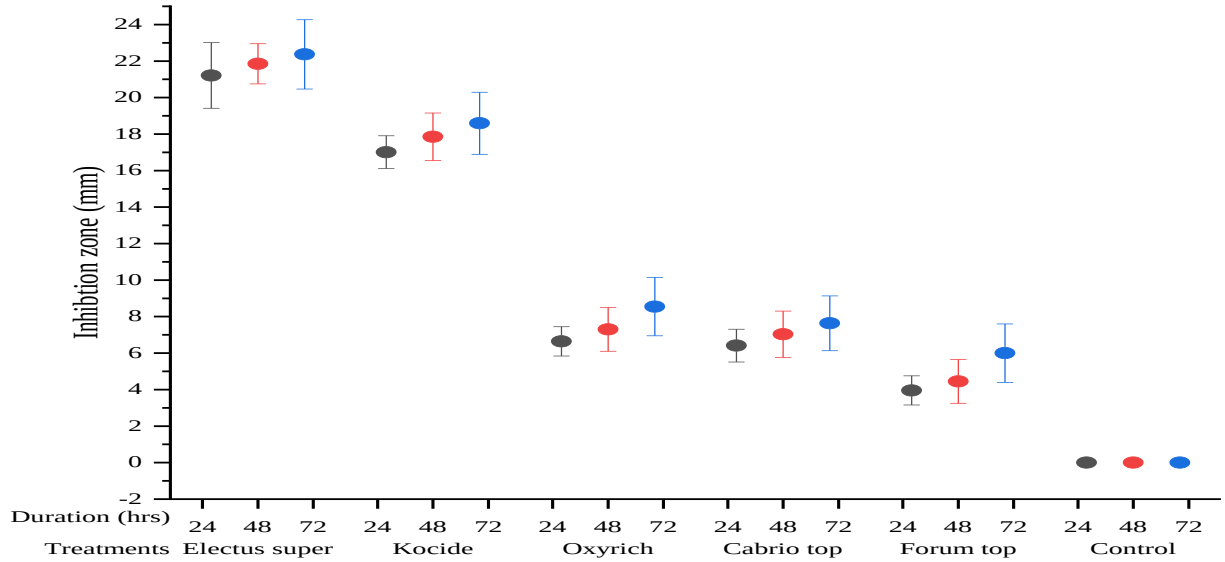

Fig 3. Evaluation of interaction between treatments and days (T×D) against Clavibacter michiganensis subsp. michiganensis under lab conditions

Forum Top (3.17, 4.53, 5.73 mm) after 24, 48, 73 h, respectively as compared to the control (Fig 3)

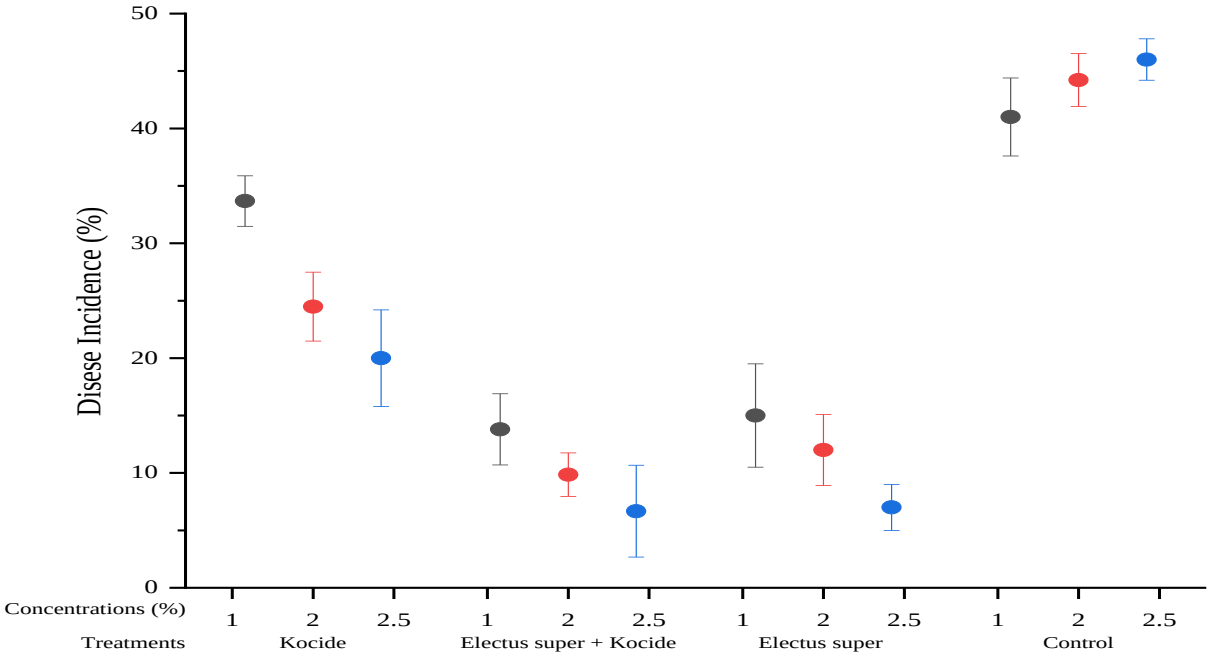

Fig 4. Evaluation of interaction between treatments and concentrations (T×C) against bacterial canker of tomato under field condition.

In-vivo evaluation of different chemicals against bacterial canker of tomato

Among all the treatments, the combination of Electus super and Kocide showed minimum disease incidence (10.10%) followed by Electus Super (14.76%) and Kocide (27.58%). (Fig 4).

Among the Interaction between Treatments×Concentrations (T×C), the combination of Kocide and Electus Super showed minimum disease incidence in the field (13.79, 9.84, 6.67%) at 1, 2 and 2..5%

concentrations, followed by Electus Super (25.19, 13.13, 5.96%) and Kocide (33.67, 24.48, 24.20%) as compared to the control (46.33, 44.21, 41.99%) at 1, 2 and 2..5% concentrations respectively.(Fig 5). Among the interactions between treatments and days (T×D) the minimum disease incidence was showed by the combination of Electus Super and Kocide (12.48, 10.57, 7.25%), after 7, 14, 21 days followed by Electus Super (21.68, 13.08, 9.53%) and Kocide (34.69, 26.40, 21.66%) after 7, 14, 21 days as compared to the control (44.81, 44.18, 43.54%).

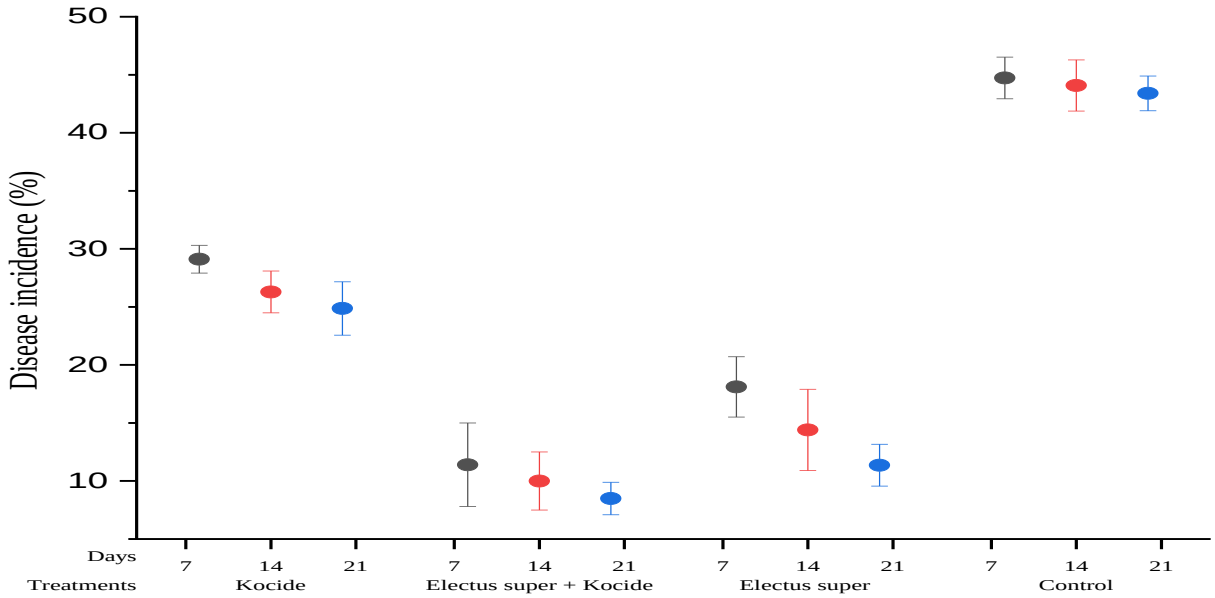

Fig. 5. Evaluation of interaction between treatments and days (T×D) against bacterial canker of tomato under field conditions.

3. DISCUSSION

Tomato is an important horticulture crop belonging to the family *Solanaceae*. In Pakistan, tomato is grown on an area of 57 thousand hectares with 0.594 million tons production (FAO, 2020). Many bacterial and fungal diseases affect tomato production, but bacterial canker is the most damaging among other bacterial diseases. Bacterial canker is caused by *Clavibacter michiganensis* subsp. *michiganensis* and causes severe economic losses each year (Takishita *et al.*, 2018). Different management strategies like use of resistant varieties, chemical and biological control are used to manage the tomato canker. Among all the management strategies, use of resistant varieties is the best method but due to unavailability of resistant varieites, farmers focus goes on the use chemicals to manage the disease because as they are easy to use, easily available, cost effective and take less time to manage the disease. In the current study, five different chemicals (Oxyrich, Electus Super, Kocide, Forum Top, Cabrio Top) were evaluated against *Clavibacter michiganensis* subsp. *michiganensis* in the lab condition. Electus super (*difenoconazole, azoxystrobin)* and Kocide (copper hydroxide) expressed maximum inhibition zone in the lab. In field trial both the chemicals showed minimum disease incidence separately and in combination. Electus Super contains *difenoconazole* in which *m*embrane lipid (ergosterol) production is inhibited, and it also blocks steroid demethylation in the pathogen. Difenoconazole is also used for increasing the production of vegetables, grains, fruit, and other field crops, as it has

strong therapeutic and defensive characteristics (Zheng *et al.*, 2020). It increases the production of APX, α-tocopherol content, and ascorbate peroxidase in tomato plants (Shanmugapriya *et al*., 2013). Superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (G-POD), and ascorbate peroxidase (APX) activities are also enhanced by difenoconazole exposure in the roots and leaves which play important role in plant defense (Liu *et al.*, 2021). Application of difenoconazole in tomatoes plants results in higher sweetness and lower acidity. Additionally, it significantly increased the expression of genes and also involved in the production of phytohormones which raised the levels of ethylene and abscisic acid (Zheng *et al.*, 2022). Active ingredient Azoxystrobin present in Electus Super works by inhibiting the mitochondrial respiration in the pathogen which blocks the process of energy conversion into ATP, pathogen become unable to respire and ultimately die (Ju *et al*., 2019). Azoxystrobin increases the production of defense enzymes in plants such as peroxidase, polyphenol oxidase and phenylalanine ammonia lyase which induce resistance in the plants against the pathogen. It also increases the production of chitinase enzyme (Tyśkiewicz *et al.*, 2022). Chitinases play important role in the production of carbon and nitrogen. It increases the plant growth by improving nitrogen metabolism, sugar content, fresh weight and photosynthetic activity (Ahmad *et al.*, 2022). Current study is supported by Kolomiiets *et al*. (2017a) who used pyraclostrobin against *Cmm* and *P. syringae* pv. tomato. Antibacterial potency of Forum Top has been evaluated against *Ralstonia solanacearum* (Atiq *et al*.,2021). The results concluded that pyraclostrobin was only effective against *Cmm* because it's a gram-positive pathogen. This chemical increases in the production of peroxidase enzyme in plant leaves which involves in response to biotic and abiotic stresses. Results of current study are supported by Chen *et al.* (2019) who used azoxystrobin and Chlorothalonil + Acibenzolar-S-methyl against *Cmm*. Azoxystrobin was the best among them to treat the pathogen and the combination of chemicals was at second best place after azoxystrobin. Copper hydroxide works by absorbing copper ions, affects nucleic acids and disrupts the active sites of enzymes. It also interferes with energy transport system and disrupt the integrity of cell membrane (Lamichhane *et al.*, 2018). It increases the amino acid metabolism which act as signal molecules and helps plants to grow well. It also increases oxidative stress response in plants which increase the activity of reactive oxygen species. Reactive oxygen species play important role in cell survival and cell signaling (Majumdar *et al.*, 2021). If oxidative stress occurs in excess, it affects lipids, proteins, cell membrane, lipoproteins, and DNA. Results of current study are supported by Kasselaki *et al.* (2011), who used copper hydroxide, and compost extracts on the tomato seeds to treat the *Cmm* infection which resulted in 100% reduction of the *Cmm* pathogen in tomato seeds. The results of current study are supported by Kolomiiets *et al*. (2017a) who used copper compounds against bacterial diseases of tomato. It was concluded that copper hydroxide is the most effective against tomato canker in the field. The results of current study are also supported by Ramos *et al.* (2022) who used copper hydroxide as a foliar spray against citrus canker in sweat oranges. Foliar application resulted in maximum reduction of disease severity by using copper hydroxide. Its control efficiency was increased more by applying it with bioactive copper. It has bactericidal properties that helps in controlling the bacterial population.

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