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Evaluation of inoculation techniques and various toxicants for the management of citrus canker under glasshouse conditions

Archana Negi, Jitendra Singh and Pradeep Kumar

Abstract
The glasshouse experiments were conducted for evaluation of inoculating techniques and various toxicants against *Xanthomonas axonopodis* pv. *citri* at, Department of Plant Pathology, College of Agriculture, G.B.P.U.A. &T., Pantnagar. The experiments were laid out in Completely Randomized Design (CRD) in glass house. In first experiment various inoculating methods were tested, among which the highest number of lesions were produced in case of carborandom abbaration method with 32.07% disease severity and was found to be most effective for inoculating the test pathogen. While in second experiment evaluation of certain chemicals, antibiotics, bioagents and their combinations were tested for the Management of citrus. The data revealed that the combination of blitox-50 and strepocycline was most effective and significantly superior among all the treatments with maximum (49.91%) decrease in disease severity followed by streptocycline (45.43%).

Keywords: citrus canker, *Xanthomonas. axonopodis* pv. *citri*, antibiotics, bioagents, streptocycline

Introduction
Citrus Canker is a serious and devastating disease affecting all types of important citrus crops in many citrus producing tropical and subtropical countries around the world. The disease is believed to have originated in South East Asia, is extremely persistent when it becomes established in an area. Citrus canker was first found in the United States in 1910 not far from the Georgia – Florida border. Citrus groves have been destroyed in attempts to eradicate the disease. Citrus Canker was first reported in India from the state of Punjab by Luthra and Sattar in 1942. There are three types of citrus canker disease caused by different pathovars and variants of the bacteria viz; Canker A caused by group of *X. axonopodis* pv. *citri* strains originally found in Asia, Canker B caused by group of strains of *X. axonopodis* pv. *aurantifolli* strains originally found in South America and third is Canker C caused by the same form as Canker B i.e. *X. axonopodis* pv. *aurantifolli* originally found in Brazil (Gottwald *et al*., 2002) [8]. The severity and significance of damages caused by infection has necessitated the development of strategies to manage the disease so as to reduce crop loss. Though the use of Bordeaux mixture, antibiotics and other copper compounds were reported in earlier 50's environmentally safe and stable chemical agents rendering control at very low concentrations are yet to be developed. Reports are on hand indicating foliar sprays with copper oxychloride and streptomycin solution at shot interval are recommended to control the disease. Ravikumar, *et al.*, 2001 [17] evaluated four antibiotics against citrus canker (caused by *X. axonopodis* pv. *tritici*) of acid lime (*Citrus aurantiifolia*) viz.; streptomycin sulfate, streptocycline, bacterimycin and paushamycin. The results showed that streptomycin sulfate (500 ppm) sprayed either alone or in combination with copper oxychloride (2000 ppm) was very effective in reducing the disease severity. Therefore the objective of the research is to evaluate various toxicants against *X. axonopodis* pv.*citri* along with the various inoculating methods used for inoculating the test pathogen under glasshouse conditions.

Materials and Methods
Experimental site and details
Experiment was conducted in glasshouse, Department of plant pathology, college of agriculture, G.B.P.U.A. & T., Pantnagar. For the glasshouse experiments, one-year-old lemon plants (variety: Pant lemon) were planted in 30cm plastic pots filled with sterilized soil and they were maintained at 25–30 °C and 60% relative humidity.

Plants were regularly irrigated with water to maintain the moisture condition. Experiments were conducted using completely randomizwd design (CRD) with three replications. Lemon plants were foliarly sprayed with different treatments. Biocontrol agents used as treatments were procured from Biological control laboratory, Department of plant pathology, college of agriculture, G.B.P.U.A. & T., Pantnagar, Uttarakhand, India.

Inoculation technique and disease assessment

Bacterial suspension was prepared from 48 hour old culture of *X. axonopodis* pv.*citri* (Pantnagar isolate). The plants were than inoculated with this bacterial suspension by six different inoculating methods. For control only sterilized water was used. Immediatly after inoculation plants were sprayed with water and covered with polythene bags for 72 h to maintain high humid condition required for the disease development. Data on the number of leaves infected after inoculation and number of lesions per leaf were collected for disease assessment. Following inoculating methods were tested:

1. Carborundum Abrasion Method (Leben *et al*., 1968) [14]

The plants were inoculated with help of cotton swab on both the surfaces of leaves. The cotton swab was soaked in inoculum containing carborundum power (300 mesh) for making gentle injury and application of inoculum simultaneously. While in control plants cotton swab was soaked with distilled water.

2. Midrib injection method (Goszynska, *et al*., 2008) [7]

Bacterial cells suspension was inject-inoculated with a hypodermic needle into the midrib of leaf of one year old susceptible plants while in control plants midrib was inject-inoculated with sterilized water**.**

3. Toothpick method (Clements, *et al*., 2003) [4]

In this method wooden tooth picks were and they were then kept in flask and autoclaved. Now the test bacterium was inoculated in a flask containing Wokimonto broth and incubated at 28 ± 2^0 C. After a rich suspension of bacterial cell was made, it was poured into flask containing autoclaved tooth picks and kept for incubation. After the tooth picks were covered with bacterial growth they were ready to inoculate citrus plants. They were intoduced into the citrus leaves obliquely. Suitable control was maintained using distilled water in place of bacterial suspension.

4. Scissor Clipping method (Kauffman *et al*., 1973) [10]

In this method sterilized surgical scissors were used for inoculation. A pair of scissors was dipped in bacterial suspension then the leaves were clipped from top and margins. Suitable control was maintained using distilled water in place of inoculum suspension.

5. Pin prick method (Di *et al*., 1991 and Akhtar *et al*., 1995) [5, 1]

In this method sterile pins dipped in bacterial suspension were used to prick the leaves on sides and in the center. While in control plants pin dipped in sterilized water was used.

6. Injection Infiltration Method (Klement, 1963) [13]

The method consists of injecting bacterial suspension into the intercellular spaces of leaves with a hypodermic needle. The hypodermic needle was inserted gently under the epidermis of the leaf. The opening face of the needle should be towards the leaf. Inoculations were made by injecting 0.1 ml of bacterial suspension in the leaf mesophyll so that tissue becomes water soaked.

Evaluation of different toxicants for management of citrus canker

The experiment was laid out in completely randomised design (CRD) with 12 treatments, and each treatment was replicated thrice. The chemicals were sprayed foliarly one weak after inoculation and observations on disease severity were recorded after appearance of primary symptoms at 7 days interval for 75 days. Data on the number of leaves infected after inoculation were collected for disease assessment. Following treatments were imposed.

Data on the number of leaves infected after inoculation were collected for disease assessment. Following treatments were imposed

S. No.	Treatments
T 1	Blitox-50
T 2	Bordeaux mixture
T 3	Streptomycin
T 4	Streptocycline
T 5	Blitox-50 + Streptomycin
T 6	Blitox-50 + Streptocyclin
T 7	Vitavax
T 8	Mancozeb
T 9	Pant bioagent 1(*Trichoderma harzianum*)
T 10	Pant bioagent 2 (*Pseudomonas fluorescens*)
T 11	Pant bioagent 3 (*Trichoderma harzianum* + *Pseudomonas fluorescens*)
T 12	Untreated / Check

Under the various treatments, each treatment was sprayed @ of 1%

Disease Status

The disease status was found out by calculating disease severity, Area under disease progress curve.

1. Disease severity values measure the amount of disease on a plant in term of intensity of symptoms or damage. Disease severity was calculated by using following formula

$$\text{Disease severity}(\%) = \frac{\text{Number of leaves infected}}{\text{Total number of leaves}} \text{ X } 100$$

2. Area under disease progress curve values are a measure for level of citrus canker attack. AUDPC was calculated by using following formula suggested by Wilcoxson *et al*. (1975) [18].

$$\text{AUDPC} = \sum_{i=1}^{k} \frac{1}{2}(S_i + S_{i-1})(T_I - T_{i-1})$$

Where,

AUDPC = Area under disease progress curve

Si = Severity of citrus canker at the end of time i

k = Number of successive evaluation of citrus canker

Ti – Ti-1 = Time interval between two evaluations i and i-1 of the disease

3. Per cent reduction in disease score was calculated by using followiing folmula

$$\text{DR}(\%) = \frac{C - T}{C} \text{ X } 100$$

Where,
DR = Per cent reduction in disease severity
C = Disease severity in control
T = Disease severity in treatment

Result and Discussion

Evaluation of inoculation techniques

The data on lesion development was recorded a month after inoculation. Results are presented in Table1 and fig.1 showed that all the inoculation methods produced cankerous symptoms and lesions with variable and non uniform pattarn. The bacterial pathogen gave differential response with respect to method of inoculation. It is revealed from the data, that among the various methods tested the highest number of lesions were produced in case of carborandom abbaration method with 32.07% disease severity and was found to be most effective for inoculating the test pathogen followed by while Pin-prick method (28.17%) and clipping method (25.49%) and least effective was Tooth-pick method (14.94%).

The results are in accordance with Maji and Nath (2015) [15] who reported that carborundum abrasion method gave quicker symptom expression as well as lesion progress on leaf of cabbage *campestris* causing black rot of cabbage by *X. campestris* pv. Jabeen *et al.*, 2011 [9] in there experiment reported that the pin prick method of inoculation proved most suitable for quantitative determination of causal bacterium. Ali *et al.*, 2009 [2] inoculated the different strains and isolates to each variety of rice through clipping method.

Table 1: Disease severity after artificial inoculation methods under glasshouse conditions.

S. No	Inoculating methods	Disease severity (%)*
1	Carborundum abbaration	32.07
2	Clipping method	25.49
3	Injection method	15.51
4	Midrib injection method	19.43
5	Pin-prick method	28.17
6	Tooth-pick method	14.94
S.Em±		1.09
CD at 5%		3.36
CV (%)		8.35

*Mean of three replications

Evaluation of fungicides, antibiotics and biocontrol agents for management of citrus canker

The effect of various treatments on citrus canker severity is obtained from the experiment conducted. The data revealed that all the treatments were significantly effective in reducing the disease severity in comparision to control (unsprayed). The combination of blitox-50 and strepocycline was turned out to be most effective with maximum (49.91%) decrease in disease severity followed by streptocycline (45.43%) and blitox-50 + streptomycin (42.49%), while the least reduction in disease severity was observed by Pant bioagent 1 (11.08%). (Table2, Fig.2)

The combination product of streptocycline and streptomycin with blitox-50 was proved to be more effective than using them (streptocycline and streptomycin) alone. This study also reveals that antibiotics and copper fungicide (blitox-50 and bordeaux mixture) either used in combination or alone are more effective than other fungicides and biocontrol agents. Thus the intensity of disease was decreased significantly in treated plants than the untreated plants.

The above results were in accordance with Ravikumar, *et al.* (2001) [17] who reported that streptomycin sulphate and streptocycline in combination to blitox-50 were very effective in reducing the disease severity as compared to the untreated control. Khan *et al.* (2003) [11] evaluated efficacy of streptomycin sulphate against *X. campestris* pv.*citri* (Hasse) dye *in vitro*, and also in greenhouse condition for the management of citrus canker. Giri, *et al.* (2008) [6] found that the combination of blitox-50 + streptocycline is most effective to manage the citrus canker disease. Khodakaramian *et al.* (2008) [12] reported that several Pseudomonads bacterial strains were effective in managing the citrus bacterial canker disease and reduced the number of disease spots between 23.8 to 64.0% under green house condition. Negi and Kumar (2015) [16] reported the effect of Streptocyclin and Streptomycin against *Xanthomonas axonopodis pv.citri in vitro.*

Progress of disease

Disease progress curve is a curved line representing progress of disease over time. The progress of citrus canker under different treatments in glasshouse condition was studied by recording disease severity at weekly interval till 75th day. Disease progress curves (DPC) for sprayed (treatments) and unsprayed (control) citrus plants were developed by plotting disease severity against time.

The DPCs in Fig.3 represented the progress of citrus canker disease for various treatments and for control. Fast disease progress was recorded in control (unsprayed) plants in comparision to treated (sprayed) plants and is represented by typical sigmoid DPC, while all the treatments exhibited the slow disease development initially with prolonged lag phase. In sprayed plants with treatments blitox-50 + streptocycline and blitox-50 + streptomycin or streptocycline, streptomycin & blitox-50 alone and bordeaux mixture with less disease severity and high reduction in disease, showed slow disease development during initial phase and prolonged lag phase was recorded. While plants treated with Pant bioagent 1, vitavax and mancozeb with more disease severity and less reduction in disease, showed faster disease progress and also their DPC lines were lie near control DPC line.

Based on data recorded on progress of disease as well as analysis of DPCs it can be concluded that natural progress of citrus canker for unsprayed (control) plants is represented by typical sigmoid DPC while in treated plants spray of various treatments interfered with progress of disease and slowed it down (prolonged lag phase), thus managed the disease.

Area under disease progress curve (AUDPC)

The AUDPC is also calculated for different treatments and are presented in Table2 and Fig.4 Maximun area under disease progress curve (AUDPC) was found in control (unsprayed) plants (128.98mm^2) in comparision to treated (sprayed) plants. Least AUDPC was recorded with treatment blitox-50 + streptocycline (64.65mm^2) which also showed least disease severity followed by streptocycline and blitox-50 + streptomycin with AUDPC 70.38mm^2 and 73.99 mm^2 respectively. Thus it was observed that more AUDPC was related to more disease severity and duration. It is directly propotional to disease severity. Hence, AUDPC is very efficient, reliable hence very frequently used parameter of host, pathogen and environment interaction over time. Behlau, *et al.*, 2008 [3] studied the effect of various treatments on disease severity and AUDPC of a citrus orchard caused by *X. axonopodis* pv. *citri* and reported that there was decrease in disease severity and AUDPC on treated plants.

Table 2: Effect of fungicides, antibiotics and bioagents spray on disease severity of citrus canker recorded at weakly interval under glasshouse conditions during 2015

S. No	Disease severity (%)* Date of observations													Reduction in disease severity (%)	AUDPC (mm^2)
Treatments	22/6	29/6	6/7	13/7	20/7	27/7	3/8	10/8	17/8	24/8	31/8	7/9	Mean		
Blitox-50	5.97	6.34	6.87	7.57	9.05	10.56	12.52	15.66	17.67	19.77	21.04	20.98	12.83	30.30	89.46
Bordeaux mixture	5.53	6.18	6.62	7.36	8.68	10.03	11.66	15.12	17.06	18.58	19.77	19.83	12.20	33.73	85.14
Streptomycin	4.94	5.57	6.11	6.83	8.36	9.46	11.56	13.32	14.99	16.56	17.54	17.63	11.07	39.86	77.41
Streptocycline	3.88	4.27	4.97	5.84	6.87	8.74	10.46	12.94	14.70	15.53	16.23	16.14	10.05	45.43	70.38
Blitox-50 + Streptomycin	4.46	5.21	5.46	6.57	7.55	9.05	10.95	13.01	14.86	15.99	16.73	17.22	10.59	42.49	73.99
Blitox-50 + Streptocycline	2.99	3.59	4.48	5.26	6.62	7.54	9.51	11.25	13.70	14.92	15.57	15.25	9.22	49.91	64.65
Vitavax	8.29	8.66	9.24	10.00	11.83	13.67	15.26	17.83	19.61	21.16	22.40	23.02	15.08	18.11	105.23
Mancozeb	8.72	9.11	10.16	11.11	12.36	14.26	16.00	18.26	20.15	21.87	23.27	23.83	15.76	14.43	110.01
Pant bioagent 1	8.95	9.59	10.27	12.17	13.76	15.23	16.47	18.56	20.93	22.48	23.95	24.14	16.38	11.08	114.55
Pant bioagenr 2	7.01	7.38	8.14	9.36	10.33	11.69	14.12	16.35	18.92	21.11	21.77	22.06	14.02	23.87	97.84
Pant bioagent 3	6.51	7.27	7.78	8.56	9.87	10.93	13.24	16.17	18.28	20.55	21.22	21.27	13.47	26.84	94.06
Control	9.74	10.26	11.24	12.99	15.03	17.27	19.47	21.47	23.68	26.06	26.79	27.01	18.42		128.98
S.Em±	0.38	0.46	0.57	0.46	0.44	0.62	0.51	0.60	0.77	0.58	0.64	1.24			
CD at 5%	1.12	1.35	1.67	1.34	1.28	1.81	1.50	1.76	2.26	1.71	1.88	3.62			
CV	10.42	11.54	13.01	9.26	7.60	9.32	6.63	6.62	7.51	5.20	5.43	9.09			

*Mean of three replications

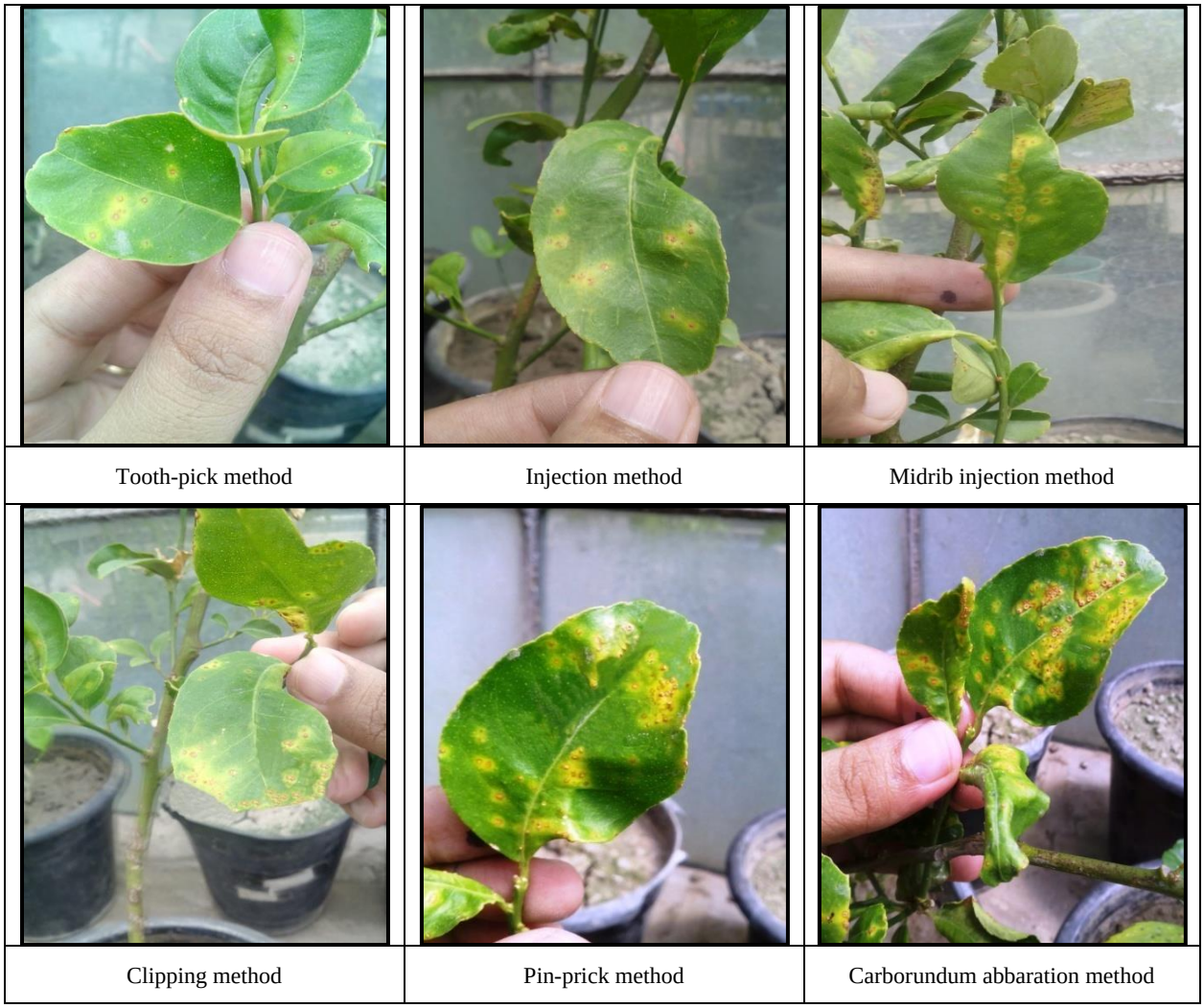

Fig 1: Evaluation of different inoculation methods under glasshouse conditions

Control citrus plant

Treated citrus plant (Blitox-50+ Streptocycline)

Fig 2: Disease in control and treated citrus plants under glasshouse conditions

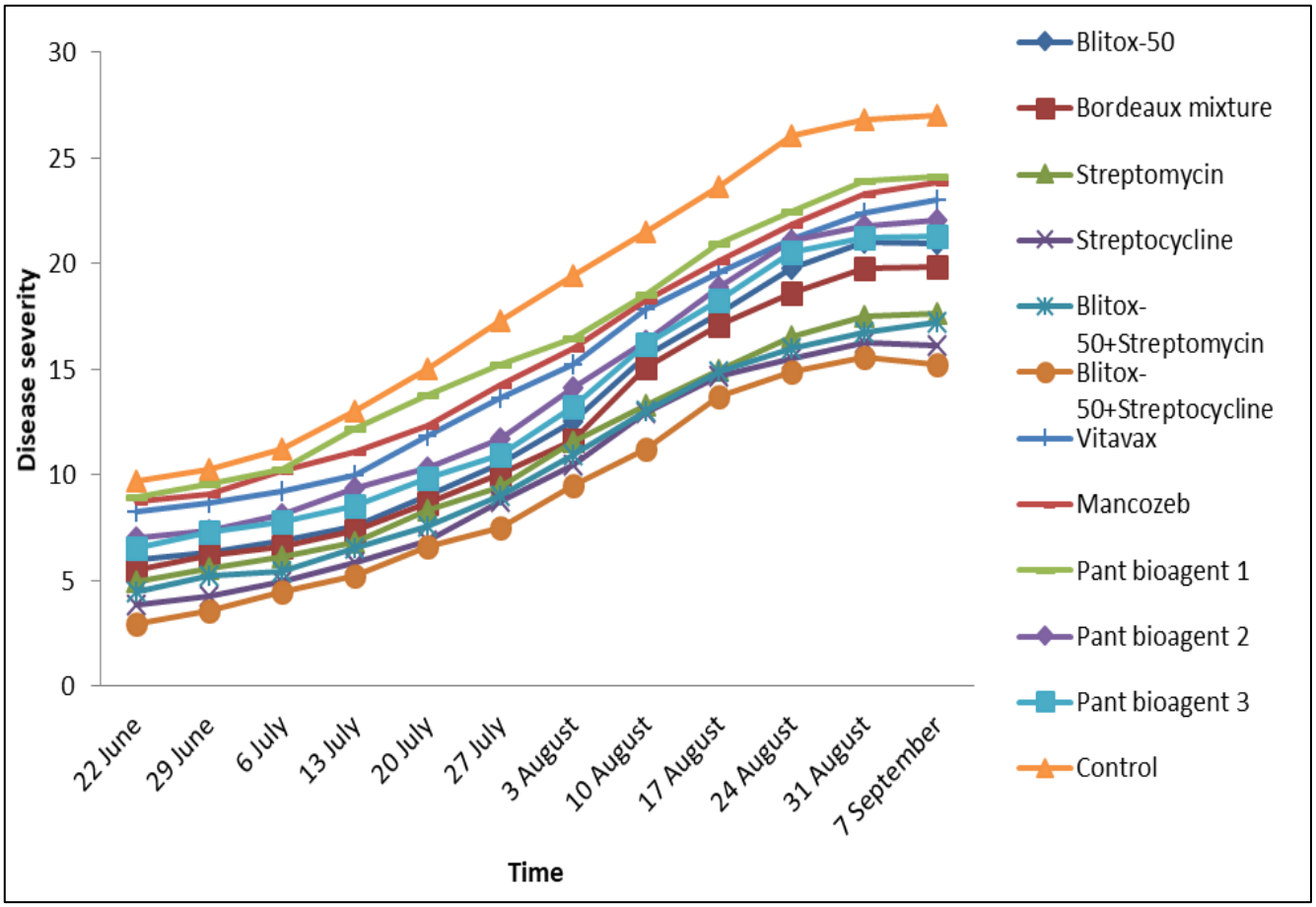

Fig 3: Effect of fungicides, antibiotics and bio agents on the progress of citrus canker in lemon

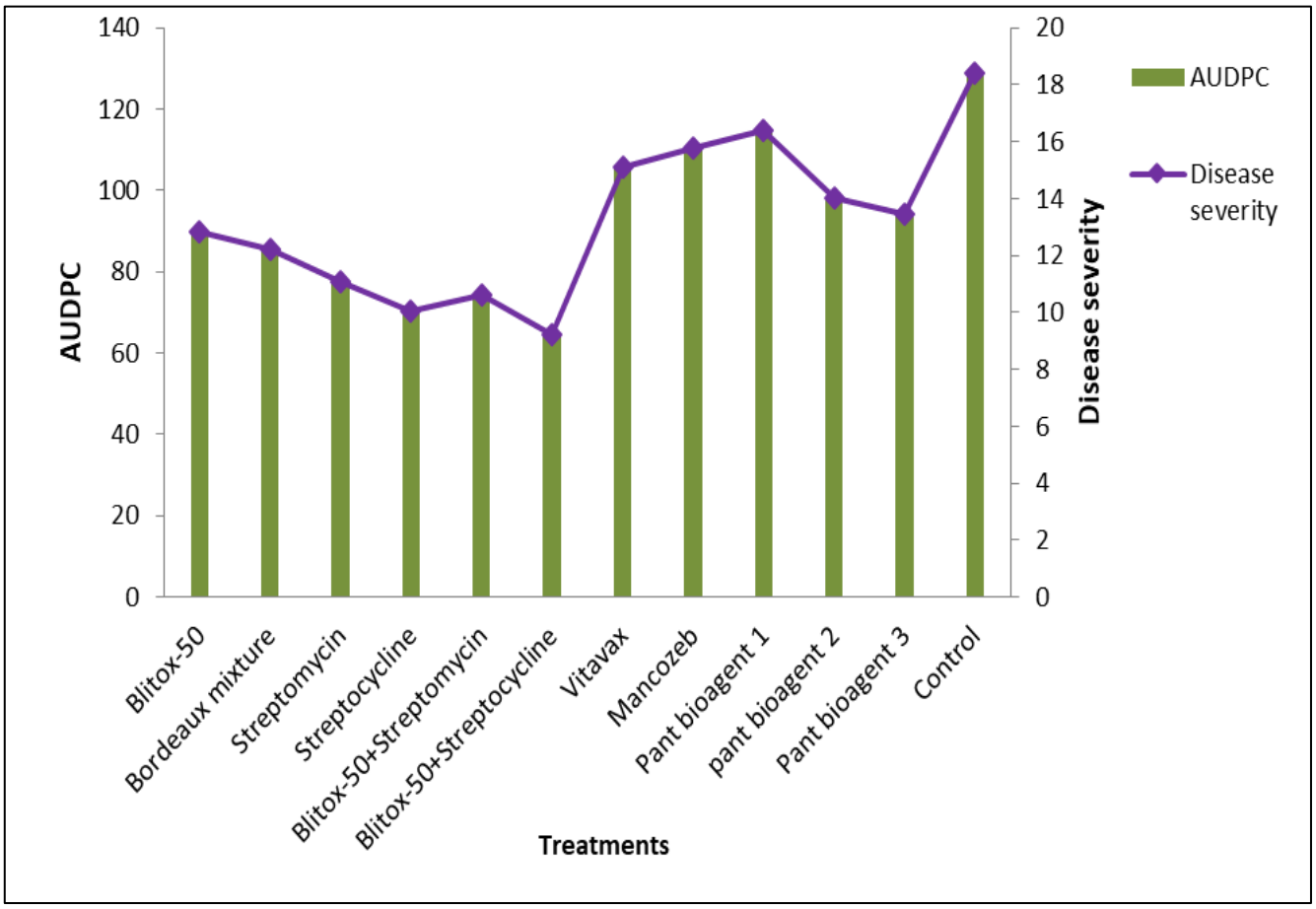

Fig 4: Effect of fungicides, antibiotics and bioagents on Disease severity and AUDPC of citrus canker in lemon

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