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In vitro bio efficacy of different antibiotics, bioagent and botanical against (*Xanthomonas axonopodis* pv. *citri*.) causing bacterial canker of acid lime

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Abstract
Four antibiotics (@100 ppm, 250 ppm and 500 ppm conc), five botanicals (@ 5%, 10% and 15% conc.) and four bioagents *viz*., *Pseudomonas fluorescens*, *Bacillus subtilis*, *Trichoderma harzianum* and *Trichoderma viride* were evaluated by *in vitro* against *Xanthomonas axonopodis* pv. *citri* (Hasse). Among the different antibiotics and antibacterial chemicals, streptomycin sulphate @ 500 ppm, among botanicals ginger (*Z. officinale*) @ 15% and among the bioagents *Trichoderma harzianum* showed maximum inhibition zone as 24.3mm, 12.3mm and 23.6mm respectively. The fungicide vitavax, turmeric (botanical) and *Bacillus subtilis* (bioagent) were found less effective against the *Xanthomonas axonopodis pv. citri*.

Keywords: Xanthomonas, pseudomonas, bacillus, trichoderma

Introduction
Citrus, including important crops like oranges, lemons, grapefruit, pomelo and lime. It grown in the world with tropical or subtropical climates. It is used as best source of vitamin C, sugars, amino acids and other nutrients. It is one of the important fruit crops of the world. It occupies an important place in the wealth and economy of India as third largest fruit industry after mango and banana. In India, citrus is grown in an area of 1,024 thousand hectares with a total production of 11,581 thousand MT (Anonymous, 2016) [2]. In Maharashtra state, citrus is grown on 287.6 thousand hectare with production of about 1725.1 MT fruits annually (Anonymous, 2016) [2]. The most important commercial citrus cultivars in India are the mandarin (*Citrus reticulata*) followed by sweet orange (*Citrus sinensis*) and acid lime (*Citrus aurantifolia*). Citrus canker is one of the most destructive and predominant disease on acid lime in Vidarbha region of Maharashtra. Citrus bacterial canker (CBC), caused by *Xanthomonas citri* subsp. *citri* (Schaad *et al.,* 2006) [14] is one of the most devastating diseases throughout the world that affects many kind of commercial citrus varieties. The origin of CBC is known but thought to have originated from south-east Asia or India and then widely distributed around the world (Civerolo, 1984 and Vernière *et al.,* 1991) [4]. According to, Fawcett and Jenkins (1933) [6] the origin of citrus canker is either from India, Java or some other region of Asia. It was first identified in Florida (USA) in 1915 and in India was reported from Punjab in 1942. The main symptoms of CBC are hyperplasia type lesions on leaves, fruit and stems. The severe infections Causes leaf abscission, twig dieback and premature fruit drop (Gottwald *et al.,* 2002) [7]. The bacterium was first named as *Pseudomonas citri* (Hasse, 1915) [8]. In 1939 it was classified as genus *Xanthomonas* spp. *(X. citri),* then reclassified in 1980 (Dye *et al.,* 1980) [5] as *Xanthomons campestris* pv. *citri* due to inadequate phenotypic data (Young *et al.,* 1978) [16].

Materials and Methods
Isolation and purification of *Xanthomonas axonopodis pv. citri* (*Xac*)
The Infected leaves of citrus canker were collected from declining orchard and cut into small pieces and crush it into a drop of distilled water. Streak that smear on NA medium plate with the help of wire loop and incubated for 24 hrs. at 30^0 C. A loop full culture was streak on another media plate for pure culture.

***In vitro* evaluation of antibiotics**

Sensitivity of the different isolates was tested by modified paper disc assay method. Desired concentration of antibiotics and chemicals *viz.*, streptomycin sulphate, bromopol, Kasugamycin, vitavax etc. were freshly prepared in sterile distilled water. The bacterium *Xanthomonas axonopodis* pv. *citri* was multiplied by inoculating a loop full culture in 250 ml conical flask containing 100 ml of nutrient broth medium and incubated at 28±2^{0}C for 72 hours. The 20 ml bacterial suspension was added to molten and cooled 1000 ml nutrient agar medium at temperature 28±2^{0}C.The seeded medium was thoroughly mixed and poured into the sterilized petriplates and allowed to solidify. The solutions of test chemicals were made with 100, 250 and 500 ppm concentrations and vitavax with 0.1%, 0.2% and 0.3% concentrations. The filter paper disc (what man no. 42) measuring 5 mm in diameter were soaked in the respective solution for 5 minutes and transferred onto the surface of the seeded NA medium in petriplates. The plates were incubated at 28±2^{0}C for 72 hours and observed for the production of inhibition zone around the filter paper discs. The results obtained were analyzed statistically. The paper disc soaked in sterile distilled water served as control.

***In vitro* evaluation of bioagents**

Four biocontrol agents *viz., Trichoderma viride, Trichoderma harzianum, Pseudomonas fluorescens* and *Bacillus subtilis* were evaluated for their efficacy against the growth of *X. axonopodis* pv. *citri* by inhibition zone assay method. The bacterium *Xanthomonas axonopodis* pv. *citri* was multiplied by inoculating a loop full culture in 250 ml conical flask containing 100 ml of nutrient broth medium and incubated at 28±2^{0}C for 72 hours. The 20 ml bacterial suspension was added to molten and cooled 1000 ml nutrient agar medium. The seeded medium was thoroughly mixed and from that 15 to 20 ml was poured into the sterilized petriplates and allowed to solidify. A loop full culture of each of the antagonistic organism was placed in the centre of petriplates containing the seeded medium. In case of fungal antagonists, a mycelial disc of 5 mm (diameter) size taken from actively growing culture was placed in the centre of the plates. The inoculated plates were incubated at 28±2°C for 72 hours and observed for the production of inhibition zone around antagonistic organism.

***In vitro* evaluation of botanicals**

Sensitivity of the different isolates was tested by modified paper disc assay method. Desired concentration of botanicals extracts *viz.*, neem leaf extract (*Azadiracta indica*), garlic clove extract (*Allium sutivum*), tulsi leaf extract (*Ocimum sanctum*), turmeric rhizome extract (*Curcuma longa*), ginger rhizome extract (*Zingiber officinales*) etc. were freshly prepared in sterile distilled water. Before preparation of extract, each botanical were dipped in 0.1% mercuric chloride ($HgCl_2$) for one minute. The extracts were prepared by grinding 100 g of washed leaf/ clove/rhizome of different species in 100ml distilled water with mixture cum grinder. These were then filtered through what man no.1 filtered paper using funnel and volumetric flask (100 ml capacity). The final clear filtrate obtained was treated as 100% concentration of these extracts. Desired quantity required for preparation of 5, 10 and 15% concentrations was taken from this 100% standard extract. These extracts were then evaluated *in vitro* against *Xanthomonas axonopodis* pv. *citri* by applying inhibition zone technique. The bacterium *Xanthomonas axonopodis* pv. *citri* was multiplied by inoculating a loop full culture in 250 ml conical flask containing 100 ml of nutrient broth medium and incubated at 28±2^{0}C for 72 hours. The 20 ml bacterial suspension was added to molten and cooled 1000 ml nutrient agar medium at temperature 28±2^{0}C.The seeded medium was thoroughly mixed and poured into the sterilized petriplates and allowed to solidify. The filter paperdisc (what man no. 42) measuring 5 mm in diameter were soaked in the respective prepared extracts solution for 5 minutes and transferred onto the surface of the seeded NA medium in petriplates. The plates were incubated at 28± 2^{0}C for 72 hours and observed for the production of inhibition zone around the filter paper discs. The results obtained were analyzed statistically. The paper disc soaked in sterile distilled water served as control.

Result and Discussion

Result (table-1) indicated that the antibiotics tested at various concentrations (each @ 100, 250 and 500 ppm) and vitavax @ 0.1%, 0.2% and 0.3% concentrations significantly inhibited the growth of *Xanthomonas axonopodis* pv. *citri* over the untreated control.

At 100 ppm, bacterial inhibition zone was ranged from 6.3 mm (Kasugamycin) to 7.6mm (streptomycin sulphate). However, it was significantly highest with streptomycin sulphate 7.6 mm (T_1 Xac-6), followed by bromopol 6.6 mm (T_7 Xac-6) and kasugamycin 6.3 mm (T_4 Xac-4).

At 250 ppm, bacterial inhibition zone was ranges from 8.3 mm (kasugamycin) to 14.6 mm (streptomycin sulphate). However it was significantly highest with streptomycin sulphate 14.6 mm (T_2 Xac-8), followed by bromopol 10.6 mm (T_8 *Xac*-1) and kasugamycin 8.3 mm (T_5 Xac-4).

At 500 ppm, bacterial inhibition zone was ranges from 15.3mm (kasugamycin) to 24.3 mm (streptomycin sulphate). However, it was significantly highest with streptomycin sulphate 24.3 mm (T_3 Xac-8), followed by bromopol 18.6 mm (T_9 Xac-5) and kasugamycin 15.3 mm (T_6 Xac-3). Jambenal *et al.* (2011) reported that streptomycin sulphate at 500 ppm is significantly best for inhibiting the growth of bacterium *Xanthomomas axonopodis* pv. *viticola*.

Jadhav *et al*. (2018) tested the efficacy of different antibiotics against *Xanthomonas axonopodis* pv. *citri* and revealed the similar result as streptomycin sulphate showed the maximum inhibition zone of inhibition followed by bromopol and kasugamycin at 100 ppm, 250 ppm and 500 ppm concentrations.

Isolates were tested with vitavax @ 0.1%, 0.2% and 0.3% concentrations and it was significantly highest inhibition in growth of bacterium as 11.0 mm (T_{12} Xac-6) at 0.3% followed by 7.6 mm (T_{11} Xac-9) and 4.3 mm (T_{10} Xac-6) at 0.1% concentration.

Table 1: Efficacy of different antibiotics and chemicals against ten isolates of *Xanthomonas axonopodis* pv. *Citri*

Tr. No.	Treatments with concentration	Zone of inhibition (mm)									
		Xac-1	Xac-2	Xac-3	Xac-4	Xac-5	Xac-6	Xac-7	Xac-8	Xac-9	Xac-10
T_1	Streptomycin sulphate 100ppm	7.3	4.6	6.0	7.3	4.0	7.6	6.3	7.3	6.6	0.0
T_2	Streptomycin sulphate 250ppm	12.3	7.3	8.3	12.6	7.6	11.3	13.3	14.6	8.3	7.3
T_3	Streptomycin sulphate 500ppm	18.3	13.3	12.6	18.6	15.6	22.3	19.3	24.3	14.3	11.6
T_4	Kasugamycin 100ppm	2.0	2.3	4.0	6.3	2.6	6.0	2.6	4.0	0.0	0.0
T_5	Kasugamycin 250ppm	7.3	7.0	6.0	8.3	6.3	7.6	7.6	8.0	6.3	2.3
T_6	Kasugamycin 500ppm	9.3	13.6	15.3	10.3	10.6	13.3	11.6	12.6	13.3	9.6
T_7	Bromopol 100ppm	6.0	4.0	4.6	6.3	6.3	6.6	4.0	6.0	2.3	0.0
T_8	Bromopol 250ppm	10.6	7.3	6.3	9.6	6.6	8.6	9.3	8.3	6.6	4.6
T_9	Bromopol 500ppm	16.6	17.0	10.6	11.6	18.6	11.6	12.6	11.3	15.3	11.3
T_{10}	Vitavax 0.1%	2.3	0.0	0.0	3.6	2.0	4.6	0.0	4.3	4.3	0.0
T_{11}	Vitavax 0.2%	4.6	4.0	2.3	6.0	4.6	7.3	2.3	7.3	7.6	2.3
T_{12}	Vitavax 0.3%	7.6	8.3	7.6	9.3	8.6	11.0	7.3	8.0	9.3	7.3
T_{13}	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	SE(m)±	0.23	0.19	0.26	0.21	0.15	0.24	0.16	0.17	0.45	0.09
	CD(P)=0.01	0.89	0.73	0.99	0.82	0.57	0.91	0.60	0.64	1.71	0.33

Efficacy of botanicals

Result (Table-2) indicated that, the botanicals tested at various concentrations (each @ 5%, 10% and 15%) significantly inhibited the growth of *Xanthomonas axonopodis* pv. *citri* over the untreated control.

At 5%, inhibition zone with test aqueous phytoextracts were ranged from 0.0 mm (turmeric) to 6.6 mm (ginger), however, it was significantly highest with ginger 6.6 mm (T_1- Xac- 6), followed by neem 5.6 mm (T_4- Xac-8), garlic 4.6 mm (T_7- Xac-1), tulsi 4.0 mm (T_{10}-Xac-9) whereas, significantly least inhibition zone was found with turmeric 0.0 mm (T_{13}- Xac-1 to 10).

At 10%, inhibition zone with test aqueous phytoextracts were ranged from 4.0 mm (turmeric) to 9.6 mm (ginger), however, it was significantly highest with ginger 9.6 mm (T_2- Xac 6) followed by neem 8.0 mm (T_5- Xac 8), garlic and tulsi 7.3mm (T_8- Xac 1 and T_{11}- Xac 9). Whereas, significantly least inhibition zone was found with turmeric 4.0 mm (T_{14}- Xac 4&9).

At 15%, inhibition zone with test aqueous phytoextract were ranged from 5.0 mm (turmeric) to 12.3 mm (ginger); however, it was significantly highest with ginger 12.3 mm (T_3 Xac-6), followed by neem 10.6 mm (T_6 Xac-8), garlic 9.6 mm (T_9 Xac-1), tulsi 8.6 mm (T_{12} Xac-1), whereas, significantly least inhibition zone was found with turmeric 5.0 mm (T_{15} Xac– 4 & 6). These observations are in the conformity with the report of Jadhav *et al.* (2018) [9], Naik *et al.* (2018) [11] Ambadkar *et al.* (2015) [1], Antre *et al.* (2016) [3] evaluated the different botanicals (@10% and 20% conc.) against *Xanthomonas axonopodis* pv. *citri* under *in vitro* condition and found that, ginger followed by neem was efficient among the different botanicals. Raju *et al.* (2012) tested the efficacy of several botanical and showed similar result as garlic bulb extract found effective against the bacterium *Xanthomonas axonopodis* pv. *pinicae.*

Efficacy of bioagents

The efficacy of bioagents was tested against ten isolates of *Xanthomonas axonopodis* pv. *citri* by agar well method and data is presented in Table-3 revealed that significant differences among the different treatment.

Table 2: Efficacy of different botanicals against ten isolates of *Xanthomonas axonopodis* pv. *Citri*

Tr. No.	Treatments	Conc.	Zone of inhibition (mm)									
			Xac-1	Xac-2	Xac-3	Xac-4	Xac-5	Xac-6	Xac-7	Xac-8	Xac-9	Xac-10
T_1	Ginger (*Zingiber officinale*)	5%	4.3	0.0	2.0	4.3	4.0	6.6	4.6	6.3	5.3	2.3
T_2	Ginger (*Z. officinale*)	10%	8.6	4.6	5.6	8.0	7.6	9.6	8.6	9.3	9.0	4.3
T_3	Ginger (*Z. officinale*)	15%	10.6	8.6	9.0	9.6	9.3	12.3	9.6	12.0	11.3	8.3
T_4	Neem (*Azadiracta indica*)	5%	4.0	0.0	0.0	4.0	2.3	5.3	4.3	5.6	4.6	0.0
T_5	Neem (*A. indica*)	10%	7.6	4.3	4.6	7.0	5.3	6.6	6.6	8.0	7.3	4.0
T_6	Neem (*A. indica*)	15%	9.6	7.6	8.3	9.6	9.0	10.0	10.3	10.6	9.6	7.3
T_7	Garlic (*Allium sativum*)	5%	4.6	0.0	0.0	2.0	0.0	4.3	4.0	4.0	4.3	0.0
T_8	Garlic (*A. sativum*)	10%	7.3	4.0	0.0	5.6	4.0	7.0	6.0	6.6	6.6	2.0
T_9	Garlic (*A. sativum*)	15%	9.6	7.3	7.0	8.3	8.0	8.3	9.3	8.6	7.0	4.6
T_{10}	Tulsi (*Ocimum sanctum*)	5%	2.0	0.0	0.0	2.0	0.0	2.0	2.0	0.0	4.0	0.0
T_{11}	Tulsi (*O. sanctum*)	10%	6.6	4.0	0.0	5.6	0.0	6.0	5.3	4.0	7.3	0.0
T_{12}	Tulsi (*O. sanctum*)	15%	8.6	7.6	5.6	7.6	6.0	7.3	6.6	5.3	8.0	4.0
T_{13}	Turmeric (*Curcuma longa*)	5%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T_{14}	Turmeric (*C. longa*)	10%	2.0	0.0	0.0	4.0	0.0	2.0	0.0	2.0	4.0	0.0
T_{15}	Turmeric (*C. longa*)	15%	4.3	0.0	2.0	5.0	0.0	5.0	0.0	0.0	4.3	0.0
T_{16}	Control		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	SE(m)±		0.13	0.09	0.11	0.20	0.17	0.21	0.14	0.15	0.22	0.13
	CD(P)=0.01		0.47	0.35	0.42	0.77	0.66	0.80	0.53	0.56	0.82	0.52

Table 3: Efficacy of bioagents against ten isolates of *Xanthomonas axonopodis* pv. *Citri*

TN.	Treatments	Zone of inhibition (mm)									
		Xac-1	Xac-2	Xac-3	Xac-4	Xac-5	Xac-6	Xac-7	Xac-8	Xac-9	Xac-10
T_1	*Pseudomonas fluorescens*	0.0	2.3	4.3	9.6	0.0	13.3	6.6	10.6	4.6	2.3
T_2	*Bacillus subtilis*	0.0	0.0	2.6	8.3	0.0	10.6	6.0	8.3	4.0	0.0
T_3	*Trichoderma harzianum*	2.0	4.6	6.3	15.6	2.6	23.6	16.3	15.6	4.6	0.0
T_4	*Trichoderma viride*	0.0	0.0	12.3	14.3	0.0	22.3	8.6	18.6	11.3	0.0
T_5	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	SE(m)±	0.05	0.14	0.20	0.19	0.14	0.20	0.15	0.23	0.14	0.11
	CD(P)=0.01	0.22	0.59	0.85	0.82	0.57	0.84	0.63	0.99	0.60	0.47

Among the four antagonistic agents tested, *Trichoderma harzianum* produced the maximum metabolites in the culture medium resulting in the production of significantly superior 23.6 mm (T_3 Xac-6) zone in inhibiting the growth of *Xanthomonas axonopodis* pv. *citri* followed by *Trichoderma viride* 22.3 mm (T_4 Xac-6), *Pseudomonas fluorescens* 13.3 mm (T_1 Xac- 6); whereas, least inhibition zone was found in *Bacillus subtilis* 10.6 mm (T_2 Xac-6). Similar finding were noted by Patil *et al.* (2017) [12] tested the antagonistic activity of three bioagents and confined that *Trichoderma harzianum* gives maximum inhibition zone (22.86 mm) followed by *Pseudomonas fluorescens* (17.20 mm) and *Bacillus subtilis* (15.00 mm).

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