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Management of citrus canker bacterium by using botanicals and chemicals

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Abstract
Citrus is the important fruit crop in the world. It occupies an important place in the wealth and economy of India as third largest fruit industry after mango and banana. Citrus canker caused by *Xanthomonas citri* subsp. *citri* is a damaging disease of acid lime. It is identified as a major threat in the region affecting leaves, twigs, petioles, branches, fruit stalk and fruits that causes considerable damage both quantitatively and qualitatively. Canker infected sample of acid lime leaves were collected from different agroclimatic regions of Vidarbha. The bacterium were isolated, identified and purified cultures were maintained on NA slants. The virulence test of causal organism was confirmed along with symptoms in pot culture experiment using syringe method. Pathogenic ability of different isolates was confirmed and observed that isolate *Xac*-2 was comparatively highly virulent to initiate water soaked lesion and fully developed symptoms within 15 to19 days.
Efficacy of different combination of chemicals, botanicals and bio-agents against *Xanthomonas citri* subsp. *citri* was assessed. Among the alcoholic and aqueous plant leaf extracts neem (*Azardiricta indica*) at all concentrations found most effective in alcoholic extracts by developing 13.45 mm, 11.70 mm, 10.12 mm and 8.2 mm of inhibition zone at 20% 15%, 10% and 5% respectively. Among fruits extracts, alcoholic extract of Kokam (*Garcinia indica*) gives highest inhibition zones 17 mm, 15.0 mm, 6.9 mm and 0 mm at 20% 15%, 10% and 5% concentrations and found to be most effective. Among flower extracts, alcoholic extract of lakh (*Lathyrus odoratus*) showed highest inhibition zones 13.14 mm, 9.23 mm, 8.00 mm and 6.23 mm at 20% 15%, 10% and 5% concentrations. Among the extracts of different vegetative parts of plants alcoholic extract of garlic (*Allium sativum*) produced highest inhibition zones 16.20 mm, 13.40 mm, 7.23 mm and 0.00 mm at 20% 15%, 10% and 5% concentrations.

Keywords: Citrus canker, botanicals, chemicals

Introduction
Citrus is one of the important fruit crop of the world as well as India. It is third largest fruit industry after mango and banana occupying an important place in the wealth and economy of country. It belongs to the family *Rutaceae* and has a great demand due to its nutritive value, aroma and taste. Citrus is popular in both fresh and processed form. It is known for its high nutritive and refreshing value, taste and attractive fragrance. In India, 10.60 million hectare area is under citrus cultivation with production of 125.10 lakh metric ton (Anonymous, 2018) [4]. In Maharashtra state, citrus is grown on 287.6 thousand hectare with production of about 1725.1 metric ton fruits annually (Anonymous, 2016) [3]. 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* is one of the most devastating diseases throughout the world that affects many kind of commercial citrus varieties.
There are number of programmers are working on the management of disease but the complete solution to the problem is yet to be found except the eradication. The antibiotics with bactericidal nature are the most commonly used for the management of disease. Residual effect of chemicals causes health hazards in human being and in recent years there has been major trust developing on residue free organic citrus production. Plants have ability to synthesize aromatic secondary metabolites, like phenols, phenolic acids, quinones, flavones, flavonoids, flavanols, tannins and coumarins. These groups of compounds show antimicrobial effect and serves as plant defense mechanisms against pathogenic microorganisms. Under

biological control of plant diseases various antagonistic organisms have been identified which fight against the pathogen by different mechanisms.
Taking the task into consideration efficient botanicals and bioagents were explored to fit into the disease management.

Material and methods

A. Isolation and purification of *Xanthomonas citri* subsp. *citri*

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. Take a loop full culture and streak on another media plate for pure culture.

B. Pathogenicity

The seedlings were sufficiently watered and exposed to sunlight. To prove the pathogenicity of each isolate collected from ten regions, isolates were separately multiplied in nutrient broth (200 ml) in conical flasks by inoculating a loopful of bacterial culture. The inoculated flasks were incubated for three days at 28±20C. The seedlings of acid lime were used for inoculation of isolate. Inoculation was done by syringe inoculation method. The plants were maintained under humid condition. The observations were recorded on the basis of number of inoculations made and number of spots exhibited diseased symptoms plants. Simultaneously plant sprayed with sterilized distilled water served as control.

C. *In vitro* evaluation of botanicals

Twenty nine botanicals were use in the present study and the information regarding the botanicals and specific part/parts used for evaluation against *Xanthomonas citri* subsp. *citri* is presented in table 1.

Table 1: Plants and their plant parts used for evaluation against *Xcc*

S. No.	Common name	Botanical name	Plant part used
1	Garlic	*Allium sativum*	Clove
2	Neem	*Azardiricta indica*	Leaf
3	Tulsi	*Ocimum indica*	Leaf
4	Ginger	*Zingiber officinale*	Rhizome
5	Turmeric	*Curcuma longa*	Rhizome
6	Onion	*Alium sepa*	Bulb
7	Chilly	*Capsicum annum*	Fruit
8	Rose	*Rosa demascina*	Flower
9	Marigold	*Tagetes erecta*	Flower
10	Marigold	*Tagetes erecta*	Leaf
11	Custard apple	*Annona reticulata*	Leaf
12	Accacia	*Acacia auriculiformis*	Leaf
13	Anole	*Emblica officinalis*	Fruit
14	Bale	*Aegle marmelos*	Leaf
15	Pomegranate	*Punica granatum*	Fruit
16	Pomegranate	*Punica granatum*	Fruit
17	Betelvine	*Piper betle*	Leaf
18	Ashok / Mast-Tree	*Polyalthia longifolia*	Flower
19	Lakh / Sweet pea	*Lathyurus odoratus*	Leaf
20	Ekdandi	*Tridax procumbens*	Leaf
21	Tamarind	*Tmarindus indica*	Fruit
22	Clove	*Eugenia caryophyllata*	Clove
23	Behda	*Terminalia bellirica*	Nut
24	Jayfal	*Myristica fragrans*	Nut
25	Kokam	*Garcinia indica*	Fruit
26	Shevga / Drumstick	*Moringa oleifera*	Leaf
27	Dalchini /Cinnamon	*Cinnamomum verum*	Bark
28	Nilgiri	*Eucalaptus citriodora*	Leaf
29	Hibiscus	*Hibicus subdariffa*	Flower

Preparation of extracts

Aqueous and organic plant extracts of botanicals used in the present study was prepared as given below.

Preparation of aqueous/alcoholic plant extracts

Sensitivity of the different isolates was tested by modified paper disc assay method. Desired concentration of botanicals extracts were freshly prepared in sterile distilled water. Before preparation extract, each botanical were dipped in one per cent sodium hypochloride for one minute. The extracts were prepared by grinding 100 g of washed leaf/ petals/ fruit of different species in 100 ml distilled water (for aqueous extract), Alcohol (for alcoholic extract) with mixture-cum grinder. These were then filtered through Whatman No.1 filter paper using funnel and volumetric flask (100 ml capacity). The final clear filtrate obtained was treated as 100 % concentration of these extracts.

D. *In vitro* evaluation of chemicals

Sensitivity of the different isolates was tested by modified paper disc assay method. Desired concentration of antibiotics & chemicals viz., streptomycin sulphate, bromopol, kasugamycin, vitavax etc. were freshly prepared in sterile distilled water. The bacterium *Xanthomonas citri* subsp. *citri* was multiplied by inoculating the loop full culture in 250 ml conical flask containing 100 ml of nutrient broth medium and incubated at 28 ± 2^0C for 72 hours. The 20 ml bacterial suspension was added to molten and cooled 1000 ml nutrient agar medium at temperature 28 ± 2^0C. 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. The filter paper disc (Whatman No. 42) measuring 5 mm in diameter were soaked in the respective solution for 5 minutes and transferred to 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.

Results and discussions

A. Isolation of Xcc from diseased specimens

Total ten isolates of *Xanthomonas citri* subsp. *citri* (Xcc) were isolated from infected leaf of citrus collected from different agro-climatic zone of Vidarbha. The isolates were purified by streak plate method. Pale yellow to yellow pigmented bacterial colonies were formed on nutrient agar medium after 72 hours of incubation at 28±20C which were identical to *Xanthomonas citri* subsp. *citri*. These isolates were maintained on NA slants and catalogued as under table 2. And used for further study.

Table 2: Location wise acid lime samples collected for isolation from agro- climatic zones of Vidarbha

S. No.	Name of district (location)	Agro climatic zone of Vidarbha
1	Bhandara	East Vidarbha zone
2	Nagpur	Central M.S zone
3	Gadchiroli	East Vidarbha zone
4	Gondia	East Vidarbha zone
5	Chandrapur	Central Vidarbha zone
6	Wardha	Central Vidarbha zone
7	Akola	Central M.S plateau zone
8	Yavatmal	Central Vidarbha zone
9	Amravati	Central M.S plateau zone
10	Washim	Central M.S plateau zone

B. Pathogenic ability of *Xanthomonas citri* subsp. *citri* on acid lime leaves

After the inoculation of bacteria, the symptoms of the disease were observed about 16-21 days depending upon the isolate. Initially the very weak symptoms were observed like slightly raised small blister- like lesions. The symptoms started turning tan to brown and a water-soaked margin appeared around the leaves surrounded by a yellow halo forming the visible lesions resembling canker symptoms later. Pathogenic ability of all different isolates of Xcc were confirmed and found that isolate Xcc-2 showed highly pathogenic to initiate minute canker lesion and fully developed symptoms after 19 days. While Xcc-4, Xcc-7 and Xcc-10, were found to produce moderate in symptoms. The isolates Xcc-3, Xcc-5, Xcc-6, Xcc-8, Xcc-9 was found to be poor in producing the canker symptoms (table 5).

Katkar *et al*. (2016) [13] categorized the fifteen isolates of Xcc on the basis of symptoms development on leaves and day taken for appearance of the symptoms as no canker (-), weak canker (+), moderate canker (++) and strong canker (+++) as presented in table 5.

Jabeen *et al*. (2011) [11] who reported that three methods of inoculation, clipping, pin prick and paint brush were tested both on detached leaves and on attached leaves in vitro and in vivo experiments. All these methods were effective for artificial inoculation, but pin prick method was found to be more efficient in detached leaf assay produced large size lesion.

Table 3: Inhibitory effect of different alcoholic leaf extract on *Xanthomonas citri* subsp. *Citri*

S. No.	Botanicals		Concentration (%) Inhibition zone (mm)			
	Scientific name	Common name	5 %	10 %	15 %	20 %
1	*Azardiricta indica*	Neem	8.2	10.12	11.70	13.45
2	*Ocimum indica*	Tulsi	6.7	8.77	10.23	11.8
3	*Tagetes erecta*	Marigold	0	0	0	7
4	*Annona reticulate*	Anole	0	0	7	8
5	*Acacia auriculiformis*	Accacia	6	7	8	11
6	*Aegle marmelos*	Bale	6	6	8	10
7	*Piper betle*	Betlevine	8	8	6.9	12
8	*Polyalthia longifolia*	Ashok	7	7.5	8	9
9	*Tridax procumbent*	Ekdandi	0	0	6	7.5
10	*Moringa oleifera*	Shevaga	0	0	0	7.5
11	*Eucalaptus citriodora*	Nilgiri	5.3	6	6.7	7
	SE(m)±		1.82	1.42	0.75	1.80
	CD(P=0.01)		5.47	4.3	2.14	5.45

The result revealed that, among the alcoholic and aqueous plant leaf extracts neem (*Azardiricta indica*) at all concentrations found most effective in alcoholic extracts by developing 13.45 mm, 11.70 mm, 10.12 mm and 8.2 mm of inhibition zone at 20% 15%, 10% and 5% respectively. The second best effective botanical tulasi (*Ocimum indica*) produced 11.8 mm, 10.23 mm, 8.77 mm and 6.77 mm of inhibition zone at 20% 15%, 10% and 5% concentrations in alcoholic extract.

The results of the present investigation are in confirmation with the results obtained by Alne and Swami (2016) [1] who evaluated antibacterial activity of plant extracts against *Xanthomonas axonopodis* pv. *punicae* causing bacterial blight of pomegranate. They found that alcoholic extract of neem and tulsi were significantly effective against *Xanthomonas axonopodis* pv. *punicae* and Prakash and Karmegam, (2012)

[14] evaluated *in vitro* antibacterial activity of certain plant extracts against *Xanthomonas campestris* pv. *citri*. They found that alcoholic extract of neem was significantly effective against *Xanthomonas campestris* pv. *citri*.

Table 4: Inhibitory effect of different alcoholic fruit extract on *Xanthomonas citri* subsp. *citri.*

S. No.	Botanicals		Concentration (%) Inhibition zone (mm)			
	Scientific name	Common name	5 %	10 %	15 %	20 %
1	*Capsicum annum*	Chilly	0	6.5	7	10
2	*Emblica officinalis*	Anole	0	6.5	7	12.5
3	*Punica granatum*	Pomegranate	0	5.8	6.5	8.2
4	*Punica granatum*	Pomegranate	0	6.5	11	12
5	*Tamarindus indica*	Tamarind	0	6	7.5	9.5
6	*Terminalia bellirica*	Behda	0	6	9	11
7	*Myristica gragrans*	Jayfal	0	7.5	11	11
8	*Garcinia indica*	Kokam	0	6.9	15	17
	SE(m)±		-	0.22	0.96	0.78
	CD(P=0.01)		-	0.65	2.89	2.35

The alcoholic extract of kokam (*Garcinia indica*) fruit among all gives highest inhibition zones 17 mm, 15.0 mm, 6.9 mm and 0 mm at 20% 15%, 10% and 5% concentrations and found to be most effective.

This result is in contrast with Chethankumar, *et al*. (2017) [8] evaluated different botanicals against *Xanthomonas citri* subsp. *citri* and stated that alcoholic and aqueous extract of *Garcinia indica* was significantly effective against *Xanthomonas citri* subsp. *citri*.

Table 5: Inhibitory effect of different alcoholic flower extract on *Xanthomonas citri subsp. citri*

Sr. No.	Botanicals		Concentration (%) Inhibition zone (mm)			
	Scientific name	Common name	5 %	10 %	15 %	20 %
1	*Rosa demascina*	Rose	6.20	6.8	7.9	9.58
2	*Tagetes erecta*	Marigold	6	6	7	7
3	*Lathyrus odoratus*	Lakh	6.23	8	9.23	13.14
4	*Hibicus subda*	Hibiscus	0	6	7.5	8.0
	SE(m)±		1.43	0.37	0.34	0.88
	CD(P=0.01)		4.37	1.10	1.01	2.68

The alcoholic extract of lakh (*Lathyrus odoratus*) among the all flowers showed highest inhibition zones 13.14 mm, 9.23 mm, 8.0 mm and 6.23 mm at 20% 15%, 10% and 5% concentrations. The results of the present investigation are in confirmation with the results obtained by Bhardwaj, *et al.* (2011) [5] Evaluated some petal extracts against *Xanthomonas campestris* pv. *campestris* and stated that extract of *Latharus odoratus* was significantly effective against *Xanthomonas campestris* pv. *campestris*

Table 6: Inhibitory effect of different alcohoslic vegetative plant parts extract on *Xanthomonas citri* subsp. *citri*

Sr. No.	Botanicals		Concentration (%) Inhibition zone (mm)			
	Scientific name	Common name	5 %	10 %	15 %	20 %
1	*Eugenia caryophyllata*	Clove	0	7	10	12
2	*Cinnamomum verum*	Dalchini	0	6	7	7.5
3	*Zingiber officinale*	Ginger	0	7.54	10.27	13.54
4	*Curcuma longa*	Turmeric	0	6.5	7.5	9.3
5	*Allium sepa*	Onion	0	6.5	10.25	12.53
6	*Allium sativum*	Garlic	0	7.23	13.40	16.20
	SE(m)±		-	0.22	0.74	0.90
	CD(P=0.01)		-	0.65	2.18	2.58

Among the extracts of different vegetative parts of plants alcoholic extract of garlic (*Allium sativum*) produced highest inhibition zones 16.20 mm, 13.40 mm, 7.23 mm and 0.00 mm at 20% 15%, 10% and 5% concentrations The results of the present investigation are in confirmation with the results obtained by Alane and Swami (2016) [1] and Ambadkar, *et al.* (2015) [2] who evaluated plant extracts against X*anthomonas axonopodis* pv. *punicae* causing bacterial blight of pomegranate and stated that alcoholic extract of *Allium sativum* was significantly effective against X*anthomonas axonopodis* pv. *punicae.*

Table 7: *In vitro* efficacy of chemicals against *Xanthomonas citri* subsp. *citri* after 72 h of incubation at 28±2^{0}C

Chemicals	Concentration (ppm)	Inhibition zone (mm)
Streptomycin sulphate	100	7.6
	250	14.6
	500	21.9
Kasugamycin	100	6.3

	250	8.4
	500	14.2
Bromopol	100	6.9
	250	9.7
	500	15.2
Carbendazim	100	8.2
	250	8.9
	500	10.7
Mancozeb	100	7.3
	250	8.9
	500	13.9
Copper oxychloride	100	10.6
	250	13.2
	500	14.1
Vitavax	100	0
	250	7.6
	500	11
SE(m)±		1.23
CD(P=0.01)		3.72

Result indicated that the antibiotics tested at various concentrations (each @ 100, 250 and 500 ppm) significantly inhibited the growth of *Xanthomonas citri* subsp. *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, followed by bromopol 6.9 mm and kasugamycin 6.3 mm.

At 250 ppm, bacterial inhibition zone was ranges from 8.4 mm (kasugamycin) to 14.6 mm (streptomycin sulphate). However it was significantly highest with streptomycin sulphate 14.6 mm, followed by bromopol 9.7 mm and kasugamycin 8.4 mm.

At 500 ppm, bacterial inhibition zone was ranges from 13.9 mm (mancozeb) to 21.9 mm (streptomycin sulphate). However, it was significantly highest with streptomycin sulphate 21.9 mm, followed by bromopol 15.2 mm and kasugamycin 14.2 mm. Jambenal, *et al.* (2011) [12] reported that streptomycin sulphate at 500 ppm is significantly best for inhibiting the growth of bacterium *Xanthomomas axonopodis* pv. *viticola*.

Conclusion

Among the botanicals extracts of garlic, neem, kokam and lathyrus was found to be most effective *in vitro* against. Further alcoholic extracts were effective, as in alcohol extract more number of phytochemicals liberated and their efficacy were enhanced against the pathogen. Hence these should be exploited as an alternate management stratergy. The most effective antibiotics against *Xanthomonas citri* subsp. *citri* were streptomycin sulphate and bromopol (each @ 100, 250 and 500 ppm) under *in vitro* condition.

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