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In vitro Potency of Antibiotics against *Xanthomonas axonopodis* pv. *citri*: the Causal Agent of Canker in Acid Lime

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A B S T R A C T

A survey was conducted in the major acidlime growing areas of Tenkasi and Madurai districts of Tamil Nadu. The causal agent of citrus canker (*Xanthomonas axonopodis* pv. *citri*) was isolated from the plant samples collected during the survey. The isolated bacterial colonies were found to be yellow pigmented with entire margin and the cells were single rod shaped under Gram's staining. The efficacy of some antibiotics namely Streptomycin, Tetracycline, Streptomycin + Tetracycline, Cefatoxime, Cefixime, Gentamycin, Amoxillin and chloramphenicol each at the concentration of 100, 250 and 500 ppm were evaluated against the virulent isolate of the pathogen. Among the various antibiotics, Cefatoxime (39.07mm) and Cefixime (34.18mm) showed maximum average inhibition zone followed by Tetracycline (29.51mm).

Introduction

Acid lime (*Citurs aurantifolia*) is the most significant fleshy, juicy and edible fruit tree belonging to the family Rutaceae. It has now been cultivated in more than 30 countries around the world and it was thought to be originated in South East Asia (Gottwald *et al*., 2002). *C. aurantifolia* is commonly called as Lime (Nigeria), Key lime, Mexican lime, Sour lime, Dayap, Indian lime, Egyptian lime (USDA,2013).

Citrus fruits are consumed worldwide in the form of fresh fruit or processed into citrus products and by-products. Approximately, one third of total citrus production is utilized for processing (Okwu, 2008). Ward and Kilmer, 1989 reported that acid lime is the richest source of Vitamin-C. Citrus

cultivation is largely affected by numerous fungi, bacteria, viruses, phloem inhabiting bacteria and phytoplasma. More than 150 diseases recorded in citrus plants from nursery level to bearing stage resulting in considerable yield loss. Canker is one of the important bacterial disease caused by the bacterium *Xanthomonas axonopodis* pv. *citri*. Cankerous fruits fetch least consumer preference and market value. Canker (corky growth) is the result of excessive mitotic cell division during pathogenesis (Gabriel *et al*., 2000). Infection causes cankerous lesions on the leaves, stems, and fruits of citrus cultivars, including lime, oranges, and grapefruits (Lakshmi *et al*., 2014). Severe infection results in defoliation, die-back, deformation of fruit and premature fruit drop (Rossetti, 1977; Civerolo, 1981; Chand and Pal, 1982; Stall and Seymour, 1983)

Jadhav *et al*., 2018a tested four antibiotics, five bactericides and nine botanicals against the pathogen of citrus cankerunder *in vitro* condition. Among these, Streptocycline (27.35 mm) and copper oxychloride (25.53 mm) showed maximum average inhibition zone and also Ginger *(Z. officinale)* was effective with the Average inhibition zone of 20.04 mm followed by Neem 19.87 mm. This experiment was also designed in such a way to identify the best antibiotic against the citrus canker pathogen.

Materials and Methods

Collection of diseased samples

Survey was carried out to collect cankerous leaf samples from the acid lime orchards at 10 different places of Tenkasi and Madurai districts of Tamil Nadu, which were used for isolating the pathogen. Also, the Per cent disease index of canker disease at various places were calculated using the expression (Mc Kinney, 1923)

$$PDI = \frac{\text{Sum of all numerical ratings X 100}}{\text{Total number of leaves graded X Maximum grade}}$$

Processing of the collected samples

The collected plant samples were surface sterilized using 70% ethanol. Small piece of leaf sample with typical lesions was selected and excised from the collected samples using a sterile scalpel and that piece was placed on a sterile glass slide with a drop of sterile distilled water and chopped into very small bits. The glass was kept undisturbed for 3-5 minutes.

Isolation of the pathogen

In the meantime, the bacteria (pathogen) oozed out from the infected samples to the water droplets resulted in cloudy appearance. The resulting suspension was streaked on the sterile Petri plates containing solidified Nutrient Agar (NA) medium [Peptone-5g/l, Beef extract-3g/l, Glucose-5g/l, Sodium chloride-5g/l, Agar-20g/l and Distilled water-1000ml]using sterile inoculation loop. These plates were incubated at 28±2°C for 72 hrs and observed for bacterial growth.

Identification and purification of the pathogen

Ten different isolates of the pathogen cultured on the NA medium were characterized based on the cultural (colony shape, margin, pigmentation) and morphological (cell shape) characters of the pathogen.

Pathogenicity test

In order to fulfil the Koch's postulates, pathogenicity test was carried out for the ten pathogen isolates namely Xac1 to Xac 10 following the procedure of Arshiya *et al*.,

2014. As a result of pathogenicity studies, the pathogenic isolate Xac1 was found to be highly virulent and this isolate was forwarded for further studies.

In vitro evaluation of antibiotics

The efficacy of antibiotics against the pathogen was evaluated by disc diffusion test (Kirby-Bauer method) (Ordax *et al*., 2009). About seven antibiotics each at 100, 250 and 500 ppm were tested *in vitro* using NA as basal medium. Twenty ml of two days old bacterial (virulent pathogen) broth (2 x 10^8 cfu/ml) was mixed with 100ml of molten sterilized NA medium and poured into Petri plates and allowed them to solidify.

The suspension of various antibiotics viz., Streptomycin, Tetracycline, Streptomycin + Tetracycline, Cefatoxime, Cefixime, Gentamycin, Amoxillin and Chloramphenicol each at 100, 250 and 500 ppm concentrations were prepared individually. Sterilized filter paper discs (Whatman No. 2) of 5mm diameter were dipped into the respective solutions separately, and then placed onto the Petri plates (2cm away from the periphery) containing solidified NA medium seeded with the test bacterium using sterile forceps. The untreated control plate was also maintained containing the solidified test bacterium seeded NA medium with a paper disc dipped in sterile distilled water. These plates were incubated at 28±1°C for 48hrs and looked for the formation of zone of inhibition around the paper disc. The results obtained were analysed statistically (Raju *et al.,* 2012).

The antimicrobial activity of antibiotics was calculated in millimetre by the expression (Bagul and Sivakumar, 2016)

Zone of inhibition = Total diameter of growth inhibited zone – diameter of paper disc

Results and Discussion

Survey of canker incidence

During the survey, it was found that the place Parankundrapuram recorded the highest incidence of the canker disease and Puliyankudi ranked second (Table 1).

Identification of the pathogen

From the observations, all the bacterial isolates were hardly distinguishable based on morphological characters but little variation was observed in pigmentation (Fig. 2). Similar results were reported by Jadhav *et al*., (2018) b, who got convex, yellow color bacterial colonies with entire margin.

Out of 10 isolates, only 2 isolates viz., Xac1 and Xac9 appeared bright with yellow pigmentation, all other isolates viz., Xac 2, 3, 4, 5, 6, 8 and 10 exhibited yellow pigmentation and the isolate Xac7 revealed dull coloration (Table 2).

All the bacterial isolates were examined for the shape of the cells under 100x magnification of compound microscope by Gram's staining technique. These cultured bacterial isolates were observed to produce single, rod shaped cells, also the cells were appeared to be pink in colour as they were gram negative bacteria (Table 2) (Fig. 3). This result correlates with the findings of Goto (1992), who have observed yellow colour colonies as a result of Xanthomonadin pigment production and rod-shaped cells while isolating citrus canker pathogen.

Potency of Antibiotics against *Xanthomonas axonopodis* pv. *citri* under *in vitro* condition

Antibiotics each at the concentration of 100, 250 and 500 ppm were found to exhibit

antibacterial activity against the virulent isolate of the pathogen compared to the untreated control (Table 3).

The average inhibition zone developed by antibiotics treatment against the pathogen ranges between 39.07 mm (Cefatoxime) and 20.98 mm (Gentamycin). Cefatoxime was observed to exhibit maximum inhibition of growth of test bacterium (30.07 mm) followed by Cefixime (34.18 mm).

Table.1 Locations of the samples collected

Isolate no.	Place	District	Geographical location		Per cent Disease Index (PDI) %
			Latitude	Longitude	
Xac 1	Parankundrapuram	Tenkasi	8°98685' N	77°4474' E	51.11%
Xac2	Kaluneerkulam	Tenkasi	8°9037' N	77°4530' E	42.22%
Xac3	Ayyanarkulam	Madurai	9°9938' N	77°8781' E	33.33%
Xac4	Kadayanallur	Tenkasi	9°0779' N	77°3452' E	35.56%
Xac5	Puliyankudi	Tenkasi	9°1725' N	77°3956' E	46.67%
Xac6	Checkanurani	Madurai	9°9420' N	77°9724' E	28.89%
Xac7	Kuruvikulam	Tenkasi	9°1780' N	77°6694' E	31.11%
Xac8	Kadayalurutti	Tenkasi	9°0268' N	77°4336' E	26.67%
Xac9	AC & RI	Madurai	9°9699' N	78°2040' E	35.56%
Xac10	Kalluthu	Madurai	10°0603' N	77°8226' E	44.44%

Table.2 Cultural and morphological features of the isolates

Isolates	Cultural characters				Morphological characters
	Colony shape	Pigmentation	Colony margin	Colony elevation	Cell shape
Xac 1	Circular	Bright yellow	Entire	Convex	Single rod
Xac2	Circular	Yellow	Entire	Convex	Single rod
Xac3	Circular	Yellow	Entire	Raised	Single rod
Xac4	Circular	Yellow	Entire	Convex	Single rod
Xac5	Irregular	Yellow	Curled	Raised	Single rod
Xac6	Circular	Yellow	Entire	Convex	Single rod
Xac7	Circular	Straw yellow	Entire	Convex	Single rod
Xac8	Circular	Yellow	Entire	Raised	Single rod
Xac9	Circular	Bright yellow	Entire	Convex	Single rod
Xac10	Circular	Yellow	Entire	Convex	Single rod

Table.3 Out-turn of in vitro treatment of Antibiotics against *Xanthomonas axonopodis* pv. *citri*

Tr. No.	Treatment	Mean Zone of Inhibition* (mm) at			Average inhibition (mm)
		100 ppm	250 ppm	500 ppm	
T1	Streptomycine	26.90 **(5.23)**	26.75 **(5.22)**	27.5 **(5.29)**	27.05[d] **(5.25)**
T2	Tetracycline	28.80 **(5.41)**	28.8 **(5.41)**	30.95 **(5.61)**	29.52 [c] **(5.48)**
T3	Streptomycin + Tetracycline	20.35 **(4.57)**	21 **(4.63)**	25 **(5.05)**	22.12[e] **(4.75)**
T4	Cefixime	31.55 **(5.66)**	33.95 **(5.87)**	37.05 **(6.13)**	34.18 [b] **(5.89)**
T5	Cefatoxime	36.00 **(6.04)**	37.65 **(6.18)**	43.55 **(6.64)**	39.07 [a] **(6.29)**
T6	Gentamycin	18.35 **(4.34)**	21.45 **(4.69)**	23.15 **(4.86)**	20.98[f] **(4.63)**
T7	Amoxillin	23.10 **(4.85)**	27.55 **(5.29)**	28.95 **(5.43)**	26.53[d] **(5.19)**
T8	Chloramphenicol	19.95 **(4.52)**	22.45 **(4.79)**	23.75 **(4.92)**	22.05[e] **(4.75)**
T9	Control	0.00 **(0.71)**	0.00 **(0.71)**	0.00 **(0.71)**	0.00[g] **(0.71)**
	CD (P=0.01)	**1.305**			

*Mean of three replications
[Figures in parenthesis are square root transformed values]

Fig.1 Cankerous growth on fruit and leaf of acid lime

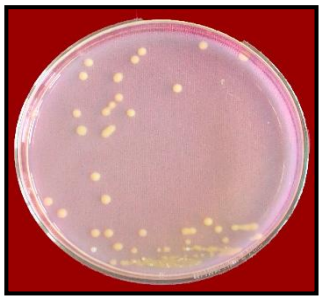


Fig.2 Isolate Xac1

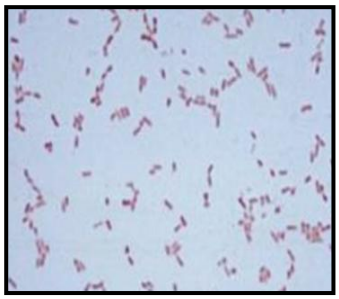
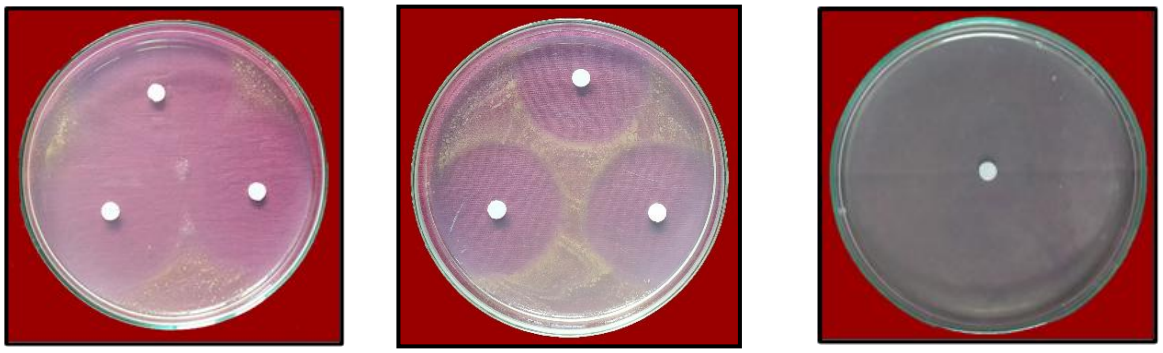


Fig.3 Gram staining



Cefatoxime Cefixime Control

Fig.4 *In vitro* efficacy of antibiotics against *Xanthomonas axonopodis* pv. *citri*

Ali *et al*., 2017, evaluated antibacterial activity of some antibiotics against the citrus canker pathogen. Highest antibiotic activity was exhibited by Gentamycin (10µg/disc) with 21 mm diameter of inhibition zone followed by both Chloramphenicol and Tetracycline (30µg/disc) showing the same inhibition zone (20.6 mm).

Mubeen *et al*., (2015) also assessed the sensitivity of various antibiotics by disc diffusion method against *Xanthomonas axonopodis* pv. *citri*. They recorded that inhibition zone of 1.8cm and 2.2cm were produced by Streptomycin sulphate and Kanamycin sulphate respectively, while Ampicillin and Chloramphenicol did not show any inhibition zone against the pathogen.

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