Original Research Article

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

A. Selva Amala¹, R. Akila¹* S. Harish² and R. Arunkumar³

¹Department of Plant Pathology, AC & RI, TNAU, Madurai
²Centre for Plant Protection Studies, AC & RI, TNAU, Coimbatore
³Coconut Research Station, Veppankulam, India

*Corresponding author

INTRODUCTION

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).

Keywords
Acid lime, Xanthomonas axonopodis pv. citri, Citrus canker, Antibiotics

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Acid lime (Citrus 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 canker under *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)

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

**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 *Xac* 1 to *Xac* 10 following the procedure of Arshiya *et al.*, 2018.
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)

\[
\text{Zone of inhibition} = \text{Total diameter of growth inhibited zone} - \text{diameter of paper disc}
\]

**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) % |
|-------------|-----------------|----------|-----------------------|-------------------------------|
| 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      | Streptomycin                   | 26.90 (5.23) | 26.75 (5.22) | 27.5 (5.29) | 27.05<sup>d</sup> (5.25) |
| T2      | Tetracycline                   | 28.80 (5.41) | 28.8 (5.41) | 30.95 (5.61) | 29.52<sup>c</sup> (5.48) |
| T3      | Streptomycin + Tetracycline    | 20.35 (4.57) | 21 (4.63) | 25 (5.05) | 22.12<sup>e</sup> (4.75) |
| T4      | Cefixime                       | 31.55 (5.66) | 33.95 (5.87) | 37.05 (6.13) | 34.18<sup>b</sup> (5.89) |
| T5      | Cefatoxime                     | 36.00 (6.04) | 37.65 (6.18) | 43.55 (6.64) | 39.07<sup>a</sup> (6.29) |
| T6      | Gentamycin                     | 18.35 (4.34) | 21.45 (4.69) | 23.15 (4.86) | 20.98<sup>f</sup> (4.63) |
| T7      | Amoxillin                      | 23.10 (4.85) | 27.55 (5.29) | 28.95 (5.43) | 26.53<sup>d</sup> (5.19) |
| T8      | Chloramphenicol                | 19.95 (4.52) | 22.45 (4.79) | 23.75 (4.92) | 22.05<sup>e</sup> (4.75) |
| T9      | Control                        | 0.00 (0.71) | 0.00 (0.71) | 0.00 (0.71) | 0.00<sup>g</sup> (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
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 1.8 cm and 2.2 cm 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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