ANTIBIOTIC SENSITIVITY, VARIABILITY AND MANAGEMENT OF *Xanthomonas axonopodis* pv. *citri*

THESIS

Submitted to
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
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DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation of the thesis entitled **"ANTIBIOTIC SENSITIVITY, VARIABILITY AND MANAGEMENT OF *Xanthomonas axonopodis* pv. *citri* "** or part there of has neither been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or Scientific Organization. The sources of material used and all assistance received during the course of investigation have been duly acknowledged.

Place : Akola. **(TAMBE SOMNATH BHIKU)**

Date : / /2018 Enrolment No. OO-3186

CERTIFICATE

This is to certify that the thesis entitled **" ANTIBIOTIC SENSITIVITY, VARIABILITY AND MANAGEMENT OF *Xanthomonas axonopodis* pv. *citri* "** submitted in partial fulfillment of the requirement for the degree of "**Master of Science in Agriculture (Plant Pathology)"** of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **TAMBE SOMNATH BHIKU** under my guidance and supervision.

The subject of thesis has been approved by the Student's Advisory Committee.

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Place: Akola (TAMBE.S.B.)

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D) Abbreviations

Abbreviations		Full form
%	–	Per cent
/	–	Per
@	–	At the rate of
^{0}C	–	Degree Celsius
µg	–	Micro grams
µl	–	Micro litre (s)
Cm	–	Centimetre(s)
CTAB	–	Cetyl Trimethyl Ammonium Bromide
dd H_2O	–	Double distilled water
Deptt.	–	Department
DNA	–	Deoxyribose Nucleic Acid
DNTPs	–	Deoxyribo nucleoside triphosphate
e.g.	–	Exampli gratia (For example)
EDTA	–	Ethylene Di-amine Tetra Acetic Acid
ERIC	–	Entrobacterial repetitive intergenic consequence
et al.	–	Et alia (and associates)
etc.	–	Et cetera
Fig.	–	Figure
G	–	Grams
i.e.	–	That is
Kbp	–	Kilo base pairs
M	–	Molar
Max.	–	Maximum
Mg	–	Milli gram(s)
min.	–	Minutes
Ml	–	Milli litre(s)
Mm	–	Milli molar
Mm	–	Millimetre
NA	–	Nutrient agar
NB		Nutrient Broth
Ng	–	Nano gram(s)

Nm	–	Nano meter
No.	–	Number
ISSR	-	Inter Simple Sequence Repeat
SSR	-	Simple Sequence Repeat
RAPD		Random Amplified Polymorphic DNA
REP	–	Repetitive extragenic palindromic
RFLP	–	Restriction Fragment Length Polymorphism
Rnase	–	Ribonuclease A
Rpm	–	Revolution per minute
Taq polymerase	–	*Thermus aquaticus* DNA polymerase Enzyme
TBE buffer	–	Tris hydroxymethyl amino methane and boric acid ethylene diamine tetra acetic acid buffer.
TE buffer	–	Tris hydroxymethyl amino methane ethylene diamine tetra acetic acid buffer
Tris	–	Tris Hydroxymethyl aminomethane
U	–	Unit
UPGMA	–	Unweighted pair group method for arithmetic mean average
UV	–	Ultra violet
viz.	–	Videlicet (namely)

F) THESIS ABSTRACT

a)	**Title of the thesis**	**:**	**" ANTIBIOTIC SENSITIVITY, VARIABILITY AND MANAGEMENT OF *Xanthomonas axonopodis* pv. *citri* "**
b)	**Full name of student**	**:**	**TAMBE SOMNATH BHIKU**
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ABSTRACT

The present investigation was carried for Management of *Xanthomonas axonopodis* pv. *citri* through antibiotic sensitivity and molecular variability of *Xanthomonas axonopodis* pv. *citri* causing citrus canker. Canker caused by *X. axonopodis* pv. *citri*, is one of the most devastating diseases of citrus growing regions. Typical cankerous

symptoms were noticed in different gardens. Eight isolates of *Xanthomonas* were obtained on NA medium from the canker infected leaves collected from different places.

The isolations were made from the symptomatic samples collected from different districts of Maharashtra viz. Nagpur(Xac1), Akola(Xac2), Amravati (Xac3), Washim (Xac4), Bhandara(Xac5), Pune (Xac6), Rahuri(Xac7), Dapoli(Xac8) and grouped as per agro-ecological zones.

The growth characteristics of all the isolates were studied on nutrient agar medium. The isolates Xac1, Xac2, Xac5, Xac6, Xac8 produced Dark yellow colour colonies however Xac3, Xac4, Xac7 showed pale yellow colour colonies. All eight isolates were found Gram –ve, short rod and positive for biochemical tests *viz* ,KOH, catalase test, starch hydrolysis, and gelatein liquefacation. The Antibiotic sensitivity was studied by paper disc method *in vitro.* In that, different antibiotics and chemicals were used namely Streptomycine sulphate, Streptocycline, Kasugamycin, and Copper oxychloride. The result revealed that the Copper oxychloride (0.3%) + Streptocycline (200 ppm) and Copper oxychloride (0.2%) + streptomycin sulphate (200 ppm) and was found significantly superior in inhibiting growth of bacteria.

The variability was studied among the eight isolates of *Xanthomonas axonopodis* pv. *citri* by using 7 ISSR primers *i.e*.ISSR8, ISSR12, ISSR817, ISSR820, ISSR 827, ISSR841, ISSR57101. 7 primers produced 22 scorable bands with an average of 3 bands per primer. Out of 22 bands, 19 bands were polymorphic and level of polymorphism was 68%.The UPGMA analysis showed that Isolate Xac6 (Pune) had higher value of similarity coefficient (0.91) with Xac7 (Rahuri), whereas Xac1(Akola), had lower value of similarity coefficient (0.50) with Xac8 (Dapoli).

CHAPTER I

INTRODUCTION

1.1 Background information

Citrus is important fruit crop of the world. Present day citrus is delectable, juicy, and seedless is of great nutritional significance as well (Khan *et al*., 1992). Additionally, it has enormous therapeutic values (Chaudhary *et al*, 1992). Citrus is a member of Rutaceae family and grown in varying areas in countries with tropical or subtropical climates. It is used as best source of Vitamin C, sugars, amino acids and other nutrients (Ahmad and Khan,1999). The most important commercial citrus cultivars in India are the mandarin followed by sweet orange and acid lime.

In India, citrus is cultivated in four major zones (1) North-West comprising arid western plains of Punjab, Haryana, Rajasthan, Gujarat and Uttar Pradesh. (2) Central includes semi-arid tracts of Maharashtra and Madhya Pradesh (3) Southern comprising hot and humid areas of Andhra Pradesh, Tamil Nadu, Karnataka (4) North-Eastern includes Assam, Meghalaya, Sikkim, Tripura and North Bengal. Commercially acid lime is grown in Khara district of Gujarat ,Akola and Amravati districts of Maharashtra and Periyakulam in Tamil Nadu.

Under the changing agriculture scenario in India, it has been realized that the horticultural sector plays a vital role in providing the livelihood security to the farmer. India is the second largest producer of fruits in the world with 92918 (000 MT) from an area of 6373 (000 ha)(Indian horticulture database 2016).Citrus is the third largest fruit industry in India after Banana and Mango (Anonymous,2016). In India, area under citrus cultivation is 985 (‘000 ha), with production of 11419 (‘000 MT). It occupies 14.9 per cent area of the total fruit area and 12.5 per cent of the production in India. Productivity of citrus is 10.3MT/hectare. In Maharashtra, area under citrus is 275(000 ha) with productivity of 6.4 MT/ha (Anonymous, 2015).

Although citrus crop is kept in great esteem, yet its present status is threatened by a number of problems. Low production caused by biotic and abiotic stresses is one of them. Citrus plant is attacked by a number of diseases like citrus canker, gummosis, citrus decline, citrus tristeza virus, greening, etc. Citrus canker is caused by *Xanthomonas axonopodis* pv. *citri* that is probably the worst enemy to citrus plants (Sahi *et al*., 2007).

Citrus canker disease is of regular occurrence on several citrus cultivars in varying degrees of incidence depending on the climatic conditions. The bacterium, *Xanthomonas* causes different symptoms like pustules to necrotic lesions consisting of erumpent corky tissue surrounded by water soaked tissues and yellow halo on leaves, stems and fruits (Zekri *et al*., 2005; Graham *et al*.,2004; Schubert and Sun, 2003; Burning and Gabriel, 2003; Das, 2003; Bergamin-Filho *et al*., 2000). As such disease severity on susceptible variety results in defoliation, premature fruit drop and blemished fruit, which consequently decrease fruit production and market value (Zekri *et al.*, 2005; Stall and Seymour, 1983).There are many types of citrus canker disease caused by various pathovars and variants of the bacterium *Xanthomonas axonopodis* (Graham *et al.,*2004). All cultivars of citrus are susceptible to canker, but grapefruit, Mexican lime and lemon are highly susceptible, whereas sour orange and sweet orange are moderately susceptible. Mandarins are moderately resistant (Gottwald *et al.,* 2002) and all young above-ground tissues of citrus are susceptible to *Xanthomonas axonopodis*. In fact, bacterial pathogen is transmitted into the plant tissues through natural openings (stomata) and mechanical injuries (wounds). However, as pathogen enters into the plant lesions, the colour changes into brown, and as such, water soaked margin, surrounded by a chlorotic halo, appears.

The genus *Xanthomonas* is gram negative, straight rod shaped bacterium motile by means of single polar flagellum, obligately aerobic with tolerance of maximum temperature for growth is 35 to 39°C (Whiteside *et al*., 1988).The member of genus *Xanthomonas* are the serious plant pathogens. As a result of cosmopolitan occurrence of citrus

canker different aspects of the disease have been potentially addressed and adequately researched in various parts of the world, there by generating substantial information on the biology and management of the disease. As such, at local conditions limited work that has been done on the identification of pathotypes epidemiology for proper management (Akhtar *et al*., 1996). It has become mandatory to substitute the conventional method of disease management (chemical control) with safer and eco-friendly management strategies (biological and genetic control). This would lead to the alleviation of the dependence on the chemicals that will be ecofriendly for the environment.

Several other workers also reported the incidence of canker on the acid lime and other varieties of citrus. Further, the disease appear as a serious problem wherever acid lime *(C. aurantifolia*) is grown on commercial scale and has become a permanent major problem to the citrus growers in the country. Recently canker has been detected in kinnow mandarin nursery in Punjab state (Anonymous, 2000).

1.2 Importance of study

The economic importance of citrus canker can be analyzed from several different points of view..As the acid lime bears thrice in a year. The citrus canker that affects the crop in all the stages and the losses are both qualitative and quantitative. When citrus infection occurs in the early growing stage, the fruits crack or become malformed as they grow, and the heavily infected fruits fall prematurely. Low infection in later growth stages may cause only scattered canker lesions on the surface of fruits which makes fresh fruits unacceptable for market although it is harmless for humans. The severity of fruit infection usually correlates with of foliage infection. Eighty to ninety percent of fruit infection is not uncommon in susceptible citrus trees that have already sustained severe foliage infection. Such heavy foliage infection often causes severe defoliation, leaving only bare twigs (Goto,1992).

The present investigation was undertaken to manage the disease through various antifungal and antibacterial antibiotics namely Streptomycine sulphate, Streptocycline, Copper oxychloride,

Kasugamycine and for existence of morphological and molecular variations among *Xanthomonas axonopodis* pv. *citri*

In order to study the molecular variations various techniques have been employed such as the Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeat (ISSR), Amplified Fragment Length Polymorphism (AFLP), Sequence-Related Amplified Polymorphism (SRAP), and Simple Sequence Repeat (SSR) analysis are well established and widely used.

The ISSR markers are DNA sequences delimited by two inverted SSR composed of the same units which are amplified by a single PCR primer. This primer is constituted by few SSR units with an anchored end, to avoid unspecific hybridization to the microsatellite region and minimize the slippage (Zietkiewicz *et al*. 1994; Blair *et al*. 1999; Weising *et al*. 2005). ISSR-PCR gives multi locus patterns which are very reproducible, abundant and polymorphic in plant genomes (Bornet nd Branchard, 2004). The major advantage of this method is its universally and ease of development (no need for sequence data) (Agostini *et al*. 2008; Jabbarzadeh *et al*. 2010), in addition to the reproducibility and low cost of the technique (Weising *et al*. 2005; Li *et al*. 2010). Inter simple sequence repeats (ISSR) markers involve amplification of the DNA segment between two identical microsatellite repeat regions

In the present investigation, the genetic diversity of the genus *Xanthomonas axonopodis* pv. *citri* was assessed by neutral ISSR markers, using universal primers based on microsatellite motifs.

1.3 Objectives of study

1) Antibiotic sensitivity among the isolates of *Xanthomonas axonopodis* pv. *citri*.
2) Molecular variability among the isolates of *Xanthomonas axonopodis* pv. *citri*. By using ISSR marker.
3) Management of *Xanthomonas axonopodis* pv. *citri.*

1.4 Hypothesis

This study showed wide variation among different strain of *Xanthomonas axonopodis* pv. *citri* on the basis of morphological, biochemical and molecular characteristics. Among different isolates collected from diverse geographical locations, *Xanthomonas axonopodis* pv. *citri* has shown genetically similar characteristics. It is concluded that distinct variability existed among the *Xanthomonas axonopodis* pv. *citri* isolates recovered from the diseased sample of different agro climatic region of Maharashtra. The study also showed that pathogen show the variation in inhibition when treated with different chemicals.

1.5 Scope and limitation

During recent days production and market value of citrus is reduced due to some limitation like heavy infection of citrus canker is one of the important constrain in production of acid lime. The development of effective chemicals for management of citrus canker has been claimed by citrus growers.

Biochemical and molecular characterization of *Xanthomonas axonopodis* pv. *citri* is necessary for detection and identification of citrus canker disease. ISSR molecular markers were used to study the molecular variability of *Xanthomonas axonopodis* pv. *citri.* The molecular variability and cluster analysis based on these molecular markers made it possible to differentiate the isolate and showed different levels of intraspecific variation, these results confirm the potential of ISSR markers as an auxiliary tool for the classification of *Xanthomonas axonopodis* pv. *citri.* isolates and also to study in intraspecific genetic variability.

CHAPTER II

REVIEW OF LITERATURE

In present investigation entitled “Antibiotic sensitivity, variability, and management of *Xanthomonas axopodis* pv. *citri.”,* all the isolates of *Xanthomonas axonopodis* pv.*citri* were characterized on the basis of biochemical and molecular characterization. Efficacy of different antibiotics and fungicides were also tested against different isolates *Xanthomonas axonopodis* pv. *citri.* Literature available on various aspect were reviewed during the period under study are given here.

2.1 Symptomatology

Hasse (1915) was the first to identify citrus canker in USA and the causal agent was described as *Pseudomonas citri*, later nomenclature was changed and proposed the name *Xanthomonas* campestris pv. *citri*

Chohan and Knorr (1970) reported that size of the canker lesion varies depending on the host variety attacked. Older lesions were corky irregular in shape and brown in colour. The fruit lesion become rough and corky and sometimes caused cracks fissures of the skin usually, severe lesions occurred on midribs and petioles resulting defoliation. On fruits the canker lesion were confined to the rind only, without affecting internal quality.

Swarup *et al*. (1991) reported that *X.citri* lesions first appeared as pin-point spots that become small, slightly raised pustules or blister-like eruptions. Initially, these appeared on the lower leaf surface about 7 days after infection. Lesions were light coloured at first and become tan or brown. As lesions develop, the epidermis ruptured and the lesions become spongy or corky.

Gottwald *et al*. (2002) first reported disease, Asiatic citrus canker (ACC) is caused by *Xanthomonas axonopodis* pv. *citri* (Xac). The disease was spread by rain splash combined with wind and was greatly exacerbated by tropical storms and hurricanes

Brunings and Gabriel (2003) found that bacteria infect leaves, stems and fruits and enter mainly through stomata and wounds. The infection was visualized as circular spots in leaves. .

Xiaoan and Stall (2004) reported that the citrus canker bacterium produced erumpent, pustule like lesions into key /Mexican lime but brownish, flat, necrotic lesion on the leaves of duncan grapefruit ,sweet orange, sour orange, citron and trifoliate orange.

Khalid *et al*. (2010) reported the bacterium, *X. axonopodis* pv. *citri*, cause different symptoms ranging from pustules to necrotic lesions consisting of erumpent corky tissue surrounded by water soaked tissues and yellow halo on leaves, stems and fruits.

2.2 Isolation of *Xanthomonas axonopodis* pv. *citri* .

Guo *et al*. (2010) reported all of the strains of *X. citri* subsp. *citri* wild-type strain 306 (rifamycin resistant) (40) and mutant strains were grown on NA medium at 28^0C and proved their pathogenicity.

Arshadi *et al*. (2013) collected the diseased samples from citrus trees which showing typical symptoms of citrus canker and bacterial suspension of each specimen was cultured on NA medium. Following incubation, colonies similar to *Xanthomonas* were subsequently subcultured on YDC semi-selective medium to maintain pure cultures of the bacteria.

Didwania *et al*. (2013) isolated black rot causing bacterium *Xanthomonas campestris* from naturally infected cauliflower plant leaves showing typical ‘V shaped symptoms. The single fresh and pure glistering yellow droplets like colonies were purified on other petriplates containing nutrient agar medium.

Mark (2013) first reported of *Xanthomonas citri* pv. *citri*-A, causing Asiatic citrus canker in Mayotte. *X. citri* pv*. citri* pathotype A, cause severe infection in a wide range of citrus species and induced erumpent, callus-like lesions with water-soaked margins evolving to corky cankers and leading to premature fruit and leaf drop and twig dieback on susceptible/very susceptible cultivars. A chlorotic halo was typically visible

around canker lesions on leaves and young fruit, but not on mature fruit and twigs.

Al-Saleh *et al.* (2014) reported 76 *Xanthomonas* like strains isolated from different citrus species showing bacterial canker symptoms from citrus commercial farms, backyard orchards and nurseries. Bacterial colonies with yellow pigmentation were picked up and transferred to new NGA plates for purification and further tests.

2.3 Morphological characters

Jayasekara and Suetsugu (1998) observed that bacterium *Xanthomonas axonopodis* pv. *citri* produced yellowish white, round, raised, convex, smooth and glistering colonies on PSA medium.

Gottwald *et al*. (2002) reported the bacteruim *Xanthomonas axonopodis* as a rod shaped, Gram negative with polar flagella. The bacterium had a genome length approximately 5 Mbp. There were a number of types of citrus canker disease caused by different pathovars and variants of the bacterium.

Manjula (2002) reported that, seven isolates of the *X. axonopodis* pv. *punicae* (Pomogranate bacterial blight) as small rods, appeared singly, rarely in pairs, Gram negative, non-capsulated and no spore forming with monotrichous flagellation.

Das (2003) reported that the citrus canker bacteria were rod shaped measuring 1.5-2.0 x 0.5-0.75µm and Gram negative.

2.4 Biochemical characterization

Raut (1990) studied 15 isolates of *Xanthomonas axonopodis* pv. *mangiferae indicae* for different physiological and biochemical properties viz. H_2S production , action on carbohydrates , gelatin test, KOH test etc. and showed that all test are positive to the *Xanthomonas axonopodis* pv.*citri.*

Kishun and Chand (1991) reported that, *Xanthomonas campestris* pv. *campestris* on radish was positive in H_2S production, starch hydrolysis, KOH solubility, gelatin liquefaction, hydrolysis of Tween 80,

sucrose utilization, indole production, growth at 3.5 per cent NaCl, milk proteolysis and acid from most of the suga.

Duraphe (2002) studied isolates of *Xanthomonas axonopodis* pv. *malvacearum* were positive in different physiological and biochemical properties viz. H_2S production , carbohydrates catabolism, liquefaction , KOH test , solubility test, catalase test .

Das (2003) reported that the bacterial cells of *Xanthomonas citri* are positive for hydrolysis of starch, liquefaction of gelatin and catalase.

Das (2005) studied different isolates of *Xanthomonas axonopodis* pv. *citri* were positive in different physiological and biochemical properties viz. H_2S production, carbohydrates , gelatin liquefaction, KOH test etc solubility test, catalase test, acid production from D-xylose, glucose, pathogenicity test.

Bhardwaj *et al.* (2014) characterized on 20 isolates of *Xanthomonas axonopodis* pv. *citri* were collected from various regions of Varanasi.Isolates were characterized with the help of morphologicaly, pathogenicity and biochemicaly. All the isolates showed similar morphological and biochemical characteristic and all were found pathogenic to citrus, which confirmed the identity of isolates belonging to stated *Xanthomonas axonopodis* pv. *citri*.

Abhang *et al.* (2015) studied on the Morphological and biochemical characteristic viz., shape, colony colour, Gram reaction, starch hydrolysis, gelatein liquefaction, indole production, acid and gas production, KOH, catalase test were performed and confirmed the identity of bacterium as *Xanthomonas axonopodis* pv. *citri.*

Mubeen *et al.* (2015) stated that *Xanthomonas axonopodis* pv. *citri* (*Xac*), which is a gram negative bacterium and performed biochemical analysis to differentiate between gram positive and gram negative. Gram staining, Starch hydrolysis, Tween 80 hydrolysis, Gelatin Liquefaction, KOH test, Kovacs' Oxidase and Fluorescent Pigmentation tests were performed to characterize the *Xac* and found that *Xac* is a gram negative bacterium.

Jabeen *et al.* (2016) studied the 25 pathogen by isolation and identified on the basis of microscopy, morphological characters and biochemical characters. 25 isolates were used for biochemical characterization of pathogen. Six biochemical tests were used for confirmation of pathogen. In Gram's staining, Kovac's oxidase and Arginin dihydrolase tests, all Xac isolates showed negative results while positive results were observed in case of starch hydrolysis, tween 80 hydrolysis and gelatin liquification tests.

Ali *et al.* (2017) were Isolated and characterized *Xanthomonas axonopodis* pv. *citri* bacteria from *Citrus aurantifolia* canker disease and evaluated antibiotic susceptibility, antibacterial sensitivity and antagonistic activity. Isolated bacterium was characterized by different biochemical test method.. The biochemical tests showed catalase positive, glucose fermenting, lactose non-fermenting and urease test negative.

Jadhav *et al.* (2018) studied the cultural and biochemical characteristics of *Xanthomonas axonopodis* pv. *citri and* showed that cultural characteristics *viz*., colony shape, margin, elevations, surface, and pigmentation help for identify action of the bacteria. Gram staining, Potassium hydroxide (KOH) solubility , Catalase, Starch hydrolysis test were performed to characterize the *Xac.* The results of all morphological, biochemical and cultural tests were confirmed the *Xac.* a gram negative bacterium

2.5 Management of *Xanthomonas axonopodis* pv. *citri* through antibiotic sensitivity

Sharma *et al.* (1981) *In vitro* evaluated eight chemicals against *Xanthomonas vesicatoria* by paper disc and turbidometric methods and observed that combination of streptocycline and copper oxychloride was most effective in inhibiting the growth of pathogenic organism in turbidometric method, but stable bleaching powder showed maximum inhibition, when tested by Thompason's method

Akhtar *et al*. (1997) evaluated 208 diffusates from various plants such as forest trees, herbs, shrubs, fruit trees, spices, vegetables,

food legumes, fodder, oil seed, fibre crops, cereals and ornamentals through agar diffusion assay to determine their inhibitory effect, against *Xanthomonas campestris* pv*. citri*. Diffusates from higher plants, appeared to be potential antimicrobial agents which could be used for the management of citrus canker disease.

Satish *et al*. (1998) evaluated *in vitro* leaf aqueous extracts of 30 higher plants, collected from different localities, for their antibacterial activity against different pathovars of the phytopathogenic bacterium, *Xanthomonas campestris*. Eight plant species showed antibacterial activity and indicated the potential of these plant extracts in the management of diseases caused by *X. campestris* in several important crop plants.

Manjula *et al.* (2002) studied efficacy of bactericides against *Xanthomonas axonopodis* pv. *punicae* and reported that Copper oxychloride @ 2000 ppm was found moderately effective and kasugamycin @ 500 ppm concentration was ineffective

Das (2005) studied efficacy of different bioagents, botanicals, chemicals against different isolates of *Xanthomonas axonopodis* pv.*citri* and found that COC (0.3%) + streptomycin sulphate 100 ppm was found more effective in reducing citrus canker *in vitro* by paper disc and turbidiometrical method.

Shahid *et al.* (2005) tested Streptomycin sulphate, Dithane M-45, Agrimycin–100, Vitavax, Benlate and Cobox at 1% concentration against *Xanthomonas campestris* pv. *citri in vitro*. Streptomycin sulphate, Vitavax, Dithane M –45 and Agrimycin–100 in the order proved effective and also in reducing the disease intensity as compared to inoculated control.

Sahi *et al*. (2007) reported that vitavax was found most effective compared to other toxicants for the control of citrus canker disease and also proved effective in reducing the disease intensity as compared to inoculated control.

Giri *et al.* (2008) studied efficacy of different botanicals, bioagent, chemicals for controlling citrus canker and found three spray of 5% neem seed extract was effective in reducing the disease intensity.

Samavi *et al.* (2009) recorded the efficiency of two novel compounds, thyme essential oil (TEO) produced from zaatar (Zataria multiflora) and Nanosilver (NS), as well as some commonly used chemicals were evaluated against citrus bacterial canker using detached-leaf assays in the laboratory and whole seedlings in the greenhouse. Treatments included 0.3% copper oxychloride (COC), 1.5% Bordeaux mixture (BO), 0.3% COC+0.04% Mancozeb (MZ), 1.5% BO+0.04% MZ, TEO at 10 -2 dilution, three concentrations of NS (100, 150 and 200 ppm) and 100 ppm Streptomycin (S). The BO+MZ, COC+MZ and TEO treatments were found most effective based on mean number of lesions and type of symptoms on detached leaves.

Basha *et al.* (2009) reported plants treated with the spray combination of 2- bromo-2-nitro propane- 1,3diol (Bactrinashak) + Streptomycin Sulphate + Tetracycline Hydrochloride + COC for four times with 30 days interval found effective in reducing the disease and recorded higher yield of 17.89 t/ha as compared to untreated control (7.69 t/ha.).

Yenjerappa (2009) reported that bactericides viz., Kcycline, copper oxychloride, and streptocycline were found moderately effective with an inhibition zone ranged from 8.40 to 10.23 mm against *Xanthomonas axonoppdis* pv. *punicae.*

Beheshti B. *et al*. (2011) reported that control of *Xanthomonas citri subsp. citri* (*Xac*),disease,by inoculating lime (*Citrus aurantifolia*) plants with *Xac* were treated with βAminobutyric Acid (BABA), ascorbic acid (vitamin C), thiamine (vitamin B1), green tea (*Camellia sinensis*), copper oxychloride and distilled water. Reduction in lesion size and lack of antimicrobial activity indicate that BABA and green tea might be useful treatments against Xcc infection.

Raghuwanshi *et al.* (2013) reported that Bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* is a major

biotic constraint in peninsular India. Six chemical treatments showed complete control under *in vitro* conditions while rest varied in their response to isolates. Complete control in all four isolates was observed with Bordeaux mixture (1%); captan (0.25%) + Copper oxychloride (0.3%), captan (0.25%) + copper hydroxide (0.3%), bromopol (500 ppm) + copper oxychloride (0.3%), streptocycline (250 ppm) + copper hydroxide (0.3%), streptocycline (500 ppm) + copper hydroxide (0.3%) during *in vitro* study.

Islam *et al*. (2014) studied isolation, identification and *in vitro* antibiotic sensitivity pattern of *Xanthomonas axonopodis* pv. *citri.* nine isolates were tested against 5 commonly used antibiotics namely, cefotaxime, bacitracin chloramphenicol, streptomycine and gentamycine and result showed that *X.axonopodis* pv *citri* was 100% resistant to cefotaxime and 77.77% to bacitracin. Chloramphenicol was found most effective as all the isolates wereshowed sensitive to this.

Mubeen *et al.* (2015) studied six antibiotics Sino Bionic, Streptomycin Sulphate, Benzyle Penicillin Sodium, Kanamycin Sulphate, Chloramyphenical Sodium and Ampicillin Sodium, were tested against *Xanthomonas axonopodis* pv.*citri In-vitro*. Sino Bionic (SB), Benzyl Penicillin Sodium (BPS), Streptomycin Sulphate (SS) and Kanamycin Sulphate (KS) were the four out of six antibiotics, showed the inhibition zone at four concentration 31 µl, 62 µl, 125 µl and 500 µl. The mean inhibition zones were 1.4cm, 1.6cm, 1.8cm and 2.2cm at 31µl, 62µl,125µl ,and 500µl respectively. Concentration of 500 µl showed the best results *in-vitro*.

Antre *et al.* (2016) investigated *in vitro* efficacy of different chemicals, botanicals and bioagent individually and in combination against *Xanthomonas axonopodis* pv*. punicae* by using disc diffusion method. Maximum zone of inhibition were recorded in chemical treatments. Copper oxychloride (0.3%) + Streptomycin sulphate (500 ppm) was found significantly superior in inhibiting the growth of bacteria with 14.33 mm zone of inhibition.

Badiger *et al.* (2016) six antibiotics, two antibacterial chemicals and five bio-agents were evaluated *in vitro* against *Xanthomonas axonopodis* pv. *citri* (Hasse). Among them Streptocycline (10.84 mm) and copper oxychloride (7.50 mm) showed maximum inhibition zone followed by K cycline (9.68 mm). Among the bio-agents, *Bacillus subtilis* found effective with inhibition zone of (16.16 mm) followed by *Pseudomonas fluorescens* (14.63 mm). The fungal bio control agents *viz., Trichoderma viride* and *Trichoderma harzianum* were also found totally in effective against the pathogen.

Savitha *et al.* (2016) were studied on integrated management of citrus canker at College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India through sanitation and pruning followed by different combinations of copper fungicides, bactericides, ISR chemical, botanicals and bioagent. The results reported that crop sanitation followed by spraying of streptomycin sulphate (500 ppm) + COC (3g L^{-1}) was found effective with percent disease index (PDI) of 30 and it was at par with sanitation followed by Bordeaux mixture (1%) spray followed by bacterinashak (0.5g $L^{-1)}$ and *Pseudomonas fluorescens* (5g L^{-1}) spray with PDI of 32.17.

Abhang *et al.* (2018) studied the different bioagents, botanicals and chemicals against *Xanthomonas axonopodis* pv. *citri* and revealed that the Copper oxychloride (0.2%) + streptomycin sulphate (200 ppm) was significantly inhibit the growth of pathogen and in botanicals and bioagents neem seed kernel extract (5%) was effective in reducing the growth of bacteria with 0.446 OD at 96 h followed by *Pseudomonas flourescence* 1x108 cell and *Bacillus subtilis* 1x108cell with 0.506 and 0.486 OD, respectively.

Jadhav *et al.* (2018) studied on three antibiotics, three antibacterial fungicides and three botanicals for the management of bacterial canker disease of kagzi lime caused by *Xanthomonas axonopodis* pv. *citri.* (Pot culture). In these antibiotics, streptocycline was found most effective with lowest PDI mean 33.77 per cent. The second and third best control recorded were mancozeb and plantomycin 60 which receded

comparatively minimum disease incidence 34.88 and 38.33 per cent respectively.

2.6 Molecular variability by using ISSR marker

Saiki *et al*. (1988) The Polymerase Chain Reaction (PCR) allows rapid, specific and sensitive detection of DNA sequences, and thus is ideally suited for the detection of plant pathogens and reported the development of a PCR based assay for *X. c*. pv *citri* based on the DNA sequence of the EcoRl insert in pFL1.

Pooler *et al*. (1996) determined genetic relationships among 25 isolates of *Xanthomonas fragariae* from diverse geographic regions by three PCR methods that rely on different amplification priming strategies: which identified nine, four and two genotypes through RAPD, REP and ERIC. RAPD, ERIC, and REP PCR assays, respectively, within *X. fragariae* isolates.

Shiotani *et al*. (2000) carried out repetative sequence-based polymerase chain reaction amplification by enterobacterial repetative intergeneric consensus (ERIC) sequences (ERIC 1R and ERIC 2) as the primers. These two groups were also distinguishable by the presence or absence of a 1.8-kb DNA fragment among identical fragments. The 1.8-kb fragment was amplified only from the strains aggressive to *C. grandis***.**

Chakraborty *et al*. (2004) reported that, RFLP analysis of *Xanthomonas axonopodis* pv. *malvacearum* strains from different geographical regions of the world exhibited wide degree of polymorphism. When clustered in three different groups and clear evidence of intra-racial variability was detected within 18 isolates of *Xanthomonas axonopodis* pv. *malvacearum.*

Siraree *et al*. (2004) generated the RAPD profiles using single decamer primers in polymerase chain reaction to study the diversity in *Xanthomonas campestris* pv. *campestris* population prevalent in the region. Amplification conditions were standardized so as to get polymorphism specific for individual isolate. Amplification products ranged from 6 to 12 with fragment size ranging from 100 bp to 5.0 kb.

Flavia Maria de Souza Carvalho *et al*. (2005) investigated *Xanthomonas axonopodis* pv*. citri* strains causing canker in citrus by pulsed field and plasmid profile analysis, the strains evaluated were collected in seven different states of Brazil, and in Argentina,Bolivia, Paraguay,and Uraguay and genetic variability found among strains of *X.axoniopodis* pv. *citri* from different geographical areas of Argentina,Bolivia,and Uraguay with similarities varying from 0.62 to 0.83 and reported that *X. axonopodis* pv. *citri* strains showed a considerable degree of diversity with regard to their extrachromosomal genetic element.

Lin *et al.* (2005) showed two types of a typical symptoms-inducing *Xac* strains differentiated from two atypical *Xac* strains A* and A^w isolated from southwest Asia and Florida by lrp sequence assay and amplified DNA profiles of PCR with primer pairs pthAP7/pthAR2, 2/3, 4/7 or ERIC1R/ ERIC2 And showed novel strains of *Xac* and designated these two types of a typical symptoms-inducing strains as Xac- Af type and Xac-Ar type, respectively.

Golmohammadi (2007) studied bacterial isolation, three conventional polymerase chain reaction (PCR) protocols, and real-time PCR with SYBR Green or a Taq Man probe, were compared. Canker-like lesions were disrupted in PBS buffer, and the extract used for bacterial isolation and DNA extraction followed by PCR amplification. Canker lesions, identified by PCR, showed viable bacteria in eleven of fifteen fruit samples. In 16 out of 130 lesions analysed from these samples, Xac was isolated, and pathogenicity on grapefruit leaves confirmed.

Abdo-Hasan *et al*. (2008) demonstrated the genetic relationships among 40 *Xanthomonas axonopodis* pv. *malvacearum* strains using RAPD and ISSR techniques and proved that both techniques were fast, sensitive, and reliable for determining of genetic variability among the same species.

Lin *et al*. (2008) reported the similarity coefficient of both a typical symptoms-inducing strains XL16 and XL38 was 0.9–1.0 to Xac reference strains XW19 or 2863 based on REP- and ERIC-PCR analysis.

Yenjerappa (2009) reported the molecular technique, Random Amplified Polymorphic DNA (RAPD) to detect the variations among the 20 isolates of *Xanthomonas axonopodis* pv. *punicae*. In the present study, the group of primers belonging to OPA, OPB and OPF series were used to determine the genetic differences among the isolates and to construct a dendrogram.

Bhavesh *et al.* (2010) analysed two DNA- based molecular marker techniques, viz., random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR), were used to assess the genetic diversity in castor genotypes. The percent age of polymorphism using ISSR primers ranged from 33.3 to 100.0, with an average of 68.1%. Clustering of genotypes within the groups was not similar when RAPD and ISSR derived dendrograms were compared, whereas, the pattern of clustering of the genotypes remained akin in RAPD and combined data of RAPD and ISSR. The similarity coefficient ranged from 0.53 to 0.91, 0.55 to 1.00, and 0.51 to 0.93 with RAPD, ISSR, and combined dendrogram, respectively.

Hussain *et al.* (2010) reported that traditional management of *X. axonopodis* pv. *citri* is brought about by chemicals which have become complicated through the development of chemical resistance, and as such, it is hazardous for health. It is necessary to identify the pathotypes of *X. axonopodis* pv. *citri* through biochemical and molecular characterization and to determine the role of different biocontrol agents (antibiotics and plant extracts), in order to find out a safer way for managing citrus canker as disease severity results in defoliation, dieback, premature fruit drop and blemished fruit that consequently decrease fruit production and market value.

Giri *et al*. (2011) analysed the 16 strains with RAPD profiles and reported that high level of genetic variability among the strains of *X. axonopodis* pv.*punicae* and showed that the variation exhibited by the strains of *X. axonopodis* pv. *punicae* were independent of geographical location.

Singh *et al*. (2011) studied genetic variability of 22 strains of *Xac* using Rep-PCR (REP-, BOX- and ERIC-PCR) and 10 strains for hrp

(hypersensitive reaction and pathogenecity) gene sequence analysis and established genetic and pathogenic variability in Indian strains of *Xac* were which will be of immense use in the development of resistant genotypes against this bacterial pathogen.

Fatima *et al.* (2012) investigated the genetic diversity among seven *Xanthomonas* isolates representing four species was assessed using randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) PCR-based techniques. Both techniques revealed high degrees of polymorphisms among the isolates. Cluster dendrogram based on combined data of RAPD and ISSR showed that genetic diversity exists in local isolates of *Xanthomonas*. In terms of percentage similarity values, the genomic variation was found to be in the range of 29.29 to 100% among the isolates. *X*. *campestris* (*Mangifera indica*) remain unclustered in cluster dendrogram and showed a unique genomic profile as compared to other isolates used in this study

Shahrestani *et al*. (2012) investigated genetic variation in 60 isolates of *Xanthomonas oryzae* pv. *oryzae,* using random amplified polymorphic DNA (RAPD) markers and reported that the RAPD markers used in study could differentiate nursery and field isolates from each other.

Rezaei *et al*. (2012) analysed genetic diversity among *Xanthomonas citri* subsp. *citri* strains through repetitive polymerase chain reaction (repPCR) and random amplified polymorphic DNA (RAPD) and reported that two primers, ERIC 1R and 232, with the highest marker index, revealed in the most genetic variability among strains.

Arshadi *et al.* (2013) isolated 25 samples of canker disease from different part of West Malaysia from three different hosts. After various diagnostic tests, the samples were identified as *Xanthomonas citri* subsp. *citri* (*Xcc*), and were also pathogenic to four tested citrus species. Molecular characterization using rep-PCR fingerprinting was carried out on the isolates. Cluster analysis using the combined banding patterns of ERIC and BOX-PCR clearly divided the isolates into different clusters according to their geographical origin, but not to their host species. A relatively high amount of genetic diversity was observed among isolates, as a group of

isolates from a more restricted part of Malaysia separated from the rest with relatively low similarity, indicating that there might be distinct pathotypes of the bacterium present in Malaysia.

Raghuwanshi *et al.* (2013) reported that bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* is a major biotic constraint in peninsular India..On Inter Simple Sequence Repeat (ISSR) analysis they formed separate clusters with Akkalkot-Solapur isolate being most divergent, while Deola-Nashik and Sangamner-Ahmednagar isolates were most similar.

Rouhrazi *et al*. (2014) assessed the genetic diversity of 40 *Xanthomonas campestris* pv. *campestris* (Xcc) strains isolated from crucifers, and were characterized their morphological, biochemical and physiological features and pathogenicity tests and studied the distribution of dispersed repetitive DNA, Enterobacterial Repetitive Intergenic Consensus (ERIC), BOX, Repetitive Extragenic Palindromic (REP) and random amplified polymorphic DNA (RAPD) sequences in the genome of Xcc using conserved primers.

Al-Saleh *et al*. (2014) investigated the distribution of 76 *Xanthomonas citri* subsp. *citri* (Xcc) pathotypes in south-western region of Saudi Arabia. These strains were subjected to biochemical, molecular and pathogenicity tests on leaves of grapefruit (*Citrus paradisi*). physiological and biochemical tests, ImmuneStrip assays and 16S rDNA analysis confirmed the identity of the two *Xcc* pathotypes: A and A* and results indicate that, the two pathogenic variants co-exist in south-western region of Saudi Arabia and this could further favour the generation of new genetic variants through recombination and horizontal genetic exchange.

Madhuri *et al.* (2016) studied on The Random Amplified Polymorphic DNA (RAPD) was used to study the variation amongst the 15 isolates of Xac. A total of 27 RAPD primers were screened. Off which 19 primers showed amplification and produced scorable bands with high degree of polymorphism. A total 220 amplicons were obtained of which 218 amplicons were polymorphic with 99.52% level of polymorphism. The banding profile varied from minimum 5 band types (OPB- 1) to maximum

21 band type (REP) indicating the high molecular variability amongst all the fifteen isolates of Xac.The similarity coefficient ranged from 0.27 to 0.68. The maximum genetic similarity was found amongst the isolate from Uttar Pradesh (Xac- V) and Shriganganagar (Xac- XIV) i.e. 0.68.

Madavi *et al.* (2017) studied the genetic diversity among eight Xanthomonas isolates was assessed using inter simple sequence repeat (ISSR) PCR based techniques and revealed high degrees of polymorphisms among the studied isolates. The genomic variation was found to be in the range of from 0.37 to 0.93 across eight isolates indicating high degree of genetic variation.

CHAPTER III

MATERIAL AND METHODS

The present investigation entitled “Antibiotic sensitivity, variability And Management of *Xanthomonas axonopodis* pv. C*itri”* was carried out in the laboratory of Department of Plant Pathology,Post Graduate Institute,Dr.PDKV,Akola (M.S.) during the year 2017 – 2018.

3.1 Material used

3.1.1 Glassware

The following glassware of borosil make were used Petri plates, reagent bottles, micropipettes, conical flasks of different capacity i.e. 2000 ml, 1000 ml, 500 ml, 250 ml and 100 ml, test tubes, pipette, beakers of Borosil make.

3.1.2 Equipments

The laboratory equipments *viz.,* Autoclave, Hot Air Oven, Laminar Air Flow, Spectronic - 20, Electronic balance, BOD incubator, Centrifuge, PCR, Gel electrophoresis, Microwave oven, Vortex were used.

3.1.3 Miscellaneous material

The inoculation needle, double distilled Water, spirit lamp, spirit, scalpel, forcep, glass slide, cover slip, eppendorf tubes, PCR tubes and tips etc. were used during the studies.

3.2 Method

3.2.1 Collection of diseased samples

The disease sample infected with citrus canker were collected during July-october from different locations of Maharashtra state. *viz.* Nagpur, Bhandara, Amravati, Akola, Washim, Pune, Rahuri,Dapoli *etc*. and isolated the bacterium on nutrient agar medium..

3.2.2 Sterilization of glassware, media, water, blotter paper etc.

Glasswares were cleaned with high quality liqid soap and rinsed with tap water. All these glassware then placed in the chromic acid solution ,cooled at room temperature and added with constant stirring to

concentration H_2SO_4 for overnight and rinsed with tap water followed by distilled water. then dried and sterilized in hot air oven at 180^0C for one h Whereas the media, distilled water and blotting papers were sterilized in autoclave at 15 psi for 15 min. The sugars and other thermo labile compounds were sterilized by bacterial proof filters. The material *viz.* inoculating needle, forcep, scarpel were disinfected by dipping in 90% alcohol and then sterilized on flame by direct heating and then cooled at room temperature before use.

3.2.3 Precautions to eliminate contamination:-

All the isolation and inoculation work of microbial cultures was carried out under aseptic condition in laminar airflow. The working area of laminar airflow was sterilized by glowing ultraviolet light for half an hour prior to commencement of work. The working surface and side glasses of laminar airflow disinfected with denatured spirit before use.

3.2.4 Isolation of *Xanthomonas axonopodis* pv.*citri*

For isolation of *Xanthomonas axonopodis* pv.*citri* Nutrient Agar (NA) medium was used.Composition of NA medium is as given below

- **Nutrient agar medium**

Beef extract	-	3 g
Peptone	-	5 g
Sodium chloride	-	5 g
Agar agar	-	15 g
Distilled water	-	1000 ml

The *Xanthomonas axonopodis* pv. *citri* (Hasse and vauterin *et al.*) was isolated by tissue isolation method.

- **Procedure**

1. Canker disease infected leaves of acid lime were surface sterilized by dipping in 70% ethyl alcohol for 1-2 min, followed by seven rinses with sterilized distilled water.

2. Infected portion of the leaves were aseptically excised with sterile scalpel and placed onto sterile mortar containing 2 ml sterile distilled water and crushed with pestle**.**
3. Sterile loop was used to streak bacterial suspension separately onto nutrient agar medium and incubated at 28^0±2C temperature for 24 h to examine periodically for colony growth.
4. One of the single yellow colony was picked by sterile bacterial loop and streaked on another media plate and incubated for 72 h at 28±2^0C temperature for pure culture.

3.2.5 Purification and maintenance of bacterial culture

The respective bacterial cultures were maintained on NA medium at 28±2^0C. The cultures were maintained by adopting subsequent sub culturing at periodical and regular intervals and three days old cultures were used for the studies.

3.3. Identification of the pathogen

The identification of the pathogen involved in causing of citrus canker in acid lime was determined by conducting studies on its morphological, biochemical, cultural and physiological features of the pathogen as per standard microbiological procedures.

3.4 Morphological and Biochemical characterization of *Xanthomonas axonopodis* pv. *citri*

The isolated yellow colour bacteria *Xanthomonas axonopodis* pv.*citri* was characterized by some morphological and biochemical tests.

3.4.1 Morphological studies

The confirmation of the *Xanthomonas axonopodis* pv.*citri* isolates were performed with the following studies.

The pure culture of eight isolates obtained from different agroclimatic regions were streaked on nutrient agar medium separately for colony development. The individual colonies of each isolate was examined visually for colony characteristics including the microscopic examination for shape.

3.4.2 Biochemical studies

The biochemical tests *viz*., Gram's reaction, KOH test, Starch hydrolysis, Gelatin liquefaction, and Catalase test etc. were carried out for confirmation of *Xanthomonas axonopodis* pv.*citri.*

3.4.2.1 Gram reaction

Gram staining test was used to differentiate bacterial species into Gram positive and Gram negative, based on the physical properties of their cell wall. Gram staining was carried out according to Jabben *et al.(*2016).

Procedure :

1. Crystal violet,ethanol,gram-iodine,and safranin were used.
2. At first,the isolated *Xanthomonas axonopodis* pv.*citri* culture was heat fixed onto glass slide.
3. Then crystal violet (main stain) was flooded to the bacterial sample for 1 min.care has been taken that stain was not dried.
4. After washing the slide, gram iodine was added in the medium for 30 sec.and then washed in the tap water. Then decolorized with 95% ethyl aclohol by flooding ethyl alcohol on bacterial sample for 10 sec and rinsed with water.
5. Then safranin was used to counter staining for 10 sec. and again washed in tap water.

3.4.2.2 KOH test

The one drops of 3% Potassium hydroxide was placed on a clean sterile glass slide. The loopful bacterial culture was picked up from the culture with the help of inoculating needle and mixed with KOH drops for 10 seconds. The needle was raised to observe the development of string/thread which was treated as positive test.

3.4.2.3 Starch hydrolysis

The Starch is a complex carbohydrate (polysaccharide) composed of two constituents – amylose, a straight chain polymer of 200-300 glucose units and amylopectin, a larger branched polymer with

phosphate groups. The positive test indicates by the presence of amalyse enzyme, an exoenzyme that hydrolyses (cleaves) starch, into maltose (disaccharide) and some monosaccharides such as glucose.

Medium

Starch Agar : Nutrient agar + 0.2% Soluble starch

Test Reagent :

Lugol's iodine

The test bacterial culture was streaked on sterilized starch agar incubated for 7 days at 28±2^{0}C. After incubation, the plates were flooded with Lugol's iodine solution and allowed to react for few min.. Presence of starch hydrolysis indicated by the appearance of clear zone. Reddish zone indicated that the starch was partially hydrolysed to dextrin.

3.4.2.4 Gelatin liquefication

Gelatin is a protein produced by hydrolysis of collagen, it dissolves in warm water (50ºC). It exists as a liquid above 25ºC and solidifies gels when cooled below 25ºC.Hydrolysis or liquefaction of gelatin is brought about by microorganisms capable of producing proteolyticexo-enzymes known as gelatinase, which acts to hydrolyze this protein to amino acids.

The ability of bacterial isolates to hydrolyze gelatin can be demonstrated by „stab inoculation of nutrient gelatin tubes" to see liquefaction of gelatin. Once the degradation of gelatin occurs in the medium by an exo-enzyme, it can be detected by observing liquefaction i.e. even at very low temperature (4ºC) it will not restore the gel characteristic.

Requirements:

1. 24-48 hrs.old culture of *X. axonopodis* pv. *citri* isolates
2. Nutrient gelatin deep tubes
3. Refrigerator
4. Inoculation needle

Procedure:

1) Prepared nutrient gelatine deep tubes and sterilized in autoclave at 15 psi for 15 min. Each gelatine deep tubes inoculated with respective *Xanthomonas axonopodis* pv. *citri* culture.
2) Using inoculating loop, make a stab inoculation from each culture into its appropriately labelled nutrient Gelatin deep tube. Un-inoculated deep tube placed as a control.
3) Incubated all the (inoculated an un-inoculated) deep tubes at 37ºC for 4-7 days.
4) After incubation, placed the tubes into a refrigerator at 4ºC for 30 minutes. Observation on the refrigerated Gelatin tubes were taken.

3.4.2.5 Catalase test

During aerobic respiration in the presence of oxygen, microorganisms produce hydrogen peroxide (H_2O_2) which is lethal to the cell. The enzyme catalase present in some microorganisms breaks down hydrogen peroxide to water and oxygen and helps them in their survival.

Medium : Nutrient broth medium

Culture of *Xanthomonas axonopodis* pv. *citri* was fixed at 2 places.one is for control and another is for treated. then 1 drop of hydrogen peroxide are dropped on treted.after few sec release of free oxygen gas bubbles indicate *Xanthomonas* positive to catalase test.

3.5. Antibiotic sensitivity against *Xanthomonas axonopodis* pv.*citri* by Paper disc method

Sensitivity of the different isolates was tested by modified paper disc assey method.Desired concentration of antibiotics & chemicals *viz* Streptomycin sulphate, Streptocycline, Kasugamycin, Copper oxychloride *etc.* were freshly prepared in sterile distilled water.The bacterium *Xanthomonas axonopodis* pv. *citri* was multiplied by inoculating the loopful culture in 250 ml conical flask containing 100 ml of nutrient broth medium and incubated at 27 ± 2^0C for 72 hours.

The 20 ml bacterial suspension was added to molten and cooled 1000 ml nutrient agar medium at temperature 40^0C.The seeded medium was thoroughly mixed and poured into the sterilized petriplates and allowed to solidify.

The concentrations of antibiotics and fungicides were prepared as mentioned in table . The filter paper disc (Whatman 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 27±2^0C for 72 hours and observed for the production of inhibition zone around the filter paper discs. The results obtained were analysed statistically.

Table 1. Treatment details

Sr.No	Treatment Detail	Concentration
1.	Streptocycline	100ppm,200ppm
2.	Streptomycine sulphate	100ppm,200ppm
3.	Kasugamycine	100ppm,200ppm
4.	Copper oxychloride	0.2% ,0.3%
5.	Copper oxychloride + Streptocycline.	0.2%+ 100ppm, 0.2%+ 200ppm
6.	Copper oxychloride + Streptomycine sulphate	0.3%+100ppm 0.3%+200ppm

3.6 Molecular variability among the isolates of *Xanthomonas axonopodis* pv. *citri*

The Inter Simple Sequence Repeat (ISSR) analysis was used to detect the variations among the different isolates of *Xanthomnas axonopodis* pv. *citri.* The ISSR technique involved the following major steps.

i. Extraction of genomic DNA (template DNA)
ii. Optimization of PCR conditions
iii. Gel electrophoresis

DNA extraction

Requirements (reagents and primers)

Lysis buffer:40 mM Trisbase,20 mM sodium acetate,1 mM EDTA,1% SDS and pH 8.0

Phenol : Chloroform : isoamyl alcohol (25:24:1) Other reagents : 1xTE buffer, 95% ethanol, 5 M NaCl.

ISSR analysis

For ISSR analysis the template DNA ISSR primers, 10 nm dNTPs (dATP, dGTP, dTTP), Taq DNA polymerase, TBE buffer 10x, nuclease free water, Taq DNA polymerase buffer (10x). Agarose loading dye (6x), Ethidium bromide, Molecular weight markers etc. were used.

Instruments

Thermal cycler, centrifuge, micropipettes, micro tips, eppendorf tubes, PCR tubes, electrophoresis unit *etc* were used.

Extraction of template DNA of the test isolates by CTAB method

Procedure

1. The pure culture of the test isolates obtained from single colony of was inoculated to 10 ml of nutrient broth taken in 100 ml flasks and incubated at 27±2^{0}C for 72 hours with vigorous shaking at a speed of 120 rpm.
2. About 1.5 ml aliquots of broth culture were taken in 2.0 ml eppendorf tubes and centrifuged at 13000 rpm for 5 minutes.
3. The supernatant was poured off, 200 µl of lysis buffer was added to the tubes containing pellet and was mixed well, 66 µl of 5 M NaCl was added and mixed well, contents were centrifuged at 13000 rpm for 10 minutes.
4. The supernatant (250 µl) obtained was transferred to a new tube, mixed well and incubated at 37^{0}C for 30 minutes.
5. An equal chloroform / isoamyl alcohol was added, mixed gently by inverting the tubes and centrifuged at 13000 rpm for 6 minutes.

6. The upper aqueous phase was transferred to a clean tube, 1.0 ml of cold 95 percent ethanol was added and gently mixed well. The tubes were then kept in deep freezer at -20^{0}C for 1 hour, centrifuged at 13000 rpm for 6 minutes.
7. The ethanol was poured off, DNA pellet was air dried by using speed vacuum for five minutes.
8. The pellet was resuspended in 50 µl of 1xTE buffer, kept in the refrigerator at 4 ^{0}C for overnight and further stored in deep freezer at -20^{0}C.

II. Optimization of PCR conditions

The PCR amplification for ISSR analysis was performed according to Bhavesh *et al.(*2010) with certain modifications. The optimum specifications followed for DNA amplifications were as follows.

Table 2-List of ISSR primers with their sequence

Sr No.	Primer Name	Primer sequence (5' – 3')	Annealing Temp (ºC)
1	ISSR-8	CACACACACACACAGC(16)	48.5
2	ISSR-816	CACACACACACACACAT	48
3	ISSR-817	CACACACACACACACAA(17)	53.7
4	ISSR-820	GTGTGTGTGTGTGTGTC(17)	52.8
5	ISSR-827	ACACACACACACACACACG(19)	48
6	ISSR-841	GAGAGAGAGAGAGAGAY(18)	52.2
7	ISSR-857101	ACACACACACACACYC(16)	50.4

Table 3- Programme for ISSR-PCR

Step	Temp. °C	Duration	Cycles	Function
1	94	5 min	1	Initial denaturation
2	94	1 min	40	Denaturation
3	*	1 min		Annealing
4	72	1 min		Extension
5	72	5 min	1	Extension of target molecules (final polishing)
6	4	Forever	1	Storage

*Annealing temperature varied from primer to primer.

Table 4- Master mix for PCR

Sr. No.	Components	Volume for one tube	Final concentration
1	10X PCR buffer	2.0 µl	1X
2	dNTPs	0.5 µl	0.2 mM (each)
3	Taq DNA polymerase	0.2 µl	1U
4	$MgCl_2$	2.0 µl	2 mM
5.	Primer	2.0 µl	36 ng
6	Sterile double distilled water	11.3 µl	---
7	Template DNA	2.0 µl	50 ng
8	Total	20.0 µl	---

Method

The master mix was prepared as detailed in theTable 3 and divided into 8 equal parts (each of 18 µl) in 8 different PCR tubes. The 2 µl (25 ng/µl) of genomic DNA of each isolate was added to master mix by changing tips to avoid contamination. The PCR tubes were then placed in

thermal cycler (Biometra PTC-200) for amplification of the genomic DNA as per the standardized protocol.

III. Gel electrophoresis

Amplified DNA products were separated by agarose gel electrophoresis.

1. Three grams of agarose was weighed and added to a conical flask containing 250 ml of 1x TBE buffer.
2. The contents were melted by heating in an oven, the solution was stirred for proper mixing and complete dissolution of agarose.
3. The agarose gel solution was cooled to about 40 to 45^0C and added 2 – 3 drops of ethidium bromide (0.5 µg/ml) was added.
4. Agarose gel was poured into the casting platform after inserting the comb in tray.While pouring, care was taken to prevent the formation of air bubbles.
5. The gel was allowed to solidify and the comb was removed. After placing the solidification gel was placed into the electrophoretic apparatus containing sufficient buffer (1x TBE), so as to cover the wells completely.
6. About 2.5 µl of loading dye was added to each tube containing amplified DNA.
7. The amplified products (10 µl) were carefully loaded into the sample wells. Electrophoresis was carried out at 60 volts until the tracking dye migrated to the end of the gel.
8. Gel was viewed under UV transilluminator for DNA bands.

IV. Analysis of fingerprints

Scoring of amplified fragments

The amplified profiles for all the primers were compared with each other and bands of DNA fragments were scored as ‘1’ for presence and ‘0’ for absence, generating ‘0’ and ‘1’ matrix. The Per cent polymorphism was calculated by using the formula.

$$\text{Per cent polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Analysis of the profile of the amplified fragments

The genetic similarity coefficient between the test isolates was estimated by DICE similarity coefficient. Clustering was done using the symmetric matrix of similarity coefficient and cluster obtained based on Unweighted Pair Group Arithmetic Mean (UPGMA) using Sequential Agglomerative Hierarical Nested (SAHN) cluster analysis of NTSYS-PC version 2.0 (Rohlf,1998).

CHAPTER IV

RESULTS AND DISCUSSION

The investigation on “Antibiotic sensitivity, variability and management of *Xanthomonas axonopodis* pv. *citri.”* was carried out during 2017-18. The results and its interpretation of the present studies are given in this chapter.

Xanthomonas isolates were isolated from diseased sample of acid lime by tissue isolation method on nutrient agar medium. Citrus canker diseased sample were collected from different location of Maharashtra region viz. Nagpur, Akola, Amaravati ,Washim , Bhandara, Pune, Rahuri, and Dapoli. Bacteria were initially identified based on colony character, morphological and biochemical characters as per Bergy’s manual of determantive bacteriology. Observations were recorded on the basis of morphological and biochemical test of isolates. The efficacy of antibiotics, chemicals were tested against *Xanthomonas axonopodis* pv.*citri. ,*also studied the molecular characterization by using ISSR marker.

4.1 Collection of diseased samples :

In the present study diseased samples were collected during July to October 2017, from different agro-ecological zone of Maharashtra. Infected part of the leaves along with some healthy portion was selected for isolation. (Plate 1) .

Table 5 . The details of samples collected for isolation

Sr.No	Location	Agro climatic zone of maharashtra
1	Nagpur	Central Vidarbha Zone
2	Akola	Central M.S Plateau Zone
3	Amravati	Central M.S Plateau Zone
4	Washim	Central M.S Plateau Zone
5	Bhandara	Eastern Vidarbha zone
6	Pune	Western Maharashtra plain zone
7	Rahuri	Western Maharashtra scarcity zone
8	Dapoli	South Konkan

4.2 Symptomatology

Symptoms observed under natural conditions

The diseased plants exhibited conspicuous raised necrotic lesion on leaves, twigs and fruits. Canker lesion initiated as pinpoint spots and attained a diameter of 3 -10 mm. The lesions were initially circular but later became irregular. They were aggregated at leaf margin or leaf tips or in restricted areas of the leaves. A diagnostic yellow halo surrounded the lesions on leaves. On fruits, lesions confined to rind only without affecting internal quality infection resulted in defoliation, die-back, deformation of fruit and premature fruit drop (Plate 1)

Canker lesion started as pinpoint spot, later attained a diameter of 3-10 mm with raised necrotic lesions surrounded by yellow halo. Under natural and artificial conditions the identical symptoms was observed as initially water soaked lesions developing raised necrotic corky spots. Present findings corroborates with findings of Xiaoan and Stall (2004) who reported that bacterium produced erumpent, pustule like lesion into key /Mexican lime but brownish. Burning and Gabriel, (2003); Khalid *et al*. (2010) reported that the bacterium, *Xanthomonas*, causes different symptoms ranging from pustules to necrotic lesions consisting of erumpent corky tissue surrounded by water soaked tissues and yellow halo on leaves, stems and fruits.

4.3 Isolation from diseased specimen

A total of eight isolates of *Xanthomonas* was isolated from infected leaf and twigs collected from different agro-ecological zone of Maharashtra state. Citrus canker was detected in collected samples. The isolates were purified by streak plate method. Pale yellow to yellow pigmented bacterial colonies identical to *Xanthomonas axonopodis* pv. *citri* were selected for further study (Plate 1).These isolates were maintained on NA slants and catalogued as under.

Plate 1a. Symptoms of citrus canker on leaves, twigs and fruits.

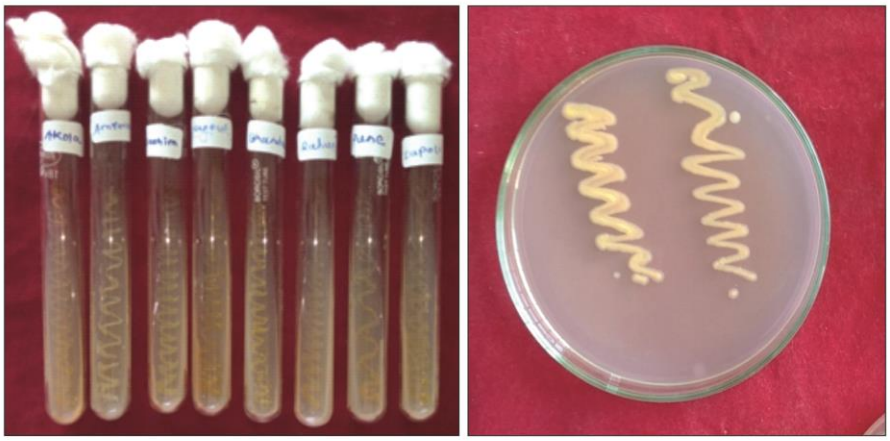


Plate 1b. Isolates of *Xanthomonas axonopodis* pv.*citri* on nutirent agar medium

Table-6. Coding of isolates

Sr. No.	Location	Code No
1	Nagpur	*Xac*1
2	Akola	*Xac*2
3	Amravati	*Xac*3
4	Washim	*Xac*4
5	Bhanadara	*Xac*5
6	Pune	*Xac*6
7	Rahuri	*Xac*7
8	Dapoli	*Xac*8

Present findings corroborates with the findings of Guo *et al*. (2010) reported that all of the strains of *X. citri* subsp. *citri* wild type strain 306 [(Rifamycin resistant) (40)] and mutant strains were grown on NA medium at 28^0C.Didwania *et al.(*2013) demonstrated that black rot causing bacterium was isolated from naturally infected cauliflower plant leaves showing typical 'V shaped symptoms. The single fresh and pure glistering yellow droplets like colonies were purified on other petriplates having nutrient agar medium.

4.5. Morphological and Biochemical characteristics of *Xanthomonas axonopodis* pv.*citri*

All the isolates were studied with respect to their colony colour, shape and Grams staining reaction. The results presented in (Table 7) revealed that bacterial cells appeared short rod and Gram negative. Isolates Xac1,Xac2, Xac5, Xac6,Xac8 produced dark yellow colonies however Xac3, Xac4, Xac7 showed pale yellow colour colonies on NA medium.(Plate 1)

All the eight isolates of *Xanthomonas axonopodis* pv.*citri* were subjected to the biochemical tests for their identification, some of the tests were performed to compared the characteristics depicted in Burgey's manual of Systematic Bacteriology. Eight isolates with respect to Gram

staining, KOH test, catalase test, starch hydrolysis, Gelatein liquefaction were studied.

4.5.1 Gram reaction

All the eight isolates tested, were showed Gram negative reaction (Plate 2).

4.5.2 Starch hydrolysis

All the isolates showed positive to starch hydrolysis, forming a clear hallow around the colony (Plate 2).

4.5.3 KOH Test

All the isolates have an ability to form mucoid thread after added KOH and found positive (Plate 2).

4.5.4 Catalase test

Catalytic activities of all the eight isolates were found positive, when culture were produced bubbles of oxygen within 10 sec after addition of H_2O_2 (Plate 2).

4.5.5. Gelatin liquefaction

All the isolates of *Xanthomonas axonopodis* pv*. citri* were found to liquefy the gelatin within seven days. (Plate-2).

The bacterium was identified as *Xanthomonas axonopodis* pv. *citri.* Variation were observed among the isolates Xac1, Xac2,Xac6,Xac7 are very strong for gelatin liquefication however isolates Xac3 Xac5, Xac8 are moderate and Xac4 are poor for gelatein liquefaction.

Manjula (2002) reported that, seven isolates of the pomegranate bacterium were small rods, appeared singly, Gram negative. Gottwald *et al.* (2002) reported *Xanthomonas axonopodis* is a rod shaped Gram negative bacterium. Das (2003) reported that the bacteria is rod shaped measuring 1.5-2.0 x 0.5-0.75µm, Gram negative.

Biochemical tests viz., Gram staining, KOH test, catalase test, starch hydrolysis, gelatein liquifaction, confirms the bacterial pathogen *Xanthomonas axonopodis* pv*. citri.* The cultured showed variation among

the isolates of *Xanthomonas axonopodis* pv.*citri.* Similar variation among the isolates has been earlier observed by Das (2003) who reported that the bacterial cells of *Xanthomonas citri* are positive for hydrolysis of starch, liquefaction of gelatin, catalase.

Abhang *et al.* (2015) studied on the Morphological and biochemical characteristic viz., shape, colony colour, Gram reaction, starch hydrolysis, gelatein liquefaction, indole production, acid and gas production, KOH, catalase test were performed and confirmed the identity of bacterium as *Xanthomonas axonopodis* pv. *citri.*

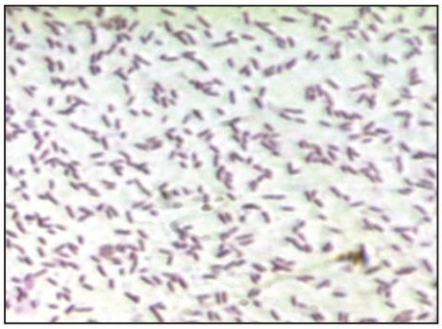
Mubeen *et al.* (2015) stated that *Xanthomonas axonopodis* pv. *citri* is a gram negative bacterium and performed biochemical analysis *viz.* Gram staining, Starch hydrolysis, Tween 80 hydrolysis, Gelatin Liquefaction, KOH test, Kovacs' Oxidase and Fluorescent Pigmentation tests and found that *Xanthomonas* is a gram negative bacterium.

Table 7. Morphological and Biochemical characteristics of *Xanthomonas axonopodis* pv*. citri* isolates

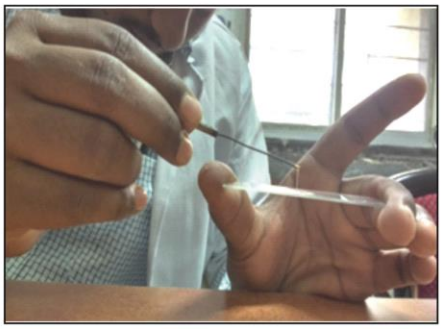
Sr. no	Isolates	*Xac1*	*Xac2*	*Xac3*	*Xac4*	*Xac5*	*Xac6*	*Xac7*	*Xac8*
1	Shape	Short Rod	Short Rod	Short Rod	Short Rod	Short Rod	Short Rod	Short Rod	Short Rod
2	Colony Colour	Dark Yellow	Dark Yellow	Pale Yellow	Pale Yellow	Dark Yellow	Dark Yellow	Pale Yellow	Dark Yellow
3	Gram Reaction	- ve	- ve	- ve	- ve	- ve	- ve	- ve	- ve
4	KOH Test	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
5	Starch Hydrolysis	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
6	Catalyse test	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
7	Gelatin Liquefaction	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve

-ve= Negative

+ve= Positive



Gram staining



KOH test

Catalase test

Starch hydrolysis

Gealtin liquification

Plate 2. Biochemical test for confirmation *Xanthomonas axonopodis* pv. *citri*

4.6. Efficacy of different antibiotic and chemicals against *Xanthomonas axonopodis* pv. *citri*

The efficacy of antibiotics and chemicals was tested against eight isolates of *Xanthomonas axonopodis* pv*. citri* (Xac 1 to Xac 8) by Paper disc method and data is Presented in Table7 revealed that significant differences among the different treatment.

The results revealed that treatment number T_{12} Copper oxychloride (0.3%)+Streptomycine sulphate (200ppm)]and T_{10} (Copper oxychoride (0.2%)+ streptocycline (200 ppm)] and were found significantly effective in inhibiting the growth of test pathogen. The maximum zone of inhibition (32 mm) was recorded in treatment no.T_{12} (Copper oxychloride (0.3%)+Streptomycine sulphate (200ppm)] for the isolate Xac7 followed in T_{10}(Copper oxychoride (0.2%)+ streptocycline (200 ppm)] for the isolate Xac1(26.66 mm). However antibiotics Kasugamycine and fungicide COC alone in different concentration were found not effective for inhibit the zone. It is also revealed from the data produced in table no. 7 that treatnent no. T_9 i.e COC +Streptocycline (Hindustan antibiotics ltd.) alone which in 90:10 (Streptomycine sulphate I.P and tetracycline hydrochloride respectively I.P)(0.2%+100 ppm)] and COC +Streptomycine sulphate (0.3%+100 ppm) i.e T_{11} were inhibit the zone of inhibition after T_{10} and T_{12}.

The lowest zone of inhibition i.e zero percent were recorded in T_4 in isolate Xac4 ,T_5 in isolate Xac1 and Xac4, T_6 in isolate Xac1 and Xac4.

The present findings are in agreement with findings of Sharma *et al*. (1981), who reported that, the combination of streptocycline and copper oxychloride was most effective in inhibiting the growth of *Xanthomonas vesicatoria* when assessed *in vitro* and similar observation were reported by Manjula *et al*. (2002) *In vitro* efficacy of bactericides against *Xanthomonas axonopodis* pv. *punicae* indicated that Copper oxychloride @ 2000 ppm was found moderately effective. Das (2003) reported that COC (0.3%) + streptomycine (100 ppm) inhibited the growth of *Xanthomonas axonopodis* pv. *citri.*

Table 8. Efficacy of different antibiotics and chemicals against eight isolates of *Xanthomonas axonopodis* pv. *citri*

Tre. no.	Treatments with conc.	Zone of inhibition (mm) × average of three replication							
		Xac1	**Xac2**	**Xac3**	**Xac4**	**Xac5**	**Xac6**	**Xac7**	**Xac8**
T_1	Streptocycline100 ppm	17.00	16.00	17.00	13.00	15.00	17.33	13.00	18.66
T_2	Streptocycline 200ppm	18.33	16.66	19.00	18.66	18.00	19.00	18.66	21.00
T_3	Streptomycine sulphate 100ppm	16.00	10.00	8.66	15.00	10.00	12.66	11.00	10.66
T_4	Streptomycine sulphate 200ppm	20.00	13.00	18.66	0.00	13.66	18.00	17.66	16.66
T_5	Kasugamycine 100ppm	0.00	14.00	5.66	0.00	4.00	16.00	10.33	5..00
T_6	Kasugamycine 200ppm	0.00	18.00	9.00	0.00	6.00	19.00	13.66	6.66
T_7	Copper oxychloride 0.2%	4.33	12.00	0.00	2.33	9.00	6.66	6.00	5.00
T_8	Copper oxychloride 0.3%	77.00	15.00	6.00	8.00	12.00	9.00	8.00	11.00
T_9	COC+Streptocycline 0.2%+100ppm	26.00	18.33	13.33	12.33	21.66	22.00	17.00	13.00
T_{10}	COC+Streptocycline 0.2%+200ppm	**26.66**	21.66	17.66	20.33	**25.00**	**24.00**	21.33	16.00
T_{11}	COC+Streptomycine sulphate 0.3%+100ppm	13.00	21.33	17.00	18.00	12.66	19.00	28.00	21.66
T_{12}	COC+Streptomycine sulphate 0.3%+200ppm	19.00	**22.00**	**20.00**	**22.66**	18.33	21.66	**32.00**	**25.00**
T_{13}	Control	0	0	0.00	0.00	0.00	0.00	0.00	0.00
	'F' test	SIG	SIG	SIG	SIG	SIG	SIG	SIG	SIG
	SE (m) ±	0.03	0.02	0.03	0.04	0.02	0.03	0.03	0.03
	CD (P) = 0.01	0.13	0.08	0.13	0.14	0.09	0.11	0.10	0.12

.*****Values in parenthesis are square root transformed

Abhang *et al.* (2018) stated that the Copper oxychloride (0.2%) + streptomycin sulphate (200 ppm) was found significantly effective

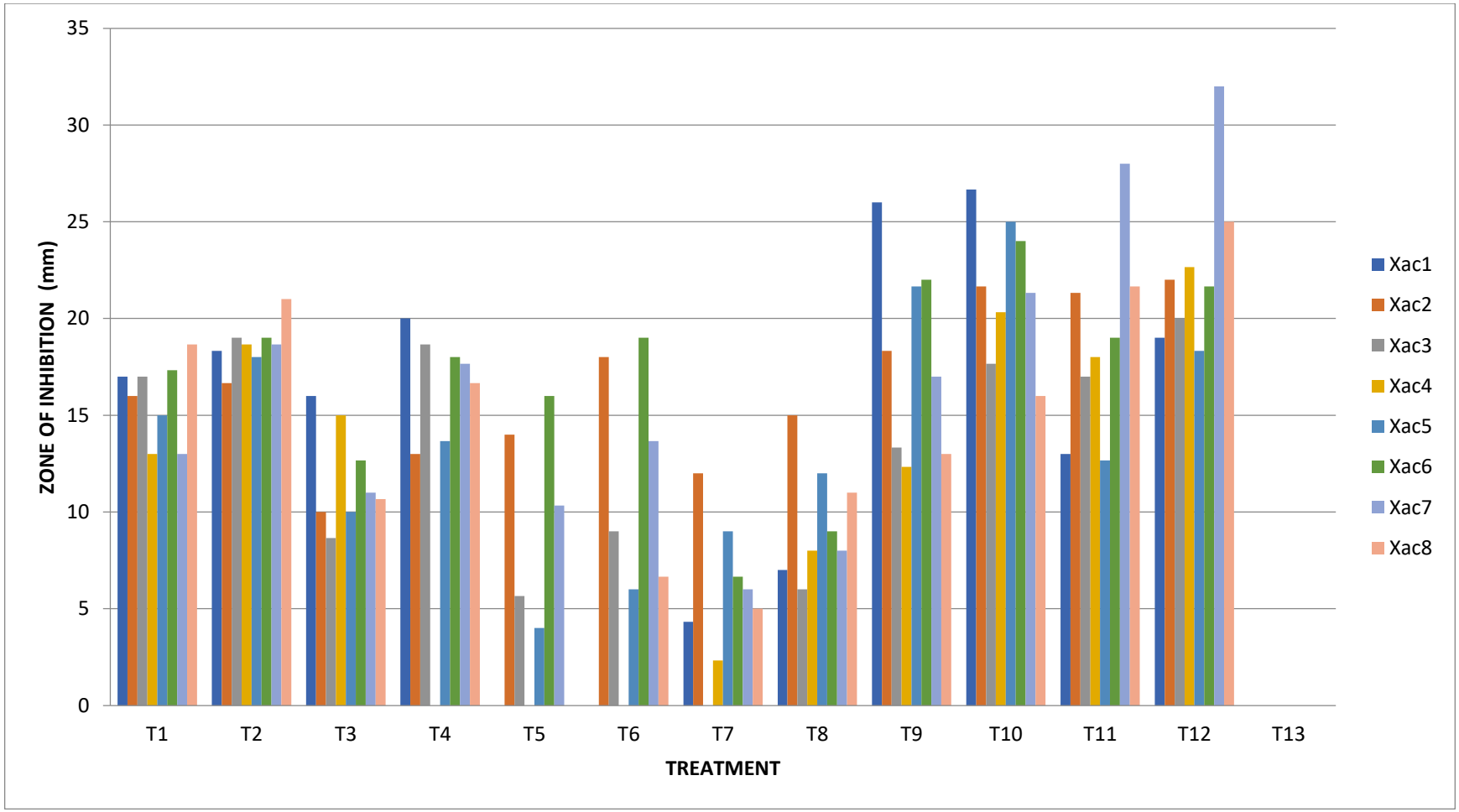
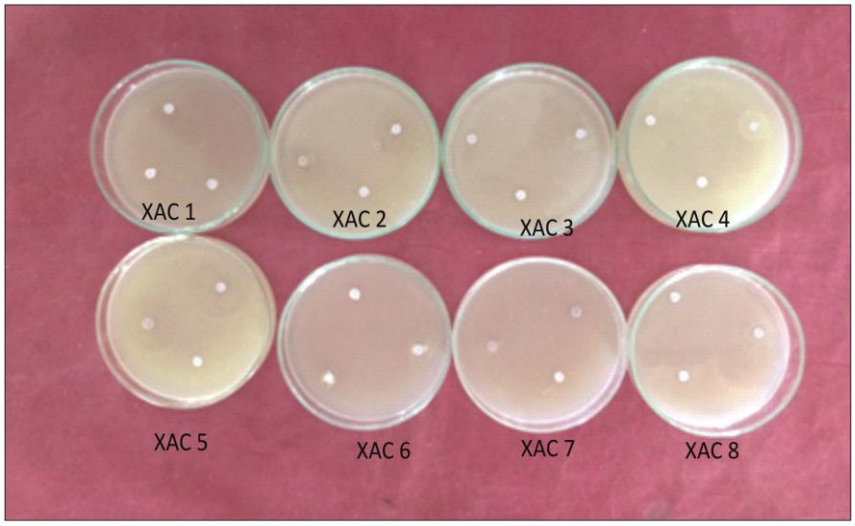
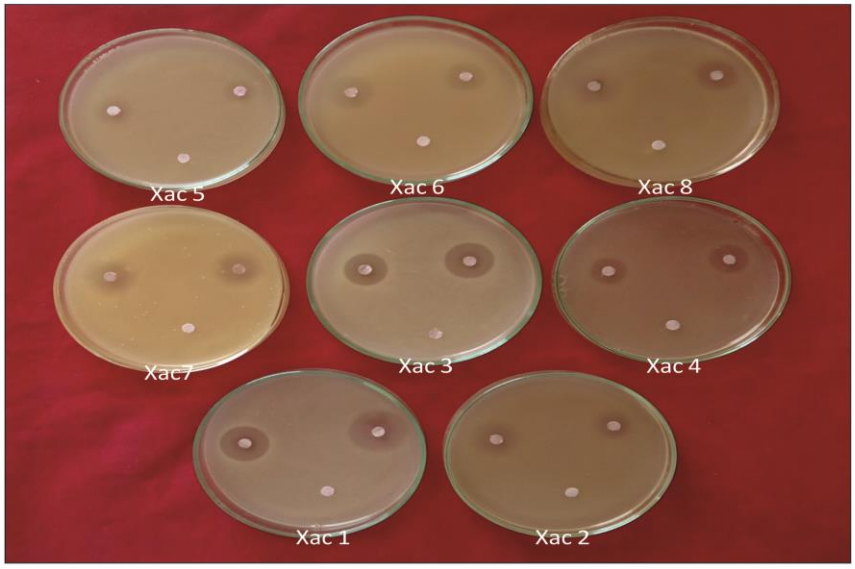


Fig. 1 Efficacy of different antibiotics and chemical against *Xanthomonas axonopodis* pv. *citri* by paper disc method

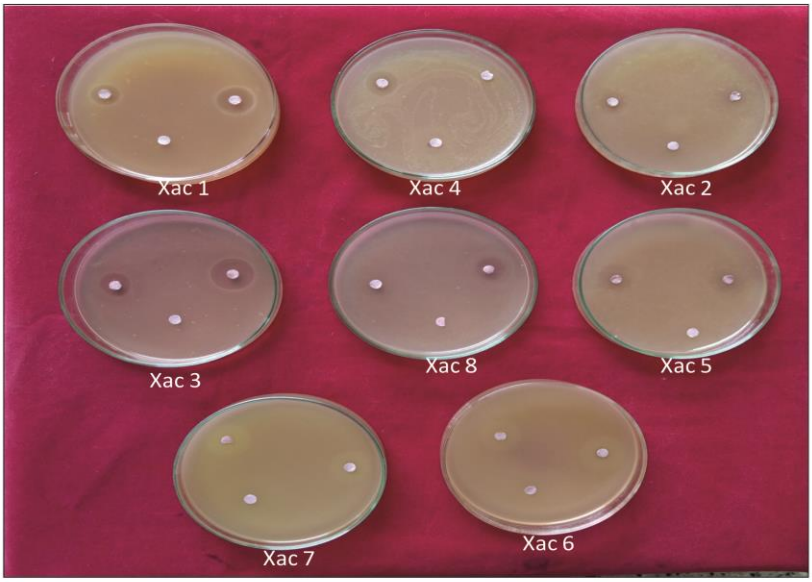


Copper oxychloride 0.2%,0.3%

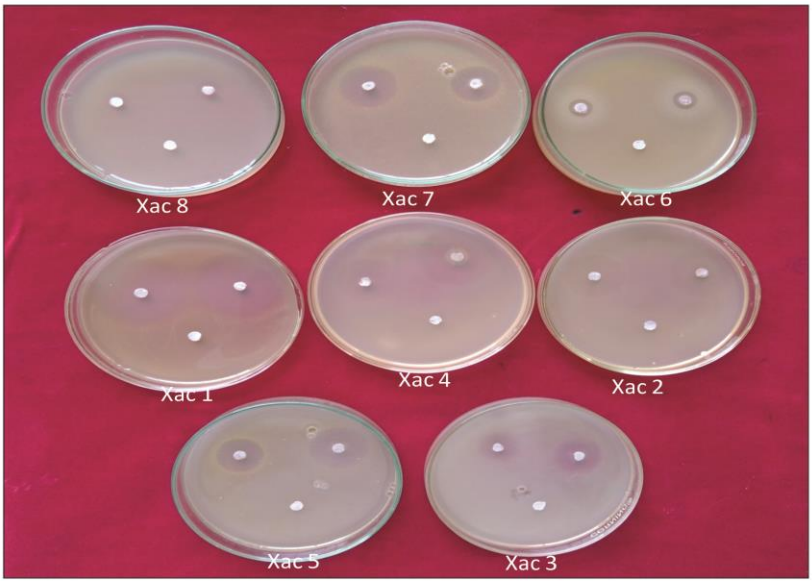


Streptocycline 100ppm,200ppm

Plate 3. The Antibiotic sensitivity against *Xanthomonas axonopodis* pv.*citri* by paper disk method

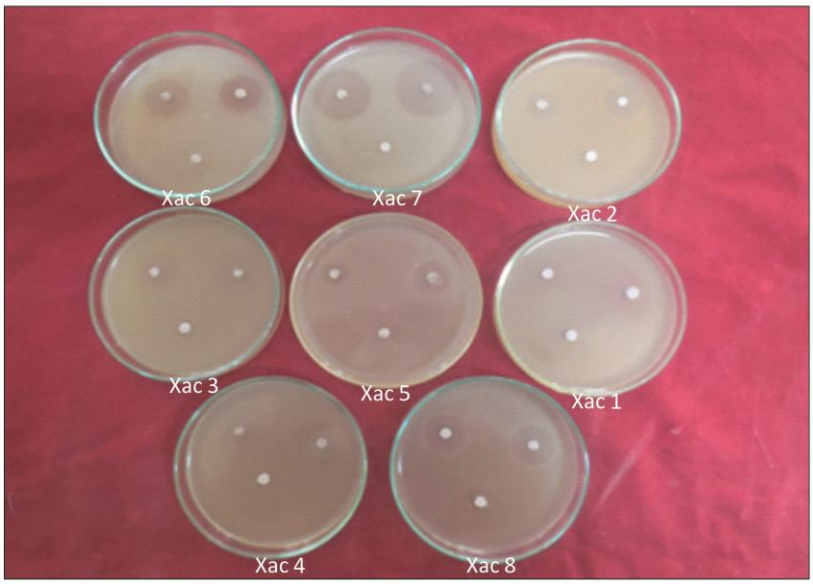


Streptocmycine 100ppm,200ppm

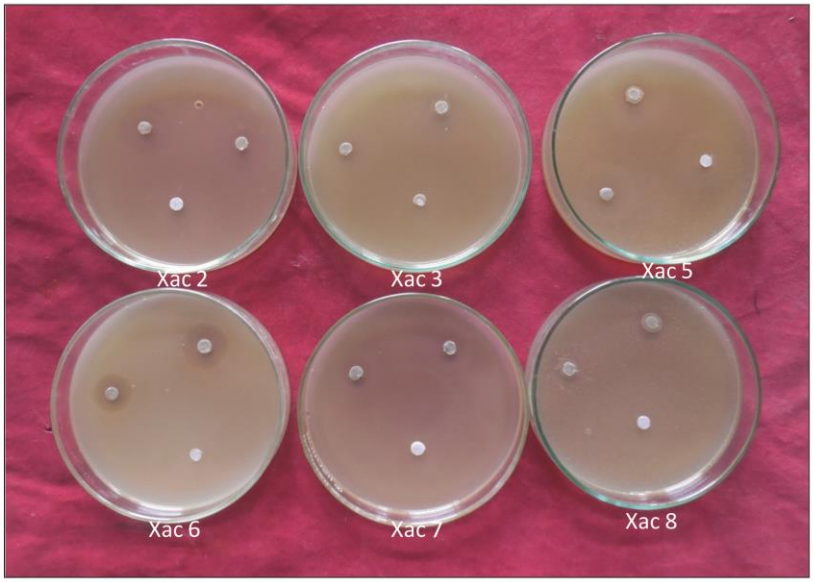


COC+Streptocycline 0.2%100ppm,0.2%+200ppm

Plate 4. The Antibiotic sensitivity against *Xanthomonas axonopodis* pv.*citri* by paper disk method



COC+Streptomycine 0.3%100ppm,0.3%+200ppm



Kasugamycine 100ppm, 200ppm

Plate 5. The Antibiotic sensitivity against *Xanthomonas axonopodis* pv.*citri* by paper disk method

in inhibiting the growth of citrus canker causing bacteria *Xanthomonas axonopodis* pv. *citri.*

Antre *et al.* (2016) investigated that Copper oxychloride (0.3%) + Streptomycin sulphate (500 ppm) was found significantly superior

in inhibiting the growth of *Xanthomonas axonopodis* pv*. punicae* with 14.33 mm zone of inhibition.

4.7 Molecular Variability

4.4.1 ISSR marker selected for molecular study

Seven ISSR primer of ISSR series were selected to evaluate the molecular variability in eight isolates of *Xanthomonas axonopodis* pv.*citri*. The PCR (Polymerases Chain Reaction) amplified product of each primer was resolved on 1.5% agarose gel electrophoresis and the amplified product was compared with DNA molecular weight marker 1 Kb of Biotechnology Grade from Fermentas.

Variation was detected among eight isolates of *Xanthomonas axonopodis* pv.*citri* using ISSR marker. Seven selected primers screened for amplification of DNA of eight isolates of *Xanthomonas axonopodis* pv.*citri.* Seven primers produced scorable bands with high degree of polymorphism. A total of 22 amplicons were obtained with the 7 primers. Out of 22 bands 19 were found to be polymorphic and 3 were monomorphic and the level of average polymorphism was 68 per cent.

Table 9 Per cent polymorphism observed in ISSR primers

Sr no.	Primer	Total Bands	Monomorphic Bands	Polymorphic Bands	% polymorphism
1	ISSR8	1	1	0	00
2	ISSR12	5	1	4	80
3	ISSR817	2	0	2	100
4	ISSR820	1	1	0	00
5	ISSR827	5	0	5	100
6	ISSR841	3	0	3	100
7	ISSR857101	5	0	5	100
Total		22	03	19	68

4.4.2 ISSR banding pattern

The banding pattern observed in primer ISSR8 is presented in table no. 9 and Plate no. 6.-.The primer amplified 1 amplicons among 8 isolates of *Xanthomonas axonopodis* pv.*citri*. The size of amplicons

amplified with primer ISSR8 on 985 bp. The details of the 1 bands types of ISSR bands were:

Band type 1	985 bp	The band observed in Xac1,Xac2,Xac3, Xac4, Xac5, Xa6, Xac7, Xac8

The banding pattern observed in primer ISSR12 are Presented in table no. 9 and Plate 6-. The primer amplified 5 amplicons among 8 isolates of *Xanthomonas axonopodis* pv.*citri* and their size amplified with primer ISSR12 ranged from 277 bp to 1995 bp. The details of the 5 bands types of ISSR bands were:

Band type 1	1995bp	The band observed in Xac2, Xac6 and Xac7.
Band type 2	983bp	The band observed in Xac1, Xac3 Xac4, Xac5,Xac6, Xac7 and Xac8.
Band type 3	896bp	The band observed in Xac1, Xac3 Xac4, Xac5, Xac6, Xac8
Band type 4	389bp	The band observed in Xac2, Xac3, and Xac7
Band type 5	277bp	The band observed in Xac1, Xac4, Xac5, Xac7, Xac8

The banding pattern observed in primer ISSR817 is Presented in table no. 9 and Plate 7-. The primer amplified 2 amplicons among 8 isolates of *Xanthomonas axonopodis* pv.*citri* and their size amplified with primer ISSR817 ranged from 164 bp to 246 bp. The details of the 2 bands types of ISSR bands were:

Band type 1	246bp	The band observed in Xac1, Xac6, Xac8 .
Band type 2	164bp	The band observed in Xac1, Xac2, Xac3, Xac4, Xac5, Xac6 and Xac7

The banding pattern observed in primer ISSR820 is Presented in table no. 9 and Plate 7-. The primer amplified 1 amplicons among 8 isolates of *Xanthomonas axonopodis* pv.*citri* and their size of amplified with primer ISSR820 ranged on 657bp. The details of the 1 bands types of ISSR bands were:

Band type 1	657	The band observed in Xac1, Xac2, Xac3, Xac4, Xac5, Xac6, Xac7, Xac8

The banding pattern observed in primer ISSR827 is Presented in table no. 9 and Plate 8-. The primer amplified 5 amplicons among 8 isolates of *Xanthomonas axonopodis* pv.*citri* and their size of amplified with primer ISSR827 ranged from 157 bp to 970bp.The banding pattern are not observed in isolate Xac4. The details of the 5 bands types of ISSR bands were:

Band type 1	970bp	The band observed in Xac8
Band type 2	615bp	The band observed in Xac1,Xac2, Xac3, Xac5, Xac6 and Xac7. Xac8
Band type3	381bp	The band observed in Xac8
Band type4	243bp	The band observed in Xac2, Xac3, Xac5
Band type 5	157bp	The band observed in Xac5, Xac6

The banding pattern observed in primer ISSR841 is Presented in table no. 9 and Plate 8-. The primer amplified 3 amplicons among 8 isolates of *Xanthomonas axonopodis* pv.*citri* and their size of amplified with primer ISSR841 ranged from 193 bp to 780 bp. However banding pattern not observed in isolate Xac4 and Xac5 The details of the 3 bands types of ISSR bands were:

Band type 1	780bp	The band observed in Xac6, Xac7
Band type 2	307bp	The band observed in Xac1, Xac6 and Xac7
Band type3	193bp	The band observed in Xac2, Xac3, Xac6,

Xac7, Xac8

The banding pattern observed in primer ISSR857101 is Presented in table no. 9 and Plate 9-. The primer amplified 5 amplicons among 8 isolates of *Xanthomonas axonopodis* pv.*citri* and their size of amplified with primer ISSR857101 ranged from 103 bp to 410 bp. The details of the 5 bands types of ISSR bands were:

Band type 1	410bp	The band observed in Xac2
Band type 2	307bp	The band observed in Xac1, Xac2, Xac3, Xac4 and Xac6.
Band type3	202bp	The band observed in Xac5
Band type4	161bp	The band observed in Xac7, Xac8
Band type5	103bp	The band observed in Xac1

4.4.3 Binary similarity matrix of ISSR analysis

A binary similarity matrix of combined data from 7 primers for the eight isolates of *Xanthomonas axonopodis* pv.*citri* were prepared by scoring bands for presence or absence and the DNA bands of same mobility (molecular weight) were assumed to be identical.

Genetic similarity estimate (jaccard's coefficient) based on ISSR banding pattern used for cluster analysis to present genetic relationship in the form of dendrogram Jaccard's coefficient value for eight isolates of *Xanthomonas axonoodis* pv. *citri* are presented in Table 10.

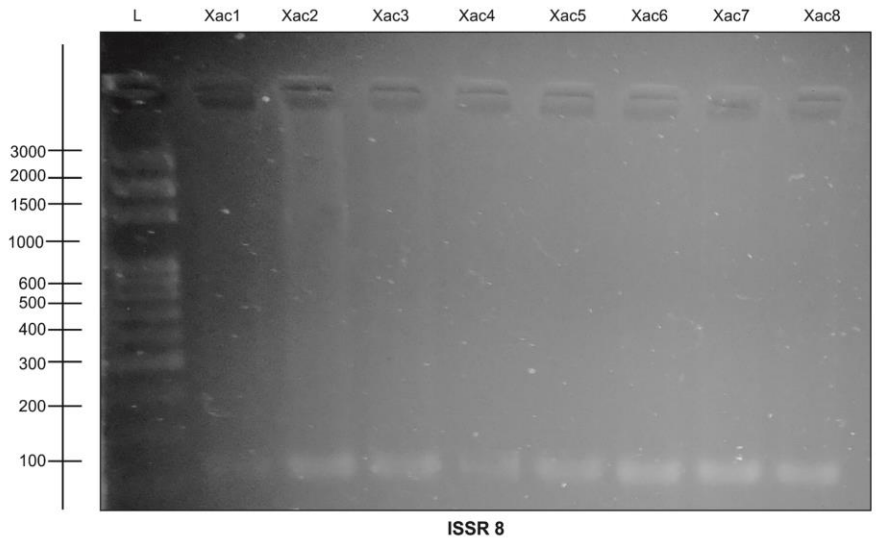
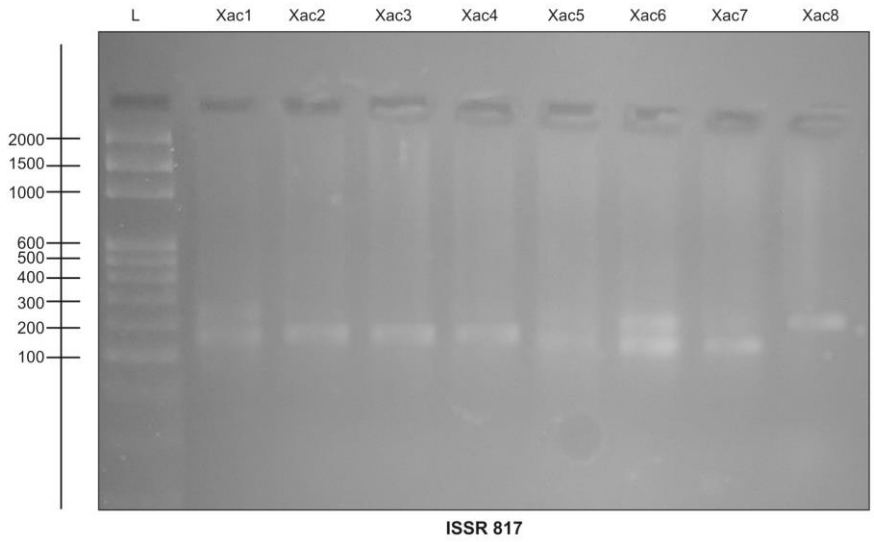
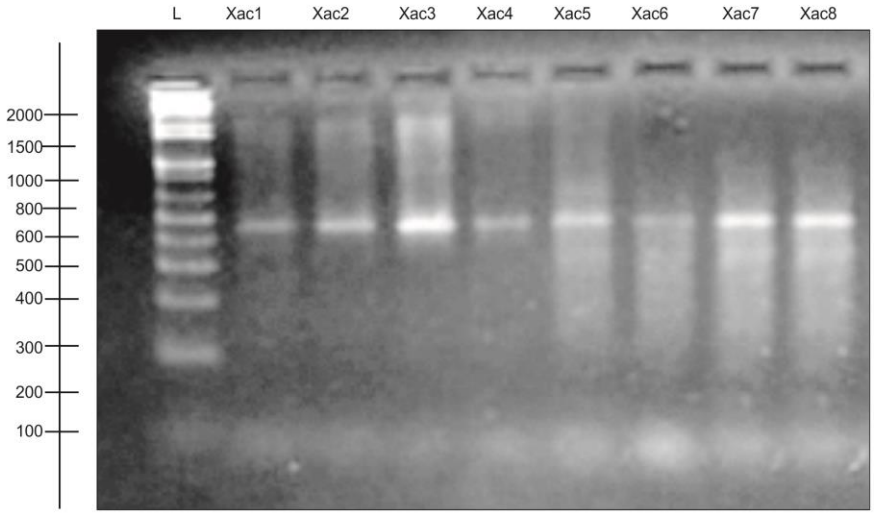


Plate 6. ISSR banding pattern of primers ISSR8 and ISSR12

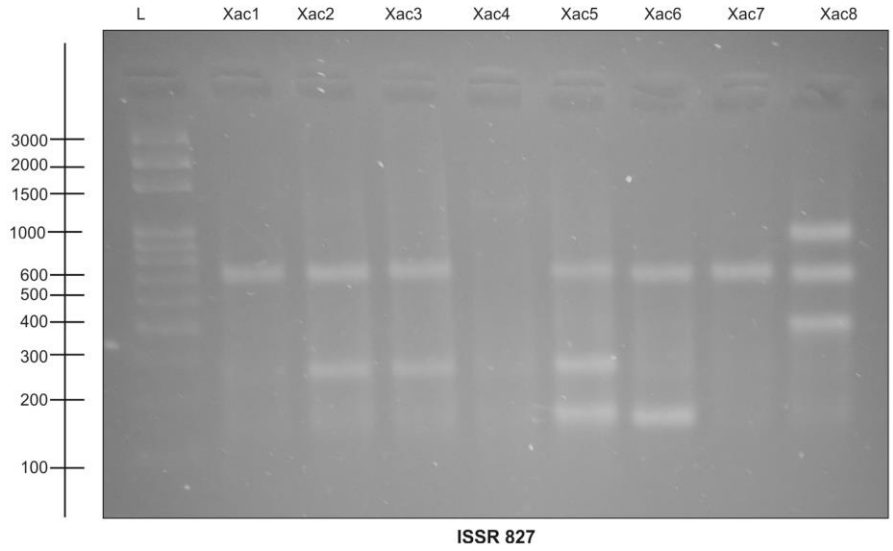


ISSR 817

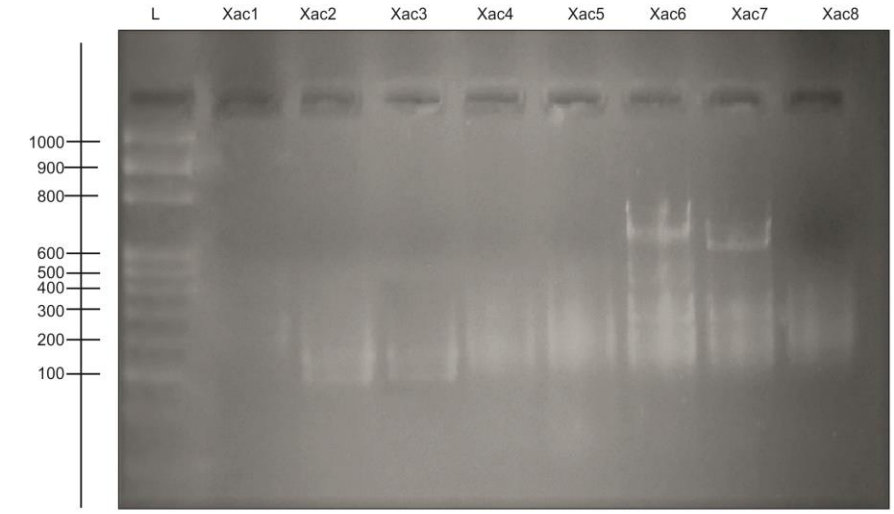


ISSR 820

Plate 7. ISSR banding pattern of primers ISSR817 and ISSR820



ISSR 827



ISSR 841

Plate 8. ISSR banding pattern of primers ISSR827 and ISSR841

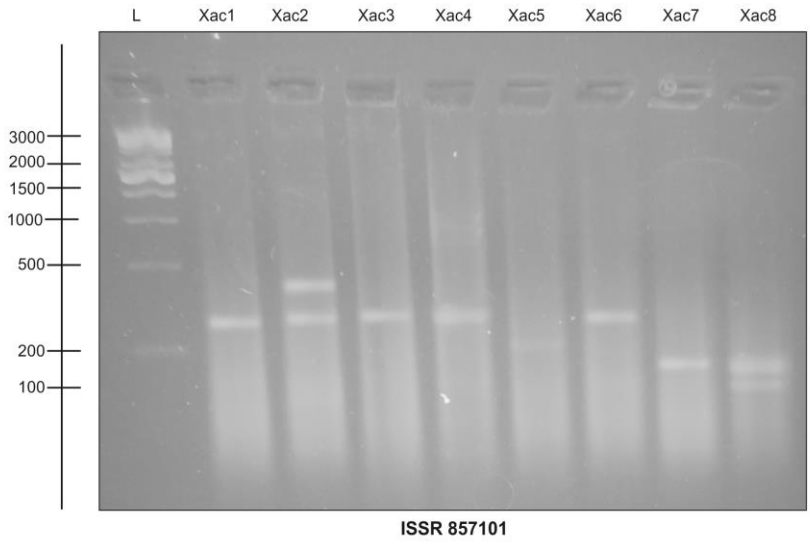


Plate 9. ISSR banding pattern of primers ISSR 857101

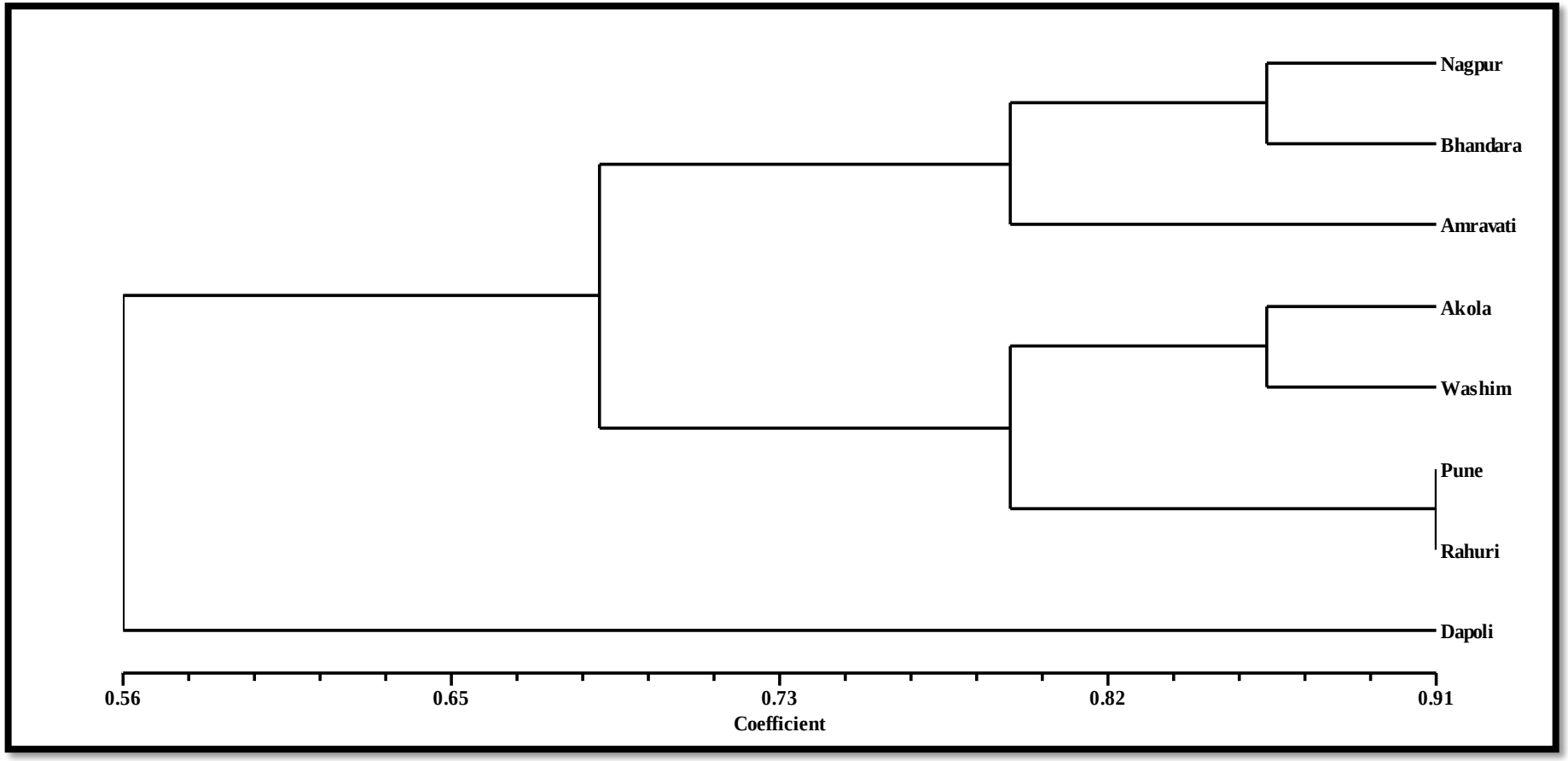


Fig. 2 The ISSR UPGMA dendrogram of eight Isolates of *Xanthomonas axonopodis* pv.*citri* based on Jaccard's similarity coefficient

Table 10 : Binary similarity matrix for ISSR analysis

Isolates	Xac1	Xac2	Xac3	Xac4	Xac5	Xac6	Xac7	Xac8
Xac1	1.00							
Xac2	0.64	1.00						
Xac3	0.77	0.86	1.00					
Xac4	0.86	0.68	0.73	1.00				
Xac5	0.77	0.68	0.73	0.82	1.00			
Xac6	0.77	0.77	0.82	0.64	0.64	1.00		
Xac7	0.68	0.77	0.82	0.64	0.64	**0.91**	1.00	
Xac8	0.59	**0.50**	0.55	0.55	0.64	0.55	0.55	1.00

In present study, the similarity coefficient value ranged from 0.50 to 0.91 among eight isolates which indicated a high range of genetic diversity. Results on differentiation of isolates revealed that, 7 primers showed the amplification. The highest genetic similarity to an extent of 91% was recorded in Xac6 and Xac7 isolates. Least genetic similarity 50% was observed in Xac2 and Xac8.

The dendrogram of ISSR analysis revealed four major clusters viz. I, II, III, IV of the test isolates. The cluster I comprised the isolates Nagpur (Xac1), Amravati (Xac3) Bhandara (Xac5) with about 0.67 similarity coefficient. Whereas The cluster II comprised of Akola (Xac2), Washim (Xac4),cluster III comprised of Pune(Xac6), Rahuri(Xac7) and cluster IV include Dapoli (Xac8) indicating their distinctness from all other isolates under study.

There are numerous DNA based molecular marker systems suitable for genetic diversity assessment. RAPD-, ISSR- and repPCR makers are most popular as prior knowledge about the sequences of genomes is not required, comparatively easy to use and inexpensive. However, RAPD is often criticised for lack of reproducibility. Hence, in the present study ISSR and rep-markers were used.The ISSR technique has widespread acceptance because it is relatively simple, well suitable assay when the nucleotide sequence is unknown.

Nagaoka and Ogihara (1997) reported the ISSR primers produced several times more information than RAPD markers. PCR was carried out with primers that annealed to simple sequence repeats. The resultant products were subjected to agarose-gel electrophoresis, and the banding patterns were compared among six wheat accessions containing diploid,ntetraploid, and hexaploid members. Out of 100 examined, 33 primers produced distinguishable as well as polymorphic bands in each of the six accessions. Although most of the primers that gave distinct bands (30 primers out of 33) contained dinucleotide repeats, each of the primers with tri-, tetra-, and penta-nucleotide motifs also yielded discrete bands. Primers based on (AC)/ repeats gave the most polymorphic bands.

Fatima *et al.* (2012) measured genetic diversity with inter-intra species variation of some indigenous *Xanthomonas* sp. using RAPD and ISSR markers and revealed that seven isolates showed 29 to 100% genetic variation among them.

Raghuwanshi *et al.* (2013) reported that Bacterial blight of pomegranate caused by *Xanthomonas axonopodis* pv. *punicae* is a major biotic constraint in peninsular India.. On Inter Simple Sequence Repeat (ISSR) analysis they formed separate clusters with Akkalkot-Solapur isolate being most divergent, while Deola-Nashik and Sangamner-Ahmednagar isolates were most similar.

Madavi *et al.* (2017) studied the genetic diversity among eight of Xanthomonas isolates using inter simple sequence repeat (ISSR) PCR based techniques and revealed high degrees of polymorphisms among the studied isolates. The genomic variation was found to be in the range from 0.37 to 0.93 across eight isolates indicating high degree of genetic variation.

Present findings are in consequences of Sabin *et al*. (2012) who studied the genetic diversity among seven *Xanthomonas* isolates representing four species using RAPD and ISSR PCR-based techniques. Both techniques revealed high degrees of polymorphisms among the test isolates. A cluster dendrogram based on the combined data of RAPD and ISSR exhibited existence of genetic diversity among local isolates of

Xanthomonas. The per cent similarity values in respect of, the genomic variations were ranged from 29% to 100 per cent among the isolates. *X*. *campestris* pv. *mangiferae indicae* remained unclustered in cluster dendrogram and revealed a unique genomic profile compared to other isolates tested.

CHAPTER V

SUMMARY AND CONCLUSIONS

The present investigation entitled Antibiotic sensitivity, variability and management of *Xanthomonas axonopodis* pv. *citri"* was undertaken to study the morphological and biochemical characteristics, to assess the efficacy of Antibiotic in the management of *Xanthomonas axonopodis* pv. *citri* and molecular characterization by using ISSR marker.

Citrus occupies an important place among the fruits in Maharashtra. The fruits are rich in vitamin'C' and contain other vitamin, sugar, acid, mineral and alkaline salt that are essential as health promoting ingredients. Citrus canker caused by *Xanthomonas axonopodis* pv.*citri* is a damaging disease of acid lime .It is identified as a major threat in the region affecting leaves, twigs, petioles, branches, stalk and fruits that causes considerable damage both quantitatively and qualitatively.

Citrus canker infected sample collected from Nagpur, Akola, Amravati, Washim, Bhandara, Pune, Rahuri and Dapoli. Associated bacterium were isolated and identified. Purified cultures were maintained on NA slants coded as Xac1, Xac2, Xac3, Xac4, Xac5, Xac6, Xac7 and Xac8 respectively.

Morphological and biochemical characteristic viz., shape, colony colour, Gram reaction, starch hydrolysis, gelatein liquefaction, KOH, catalase test were performed and confirmed to identity the bacterium as *Xanthomonas axonopodis* pv. *citri*

Efficacy of different combination of antibiotics and chemicals against *Xanthomonas axonopodis* pv. *citri* was assessed *in vitro* by measuring the growth by paper disc method. The result revealed that treatment no.T_{10} (Copper oxychoride (0.2%)+ streptocycline(200 ppm)] and T_{12} Copper oxychloride (0.3%)+Streptomycine sulphate (200ppm)] were found significantly effective in inhibiting the growth of test pathogen. The maximum zone of inhibition (32 mm) was recorded in treatment no.T_{12} (Copper oxychloride (0.3%)+Streptomycine sulphate (200ppm)] for the

isolate Xac7 followed in T_{10}(Copper oxychoride (0.2%)+ streptocycline (200 ppm)] for the isolate Xac1(26.66 mm). It is also revealed that treatment no. T_9 i.e COC +Streptocycline alone which in 90:10 (Streptomycine sulphate I.P and tetracycline hydrochloride respectively I.P)(0.2%+100 ppm)] and COC +Streptomycine sulphate (0.3%+100 ppm) i.e T_{11} were inhibit the zone of inhibition after T_{10} and T_{12}.

The variability was studied among the eight isolates of *Xanthomonas axonopodis* pv. *citri* by using 7 ISSR primers *viz* ISSR8,ISSR12, ISSR817,ISSR820,ISSR827,ISSR841, ISSSR857101 which produced 22 scorable bands with an average of 3 bands per primer. Out of 22 bands, 3 bands were monomorphic and 19 bands were polymorphic and level of polymorphism was 68%.

Isolate Xac6 had higher value of similarity coefficient (0.91) with Xac7, whereas Xac2, had lower value of similarity coefficient (0.50) with Xac8.

Conclusions

1. All the eight isolates of *Xanthomonas axonopodis* pv.*citri* , were rod shaped ,Gram negative and produced yellow pigmentation and exhibited positive reaction to gelatin liquefication, starch hydrolysis, catalase and KOH test.
2. Treatment no.T_{10} (Copper oxychoride (0.2%)+ streptocycline(200 ppm)] and T_{12} Copper oxychloride (0.3%)+Streptomycine sulphate (200ppm)] were found significantly effective in inhibiting the growth of test pathogen. The maximum zone of inhibition (32 mm) was recorded in treatment no.T_{12} (Copper oxychloride (0.3%)+ Streptomycine sulphate (200ppm)] for the isolate Xac7 followed in T_{10}(Copper oxychoride (0.2%)+ streptocycline (200 ppm)] for the isolate Xac1(26.66 mm).
3. In ISSR analysis, all seven selected primers were screened against the isolates of *X. axonopodis* pv.*citri*, amplified scorable banding pattern of 22 amplicons of which 19 bands were polymorphic and 3

were monomorphic and the Level of polymorphism for ISSR analysis was about 68%.

4. Isolate Xac6 had higher value of similarity coefficient (0.91) with Xac7, whereas Xac2, had lower value of similarity coefficient (0.50) with Xac8

CHAPTER VI

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Appendix

List of bands amplified against ISSR primers:

ISSR primer	Xac1	Xac2	Xac3	Xac4	Xac5	Xac6	Xac7	Xac8	Bp
ISSR8	1	1	1	1	1	1	1	1	985bp
ISSR12	0	1	0	0	0	1	1	0	1995bp
	1	0	1	1	1	1	1	1	983bp
	1	0	1	1	1	1	0	1	895bp
	0	1	1	0	0	0	1	0	390bp
	1	0	0	1	1	0	1	1	277bp
ISSR817	1	0	0	0	0	1	0	1	246bp
	1	1	1	1	1	1	1	0	164bp
ISSR820	1	1	1	1	1	1	1	1	657bp
ISSR827	0	0	0	0	0	0	0	1	970bp
	0	1	1	0	1	1	1	1	615bp
	0	0	0	0	0	0	0	1	381bp
	0	1	1	0	1	0	0	0	243bp
	0	0	0	0	1	1	0	0	157bp
ISSR841	0	0	0	0	0	1	1	0	780bp
	1	0	0	0	0	1	1	0	307bp
	0	1	1	0	0	1	1	1	193bp
ISSR857101	0	1	0	0	0	0	0	0	410bp
	1	1	1	1	0	1	0	0	307bp
	0	0	0	0	1	0	0	0	202bp
	0	0	0	0	0	0	1	1	161bp
	1	0	0	0	0	0	0	0	103bp