STUDIES ON BACTERIAL WILT OF BRINJAL CAUSED BY *Ralstonia solanacearum* (Smith) Yabuchi *et al*

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in

PLANT PATHOLOGY

BANGALORE **JULY, 2014**

AFFECTIONATELY DEDICATED

to

My

beloved Parents

Brothers & Family

DEPARTMENT OF PLANT PATHOLOGY
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GKVK, BANGALORE – 560 065

CERTIFICATE

This is to certify that the thesis entitled "**Studies on bacterial wilt of brinjal caused by *Ralstonia solanacearum* (Smith) Yabuchi *et al*"** submitted by **Mr. MUHAMMAD RAFI BAWARI, ID. No. PALB 2265** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture) in PLANT PATHOLOGY** to the University of Agricultural Sciences, GKVK, Bangalore is a record of research work done by him during the period of his study in this university under my guidance and supervision and the thesis has not previously formed the basis for the award of any other degree, diploma, associateship, fellowship or other similar titles.

Bangalore,
July, 2014

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"In the Name of Allah, the Merciful, the Compassionate"

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Bangalore

July, 2014 ***(Muhammad Rafi Bawari)***

Studies on bacterial wilt of brinjal caused by *Ralstonia solanacearum* (Smith) Yabuchi *et al*

Muhammad Rafi Bawari

THESIS ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* is a lethal disease of brinjal in tropical, subtropical and warm temperate regions of the world. In present investigations, isolation of the pathogen and antagonists/ bioagents, biochemical characterization and pathogenicity of the wilt bacterium along with *in vitro* & *in vivo* evaluation of selected antibiotics and bioagents against *R. solanacearum* were conducted. The bacterial wilt pathogen of brinjal was isolated by serial dilution technique and characterized through morphological and cultural characteristics. The bacterium was Gram negative, rod shaped, non-capsulated and the colonies on TZC medium were white with light-pinkish centre and highly fluidal producing copious slime. Growth of bacterium at different temperature and pH levels indicated significant differences and the temperature of 30^oC and pH 6 to 7 were found optimum for maximum growth of *R. solanacearum. In vitro* studies carried out to find the effectiveness of antibiotics and antagonists in inhibiting the growth of *R. solanacearum*, the antibiotics *viz*., Amoxycilin, Cefixime, Ciprofloxacin, Tetracyclin, Norfloxacin and Streptomycin were highly effective at 500 – 750 ppm with the maximum inhibition. Among bacterial antagonists, *Bacillus subtilis* was very effective in inhibiting the growth of pathogen followed by *Pseudomonas fluorescens* and *Bacillus megaterium*. Among the antibiotics evaluated under pot culture in the green house the treatments *viz.*, Streptomycin, Cefixime and Tetracycline at 500 – 750 ppm were found very effective in reducing the population of *R. solanacearum* ($0.0x10^4$cfu/g of soil). Bacterial antagonists *viz.*, *P. fluorescens; B. megaterium* and *Bacillus subtilis* reduced the pathogen population from (686.66 x 10^4cfu/g of soil) to $3.33x10^4$, $4.33x10^4$ and $7.33x10^4$cfu/g of soil respectively.

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Major Advisor

ಬದನೆಯಲ್ಲಿ <u>ರಾಲ್‌ಸ್ಟೋನಿಯಾ</u> <u>ಸೊಲನೇಶಿಯಾರಂ</u> (ಸ್ಮಿತ್) ಯಬೂಛಿ ಇತರರು ನಿಂದ ಉಂಟಾಗುವ ದುಂಡಾಣು ಸೊರಗು ರೋಗದ ಅಧ್ಯಯನ

ಮಹಮದ್ ರಫಿ ಬವಾರಿ

ಪ್ರಬಂಧದ ಸಾರಾಂಶ

ವಿಶ್ವದ ಬದನೆ ಬೆಳೆಯುವ ಪ್ರದೇಶಗಳಲ್ಲಿ ಕಂಡುಬರುವ ದುಂಡಾಣು (<u>ರಾಲ್‌ಸ್ಟೋನಿಯಾ ಸೊಲನೇಶಿಯಾರಂ</u>) ಸೊರಗು ರೋಗವು ಒಂದು ಮಾರಕ ರೋಗವಾಗಿದೆ. ಪ್ರಸ್ತುತ ಅಧ್ಯಯನದಲ್ಲಿ, ರೋಗಾಣುವನ್ನು ರೋಗಯುಕ್ತ ಗಿಡದಿಂದ ಪ್ರತ್ಯೇಕಗೊಳಿಸಿ, ಅದರ ಆಕೃತಿ ಮತ್ತು ಜೀವಸಾಕಾಣಿಕೆಯ ಲಕ್ಷಣಗಳಿಂದ <u>ರಾಲ್‌ಸ್ಟೋನಿಯಾ</u> <u>ಸೊಲನೇಶಿಯಾರಂ</u> (ಸ್ಮಿತ್) ಯಬೂಛಿ ಎಂದು ಗುರುತಿಸಲಾಯಿತು. ಈ ದುಂಡಾಣುವು 'ಗ್ರಾಂ ಪ್ರತಿಕ್ರಿಯೆಯಲ್ಲಿ' ನಕಾರಾತ್ಮಕವಾಗಿದ್ದು, ಕೋಶರಹಿತ ಮತ್ತು ಸಲಾಕೆಯ ಆಕಾರದಲ್ಲಿತ್ತು. ಟಿ.ಝಡ್.ಸಿ. ಮಾಧ್ಯಮದಲ್ಲಿ ಬೆಳೆದಾಗ ದುಂಡಾಣು ಸಮೂಹಗಳು ಬಿಳಿಬಣ್ಣದಿಂದಿದ್ದವು ಮತ್ತು ಅದರ ಕೇಂದ್ರ ಭಾಗದಲ್ಲಿ ತಿಳಿ ಗುಲಾಬಿ ಬಣ್ಣವನ್ನು ಹೊಂದಿದ್ದವು. ರೋಗಾಣುವು ಮೂವತ್ತು ಡಿಗ್ರಿ ಉಷ್ಣತೆ ಹಾಗೂ ಆರರಿಂದ ಏಳು ರಸಸಾರದಲ್ಲಿ ಅತ್ಯುತ್ತಮವಾಗಿ ಬೆಳೆಯುವುದು ಕಂಡು ಬಂದಿತು. ಪ್ರಯೋಗಾಲಯದಲ್ಲಿ ನಡೆಸಿದ ಅಧ್ಯಯನದಿಂದ ತಿಳಿದುಬಂದ ಅಂಶವೆಂದರೆ, ಜೀವವಿರೋಧಾತ್ಮಕಗಳಾದ ಅಮೋಕ್ಸಿಸಿಲಿನ್, ಸೆಫಿಕ್ಸಿನ್, ಸಿಪ್ರೊಫ್ಲೊಕ್ಸಾಸಿನ್, ಟೆಟ್ರಾಸೈಕ್ಲಿನ್, ನೋರಾಫ್ಲೊಕ್ಸಾಸಿನ್ ಮತ್ತು ಸ್ಟ್ರೆಪ್ಟೋಮೈಸಿನ್‌ಗಳು ಏಳುನೂರು ಪಿ.ಪಿ.ಎಮ್ ಸಾಂದ್ರತೆಯಲ್ಲಿ ಅತೀ ಹೆಚ್ಚು ರೋಗದ ದುಂಡಾಣುವಿನ ಬೆಳವಣಿಗೆಗೆ ತಡೆಹೊಡ್ಡಿದ್ದವು. ಹಾಗೆಯೇ ಜೀವಾಣು ಪ್ರತಿರೋಧಕಾತ್ಮಕಗಳಾದ <u>ಬ್ಯಾಸಿಲಸ್</u> <u>ಸಬ್‌ಟಿಲಿಸ್</u> ಮತ್ತು <u>ಸುಡೊಮೊನಾಸ್</u> <u>ಫ್ಲೋರೊಸೆನ್ಸ್</u> ಪರಿಣಾಮಕಾರಿಯಾಗಿ <u>ರಾಲಸ್ಟೋನಿಯಾ</u> <u>ಸೋಲನೇಶಿಯರಂ</u> ರೋಗಾಣು ಬೆಳವಣಿಗೆಯನ್ನು ಕುಂಠಿತಗೊಳಿಸಿದವು. ಗಾಜಿನ ಮನೆಯಲ್ಲಿ ನಡೆಸಿದ ಪ್ರಯೋಗದಿಂದ ತಿಳಿದು ಬಂದಿದ್ದೇನೆಂದರೆ, ಮೌಲ್ಯಮಾಪನ ಮಾಡಿದ ಜೀವ ವಿರೋಧಾತ್ಮಕಗಳಲ್ಲಿ, ಸ್ಟ್ರೆಪ್ಟೋಮೈಸಿನ್, ಸೆಫಿಕ್ಸಿನ್ ಮತ್ತು ಸ್ಟ್ರೆಪ್ಟೊಸೈಕ್ಲಿನ್ ಐದುನೂರರಿಂದ–ಏಳುನೂರು ಪಿಪಿಎಮ್ ನಲ್ಲಿ (೦.೦ x ೧೦^{೪} ಸಿಎಫ್‌ಯು/ಗ್ರಾಂ ಮಣ್ಣಿನಲ್ಲಿ) ಪರಿಣಾಮಕಾರಿಯಾಗಿ ದುಂಡಾಣುಗಳ ಸಂಖ್ಯೆಯನ್ನು ಕಡಿಮೆಮಾಡಿದೆ. ದುಂಡಾಣು ಪ್ರತಿರೋಧಾತ್ಮಕಗಳಲ್ಲಿ <u>ಸುಡೊಮೋನಾಸ್</u> <u>ಫ್ಲೊರೊಸೆನ್ಸ್</u>, <u>ಬ್ಯಾಸಿಲಸ್</u> ಮೆ<u>ಗಾಥೀರಿಯಂ</u> ಮತ್ತು <u>ಬ್ಯಾಸಿಲಸ್</u> <u>ಸಬ್‌ಟಿಲಿಸ್</u> ಪರಿಣಾಮಕಾರಿಯಾಗಿ ಮತ್ತು ದುಂಡಾಣು ಜೀವಸಂಖ್ಯೆಯನ್ನು (೬೮೬.೬೬ x ೧೦^{೪} ಸಿಎಫ್‌ಯು ಯಿಂದ ೩.೩೩ x ೧೦^{೪}, ೪.೩೩ x ೧೦^{೪} ಮತ್ತು ೭.೩೩ x ೧೦^{೪} ಸಿಎಫ್‌ಯು/ಗ್ರಾಂ ಮಣ್ಣಿನಲ್ಲಿ) ಕಡಿಮೆಗೊಳಿಸಿದೆ.

ಸಸ್ಯರೋಗಶಾಸ್ತ್ರ ವಿಭಾಗ
ಯುಎಎಸ್, ಜಿಕೆವಿಕೆ, ಬೆಂಗಳೂರು–೬೫

ಪ್ರಧಾನ ಮಾರ್ಗದರ್ಶಕರ ಸಹಿ
(ಟಿ. ನರೇಂದ್ರಪ್ಪ)

Studies on bacterial wilt of brinjal caused by *Ralstonia solanacearum* (Yabuchi)

Muhammad Rafi Bawari

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INTRODUCTION

Bacterial wilt of brinjal and other solanaceous vegetables caused by *Ralstonia solanacearum* is emerging as an economically important bacterial pathogen in India and the world.

The pathogen has wide host range and it infects several crop species including of families of *Solanaceae* and *Cucurbitaceae.*

Sudden wilting characterized by drooping down of the young top leaves and shoots of plants are the typical symptoms.

OBJECTIVES:

- Isolation, identification and pathogenicity of brinjal bacterial wilt pathogen.
- Cultural and biochemical characterization of *R. solanacearum* .
- Physiological studies of the bacterial wilt pathogen *R. solanacearum.*
- *In-vitro* and *in vivo* evaluation of antibiotics and bio agents against the bacterial wilt pathogen/ disease.

RESULTS

- **The pathogen isolated was Gram negative and non capsulated. Colonies appeared dull white with light pink colored centre on TZC medium and pathogenic to brinjal seedlings.**
- **Based on biochemical (nitrate reduction test, starch hydrolysis and gelatine hydrolysis) and physiological characteristics pathogen was identified as *Ralstonia solanacearum* (Yabuchi). The pathogen grew well at optimum temperature of 30°C and pH 6 to 7.**
- **Among the antibiotics tested, Amoxycilin (36.00 mm), Cefixime (35.66 mm), Ciprofloxacin (32.66 mm), Tetracycline (27.16 mm), Norfloxacin (22.50mm) and Streptomycin (16.33 mm), at 750 ppm were found very effective in reducing the population of *R. solanacearum* in *in vitro.***
- **Among them, Cefixime, Streptomycin and Tetracycline were effective at 500and 750 ppm in *in vivo*.**
- **The bacterial antagonist *Pseudomonas fluorescens* , *Bacillus megaterium,* and *B. subtilis* were more effective in reducing the wilt incidence.**

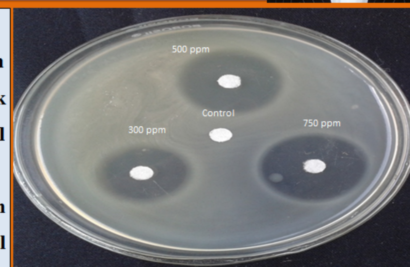

Fig1: Inhibitions zone formed by Tetracycline at different concentrations in *in vitro* on CPG medium

Fig2: Effect of antibiotics on brinjal wilt incidence.

Fig3: Effect of bioagents on brinjal wilt incidence.

MATERIAL & METHODS

❖**Isolation, identification and pathogenicity** the bacterial pathogen was isolate by serial dilution techniques. Identification was done through cultural studies. Pathogenicity was proved by Koch's postulates.

❖**Cultural characterisation** by studying colony morphology on TZC, CPG and King's B media and Gram staining.

❖**Biochemical characterisation of bacterial pathogen** through Starch hydrolysis, Nitrated reduction test and Gelatine hydrolysis.

❖**Physiological studies** through growth of the pathogen at different temperatures and pH levels.

❖***In vitro* and *In vivo* studies** by testing different antibiotics and bio agents in laboratory and pot culture experiments.

DISCUSSION

Morphological studies of the pathogen revealed Gram-negative rods and non-capsulated which is in conformity with earlier description of *R. solanacearum* as reported by Smith (1896), Khan (1974) and Prasannakumar *et al*(2003)

Maximum number of colonies were recorded at a temperature 30°C with highest growth at a pH 6 to 7 as reported by Narumol *et al*(2007).

Cefixime was found very effective on *R. solanacearum* both *in vitro* and *in vivo.* Similarly, Streptomycin and Tetracycline were effective in reducing the disease severity and suppressing the pathogen population similar results were reported by Das *et al.*(1995).

Antagonists, *P. fluorescens, B. megaterium,* and *B. subtilis* were more effective *in vitro* and *in vivo* the studies conducted by Kempe *et al*(1983) and Shree *et al*(2005).

SUMMARY

Bacterial wilt of brinjal showing typical wilt symptoms was identified as *Ralstonia solanacearum* based on morphological, biochemical and physiological studies.

Optimum temperature and pH required for this pathogen were 30^{0}C and 6-7 respectively.

Cefixime, Streptomycin and Tetracycline were more effective in reducing the disease severity and suppression of pathogen population under *in vitro* and *in vivo* conditions.

Among the different bio agents tested *P. fluorescens, B. megaterium,* and *B. subtilis* were effective in managing the bacterial wilt disease on brinjal.

ADVISORY COMMITTEE:

Dr. T. Narendrappa	Chairman
Dr. N.G.Ravichandra	Member
Dr. Venkatesh	Member
Dr. Suvarna, V. Chavannavar	Member
Dr. V. B. Sanath Kumar	Member

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I. INTRODUCTION

Bacterial wilt of brinjal and other solanaceous vegetables caused by *Ralstonia solanacearum* (Smith) Yabuchi *et al* (Yabuchi *et al.,* 1995) is the most destructive disease in the tropical, subtropical and temperate regions of the world, causing heavy economic loss. Representatives of 50 families comprising of more than 350 host plants are affected by this disease and the number of new species continue to increase (Hayward, 1991). The bacterial wilt disease is wide spread, affecting many solanaceous vegetable crops in India, especially in Karnataka. The major hosts affected by this disease in India include tomato, potato, brinjal, chilli, ginger, groundnut, tobacco, banana and other floricultural plants. In India, brinjal is cultivated in an area of 4,74,400 ha with a production of 76,61,510 tonnes and in Karnataka it is grown in an area of 22,481 ha with production of 4,49,620 tonnes (Anon., 2004).

One of the major factors limiting the cultivation of brinjal crop in Karnataka is the incidence of bacterial wilt caused by *Ralstonia solanacearum*. The soil-borne pathogen causes substantial economic loss to crops. This disease appears in the field when the plants come to flower and begin to bear fruits. Sudden wilting is characterized by drooping down of the young top leaves and shoots of plants. The attacked plants die within 3 to 5 days of the infection, that in the first recognizable symptom of the disease. The disease is more severe in the year of heavy monsoon when the fields become very frequently water-logged (Das and Chattopadhyay, 1955).

Ralstonia solanacearum is an aerobic non-sporeforming, Gram-negative, plant pathogenic bacterium. *R. solanacearum* is motile with tuft of polar flagella. It colonises the xylem, causing bacterial wilt in a very wide range of potential host plants. Bacterial wilt of tomato, pepper, eggplant and Irish potato caused by *R. solanacearum* were among the first diseases that Erwin Frank Smith proved to be caused by a bacterial pathogen. Because of its devastating lethality, *R. solanacearum* is now more intensively studied phytopathogenic bacteria and bacterial wilt of tomato is a model system for investigating mechanisms of pathogenesis. *Ralstonia* synonymous to *Pseudomonas* with a similarity in most aspects except, it does not produce fluorescent pigment. It belongs to Kingdom: Bacteria; Phylum: Proteobacteria; Class: Beta- Proteobacteria; Order: Burkholderiales; Family: Ralstoniaceae and Genus: *Ralstonia*.

The wilt disease can bring about almost total destruction during rainy season in all brinjal growing areas of Karnataka. In India, an average loss in yield due to brinjal and tomato wilt is as high as 80 and 90 per cent, respectively (Rao, 1976). Kishun (1987) recorded loss in yield ranging from 10 to 90 per cent, and plant mortality from 10 to 100 percent in tomato. The destructiveness of the pathogen is attributed to its widespread occurrence, the existence of different strains, its exceptional ability to survive in soil and its broad host range.

Although some progress have been made in understanding the biology of the pathogen and measures for control of bacterial wilt, but the disease continue to pose serious problem to farmers. At present, no effective control measures are available except resistant cultivars, crop sanitation, crop rotation and other cultural practices. The use of resistant varieties is the most popular way of controlling the disease, but the development of resistance has been hampered by high degree of variability of the pathogen (Aspires and Cruz, 1985).Not much effort has been directed towards the production of plant bactericides and as a result, very few effective ones are available today for controlling plant bacterial diseases. Use of antibiotics has been recommended for the control of disease, but there are obvious disadvantages, hence discouraged (Egli and Sturn, 1980).

In recent years, owing to the increased awareness to the hazards of chemical control, the biological control is gaining popularity. Various fungi, actinomycetes and bacteria exhibited antagonistic effects against *R. solanacearum* (Kelman, 1953).Many kinds of beneficial antagonistic bacteria inhabit the soil and the rhizosphere of plants and survive within the plant. These antagonistic bacteria may reduce the effect of pathogen, may exceed the number and weight in soil with their rapid growth and ability to utilize varied substrates under different conditions. While some bacteria are antibiotic producers and others are effective fast growing colonizers. Since, *Bacilli* and *Pseudomonas* are abundant in the rhizosphere and they could prove to be important competitors with the root pathogens (Baker and Cook, 1974).

Other biological agents that have been used to control bacterial wilt include bacterial viruses or bacteriophages (phages). Bacteriophages have long been proposed as plant disease control agents (Moore, 1962) and have been used in several plant bacterium pathosystems. However, reliable disease control was not achieved with them in the past. Jackson (1989) proposed an approach that uses a mixture of host-range, mutant bacteriophages and in subsequent fields trials, weekly two sprays of the phage mixture reduced the disease severity of bacterial tomato wilt caused by *R. solanacearum* by 68.75 per cent (Manjunath and Khan,2003).

Some of the studies conducted in India and abroad (Ganesan and Gnanamanickam, 1987; Savithry and Gnanamanickam, 1987 and Vasantha Devi *et al*, 1989) have shown the potential of root colonizing microorganisms that inhibit or displace rhizosphere pathogenic microorganisms and thereby protect the health of perennial and annual crops (Anuratha and Gnanamanickam, 1990). Considering all these factors, the biological control of *R. solanacearum* would be ideal if a suitable antagonist is identified, which is very effective, economical and eco-friendly and highly specific in the context of the bacterial diseases such as bacterial wilt.

The investigations were taken up with the following objectives:

- Isolation, identification and pathogenicity of *Ralstonia solanacearum* causing wilt of brinjal
- Cultural and biochemical characterization of the bacterial wilt pathogen
- Physiological studies of the bacterial wilt pathogen
- *In vitro* and *in vivo* evaluation of antibiotics and bioagents against the bacterial wilt pathogen/ disease

II. REVIEW OF LITERATURE

Bacterial wilt caused by *Ralstonia solanacearum* is a major plant disease of economic importance since it destroys a variety of plants of commercial value in most of the tropic and subtropical countries. A century has elapsed since Smith (1896) first described the bacteria affecting potato and tomato. A voluminous literature has become accumulated on this disease. The literature pertaining to various aspects of bacterial wilt caused by *R. solanacearum* on brinjal and other related hosts *viz.* morphology, cultural, physiological and biochemical characteristics and management of disease has been described briefly in this chapter.

2.1 *Ralstonia solanacearum* as a pathogen

The earliest report of the disease was proposed by Burill (1890), in connection with an unidentified bacterial pathogen affecting southern potato tubers in the United States. Later, in 1896 Smith began the investigation, which resulted in the first ever description of the disease and the casual agent. In India, the disease was first recorded from Pune district of Maharastra by Cappel (1892) and the nature of the bacterial disease was described by Butler (1903). Ansari and Ram (1987) reported the wilt causing bacterium from sesamum. In Karnataka, the disease was recorded by Coleman in 1909 and the pathogen was reported as *Pseudomonas solanacearum* but later, regrouped as *Ralstonia solanacearum* (Yabuchi *et al*., 1995).

2.2 Economic importance of wilt disease

In brinjal, the disease can bring about total destruction of the crop during rainy season in all brinjal growing areas of Karnataka. In India, the loss in yield due to the disease in brinjal and tomato is as high as 80 and 90 per cent, respectively (Rao, 1976). Kishun (1987) recorded loss in yield ranging from 10 to 90 per cent, and plant mortality from 10 to 100 percent in tomato. Shekhawat *et al.* (1992) reported that the disease was wide spread, endemic throughout India. However, the disease is more severe in parts of Kerala, Gujarat, Karnataka, Western Maharastra, Madhya Pradesh, Eastern plains of Assam and Nicobar islands. In Karnataka, the disease is locally known as Parrya and Bangadiroga. The disease occurs in both *kharif* and *rabi* seasons or incidence varies from 0 to 98 per cent (Gadewar *et al.,* 1991).

2.3 Morphology of *R. solanacearum*

Smith (1896) was the first to describe the shape and size of the bacterium. He reported rod shaped bacterium measuring 0.5 x 1.5µm when 48 hours old culture was stained with methyl violet. The variations among the isolates infecting tomato, potato, brinjal and chilli were studied by measuring the size of the cell and they were found to be motile with lophotrichous flagellum, non-spore forming, gram negative and cell size ranged from 1.3 to 1.02 x 1.02 to 1.78 μm (Khan, 1974).

Ansari and Ram (1987) reported that the sesamum wilt bacterium was Gram negative, rod shaped with one or more flagella. Singh and Hussain (1991) reported that, *R. solanacearum* from groundnut measuring 0.5 to 0.7 x 1.2 to 2.5 μm in sizewas gram negative, rod shaped with one polar flagellum. Estelitta *et al.* (1997) reported that the bacterium causing wilt on *Moringa oleifera* was Gram negative and motile rods. Norman and Yuen (1988) described similar bacteria from wilted plants of potato.

2.4 Cultural characteristics of *R. solanacearum*

Kelman (1954) described the colony morphology of *R. solanacearum* mainly into three types based on his observation on Triphenyl Tetrazolium Chloride medium (TZC). Colonies of normal or wild type were irregularly round smooth fluidal and opaque. The mutant type formed round, smooth, butyrous and translucent colonies. Third type formed butyrous translucent colonies with a rough surface.

Two distinct subgroups of the American moko strains are identified *i.e.,* the A type or SFR (Small Fluidal Round) based on its distinctive colony morphology on Kelman's medium and B (Banana) strain (French and Sequeira, 1970).

Khan (1974) described the colonies of the four isolates of *R. solanacearum* from chili, tomato, brinjal and potato on TZC. All the isolates produced fluidal, shiny, slightly convex colonies with slight pink center. However, the brinjal isolate produced copious slime and the colony was highly fluidal. Currier and Morghan (1981) grew isolates of *Ralstonia solanacearum* in TZC broth without TZC at 29^0C, shaken at 200 rpm to get fluidal colonies and without shaking to get non-fluidal colonies.

French *et al.,* (1993) used colony phenotype to distinguish mixtures of *Ralstonia solanacearum* in Peru and found that strains of biovar 1 and biovar 2 differed consistently in colony size on Kelman's medium with average diameters of 1.0 to 1.5 and 1.8 to 2.5 mm, respectively with optimum temperature of 34.5 to 36 ^{0}C for growth. Hussain (1995) revealed the presence of purplish pink centered fluidal colonies of *R. solanacearum* on TZC medium isolated from potato in Pakistan.

2.5 Biochemcial and physiological characteristics of *R. solanacearum*

Bhide (1948) reported that *R. solanacearum* was positive for nitrate reduction, citrate utilization, utilization of inorganic nitrogen and were negative for gelatin liquefaction, indole production, starch hydrolysis and production of hydrogen sulphide.

Mathew *et al.* (1979) recorded *R. solanacearum* on *Zingiber offiicinale.* The pathogen was positive for catalase, nitrate reduction, ammonia production and utilized sucrose, dextrose and glucose. It was negative for starch hydrolysis, arginine dihydrolase and production of hydrogen sulphide.

Singh and Hussain (1991) observed that the isolate from bacterial wilt of groundnut produced hydrogen sulphide and ammonia, reduced nitrate, hydrolyzed starch and was negative for arginine dihydrolase activity and grew well in two per cent sodium chloride.

Marin and Nashaar (1993) reported that biovar 2 strains of *R. solanacearum* from Peru were positive for oxidase, catalase and solubility in three per cent KOH for nitrate reduction. Alvarez *et al.* (1993) showed that, the strains of *R. solanacearum* from *Heliconia* and *Musa* sp. in Hawaii were positive for nitrate reduction and glucose utilization and were negative for tryptophan, arginine dihydrolase, urease, gelatinase, *esculin* hydrolysis and hydrolysis of p-nitro-β-galactopyranoside.

Date *et al.* (1994) observed that, the isolate from wilted *Eustoma russellianum* accumulated poly-β-hydroxybutyrate granules and was positive for nitrate reduction, catalase, tyrosine, oxidase and negative for hydrolysis of arginine, starch and esculin.

Rahman and Main (1996) recorded that, the *R. solanacearum* isolates from Bangladesh were positive for oxidase, catalase and urease test and oxidized glucose. Estelitta *et al.* (1997) recorded *R. solanacearum* from *Moringa oleifera* to be positive for catalase and nitrate reduction. It was negative for starch hydrolysis, gelatin liquefaction and arginine dihydrolase activity. Further, milk was slightly curdled with production of acid and growth was slightly inhibited with 2 per cent sodium chloride. Subhalaxmi (1999) reported that, the isolate *R. solanacearum* from Bird of paradise could reduce nitrate, produced levan and curdled skimmed milk with production of sodium chloride.

Temperature is an important environmental factor that affects *R. solanacearum* multiple plant pathosystems and their interactions with their hosts (Hayward, 1991). High ambient temperatures have been shown to induce growth of *R. solanacearum* incidence at a faster rate than moderate temperatures (Ciampi and Sequeira, 1980). An increase of temperature to a range of 30 to 35ºC is associated with an increase in severity of the disease caused by *R. solanacearum* in several hosts. That is, plants resistant to *R. solanacearum* at moderate temperatures become more susceptible at high ambient temperatures (Hayward, 1991). For instance in tomato, the *R. solanacearum* pathogen rapidly moves through the plant at temperatures above 28º C and thus cultivars that seem resistant in lower temperatures become susceptible when exposed to temperatures higher than 28ºC (Prior *et al.*, 1996). Another study has shown that disease severity caused by *R. solanacearum* to be significantly greater at 32.2ºC than at 26.6ºC in resistant tomato cultivars (Krausz and Thurston, 1975).

Understanding host-pathogen interactions and the effect of temperature on disease caused by *R. solanacearum* development may offer information to advance breeding and disease management strategies. These results confirm that temperatures between 30 and 35ºC significantly increase wilting severity in tobacco as it has been reported in other hosts (Hayward, 1991).High temperatures (*i.e.* 30-35°C) promote occurrence of these disease caused by *Ralstonia solanacearum*, whereas soil temperatures below 20°C are not suitable for the disease (Gadewar *et al*., 1999; Wang and Lin, 2005a).

At pH 8.0 and above, despite the previous findings of suppression effect of *R. solanacearum* (Michel and Mew, 1998), the availability of many nutrients were likely very low in hydroponics and that may have limited plant growth and development. Vincent *et al.* (1997) studied, the *in-vitro* experiments growth of all strains of *R.*

solanacearum was suppressed at pH 3, 10, and 11, and strongly reduced at pH 4 and 9 (*P* < 0.001). At pH 5 and 8, growth reduction was weak or it did not occur at all. Normal growth of all strains occurred at pH 6.

2.6. Ecology/Survival of *R. solanacearum*

The ability of the pathogen to survive depends on the initial inoculum and its ability to tide over the adverse condition. It was found that discontinuity of cropping pattern forces the pathogen to lead a saprophytic existence. The saprophytic survival of the pathogen takes place in the soil, plant parts, seeds and insects (Schuster and Coyne, 1974).

2.6.1 Survival in Soil

Survival of *R. solanacearum* in soil is based on soil temperature, moisture, pH, aeration, competition and antagonism. Buddenhagen (1965) proposed the status of phytopathogenic bacteria in soil as transient visitors, resident visitors and residents. The first group is represented by pathogen like *Erwinia stewartii* and *E. tracheiphila* that find their way to soil at some stage. *Agrobacterium tumifaciens* and *R. solanacearum* represents the second group that comprises of facultative pathogens capable of saprophytic survival. The third group are those organisms that are saprophytes, under certain circumstances turn pathogenic, *Erwinia* causing soft rot and fluorescent *Pseudomonas.*

Coleman (1909), observed that pathogen persisted in the soil for two and half years in Karnataka. Kelman (1953) found that the survivability of *R. solanacearum* varied with soil. Some soil can support the pathogen for longer periods, in other inspite of presence of the susceptible host, the organism did not survive. Das and Chattopadhayay (1955) observed that the bacterium survived for 16 months in pot soil. Rangaswami and Thirunakarasu (1964) reported that *Ralstonia solanacearum* survived upto 250 days in free-state in soil or in the infected plant parts indicating there by it could over winter from season to season. Crosse (1968), however found that as a free propagule in the soil *R. solanacearum* survives upto 6 years in the bare soil.

Field trials conducted by Central Potato Research Institute (CPRI) in hills and peninsular India showed that bacterium persisted in soil for at least two years even in the absence of the host, but the population was declined with time (Shekhawat *et al.,* 1982). Granada and Sequeira (1983) studied the survival of *R. solanacearum* in artificially infested soil and found that race 1, 2 and 3 survived upto 21, 16 and 8 weeks respectively and are in accordance with well established notion that race 1 survived in the soil longer than race 2 and race 2 longer than race 3.

2.6.2 Survival in rhizosphere

Sequeria and Averre (1961) found that even in virgin forest land of Costa-Rica, a species of *Heliconia* plants were infected extensively with banana race of *R. solanacearum*. In 1969, Okabe reported the survival of *R. solanacearum* in the rhizosphere of many weeds. Goto (1971) compared the survivability of true soil-borne plant pathogens and found that the pathogen tends to survive better in rhizosphere and rhizoplane rather than as saprophytes.

Balasubramanian (1972a) reported the effect of spermosphere and rhizosphere of sorghum and sunhemp on the survival and growth of four plant pathogens namely *Ralstonia solanacearum, Xanthomonas malvacearum, Fusarium moniliforme* and *Helminthosporium sativum* in artificially inoculated soils. He found the suppression of all the pathogens in rhizosphere of sorghum, while in the rhizosphere of sunhemp all the four pathogens were stimulated and their number increased with time upto 20 days and declined later. Population of all the pathogen was much higher in the sterile soils as compared to those in non-sterile soil. He attributed this to increased competition and antagonism in the sun hemp rhizosphere. He further noted (1972b) the inhibition of *R. solanacearum*in the diffusates of sorghum seeds. He also reported that these exudates contained thermolabile compounds, which are toxic to the microorganism.

Gowda *et al.* (1974) reported that the pathogen *R. solanacearum* population from brinjal, population was higher in the rhizosphere of wilting plants than in the rhizosphere of non-wilting plant. The virulent population in rhizosphere of the wilted plants was 4 to 50 folds higher than the avirulent population. The avirulent population was 1 to 20 folds higher in the rhizosphere of non-wilted plants than that of wilted plants. The rhizosphere soils of wilted plants showed a general increase in dehydrogenase activity.

2.7 Management of bacterial wilt

2.7.1 Antibiotic in the control of bacterial wilt

As early as 1955, Morgan arid Goodman reported the *in vitro* sensitivity of plant bacterial pathogens to antibiotic. It was found that the antibiotics varied considerably in their capacity to inhibit the entire spectrum of the test organisms and none was considerably superior to the others. *R. solanacearum* was effectively inhibited by the lower concentration of Aureomycin and Terramycin and relatively insensitive to the other two antibiotics, Streptomycin and Polymixin. Hidaka and Murano (1956) studied the effect of streptomycin on the behavior of *R. solanacearum in vitro.* They found that streptomycin at 0.3µg /ml of water inhibited and subsequently killed *R. solanacearum.* Dutta and Verma (1969) studied the efficacy of Streptocycline in controlling bacterial wilt of egg plant and found that seedling treatment of variety Pusa purple long with the antibiotic (1g/1) for 30 minutes before planting gave best results.

Shetty and Rangaswami (1969) found that antibiotic C-6 similar to Erythromycin effectively inhibited the pathogen in diseased potato tuber. Tuber treated with 500-1000 ppm of C-6 showed better and quicker germination than the control. Mondal and Mukherjee (1978) reported the protection of tomato plants against *R. solanacearum* by 6-aminopenicillic acid and Streptomycin sulphate. Khan (1980) conducted field trials to control bacterial wilt of tomato and reported that Agrimycin-100 was effective in decreasing wilt. Percentage of wilting in treated plots for three seasons were 30.4, 35.8 and 35.8 while in untreated plots, it was 59.9, 72.5 and 82.8 respectively.

Indersena *et al.* (1981) reported that treatment of ginger rhizomes with Plantomycin at 1000 ppm was effective against the seed borne bacteria *R. solanacearum*. Farag *et al.* (1986) studying the effect of Streptomycin in relation to potato bacterial wilt found that both virulent and avirulent forms of pathogen were sensitive to streptomycin and dihydro streptomycin. However, application of the antibiotic at 150 and 400 ppm increased wilt incidence. The increase in disease incidence after Streptomycin application was correlated with increased root exudation at early stages of growth. Ojha *et al.* (1986) observed that rhizome treatment of ginger with organomercurials like Emisson-6 (0.2%) plus Plantomycin (0.05%) for 30 minutes in addition to three sprays, first at 30 days after planting followed by 15 days interval resulted in 100 per cent control of the disease.

Gunawan (1987) reported that best control of bacterial wilt of potato was obtained with 100 ppm Agrimycin-15 applied four times at seven days intervals, starting at 30 days after planting. Later, in 1988 he sprayed tomato crop with 200 ppm streptomycin sulphate at interval of 4, 7, 10 and 14 days and disease incidence was evaluated each week from 49.77 days after planting. He found that spraying at four and seven days intervals gave the highest degree of disease control.

Among 14 antibiotics tested against the *Ralstonia solanacearum* sensitive to Tetracycline, Streptomycin, Chloremphenical, Riframpicin, Spirmycin and vancomycin. They concluded that Tetracycline was the most effective (Das *et al.* 1995).

Draz *et al.*, (2003) reported that, the soil amended with commercial compost and dolomite in the field condition, field experiment showed no significant differences among the treatments with respect to disease severity and incidence. The *Ralstonia solanacearum* population were significantly reduced in the soil.

Venkatesh *et al.*(2003) reported that, potato planting after finger millet, followed by autumn ploughing and pre-planting application of bleaching powder (18 kg/ ha) to soil was also effective in the control of bacterial wilt potato caused by *R. solanacearum.*

2.7.2 Biological control of bacterial wilt

Biological control has an immense potential in management of bacterial wilt. The basic idea in biological control is that a rhizosphere colonizer that would antagonize *R. solanacearum*at the root infection site could be applied to the system, which would result in reduced infection.

Attempts were made by Celino *et al.* (1952) to control bacterial wilt or tomato by adding antagonistic microorganisms to soil. One isolate B_3 A, a bacterium obtained from Phillipine soil, gave significant control when added to soil infected with *R. solanacearum.* This antagonist inhibited the growth of the pathogen on Petri-Plates and gave large inhibition zones on assay plates. Soil which had been previously infested with the pathogen was treated by adding a broth culture of isolate B_3A after 19 days Bonny best tomato seedlings were planted both in the pots which had received the antagonist and the control pots which contained only the pathogen, a marked decrease in the disease was apparent in treated soil, the controls had 79 per cent wilt incidence whereas the pots that had the antagonist only 33 per cent wilt was observed Isolate B_3A was tentatively identified as *Bacillus polymyxa* and they found that the organisms produced an antibiotic.

Kelman (1953) reported that *Bacillus megatierium, B. subtilis, B. prodigiosus, B. mesentricus* and many unidentified bacteria, which were antagonistic to *R. solanacearum.* When these bacteria were added to wilt sick soil, the disease failed to spread. They further reported that species of *Aspergillus* and actinomycetes inhibited *R. solanaearum.* Kempe and Sequeria (1983) obtained control of bacterial wilt of potato by treating tubers with bacteria. Induced resistance and microbial antagonism were investigated as possible methods for control of bacterial wilt of potato caused by *R. solanacearum.* The bacterium tested included avirulant and incompatible strains of *Pseudomonas fluorescens* and strains *P. syringae* pv. *glycinea* (S9-4) and *P. syringae* pv. *lachrymans* (PHW 214-6).

Aspires and Cruz (1986) reported biological control of bacterial wilt in tomato and potato with *B. polymyxa*FU 6 and *P. fluorescens.* They observed that these rapidly growing bacteria were highly effective in reducing the incidence of *R. solanacearum* in tomato and potato under experimental conditions. The antagonists were able to colonize aggressively on the roots of young plants and prevented the entry of the pathogen. It was found convenient to apply *B. polymyxa* FU 6 as a spore preparation during sowing or planting. Suresh (1986) reported that, *Azotobacter* did not inhibit the *R. solanacearum* and hence, its role as a biocontrol for *R. solanacearum* was ruled out.

Wakimoto (1987) reported the control of bacterial wilt of tomato by non-pathogenic strains of *Pseudomonas glumae.* He observed that the protective effect of selected strains of *P. glumae* against *R. solanacearum* on tomato seedling roots dipped in suspension of the bacterium was not only due to antibacterial substances produced by the antagonists but also due to factor such as occupation of attachment site and induction of resistance in the host tissue.

Gallardo *et al.* (1989) reported the inhibition of *R. solanacearum in vitro* by using *P. fluorescens* strain BC-8. The growth of *R. solanacearum* on agar was inhibited by inoculation with *P. fluorescens.* It was found that extracts contained rod shaped particles with a helical structure, 150 nm long and 25 nm diameters, these were interpreted to be bacteriocins and designated as Fluocin BC-8. Bacteriocin activity was found to be associated with plasmids, since plasmid DNA could not be detected electro phoretically.

Gallardo and Panno (1989) reported the biological control of bacterial wilt of potato caused by *R. solanacearum.* To test the activity, potato seedlings were grown in sterile soil in a growth chamber, when the seedlings were eight cm tall the soil was inoculated with a suspension of *P. fluorescnes* (strain BC-8) and 15 days later *R. solanacearum* was inoculated. They observed that prior inoculation of *P. fluorescens* resulted in saving 90 per cent of the seedlings from wilt disease.

Ciampi *et al.* (1989) reported that biological agent designated as BC-6 caused strong inhibition of *R. solanacearum* in both *in vitro* assay and growth chamber condition. They also treated this biological agent to seed tubers and planted in infested soil and found that treatments that included isolate BC-6 had the lowest number of wilted plants and fewer latently infected tubers.

Anuratha and Gnanamanickam (1990) tested 125 strains of fluorescent and 52 strains of non-fluorescent bacteria against the *R. solanacearum* initially in the laboratory for their antibiosis. They evaluated strain PfCP of *P. fluorescens* and strains of B33 and B36 of *Bacillus* species under green house and field conditions. *P. fluorescens* treated to banana, brinjal and tomato plants were protected from wilt upto 50, 61 and 95 per cent in green house and upto 50, 49 and 36 per cent, respectively in the field.

Aoki *et al.* (1991) isolated and identified an antimicrobial substance produced by *P. cepacia* (B5) against *R. solanacearum.* The antimicrobial substance was identified as 2-Keto-D-gluconic acid (2KGA). The production of 2KGA and the destruction of the disease pathogen in a contaminated soil suspension, to which the B5 strain was added, together with wheat bran, suggested that 2KGA is involved in disease suppression by *P. cepacia* (B5).The trials conducted at Central Potato Research Institute, Shimla, India showed that an isolate of *Bacillus* species reduced wilt incidence by 74 per cent and increased the incubation period by 29-30 days. Field trials at Bhowali (Kumaon hills) showed that isolates of *Bacillus* species (BNS_1 and BNS_2) reduced wilt incidence by 87 and 89 per cent and tuber rot by 83 and 84 per cent respectively (Shekhawat *et al.,* 1992).

Khan *et al.* (1997) reported that both bacteriocin producing and non- producing avirulent strain of *R. solanacearum* reduced the wilt incidence of tomato. Further they observed that pathogen population of virulent strain was reduced both in rhizosphere and non-rhizosphere soil after application of avirulent strain of *R. solanacearum.*

Ciampi *et al.* (1996) isolated siderophore like substaces from *Pseudomonas fluorescens* responsible for inhibiting the growth of *R. solanacearum* and reported that these substances are responsible for inhibiting the growth of the pathogen and this siderophores activity is more when iron in the medium is limited which chelate the iron compound required for the growth of *R. solanacearum.*

Sunaina *et al.* (1997) studied the effect of different biological agents *viz. Bacillus cereus, B. subtilis* and avirulent strain of *R. solanacearum* on bacterial wilt of potato both in glass house and in naturally infested field and found that by treating these agents the wilt incidence was reduced to 23.51, 17.09 and 13.10 per cent, respectively compared to control 74.41 per cent. Correspondingly substantial increase in yields to an extent of 22.72, 64.47 and 62.77 per cent was obtained as compared to control.

Pretreating tomato roots with killed cells of *P. auregenosa* also gave protection suggesting that mechanisms other than antibiotic production such as induced resistance and infection sites competition were involved in suppression of the disease (Furuya *et al.,* 1997).Singh and Rana (2000) reported that the tuber bacterization with *B. cereus, Bacillus* spp. and *B. subtilis* gave significant reduction of bacterial wilt incidence and increase in yield over check. All the *Bacillus* spp. were equally effective, as these treatments did not show significant difference in wilt incidence and yield. An avirulent strain of *R. solanacearum* was found least effective.

Kumar and Sood (2001) reported that soil solarization for 8 and 10 weeks during March to May increased the soil temperature at 5 cm depth in wilt sick plots at Palampur by 5.2 ^{0}C and 6.2 ^{0}C during 1998 and 1999 respectively. The increased soil temperature promoted the abundant germination and profuse growth of weeds in the tarped (solarized) plots during early stages of tarping followed by their complete death during later on in both the 8 and 10 weeks solarized plots. The bacterial wilt incidence in such plots was significantly suppressed by 65.9 per cent and 71.6 per cent during 1998 and 68.9 per cent and 73.4 per cent 86.9 per cent wilt in non-solarized plots. The significant reduction in wilt incidence was attributed to the incorporation of antagonist rhizobacteria (*P. fluorescens* and *B. cereus*) in soil prior to solarization as the population of antagonists was considerably enhanced after solarization where as that of *R. solanacearum* was significantly reduced and resulted in increased vigour of tomato plants in solarized plots as compared to non-solarized plots.

Rahman and Khan (2002) reported that among 69 isolates of endophytic and plant pathogenic bacteria tested, five isolates (SS 21, ST 26, AC 53 and IB 55) showed antagonistic effect against *R. solanacearum.* Isolates SS 21 and IB 55 were effective against all the isolates of *R. solanacearum*, three other isolates were antagonistic against *R. solanacearum* isolated from tomato and brinjal but ineffective against *R. solanacearum* isolated from potato.

The bioagent treated tomato seedlings of moderately resistant cultivar (cv.) EC 191536 grown in soil amended with sodium nitrate ($NaNO_3$) and potassium nitrate (KNO_3) resulted in complete control of wilt. In the case of susceptible cv. Solangola, least incidence (27.77%) was observed when NaNO3 was combined with *P. fluorescens* followed by *Bacillus cereus* + Na NO_3 (33.37%), *P. fluorescens* + KNO3 (33.33%) and *Bacillus cereus* + KNO_3 (38.88) (Kumar and Sood, 2003).

Li *et al.* (2003) evaluated 36 strains of antagonists under *in vitro* condition against *R. solanacearum* among them, strain B 47 showed highest antagonistic activity and highest efficiency to the disease in the green house and field condition. El-Sayed *et al.* (2003) evaluated *Pseudomonas fluorescens, Streptomyces fumigatisclerotis, S. griseviridis, S. rochei, S. violaceusniger* and *Bacillus subtilis* against *R. solanacearum* in *in vitro* and *in vivo* conditions. All the above agents were most effective as soil drench and tuber treatment, reduced the population of *R. solanacearum* in the rhizosphere of potato. *P. fluorescens* and *S. griseiviridis* were the most effective in the reduction of disease severity.

Anith and Momol (2004) reported that the application of PGPR, *Pseudomonas putida*89B61 and *Bacillus pumilis* 8E 34 reduced bacterial wilt incidence significantly in tomato under green house conditions. Here *P. putida* was found more consistent and effective against tomato bacterial wilt. In green house experiment three strains of plant growth promoting rhizobacteria, *Serratia* species (I_2), *Pseudomonas fluorescens* (T_3) and

Bacillus species (BB11) were evaluated for biological control of bacterial wilt of tomato caused by *R. solanacearum.* Biological efficiencies of 71.3, 63.6 and 78.2 per cent were recorded by I_2, T_3 and BB11 respectively; yield increase ranged from 53.5 to 76.2 (Jianhua *et al.,* 2004).

Vanitha *et al*. (2009) reported that, the bacterial wilt incidence was reduced significantly in all 20 cultivars tested, in plants raised from seeds treated with *P. fluorescens* followed by challenge inoculation with *R. solanacearum*. The bacterial wilt incidence in the untreated control ranged from 12 to 85 per cent wherein it was reduced to 2 to 30 per cent in the treated cultivars.

III. MATERIAL AND METHODS

In the present investigation, laboratory and green house studies were conducted at the Department of Plant Pathology, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra, Bangalore. The details of materials used and methodology followed during the course of investigation described in this chapter:

3.1 Cleaning and sterilization of materials and reagents

All the glass wares and plastic wares were soaked overnight in potassium dichromate sulphuric acid solution. After 24 hours the washing solution was drained off and the materials were washed thoroughly in running tap water and rinsed with double distilled water. The plastic wares were air-dried and the glass wares were dried in hot air oven. The cleaned and dried glass wares were wrapped in aluminum foil, covered with paper and autoclaved at 121^0C, 15 PSI for 20 minutes.

3.1.1 Sterilization of the laminar air flow

All the experiments namely isolation, cultural studies and *in-vitro* evaluation of antibiotics or bactericides were conducted under aseptic conditions in the laminar air flow cabinet. Before working under the hood, the working surface was uniformly sterilized by swabbing with 70 per cent alcohol. Any material coming outside was also sterilized with alcohol. In case of glassware, mouth of the bottles, were, of lamed before and after use. The blades, forceps, inoculation loop *etc.* were sterilized by heating in the flame. Also before starting the experiments the hands were cleaned well with alcohol.

3.2 Collection of wilted brinjal plants and soil from fields and isolation of *Ralstonia solanacearum*

3.2.1 Collection of wilted plants and soil samples

The plant and soil samples from wilt affected brinjal plants showing typical symptoms of bacterial wilt were collected from infested fields of Department of Horticulture, University of Agricultural Sciences, Bangalore. The collected plant samples were packed in polythene bags and kept at 4^0C for isolation of causal organism.

3.2.2 Isolation of *R. solanacearum* from bacterial wilt affected brinjal plant and soil

The plant and soil samples were collected from Department of Horticulture, University of Agricultural Sciences, Bangalore. The discolored vascular tissues of plant samples were cut into small pieces and they were kept in the glass beaker containing clean water taking care that the infected tissue is in contact with the water surface. After five minutes the water in the glass beaker becomes turbid due to oozing of bacterial cell from the cut ends of the diseased tissue and thus confirming the bacterial nature of the wilted plants.

The small pieces of discolored vascular tissue measuring 4-5 mm in length were cut from the discolored stem. The tissue was surface sterilized by immersing bits in one per cent sodium hypochloride for 30 seconds followed by repeated washing in sterile water for 5 minutes to remove traces of sodium hypochloride. The surface sterilized bits were then suspended in the ten-milliliter sterile distilled water taken in test tube for ten minutes. After the water in the test tube becomes turbid due to oozing of bacterial cells from cut ends of diseased tissue, the bacterial suspension was serially diluted in 9 ml sterile distilled water. One hundred microliter of the diluted bacterial suspension was poured onto the surface of solidified Triphenyl Tetrazolium Chloride agar (TZC) medium (Kelman, 1954) (Appendix) in sterilized petri plates. The bacterial suspension was spread on the surface of TZC medium with a sterilized spreader. The inoculated plates were inocubated at 28^{o}C for 48 hours.

To isolate the pathogen from soil, the soil samples were serially diluted and the pathogen was isolated using TZC medium. At the end of the incubation period, the plates were observed for the development of both the virulent and avirulent colonies of *R. solanacearum*. The virulent colonies are irregularly shaped, fluidal, dull white colonies with slight red center. Whereas, avirulent colonies were small, round, convex, butyrous with large red pigment and narrow bluish margins as described by Kelman (1954).

3.3 Purification of pathogen isolated from wilted plants

Typical virulent colonies of *R. solanacearum* on TZC medium were picked and streaked separately on TZC medium in sterilized petri plates. The plates were incubated at 28^{o}C for 44 to 48 hours. The well separated virulent colonies of *R. solanacearum* were picked up with sterile inoculated loop and suspended in sterile distilled water culture collection tubes and stored at 4^{o}C in refrigerator for further use as stock culture.

3.4 Identification of the bacterial isolate obtained from bacterial wilted plants

The identification of the bacteria isolated from brinjal was based on the morphological, cultural, biochemical characteristics and pathogenicity studies.

3.4.1 Morphological and cultural characters

The morphological characteristics such as cell shape, gram reaction and capsule staining were carried out as per the methods described by Kelman (1954) and Schaad (1992).

3.4.2 Biochemical characters

Some of the biochemical tests *viz.* Gram staining, starch hydrolysis, nitrate reduction test and gelatin hydrolysis in the department of Plant Pathology.

3.4.3 Effect of Physical factors on the growth and multiplication of *R. solanacearum*

3.4.3.1 Temperature requirement

The effect of temperature on the growth of the bacterial isolates was studied in different temperatures. The bacterial cultures were multiplied separately in Casmino acid Peptone Glucose (CPG) broth. The cultures were prepared by inoculating a loopful of bacterial cultures from stock culture to 100 ml CPG broth contained in 250 ml conical flask and incubated for 48 hours at 28^{o} C. Bacterial cultures (50 µl) diluted to a concentration of 5 x 10^5cfu/ ml was poured on to the surface of CPG agar medium taken in sterilized petri dishes. The bacterial suspension was uniformly spread with the help of a sterilized glass spreader so as to obtain well distributed bacterial colonies. The inoculated plates were incubated at different temperatures *viz.,* 5^o C, 10^o C, 15^o C, 20^o C, 25^o C, 30^o C and 35^o C for 48 hours. Observation was recorded on the number of colonies appearing after the incubation period at each temperature.

3.4.3.2 pH requirement

The effect of hydrogen ion concentration on the growth of the bacterial isolates was studied by adjusting the pH of the CPG agar. The pH was adjusted to 4, 5, 6, 7, 8, 9 and 10. A loopful of 48 hours old bacterial culture was serially diluted in 9 ml sterilized water blanks. One ml of 10^5 dilution was plated separately on to the surface of the medium having varied pH levels. The suspension was spread uniformly over the medium with the help of sterilized spreader. Inoculated plates were incubated at 28^oC temperature for 48 hours. After the incubation period, observations were recorded for the development of colonies on the media having different pH. Colonies were counted and recorded. Data was analyzed statistically.

3.4.4 Pathogenicity test

Pathogenicity test was carried out to establish that the bacterial isolates were pathogenic on the host plant under infested conditions. The brinjal seedlings of variety Maya 9 were raised in polythene bags containing sterilized soil. The plants were watered regularly and Hogland's nutrient solution (Hogland, 1920) was supplied for good plant growth. The twenty day old seedlings of brinjal were uprooted and the roots were washed in water and few tertiary roots were sheared with sterilized scissors. The injured roots of the seedlings were dipped in the bacterial suspension (7.66 x 10^5cfu/ml of culture broth) of *R. solanacearum* for ten minutes. Then they were transplanted to plastic bags containing sterilized soil. Plants similarly dipped in sterile water served as negative control and un-inoculated apparently healthy plants planted in sterilized soil served as positive control. The nutrients required for the plant growth were supplied through nutrient solution at an interval of 15 days. The plants were watered regularly and observations on appearance of wilt symptoms were recorded. The plants expressing wilt symptoms were selected and the bacterium was re-isolated as explained under 3.2.2 and compared with the original culture of *R. solanacearum* so as to satisfy the Koch's postulates.

3.5 Collection of biocontrol agents from Department of Agricultural Microbiology, University of Agricultural Sciences, Bangalore

The biocontrol agents obtained from Department of Agricultural Microbiology, UAS, Bangalore were as follows:

1. Bacterial biocontrol agents *viz., Bacillus subtilis, Bacillus megaterium* and *Pseudomonas fluorescens.*
2. Fungal biocontrol agents such as *Trichoderma viride, T. virens* and *T. harzianum.*

3.6 *In-vitro* evaluation of antibiotics and bactericides, bioagents against *R. solanacearum*

3.6.1 Evaluation of antibiotics and bacteroicides

Antibiotics and bacteriocides were evaluated at different concentrations (Table 3) to test their efficacy in inhibiting the growth of *R. solanacearum*, by inhibition zone assay method. The bacterium was multiplied by inoculating the culture into the 250 ml of CPG broth taken in flask. The inoculated flasks were incubated at 28^{o}C for 48 hours. The bacterial suspension was then seeded to the CPG agar medium. The seeded medium was poured onto the sterilized Petri Plates and plates were allowed to solidify.

The bacteriocides solutions were prepared at different concentrations as mentioned in the list. The filter paper discs (Whatman No. 42) measuring 5 mm in diameter were soaked in the respective chemical solution for 5 – 10 minutes and transferred onto the surface of the bacterium seeded medium in petriplates. The inoculated plates were kept in the refrigerator at 4^{o}C for 4 hours to allow the diffusion of chemical into the medium. Then plates were incubated at 28^{o}C for 48 hours and observed for the production of inhibition zone around the filter paper discs. The results obtained were analyzed statistically.

3.6.2 Evaluation of bioagents

3.6.2.1 Bacterial antagonists against *R. solanacearum*

Three isolates of bacteria *viz., Bacillus subtilis, B. megaterium*and *Pseudomonas fluorescens* isolates collected from Department of Agricultural Microbiology were tested for their efficacy in inhibiting the growth of *Ralstonia solanacearum* by the paper disc method. The virulent isolate of *R. solanacearum* was multiplied in Casmino acid Peptone Glucose broth (CPG). The 48 hour old culture of *R. solanacearum* containing $7x10^8$cfu/ml and was mixed with molten (50^{o}C) CPG agar, so as to get a thick lawn of bacteria on the surface of agar medium. The seeded medium was poured onto sterilized petridishes and allowed to solidify. Previously sterilized filter pater (Whatman No. 42) measuring 5 mm diameter was soaked in different antagonist broth for 10 minutes and placed in the petridplates. The excess solution from the filter paper discs was removed by touching side of the paper discs to the lid of petridishes containing broth of the same organism. Then the filter discs were placed in a marked position on the surface of the seeded agar medium. The inoculated plates were incubated at 28^{o}C for 48 hours. The zone of inhibition produced around the filter paper disc was recorded. Filter paper discs dipped in sterile water served as check.

3.6.2.2 Fungal antagonists against *R. solanacearum*

Three fungal isolates collected from the Department of Agricultural Microbiology were tested for their inhibitory effect on *R. solanacearum in vitro* by inhibition assay method. All the fungal isolates were grown separately on Potato Dextrose Agar. Molten sterilized PDA (15 ml) was poured in sterilized peteriplates and allowed to solidify. Fungal culture discs of 5 mm diameter from margin of actively growing four days old culture were removed and placed in the center of the plates containing PDA. The plates were incubated for three day at 30°C. Discs (5 mm) were cut from these plates and used in the experiment.

A heavy suspension of *R. solanacearum* (6.8 x 10^8cfu/ml) was mixed with molten (50°C) CPG Agar contained in 500 ml conical flask so as to get a thick growth of bacteria on the medium. The seeded medium was poured into sterilized peteriplates and allowed to solidify. The actively growing 5 mm size of agar mycelial discs of fungal isolate was placed at the center of petridishes containing the seeded medium. These plates were incubated at 28°C for four days. The observations on the zone of inhibition around the mycelial disc against *Ralstonia solanacearum* were recorded after the incubation period.

3.7 Evaluation of selected biocontrol agents against *R. solanacearum*

A pot culture experiment was conducted in the glass house of Department of Plant Pathology, GKVK, Bangalore to evaluate antagonistic property of selected biocontrol agents against *R. solanacearum.* The three selected biocontrol agents were considered as treatments and each treatment was replicated twice and in each replication two plants were transplanted.

3.7.1 Experimental details

Crop: Brinjal cv. Maya 9	Design: Completely Randomized Design (CRD)
Soil: Red soil	Location: Department of Plant Pathology
Treatment: a. Antibiotics	b. Biocontrol agents

3.7.2 Details of treatments imposed

3.7.2.1 Antibiotics and biocontrol agents *R. solanacearum*

T1 – *R. solanacearum* (Control)

T2 – *R. solanacearum* + Streptomycin (500ppm)

T3 – *R. solanacearum* + Cefixime (500ppm)

T4 – *R. solanacearum* + Amoxycillin (500ppm)

T5 – *R. solanacearum* + Norfloxacin (500ppm)

T6 – *R. solanacearum* + Teteracyclin (500ppm)

T7 – *R. solanacearum* + Ciprofloxacin (500ppm)

T8 – *R. solanacearum* + Streptomycin (750 ppm)

T9 – *R. solanacearum* + Cefixime (750 ppm)

T10 – *R. solanacearum* + Amoxycillin (750 ppm)

T11 – *R. solanacearum* + Norfloxacin (750 ppm)

T12 – *R. solanacearum* + Teteracyclin (750 ppm)

T13 – *R. solanacearum* + Ciprofloxacin (750 ppm)

T14 – *R. solanacearum* + *Bacillus subtilis*

T15 – *R. solanacearum* + *Bacillus megaterium*

T16– *R. solanacearum* + *Pseudomonas fluorescens*

3.7.2.2 Method of application of antibiotics

Soil drenching method of application of antibiotics was followed.

Soil drenching: The seedlings were transplanted to the sick soil after adding a different concentration of antibiotics to the sick soil *viz.,* 500 ppm and 750 ppm of different antibiotics

3.7.2.3 Method of application of biological control agents

Seedling dip method was followed.

Seedling dip alone: The seedlings were dipped in suspension of antagonist for 20 minutes and used for transplanting.

3.7.3 Preparation of pots

The soil was collected from farm land GKVK, Bangalore. Soil was sterilized by autoclaving for 30 minutes at 121^{o}C / 15 psi. The sterilized soil was filled into pots at the rate of 2 kg per pot.

3.7.4 Inoculation of *R. solanacearum* to soil

R. solanacearum was used from the stock culture as mentioned under Sec 3.3. The stock culture was tested for its virulence before mixing into soil by plating the culture of *R. solanacearum* on TZC medium. The inoculated plates were incubated at 28^{o}C for 48 hours. The highly virulent colonies of *R. solanacearum* were picked up and used for multiplication of culture. The culture was multiplied by inoculating or the virulent culture of *R. solanacearum* to CPG broth in 500 ml conical flasks. The inoculated flasks were incubated for 48 hours at 28 ^{o}C. Subsequently the bacterial culture was added to each bag containing sterilized soil at the rate of 200ml per bag. The initial population of the pathogen in the culture broth was estimated before filling to the bag. Then brinjal seedlings (cv. Maya 9) raised in the nursery were transplanted to the infested soil.

3.7.5 Observations recorded

3.7.5.1 Population of *R. solanaceaarum*

The population build up of the pathogen in the rhizosphere was estimated at flowering stage. The soil samples for the rhizosphere estimation of pathogen population were obtained by drawing soil plugs with the help of soil sampler. The population was estimated by standard count technique on CPG medium.

The inoculated plates were incubated at 28°C for 48 hours. At the end of the inoculation period the plates were observed for the development of well separated circular shaped, meist colonies typical of *R. solanacearum.*

3.7.6 Statistical analysis of data

The data collected in this study was subjected to statistical analysis by Complete Randomized Design (CRD).

IV. EXPERIMETNAL RESULTS

The experimental results obtained from studies on isolation of the pathogen *R. solanacearum*, and antagonists/ bioagents, *in-vitro* evaluation of selected antibiotics and bioagents and evaluation of the effective antibiotics and biocontrol agents against *R. solanacearum* under *in-vivo* are presented here below.

4.1 Isolation of *R. solanacearum* from bacterial wilt affected brinjal plants

Wilted brinjal plants showing typical symptoms of bacterial wilt *viz.* the lower leaves turning pale yellow, loss of turgidity of the leaves, followed by drooping of the leaves and sudden wilting of plants and brown discoloration of vascular bundles of the infected plants (Plate-1) were collected from Department of Horticulture, UAS, Bangalore.

Tentative diagnosis of the disease was made by ooze test. The ooze test was conducted by placing longitudinal section of diseased vascular tissue from the identified plant in a glass beaker containing clean water, within a few minutes; fine milky exude clouded the water streaming out from the margin of the cut end which revealed the presence of bacteria in the discoloured vascular tissue.

Isolation was made from the bacterial ooze obtained from the infected discolured tissue of the plants by serially diluting the bacterial suspension in sterile distilled water and planting on TZC medium (Kelman, 1954).

4.2 Purification and Preservation of *R. solanacearum* isolated from wilted plants

Typical virulent colonies of *R. solanacearum* developed within 48 hours. The colonies were well-separated, fluidal, dull white colored with slight pink to red colored center (Plate 2). The well separated colonies were picked up and purified further by single colony isolation technique and then suspended in sterile distilled water in sterile water in sterile polypropylene tubes and stored at 4^{o} C this served as stock culture for further use.

4.3 Identification of the pathogen isolated from wilted plants collected from bacterial wilt infested brinjal plants

The bacteria isolated from brinjal were identified as per standard morphological procedures *i.e.,* on the basis of morphological, cultural, biochemical and pathogenic characteristics.

4.3.1 Morphological and Cultural characters

The results of the various morphological and cultural characters were studied. The bacterium isolates were rod shaped, Gram negative, non-capsulated. They appeared dull white with light pink colored center on TZC medium and the colonies were highly fluidal producing copious slime.

4.3.2 Biochemical characters

The bacterium was positive for nitrate reduction test and negative for starch hydrolysis and gelatin liquification.

4.3.3 Effect of temperature and pH on the growth and multiplication of *R. solanacearum*

4.3.3.1 Temperature

The effect of temperature on the growth of *R. solanacearum* was studied as per the procedure described in material and methods. The results are presented in Table 1, Fig. 1 and Plate 3.

The effect of varied temperature levels on the growth of *R. solanacearum* was significant. The temperature of 30^{o} C was found optimum for the growth of pathogen as the highest population (196.66 x 10^{5}cfu/ml) were recorded at this temperature level followed by 25^{o}C (114.66 x 10^{5}cfu/ml) and 35^{o}C (103.33 x 10^{5}cfu/ml). The growth of bacterium at these two temperature levels differed significantly and also found superior to other temperature levels tested. However, the pathogen grew at temperature of 20^{o} C, but it failed to grow at the lowest extreme temperatures of 5^{o} C to 15^{o} C.

4.3.3.2 pH requirement

This experiment was carried out to know the effect of pH on the growth of *R. solanacearum.* The effect of pH on the growth was studied at varied pH levels from 4 to 10 as mentioned in material and methods (3.3) and the results obtained are presented in Table 2, Fig. 2 and Plate 4.

The data revealed that, number of bacterial colonies increased with increase in pH to certain range from 5 to 7 and thereafter colony numbers decrease with increase in pH indicating 6 and 7 were the optimum pH for maximum growth of the pathogen *R. solanacearum*. The maximum number of bacterial colonies were recorded at pH 7 (154.00 x 10^{5}cfu/ml). The next best pH levels, which significantly supported the good growth were 6 (52.33 x 10^{5}cfu/ml) and 8 (46.00 x 10^{5}cfu/ml). The least growth of the pathogen was observed at the lowest and the highest pH levels of 5 (11.66 x 10^{5}cfu/ml) and 9 (8.33 x 10^{5}cfu/ml) respectively. However no growth of the pathogen was recorded at pH level of 4 and 10.

4.3.4 Pathogenicity test

The bacterium was inoculated to the host plant (Brinjal) under artificial condition by root injury technique. The inoculated plants showed wilting symptom 15 days after the inoculation. The isolate was found to be pathogenic to host plant, expressing wilt symptoms. The inoculated plant lost turgidity; leaves started dropping and plant wilted suddenly.

Re-isolation of the bacterium was done from the artificially inoculated wilted plants. The bacterium re-isolated were similar to the ones used for inoculation purpose.

Plate 1a: Brinjal plant showing typical wilting symptoms

Plate 1b: Bacterial ooze from vascular bundle of brinjal plant

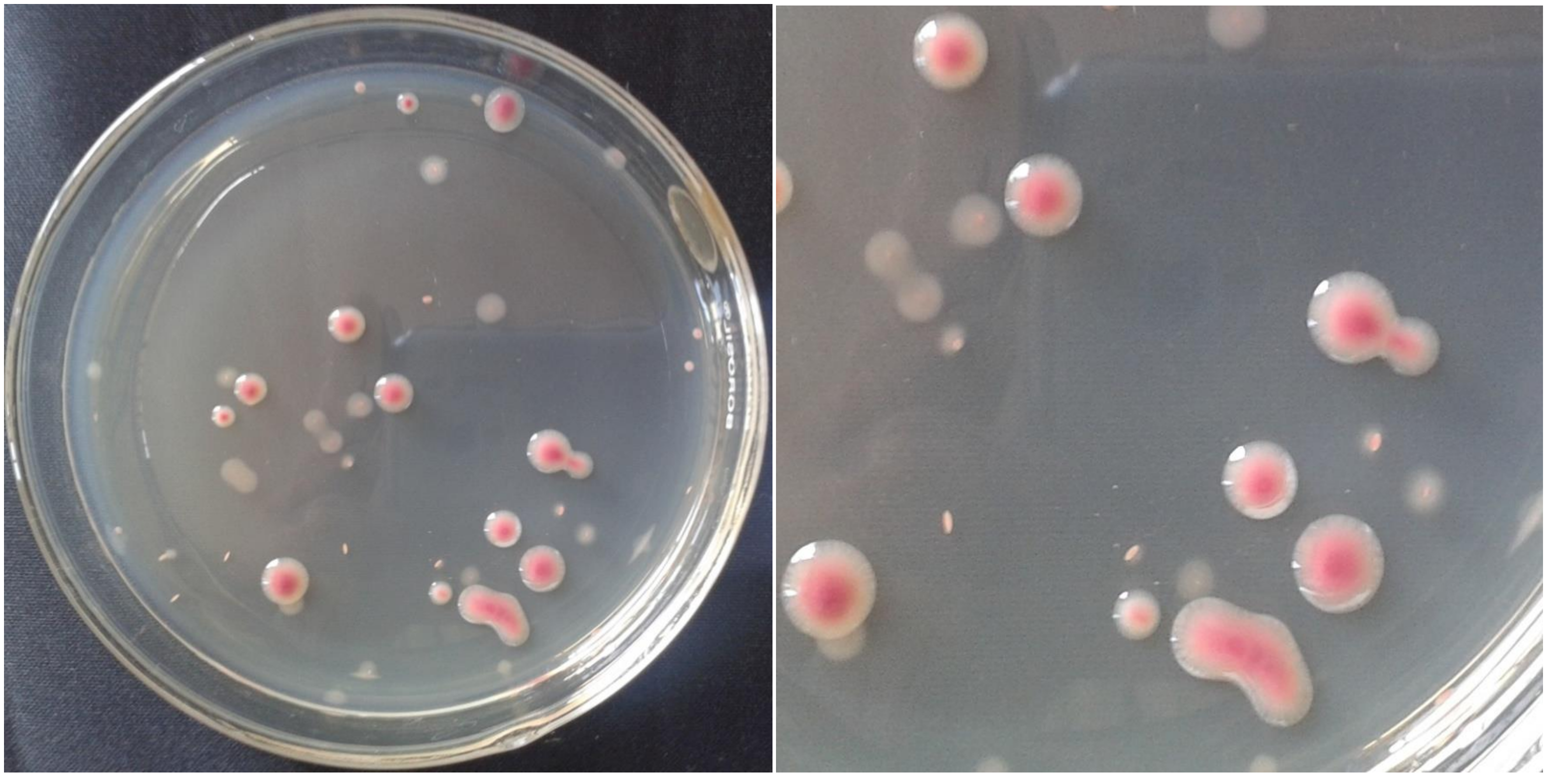

Plate 2: Typical virulent colonies of *Ralstonia solanacearum* on TZC medium

Table 1: Effect of temperature on the growth of *R. solanacearum* on CPG agar medium

Sl. No.	Temperature (^{o} C)	Colony Number (10^5cfu/ml)
1	**5.00**	0.00 (1.00) *
2	**10.00**	0.00 (1.00)
3	**15.00**	0.00 (1.00)
4	**20.00**	34.33 (6.85)
5	**25.00**	114.66 (11.70)
6	**30.00**	196.66 (15.02)
7	**35.00**	103.33 (11.16)
S.Em ±		**0.14**
CD @ 1%		**0.60**
CV (%)		**4.11**

*Transformed values

Table 2: Effect of pH on the growth of *R. solanacearum* on CPG agar medium

Sl. No.	pH	Colony Number (10^5 cfu/ml)
1	**4**	0.00 (1.00)*
2	**5**	11.66 (4.40)
3	**6**	52.33 (8.23)
4	**7**	154.00 (13.41)
5	**8**	46.00 (7.78)
6	**9**	8.33 (3.89)
7	**10**	0.00 (1.00)
S.Em ±		**0.15**
CD @ 1%		**0.63**
CV (%)		**5.26**

*Transformed values

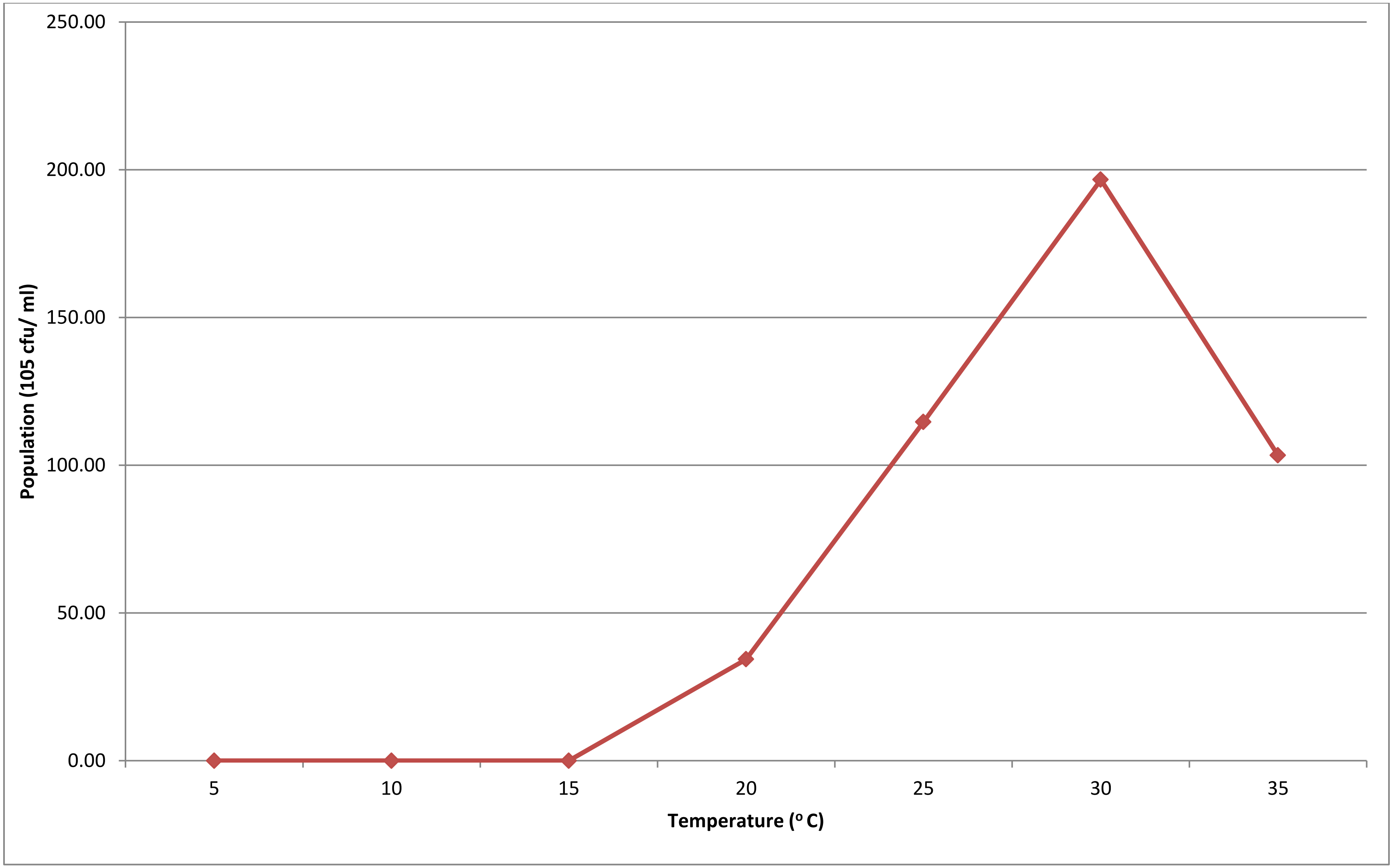

Fig. 1: Effect of temperature on growth of isolates of *R. solanacearum* on TZC medium

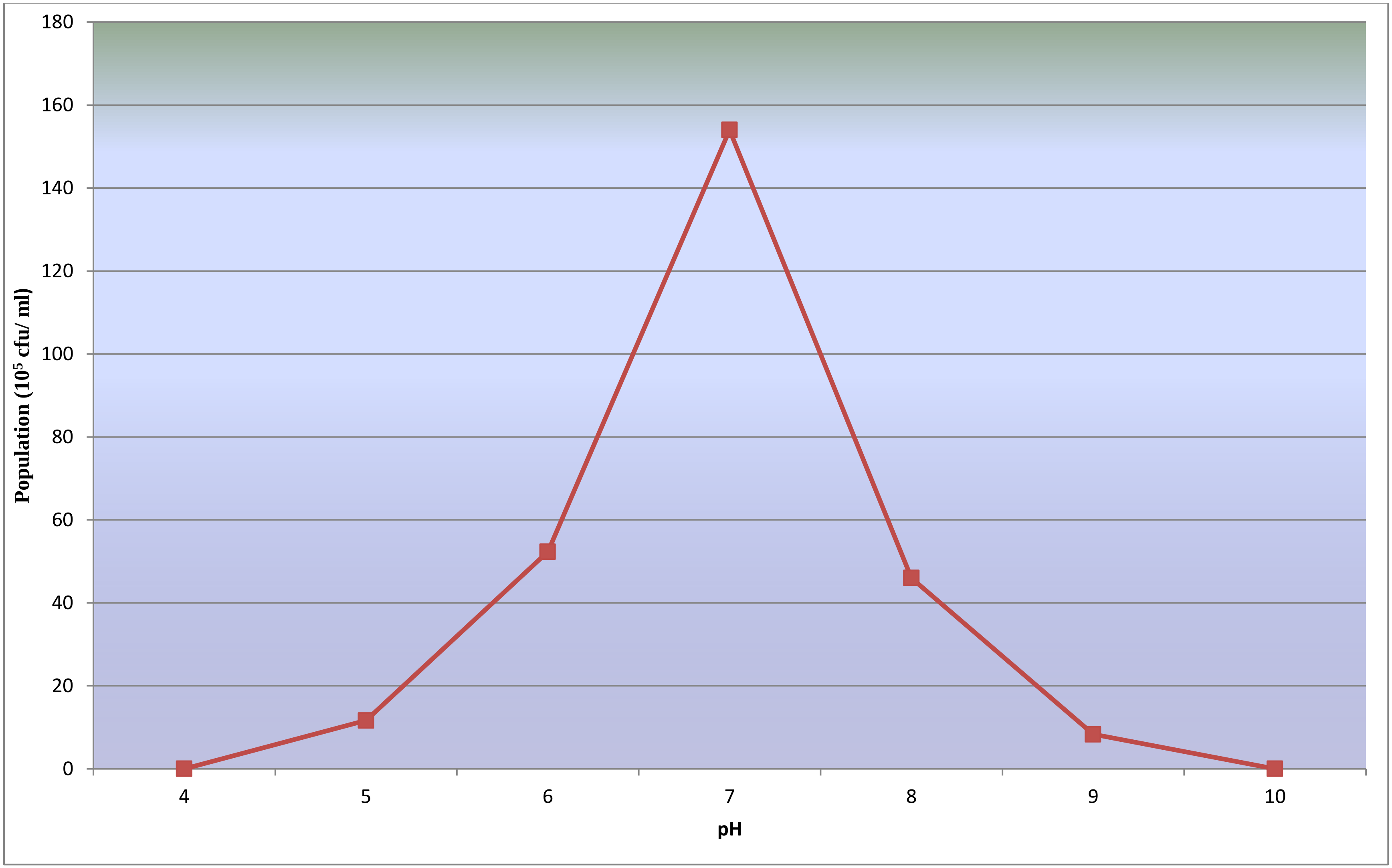

Fig. 2: Effect of pH on growth of isolates of *R. solanacearum* on TZC medium

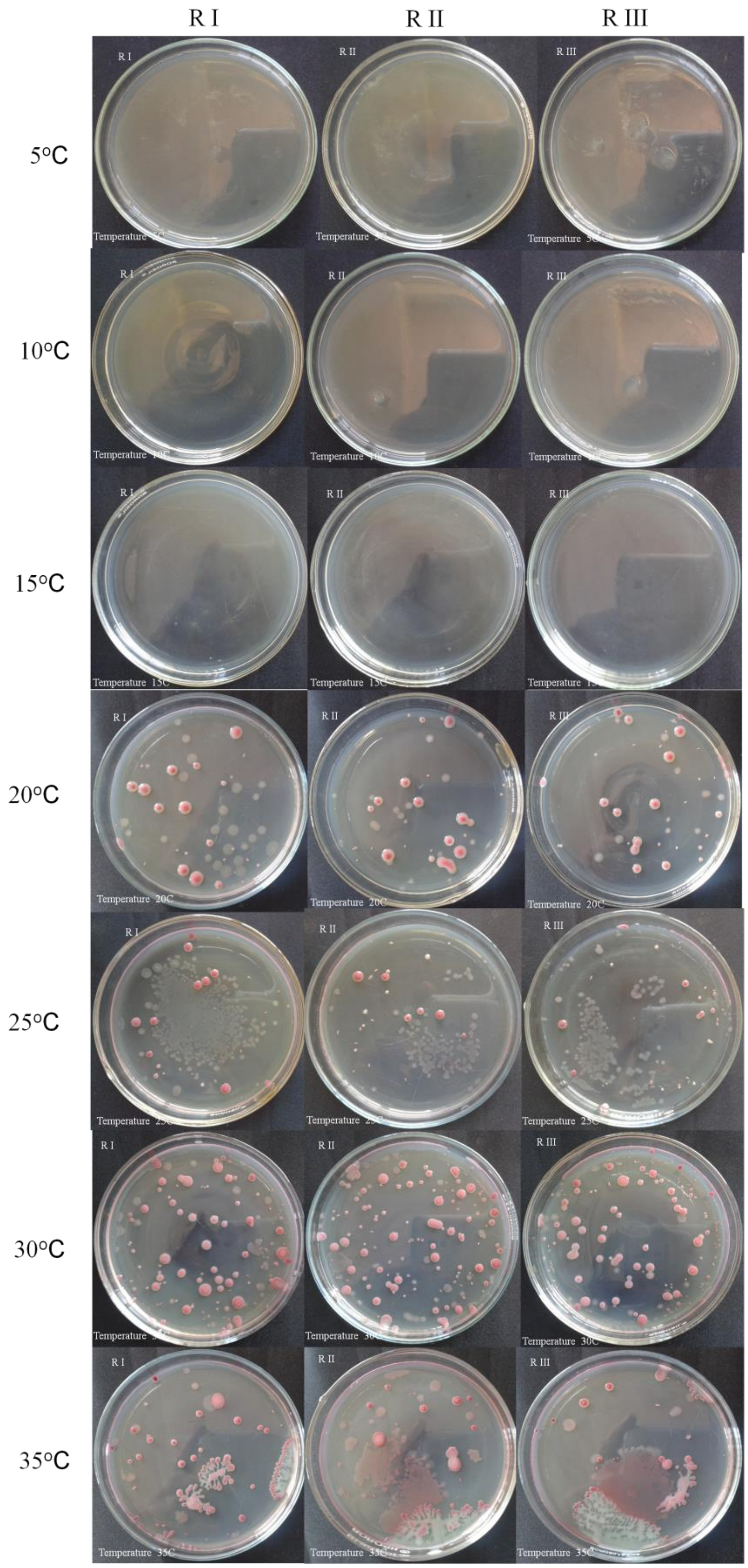

Plate 3: Effect of different temperature levels on the growth of *R. solanacearum*

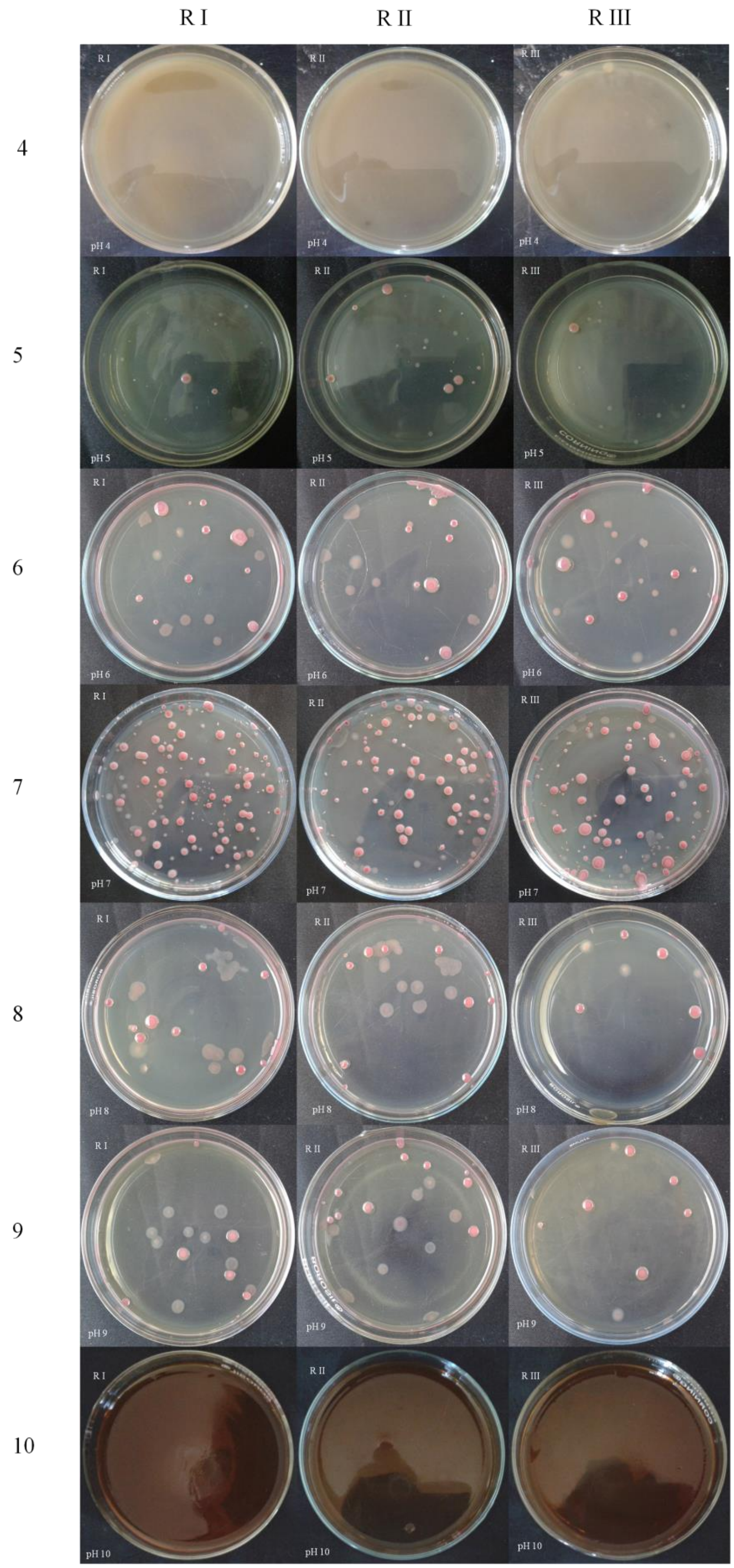

Plate 4: Effect of different pH on the growth of *R. solanacearum*

Thus on the basis of morphological, cultural, biochemical and pathogenicity characteristics and on comparison of these characters with the characters reported for *R. solanacearum* by Kelaman (1954), Buddenhagen *et al.,* (1962), and Schaad (1992) the isolate was identified as *R. solanacearum.*

4.4 *In vitro* evaluation of antibiotics on the growth of *R. solanacearum*

Seven antibiotics were evaluated at different concentrations (Table 3) to test their efficacy to inhibit the growth of *R. solanacearum*, by inhibition zone assay method.

Among the antbiotics tested (Table 3, Fig. 3 and Plate 5), Amoxycillin was found to be highly effective with the maximum inhibition range of 31.25 to 36.00 mm at 500 to 750 ppm respectively and this was also on par with Cefixime which recorded inhibition zone of 31.25 to 35.50 mm, Ciprofloxacin was next with inhibitory zone of 28.75 to 32.75 mm, followed by Tetracyclin 24.25 to 27.25 mm, Norfloxacin 20.75 to 22.75 mm and Streptomycin (14.75to 16.25mm). The least inhibitory zone of 7.25 to 10.00 mm was recorded by Bactrimil at 500 and 750 ppm.

Between the concentrations of antibiotics, efficacy significantly increased from lower to higher concentration with greater efficacy of higher concentration.

4.5 *In vitro* evaluation of antagonists on the growth and multiplication of *R. solanacearum*

4.5.1 Bacterial antagonists against *R. solanacearum*

The anatagonistic bacteria *viz., Bacillus subtilis, B. megaterium* and *P. fluorescens* were evaluated for their bio efficacy on *R. solancearum* and the data obtained are presented in the Table 4.

Of the three bacterial antagonists tested, *B. subtilus* was found to be the most effective in inhibiting the growth of *R. solanacearum* by producing inhibition zone. The other antagonist *viz., P. fluorsecnes, B. megaterium* also inhibited the growth of *R. solanacearum*.

4.5.2 Fungal antagonists against *R. solanacearum*

Investigations carried out (Table 4) to test the efficacy of different fungal antagonists obtained from Department of Agricultural Microbiology, UAS, Bangalore *viz., Trichoderma harzianum, T. viridae* and *T. virens.*

The results from this experiment revealed that fungal antagonists had no effect on the growth of the *R. solanacearum.*

Table 3: ***In vitro*** **evaluation of antibiotics against** ***R. solanacearum***

Sl. No.	Antibiotics	Inhibition zone (mm)		
		300 ppm	**500 ppm**	**750 ppm**
1	**Streptomycin**	13.50 (4.70)*	14.75 (4.85)	16.25 (5.04)
2	**Tetracyclin**	21.25 (5.62)	24.25 (5.97)	27.25 (6.21)
3	**Amoxycillin**	28.50 (6.27)	31.25 (6.60)	36.00 (7.00)
4	**Cefixime**	28.50 (6.27)	31.25 (6.60)	35.50 (6.94)
5	**Ciprofloxacin**	26.75 (6.18)	28.75 (6.34)	32.75 (6.72)
6	**Norfloxacin**	18.75 (5.28)	20.75 (5.51)	22.75 (5.74)
7	**Bactrimil**	6.75 (3.55)	7.25 (3.74)	10.00 (4.26)
8	**Control**	0.00 (1.00)	0.00 (1.00)	0.00 (1.00)
S.Em±		**0.06**	**0.04**	**0.07**
CD @ 1%		**0.23**	**0.15**	**0.27**
CV (%)		**1.98**	**1.24**	**2.11**

*Transformed values

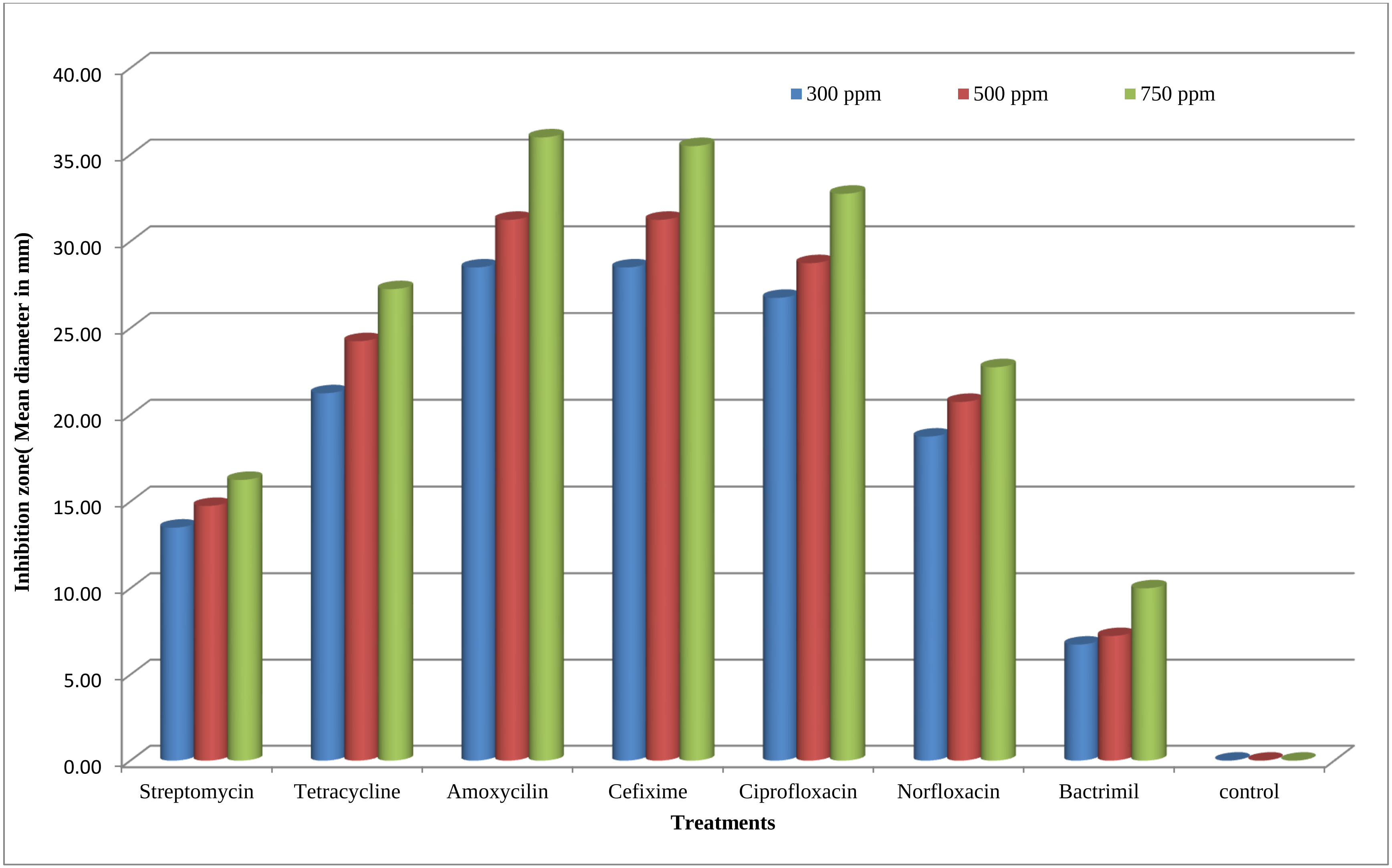

Fig. 3: *In vitro* evaluation of antibiotics against *Ralstonia solanacearum*

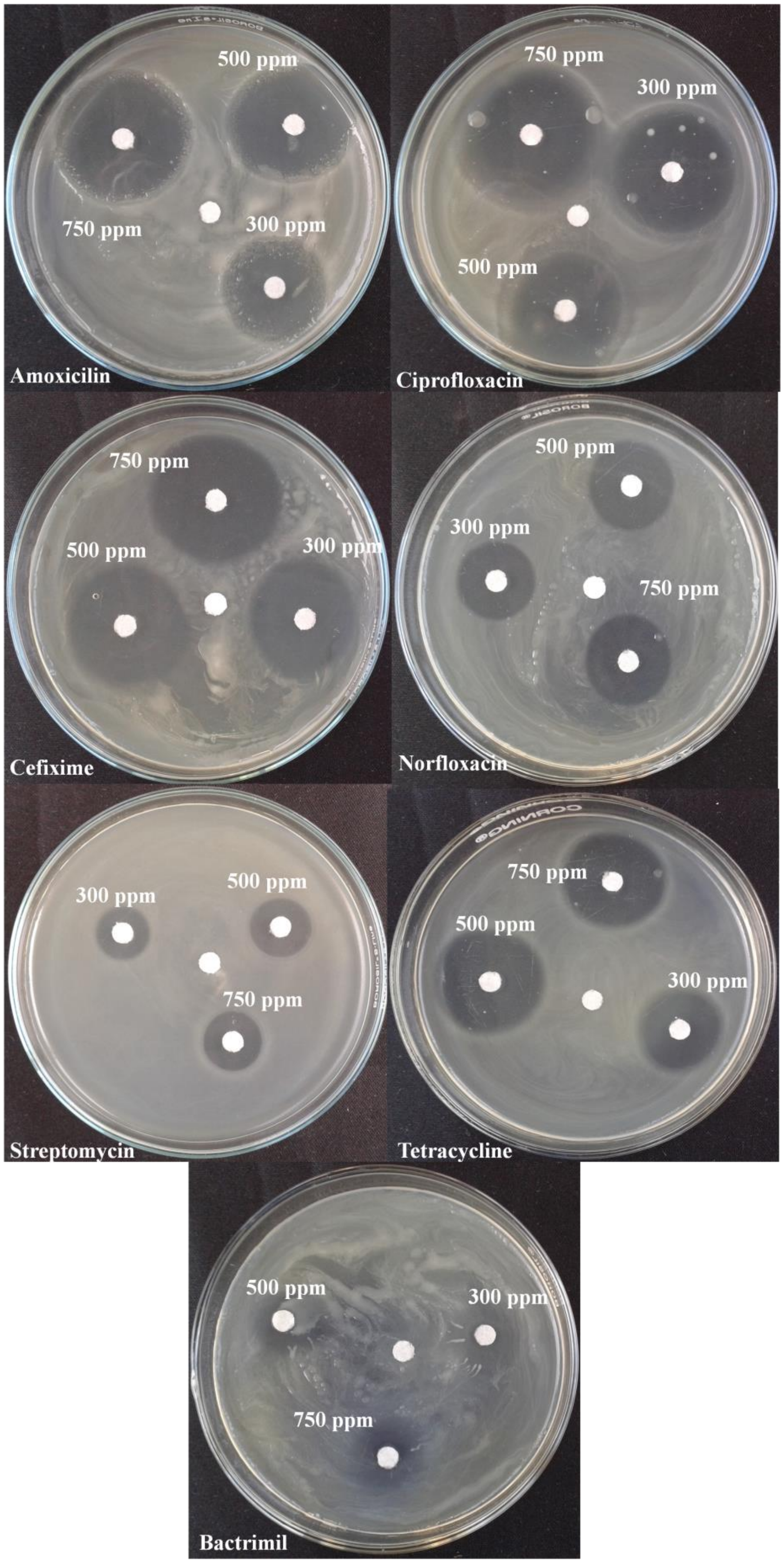

Plate 5: *In vitro* evaluation of antibiotics against *Ralstonia solanacearum*

Table 4: *In vitro* evaluation of bacterial and fungal antagonists against *R. solanacearum*

Sl. No.	Antagonist	Inhibition zone (mm)
1	***Bacillus subtilis***	12.17 (4.49)*
2	***Bacillus megaterium***	9.67 (4.11)
3	***Pseudomonas fluorescens***	9.83 (4.13)
4	***Trichoderma harzianum***	0.00 (1.00)
5	***Trichoderma viridae***	0.00 (1.00)
6	***Trichoderma virens***	0.00 (1.00)
7	**control**	0.00 (1.00)
S.Em ±		**0.03**
CD @ 1%		**0.12**
CV (%)		**2.39**

*Transformed values

4.6 Pot culture evaluation of selected antibiotics and antagonists on *R. solanacearum*

A glass house experiment was conducted to evaluate the efficacy of the most effective antibiotics and antagonists on *R. solanacearum* in the department of Plant Pathology, University of Agricultural Sciences, Bangalore. The observations on wilt incidence, population of *R. solanacearum* at flowering stage were recorded.

4.6.1 Population of *R. solanacearum* in rhizosphereof brinjal

The data on the effect of antibiotics and bacterial antagonists and their application on the survival of *R. solanacearum* at flowering stage are presented in the Table 5, Fig. 4 and Plate 6 and 7.

The population of the pathogen in the rhizosphere was suppressed drastically by the antibiotics and antagonistic bacteria. Among the antibiotics tested the treatments with pathogen + antibiotics Streptomycin, Cefixime and Tetracyclin were found to be very effective in reducing the population of *R. solanacearum* to 0.0 x 10^4cfu/g of soil respectively. Among the bacterial antagonists tested the treatment with Pathogen + *P. fluorescens*, Pathogen + *B. megaterium* and Pathogen + *Bacillus subtilis* were found to be very effective in reducing the population of *R. solanacearum* to 3.33 x 10^4, 4.33x 10^4 and 7.33x 10^4cfu/g of soil respectively. There was 100 per cent incidence of bacterial wilt observed at 30 days after planting in the treatment of pathogen control.

However, maximum population (686.66 x 10^4cfu/g of soil) of *R. solanacearum* was found in the sick soil of pots.

Table 5: Effect of antibiotics and bacterial antagonists on the survival of *Ralstonia solanacearum* in the rhizophere of brinjal

Sl. No.	Treatments	Population of *Ralstonia solanacearum* (10^4cfu/g of soil)
1	Pathogen control	686.66 (27.20)*
2	Pathogen + Streptomycin (500 ppm)	0.00 (1.00)
3	Pathogen + Streptomycin (750 ppm)	0.00 (1.00)
4	Pathogen + Cefixime(500 ppm)	0.00 (1.00)
5	Pathogen + Cefixime(750 ppm)	0.00 (1.00)
6	Pathogen + Teteracyclin(500 ppm)	0.00 (1.00)
7	Pathogen + Teteracyclin(750 ppm)	0.00 (1.00)
8	Pathogen + Amoxicillin (500 ppm)	18.33 (5.26)
9	Pathogen + Amoxicillin (750 ppm)	11.66 (4.37)
10	Pathogen + Ciprofloxacin (500 ppm)	196.33 (14.90)
11	Pathogen + Ciprofloxacin (750 ppm)	144.66 (12.80)
12	Pathogen + Norfloxacin(500 ppm)	96.00 (10.68)
13	Pathogen + Norfloxacin(750 ppm)	59.33 (8.69)
14	Pathogen + *Pseudomonas fluorescens*	3.33 (2.76)
15	Pathogen + *Bacillus megaterium*	4.33 (3.06)
16	Pathogen + *Bacillus subtilis*	7.33 (0.00)
S.Em ±		**0.62**
CD @ 1%		**2.38**
CV(%)		**8.58**

*Transformed values

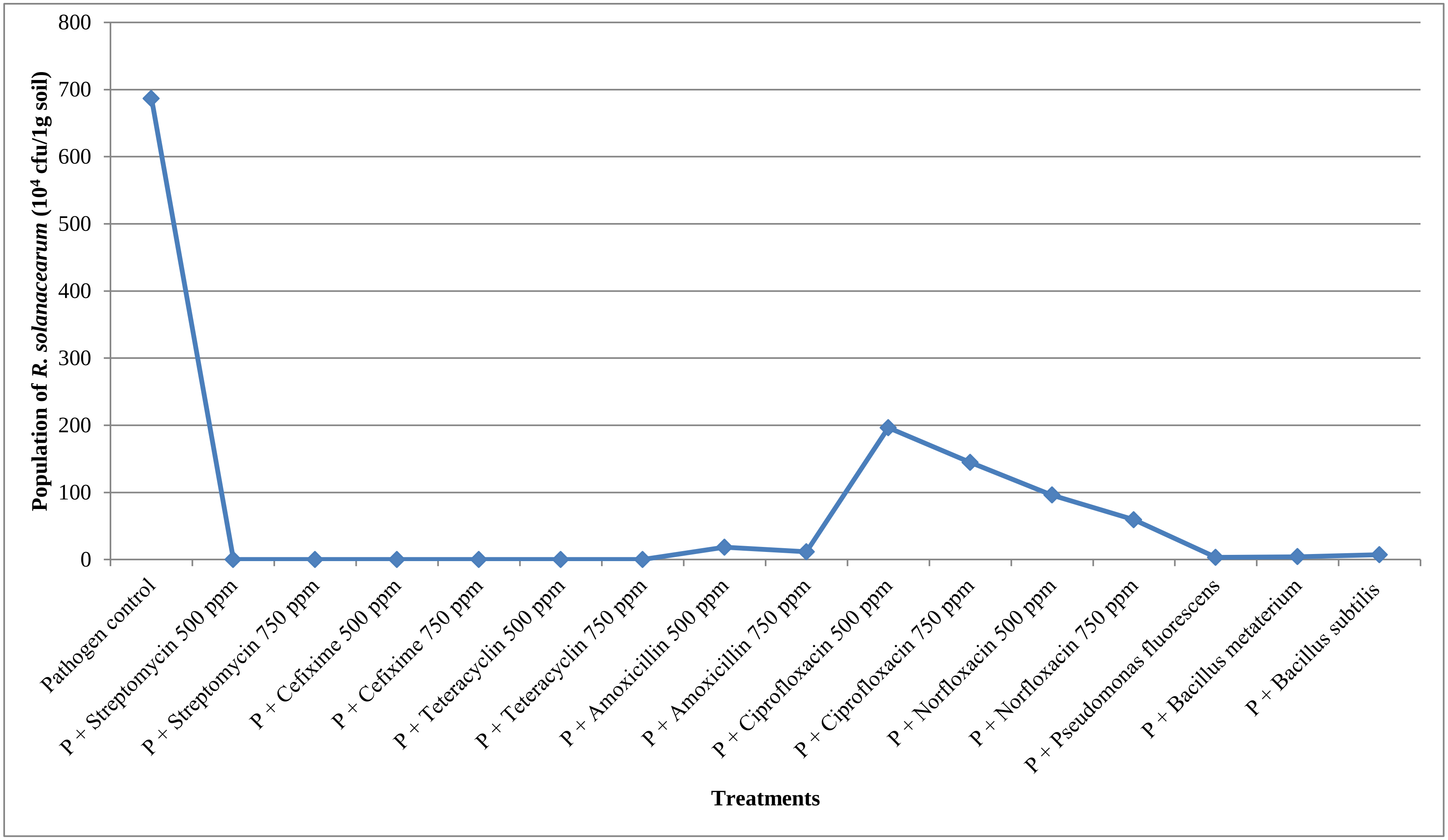

Fig. 4: Effect of antibiotics and bacterial antagonists on the survival of *Ralstonia solanacearum* in the rhizophere of brinjal

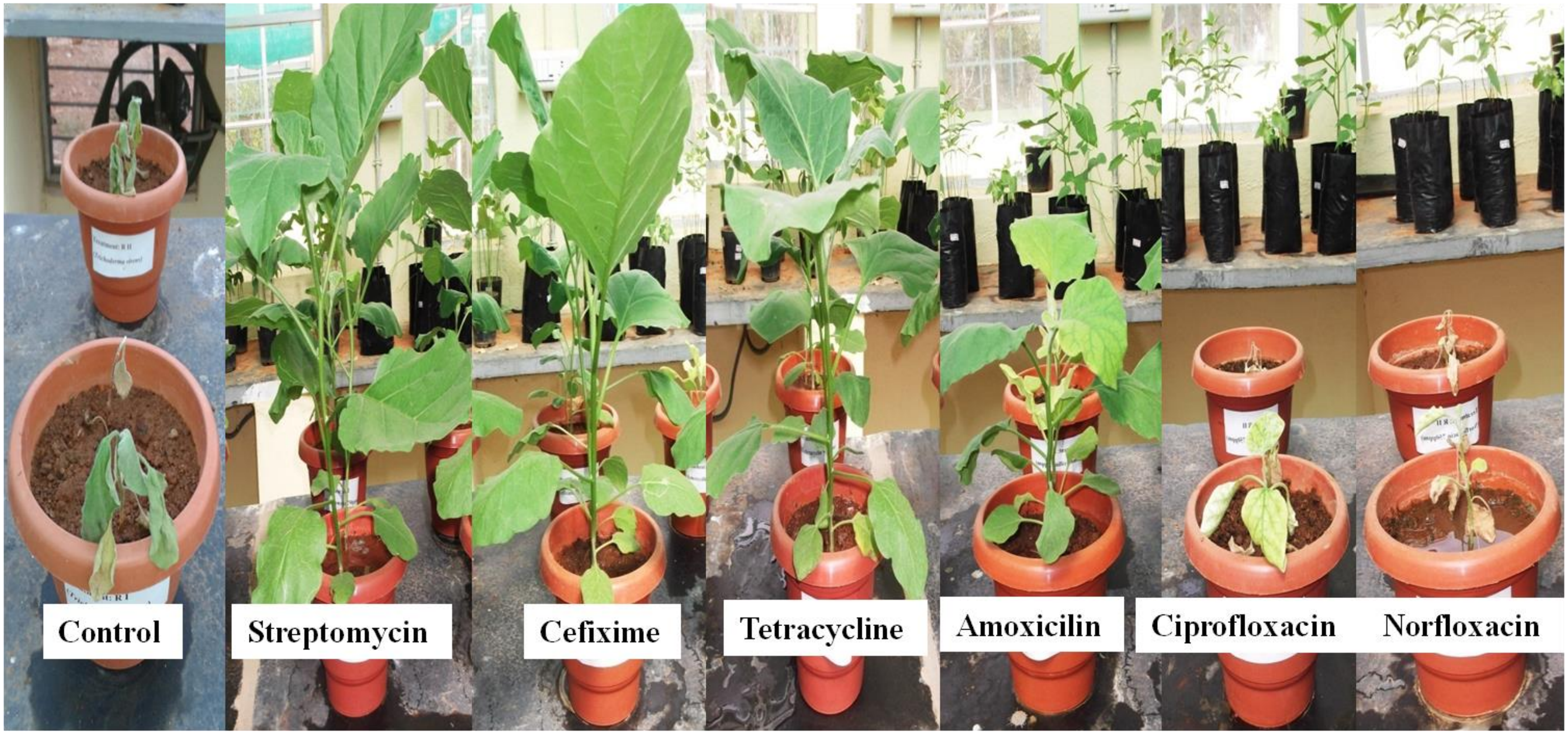

Plate 6: Effect of antibiotics on the survival of *Ralstonia solanacearum* in the rhizophere of brinjal

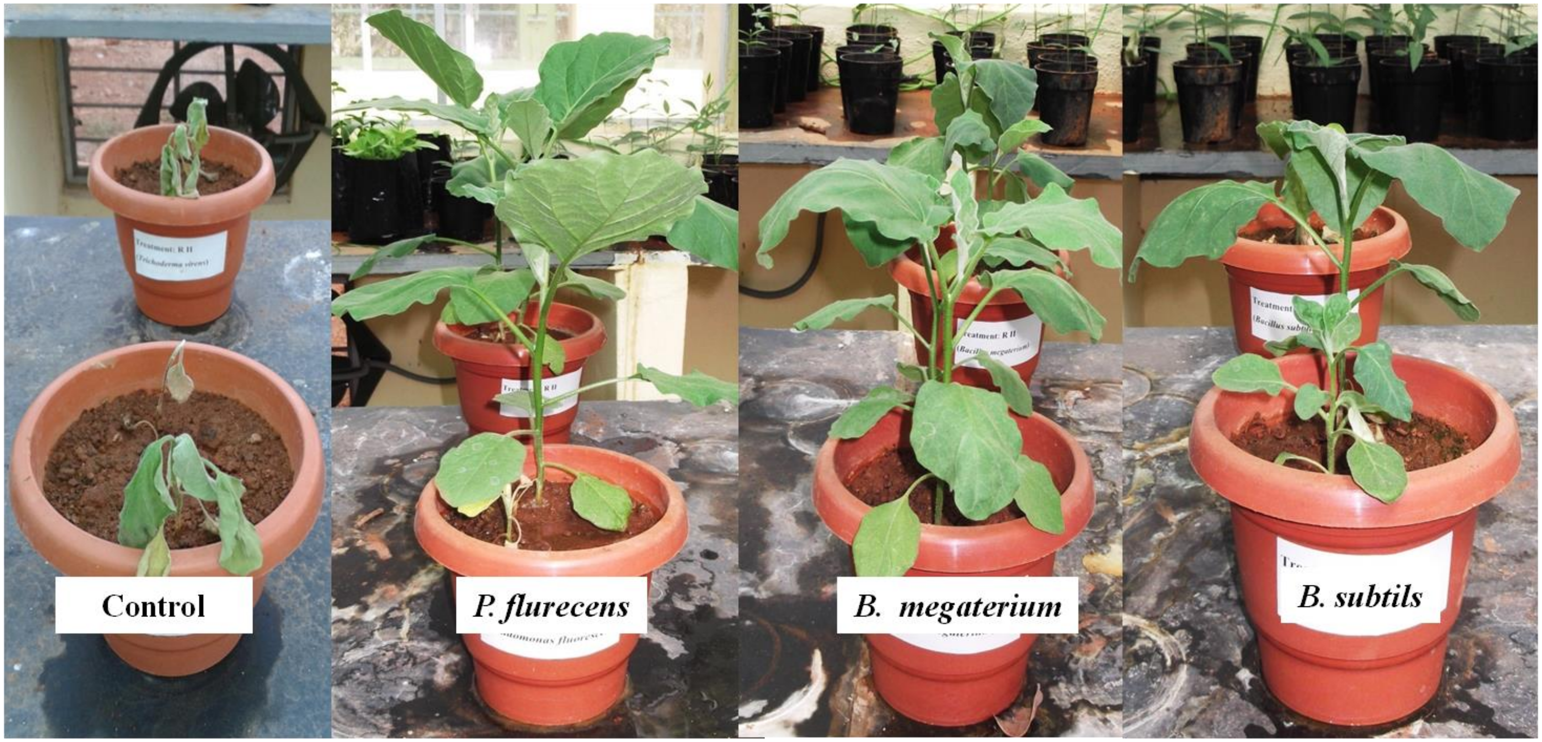

Plate 7: Effect of bacterial antagonists on the survival of *Ralstoniasolanacearum* in the rhizophere of brinjal

V. DISCUSSION

The bacterial wilt caused by *R. solanacearum* (Smith) Yabuchi *et al* is a major constraint in the cultivation of brinjal and other solanaceous crops in tropical, subtropical and warm temperature regions of the world (Yabuchi, *et al*., 1995). The yield loss in brinjal due to this disease ranges from 15 to 95 per cent (Javier, 1994).

At present, there is no effective control measure to manage the bacterial wilt. However, several antibiotics are being used in the field and several other non-antibiotics which are effective against other Gram negative bacteria are being tested in different concentrations against *R. solanacearum*. These antibiotics are effective under *in vitro* but fail to control the diseases under field condition. Also, continuous application of antibiotics to soil is known to affect other beneficial micro flora. Further, most of the antibiotics tested/ used are not developed for agricultural use and there is a need to evaluate the antibiotics which are less hazardous to soil micro-flora or the environment.

Biocontrol is regarded as one of the important IDM strategy for control of bacterial wilt. There are contradictory reports about the efficiency of various biological agents in controlling bacterial wilt under field conditions. Hence, in the present investigation attempts were made to evaluate the new antibiotics and potential antagonistic bacteria and fungi under *in vitro* and *in vivo* conditions.

The results obtained on isolation of the causal agent from the wilt affected brinjal plant and mass production, formulation and *in vitro* and *in vivo* evaluation of selected antibiotics and biocontrol agents against the pathogen/ disease are discussed below.

5.1 Isolation and identification of *R. solanacearum* from bacterial wilt infected brinjal plants

The pathogen isolation by serial dilution technique yielded *R. solanacearum* colonies which were fludial, dull-white colored with slight pink to red colored centers. The results of morphological studies revealed that the pathogen was Gram-negative rod and non-capsulated. Several workers have confirmed the morphological of *R. solanacearum* as rod shaped and non-capsulated (Smith, 1896; Khan, 1974; Ansari and Ram, 1987; Norman and Yuen, 1988).

The pathogen colonies on TZC medium was highly fluidal with copious slime and appeared white with light pink colored centre. Pathogenic and biochemical characteristics *viz.,* positive to nitrate reduction test; negative to starch hydrolysis and gelatin liquification also confirmed the identity of the pathogen as *R. solanacearum* (E. F. Smith, Yabuchi *et al*., 1995). Khan *et al*. (1974) and Shobha (2002) observed similar colony characters in brinjal isolate on TZC medium and further also identified that, the bacterium as *R. solanacearum* based on standard morphological procedures. These results are in accordance with other reports of Kelman, 1954; Buddenhagen *et al*., 1962; Singh and Hussain (1991).

5.2 Physiological characters

The temperature of 30^o C was found optimum for the growth of pathogen. The maximum number of colonies (196.66 x 10^5cfu/ml) were recorded at this temperature level followed by 25^o C (114.66 x 10^5cfu/ml) and 35^{o}C (103.33 x 10^5cfu/ml). The growth of bacterium at these two temperature levels differed significantly and also found superior to other temperature levels tested. However, the pathogen grew at a temperature, 20^o C, but it failed to grow at the lowest extreme temperature 5^o C to 15^o C. Wang and Lin (2005) reported that temperature of 30 - 35^o C is conducive for the disease occurrence of bacterial wilt whereas, soil temperature < 20^o C was not found suitable for the disease. Further, Prior *et al*., (1996) observed that *R. solanacearum* rapidly moves through the plant at temperature above 25^o C, thus the temperature of >28^o C is ideal for the growth of the bacteria both in plant and on nutrient medium as evident in the present study. These results are in conformity with the results of Hayward, (1991) that the temperature between 30 and 35º C significantly increases wilt severity in tobacco and other hosts. Also, in another study *R. solanacearum* caused significantly greater disease in resistant tomato cultivars at 32.2º C than at 26.6º C (Krausz and Thurston, 1975).

Ability of *R. solanacearum* to survive and grow depends on pH of the environment. The data revealed that, number of bacterial colonies increased with increase in pH to certain range from 5 to 7 and thereafter colony numbers decrease with increase in pH indicating 6 and 7 were the optimum pH for maximum growth of the pathogen *R. solanacearum*. The maximum number of bacterial colonies recorded were 154.00 x 10^5cfu/ml at pH 7 and the next best pH levels, which significantly supported the good growth were 6 (52.33 x 10^5cfu/ml) and 8 (46.00 x 10^5cfu/ml). The least growth of the pathogen was observed at the lowest and the highest pH levels of 5 (11.66 x 10^5cfu/ml) and 9 (8.33 x 10^5cfu/ml) respectively and no growth of the pathogen was recorded at pH of 4 and 10.Vincent and Mew (1997) reported that, growth of *R. solanacearum* was suppressed at pH 3, 10, and 11, and strongly reduced at pH 4 and 9. At pH 5 and 8, growth reduction was weak or it did not occur at all. Normal growth of all strains occurred at pH 6.

5.3 *In vitro* evaluation of antibiotics on the growth of *R. solanacearum*

Among different antibiotics tested, Amoxycillin was found to be highly effective with the maximum inhibition range of 31.25 to 36.00 mm at 500 to 750 ppm and was on par with Cefixime which recorded inhibition zone of 31.25 to 35.50 mm. Ciprofloxacin recorded inhibitory zone of 28.75 to 32.75 mm followed by Tetracyclin (24.25 to 27.25 mm), Norfloxacin (20.75 to 22.75 mm) and Streptomycin (14.75 to 16.25 mm). The least inhibitory zone of 7.25 to 10.00 mm was recorded by Bactrimil at 500 and 750 ppm. Dutta and Verma (1969) studied the efficacy of Streptocycline in controlling bacterial wilt of egg plant and found that seedling treatment of variety Pusa purple long with the antibiotic (1g/1) for 30 minutes before planting gave the best results.

Farag *et al.* (1986) studied the effect of Streptomycin and dihydro streptomycinin relation to potato bacterial wilt and found that both virulent and avirulent forms of pathogen were sensitive to Streptomycin and dihydro streptomycin. Similarly, Das *et al.* (1995) tested 14 antibiotics and amongst them Tetracycline was the most effective

5.4 *In vitro* evaluation of a bioagents on the growth of*R. solanacearum*

5.4.1 Bacterial antagonists against *R. solanacearum*

The studies carried out to test the effect of antagonistic bacteria on *R. solanacearum* under *in- vitro* revealed that *B. subtilus,* was found to be most effective in inhibiting the growth whereas, other antagonists *viz., P. fluorsecnes, B. megaterium* also inhibited the growth of *R. solanacearum.*

Gallardo and Panno, (1989) reported the inhibition of *R. solanacearum in vitro* by using *P. fluorescens* strain BC-8. The growth of *R. solanacearum* was inhibited by inoculation with *P. fluorescens* on the medium. Anuratha and Gnanamanickam (1990) reported that, the 125 fluorescent strains of bacteria (which included 117 strains of *P. fluorescens*) screened against *R. solanacearum*, 68 strains (54.4%) inhibited the growth of the pathogen. Ciampi *et al.* (1989) reported that, the biological agent designated as BC-6 caused strong inhibition of *R. solanacearum* in both *in vitro* assay and growth chamber condition.

5.4.2 Fungal antagonists against *R. solanacearum*

Three fungal antagonists *viz., T. harzianum, T. viride* and *T. virens* were not effective against *R. solanacearum* under *in vitro* that could be due to the inability of the fungal antagonists to produce antibiotics. However, Nesmith and Jenkins (1985) reported that *Trichoderma* was inhibitory to *R. solanacearum* under *in vitro* conditions.

5.5 Evaluation of selected antibiotics and antagonists against *R. solanacearum* under *in vivo*

The population of the pathogen in the rhizosphere was suppressed drastically reduced by the antibiotics and antagonistic bacteria. Among the antibiotics tested the treatments with pathogen + antibiotics Streptomycin, Cefixime and Tetracyclin were found to be very effective in reducing the population of *R. solanacearum* to 0.0 x 10^4cfu/g of soil. Among the bacterial antagonists tested, the treatment Pathogen + *P. fluorescens*, Pathogen + *B. megaterium* and Pathogen + *Bacillus subtilis* were found to be very effective in reducing the population of *R. solanacearum* to 3.33 x 10^4, 4.33x 10^4 and 7.33x 10^4cfu/g of soil respectively. However, under pathogen alone inoculated condition, all the plants exhibited typical wilting symptoms within 30 days after inoculation in the pots. The population in pots without antibiotics was686.66 x 10^4cfu/1g of soil at the time of flowering.

Antibiotic Cefixime has been tested for the first time against *R. solanacearum* which was found very effective as no brinjal plant found wilting symptoms in the pot culture experiment. The pathogen did not multiply in the inoculated soil with the population of zero. However, further studies are required to test its field efficiency.

Dutta and Verma (1969) studied the efficacy of Streptocycline in controlling bacterial wilt of egg plant and found that seedling treatment of variety *Pusa purple* long with the antibiotic (1g/1) for 30 minutes before planting gave the best results. Mondal and Mukherjee (1978) reported the protection of tomato plants against *R. solanacearum* by 6-aminopenicillic acid and streptomycin sulphate. Khan (1980) conducted field trials to control bacterial wilt of tomato and reported that Agrimycin-100 was effective in decreasing the wilt. Wherein, the percentage of wilting in treated plots for three seasons was 30.4, 35.8 and 35.8 while in untreated plots it was 59.9, 72.5 and 82.8 percentages respectively.

Anuratha and Gnanamaickam (1990) reported that the application of antagonistic bacteria like *P. fluorescens* (strain PfCP) and strains of B33 and B36 of *Bacillus* spp. along with pathogen inoculation is known to enhance the height and biomass of brinjal, banana and tomato. The growth promotion was observed in these treatments appear to be due to elimination of effects of wilt pathogen by the antibiotics producted, by the antagonists than due to siderophores production.

The trials conducted at Central Potato Research Institute, Shimla, India reported that an isolate of *Bacillus* sp reduced wilt incidence by 74 per cent and increased the incubation period by 29-30 days. Field trials at Bhowali (Kumaon hills) showed that, isolates of *Bacillus* species (BNS_1 and BNS_2) reduced wilt incidence by 87 and 89 per cent and tuber rot by 83 and 84 per cent respectively (Shekhawat *et al.,* 1992).

Suvarna *et al.* (1997) studied the effect of different biological agents *viz., Bacillus cereus, B. subtilis* and avirulent strain of *R. solanacearum* on bacterial wilt of potato both in glass house and in naturally infested field and reported that by treating with these bio agents the wilt incidence was reduced to 23.51, 17.09 and 13.10 per cent respectively compared to control 74.41 per cent. Correspondingly substantial increase in yields to an extent of 22.72, 64.47 and 62.77 per cent were observed as compared to control.

Singh and Rana (2000) reported that the tuber bacterization with *B. cereus, Bacillus* spp. and *B. subtilis* gave significant reduction of bacterial wilt incidence and increase in yield over check. All the *Bacillus* spp. were equally effective as these treatments did not show significant difference in wilt incidence and yield. An avirulent strain of *R. solanacearum* was found the least effective.

Vanitha *et al*. (2009) reported that, the bacterial wilt incidence was reduced significantly in all 20 cultivars tested, in plants raised from seeds treated with *P. fluorescens* followed by challenge inoculation with *R. solanacearum*. The bacterial wilt incidence in the untreated control ranged from 12 to 85 per cent wherein it was reduced to 2 to 30 per cent in the treated cultivars.

VI. SUMMARY

Bacterial wilt caused by *Ralstonia solanacearum* is a lethal disease of economically important crops *viz.,* potato, tomato, brinjal, banana, ginger *etc*., in tropical, subtropical and warm temperate regions of the world. In present investigation, isolation of the pathogen and antagonists/ bioagents, biochemical characterization and pathogenicity of the wilt bacterium along with *in-vitro* and *in-vivo* evaluation of selected antibiotics and bioagents against *R. solanacearum* was conducted.

The pathogen was isolated from the wilt infected plant samples by serial dilution technique and the isolate was characterized through morphological and cultural characteristics. The bacterium was Gram negative, rod shaped non-capsulated and the colonies on TZC medium were white with light-pinkish centre and highly fluidal producing copious slime. On the basis of these morphological, cultural, biochemical and pathogenicity tests, the isolate was identified as *Ralstonia solanacearum* (E. F. Smith, Yabuchi *et al*., 1995).

The growth of bacterium at different temperature and pH levels indicated significant differences and the temperature of 30^{o}C and pH 6 to 7 were found optimum for the maximum growth of *R. solanacearum*.

In vitro studies carried out to find the effectiveness of antibiotics and antagonists in inhibiting the growth of *R. solanacearum.* It was revealed from the evaluation that the antibiotics *viz.,* Amoxycillin, Cefixime and Ciprofloxacin were highly effective with the maximum inhibition range than other antibiotics like Tetracyclin, Norfloxacin and Streptomycin. Among bacterial antagonists, *Bacillus subtilis* was very effective in inhibiting the growth of *R. solanacearum.* The other bacterial antagonists *Pseudomonas fluorescens* and *Bacillus megaterium* also showed inhibition of *R. solanacearum.* However, the fungal isolates were not found effective against *R. solanacearum* under *in vitro* condition.

Among the antibiotics evaluated under pot culture in the green house, the treatments with pathogen + antibiotics Streptomycin, Cefixime and Tetracyclin were found very effective in reducing the population of *R. solanacearum* (*0.0* x *10^4cfu/g* of soil). Among the bacterial antagonists tested, the treatment with Pathogen + *P. fluorescens*; Pathogen + *B. megaterium;* and Pathogen + *Bacillus subtilis* were found very effective in reducing the population of *R. solanacearum* to 3.33 x 10^4, 4.33x 10^4 and 7.33x 10^4cfu/g of soil respectively compared to pathogen control 686.66 x 10^4cfu/g of soil.

Future line of work

The investigation need to be carried out under field conditions to arrive at a more reliable data and performance assessment of antibiotics and bio-agents. Further there is need to identify new generation of antibiotics (bactericides) and potential bio control agents viz., bacteriophages and other Gram positive and Gram negative bacteria for effecting management of bacterial wilt of brinjal.

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APPENDIX

COMPOSITION OF THE MEDIA USED IN THE EXPERIMENT

Nutrient Agar

Beef extract	: 3.0 g
Peptone	: 5.0 g
Agar	: 17 g
Distilled water	: 1000 ml

Nutrient Broth

Beef extract	: 3.0 g
Peptone	: 5.0 g
Distilled water	: 1000 ml

Potato Dextrose Agar

Peeled potato	: 200 g
Dextrose	: 20 g
Distilled water	: 1000 ml
Agar	: 17 g

Slice potato and boil. Take the infusion and make the volume to one liter.

Potato Dextrose Broth

Peeled potato	: 200 g
Dextrose	: 20 g
Distilled water	: 1000 ml

Slice potato and boil. Take the infusion and make the volume to one liter.

CPG Agar

Casein hydrolysate	: 1.0 g
Peptone	: 10.0 g
Glucose	: 10.0 g
Agar	: 20.0 g
pH	: 7.0

CPG Broth

Casein hydrolysate	: 1.0 g
Peptone	: 10.0 g
Glucose	: 10.0 g
pH	: 7.0

Triphenyl Tetrazolium Chloride medium

Peptone	: 10 g
Casein hydrolysate	: 1 g
Glucose	: 5 g
Agar	: 17 g
2,3,5- TTC	: 0.05 g
Distilled water	: 1000 ml
pH	: 7.0

stock solution of 0.005% of TTC at the rate of 1 ml / 200 ml of media was added before pouring the media into petriplates.

King's B Agar

Proteose petone No.3	: 20 g
Dipotassium hydrogen phosphate	: 1.5 g
Magnessium sulphate heptahydrate	: 20 g
Agar	: 20 g
Distilled water	: 1000 ml
pH	: 7.2 ± 0.2

King's B Broth

Proteose petone No.3	: 20 g
Dipotassium hydrogen phosphate	: 1.5 g
Magnessium sulphate heptahydrate	: 20 g
Distilled water	: 1000 ml
pH	: 7.2 ± 0.2