

**“STUDIES ON BIOCHEMICAL CHANGES IN
PADDY ON INOCULATION WITH
ENDOPHYTE AGAINST BACTERIAL LEAF
BLIGHT (*Xanthomonas oryzae* pv. *oryzae*)”**

BY
B. RAJYALAKSHMI
B.Sc. (Ag.)

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CHAIRPERSON: Dr. P. NAGAMANI



**DEPARTMENT OF PLANT PATHOLOGY
SRI VENKATESWARA AGRICULTURAL COLLEGE, TIRUPATI
ACHARYA N.G. RANGA AGRICULTURAL UNIVERSITY
GUNTUR – 522 509, ANDHRA PRADESH**

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DECLARATION

I, Miss. **BOBBALA RAJYALAKSHMI**, hereby declare that the thesis entitled “**STUDIES ON BIOCHEMICAL CHANGES IN PADDY ON INOCULATION WITH ENDOPHYTE AGAINST BACTERIAL LEAF BLIGHT (*Xanthomonas oryzae* pv. *oryzae*)**” submitted to the **Acharya N. G. Ranga Agricultural University** for the degree of **Master of Science in Agriculture** is the result of original research work done by me. I also declare that no material contained in this thesis has been published earlier in any manner.

Place: Tirupati

Date:

(B. RAJYALAKSHMI)
I.D. No. TAM/2018-40

CERTIFICATE

Miss. BOBBALA RAJYALAKSHMI has satisfactorily prosecuted the course of research and that the thesis entitled “**STUDIES ON BIOCHEMICAL CHANGES IN PADDY ON INOCULATION WITH ENDOPHYTE AGAINST BACTERIAL LEAF BLIGHT (*Xanthomonas oryzae* pv. *oryzae*)**” submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that neither the thesis nor its part thereof has been previously submitted by her for a degree of any university.

Date:

(P. NAGAMANI)
Chairperson
Subject Matter Specialist (PP),
Krishi Vigyan Kendra,
Nellore-524004,
Andhra Pradesh.

CERTIFICATE

This is to certify that the thesis entitled “**STUDIES ON BIOCHEMICAL CHANGES IN PADDY ON INOCULATION WITH ENDOPHYTE AGAINST BACTERIAL LEAF BLIGHT (*Xanthomonas oryzae* pv. *oryzae*)**” submitted in partial fulfillment of the requirements for the degree of ‘**Master of Science in Agriculture**’ of the Acharya N. G. Ranga Agricultural University, Guntur is a record of bonafide original research work carried out by **Miss. BOBBALA RAJYALAKSHMI** under our guidance and supervision.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part and all assistance received during the course of the investigation have been duly acknowledged by the author of the thesis.

Thesis approved by the Students Advisory Committee

Chairperson : **Dr. P. NAGAMANI** _____
Subject Matter Specialist (PP),
Krishi Vigyan Kendra, Nellore.

Member : **Dr. P. MADHUSUDHAN** _____
Scientist,
Dept. of Plant Pathology,
Agricultural Research Station,
Nellore.

Member : **Dr. P. LATHA** _____
Senior Scientist,
Department of Crop physiology,
Institute of Frontier Technology,
Regional Agricultural Research
Station, Tirupati.

Date of final viva – voce:

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LIST OF ABBREVIATIONS AND SYMBOLS

%	:	Per cent
°C	:	Degree Celsius
±	:	Plus or minus
µl	:	Microlitre
ANOVA	:	Analysis of variance
AR	:	Analytical reagent
BOD	:	Bio-oxygen demand
BP	:	Base pairs
CD	:	Critical Difference
Cfu	:	Colony forming units
cm	:	Centimetre
Conc.	:	Concentrations
CRD	:	Completely Randomized Design
CTAB	:	Cetyl-Trimethyl-ammonium
CV	:	Coefficient of Variation
DAS	:	Days After Sowing
DAT	:	Days After Transplanting
Dia.	:	Diameter
DMRT	:	Duncan Multiple Range Test
DNA	:	Deoxyribonucleic acid
Dt	:	District
EDTA	:	Ethylene-Diamine- Tetra - Acetic acid
<i>et al.</i>	:	And other people
etc.	:	And so on; and other people/ things
Fig.	:	Figure
g ha ⁻¹	:	Grams per hectare
g	:	Gram
GR	:	Guarenteed reagent
HCl	:	Hydrochloric acid

h	:	Hours
hapi	:	Hours after post inoculation
<i>i.e.</i>	:	That is
IAA	:	Indole acetic acid
IRRI	:	International Rice Research Institute
kg ha ⁻¹	:	Kilogram per hectare
kg	:	Kilogram
kb	:	Kilobyte
LAF	:	Laminar air flow chamber
lbs	:	Pound by weight
m	:	Metre
M	:	Molarity
min	:	Minute
ml	:	Milli litre
mm	:	Milli metre
16S rDNA	:	16S Ribosomal DNA
mg	:	milli gram
Mm	:	Milli molar
NaCl	:	Sodium chloride
NaOH	:	Sodium hydroxide
ng	:	Nano grams
nm	:	Nano metre
p.s.i	:	Pounds per square inch
pH	:	Potential of hydrogen ion concentration
PCR	:	Polymerase chain reaction
PGP	:	Plant growth promotion
PM	:	Pico moles
ppm	:	Parts per million
pv.	:	Pathovar
RBD	:	Randomized block design
RNA	:	Ribose nucleic acid

rpm	:	Revolutions per minute
S.No.	:	Serial number
SDS	:	Sodium dodecyl sulphate
SDW	:	Sterile distilled water
SE _{m±}	:	Standard error of mean
SES	:	Standard evaluation system
Sp.	:	Species (singular, plural)
SPSS	:	Statistical Package for the Social Sciences
TBE	:	Tris - Borate- EDTA
TE	:	Tris-EDTA
Tris	:	Trisaminomethane
U	:	Units
U.V.	:	Ultra violet
V	:	Volts
<i>viz.</i> ,	:	Namely

ABSTRACT

Author of the thesis : **B. RAJYALAKSHMI**
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Rice (*Oryza sativa* L.) is life for most of the Asians with more than three billion people around the world consume rice every day. Rice diseases have always had a notable influence on rice supply. Among rice diseases, Bacterial Leaf Blight (BLB) of paddy caused by *Xanthomonas oryzae* pv. *oryzae* is the most destructive problem and threat to rice production in rice growing regions. BLB pathogen was isolated from diseased leaf sample and the pathogenicity of the isolated culture was established by clip inoculation method on rice cultivar NLR-34449.

Endophytic bacteria were isolated from the samples collected from four mandals of Chittoor district of Andhra Pradesh. A total of 29 bacterial endophytes were isolated from healthy rice plants of popularly grown cultivars in Chittoor viz., MTU-1010, BPT-5204, ADT-37 and RNR-15048.

All the bacterial endophytes were screened for their antagonistic efficacy against bacterial leaf blight (BLB) pathogen *Xanthomonas oryzae* pv. *oryzae* under *in vitro* by agar well diffusion method. The inhibition diameter among the 29 isolates varied from 0.0 to 14.5 mm. Among 29 isolates, four isolates viz., EYK-3, ECP-1, ESR-5 and ECT-3 recorded antagonistic efficacy of more than 10.0 mm zone of inhibition with 14.3, 12.3, 11.43 and 10.53 mm respectively and were considered as effective antagonistic endophytic bacterial isolates.

The increased levels of expression of defense related enzymes viz., Peroxidase (PO), Polyphenol oxidase (PPO) and Phenylalanine ammonia lyase (PAL) was observed in rice plants treated with effective antagonistic endophytic bacterial isolates (EYK-3, ECP-1, ECT-3 and ESR-5) on challenging with *Xoo* than in untreated control. Among the four isolates

(EYK-3, ECP-1, ECT-3 and ESR-5), EYK-3 recorded highest level of expression of defense related enzymes (PO, PPO and PAL).

Molecular characterization of the effective antagonistic endophytic isolates (EYK-3 ECP-1, ECT-3 and ESR-5) was carried out for identification of bacterial endophytes by amplification of 16S rRNA region of the effective bacterial isolates Primers 27F and 1492R produced amplicon having size of approximately 1400 bp observed on 0.8 per cent agarose gel.

The isolate EYK-3 with highest antagonistic activity and increased level of expression of defense related enzymes was used for the management of BLB under field condition.

Among all the treatments, treatment T₃ (Seed treatment with EYK-3 @ 10⁹ Cfu /ml + Seedling root dip with EYK-3 @10⁹ cfu /ml) recorded lowest per cent disease index (7.3 %), with increased plant height (85.3 cm), no. of tillers (24.3), no. of productive tillers (22.3) and highest grain yield (6373 Kg ha⁻¹) over untreated control.

Biocontrol agents having plant growth promoting traits may be used in addressing the complicated and integrative phenomena of plant disease suppression and growth promotion through plant augmentation. The results strongly make a point that endophytic bacterial isolate (EYK-3) could be efficiently used for the management of bacterial leaf blight and as well as growth promotion of the plant for the sustainable rice cultivation.

Chapter –I

Introduction

Chapter - I

INTRODUCTION

Rice is life for most of the Asians with more than three billion people around the world consume rice everyday. It decides the diets and economic status of the millions of people (Hossain, 1997). Of the total world's rice production, more than 90 per cent is produced and consumed in Asia (FAO, 2011). Rice aggregates upto 20 per cent of the world's dietary energy source of the people.

In India, rice is cultivated in an area of 42.94 M ha with 112.95 MT of production and 2585 Kg ha⁻¹ of productivity. In Andhra Pradesh, area under cultivation of rice is approximately 2.15 M ha with 8.05 MT of production and 3741 Kg ha⁻¹ of productivity (Directorate of Economics & Statistics, 2018).

Paddy crop is cultivated over range of environments characterized by differences in temperature, soil and available water. The crop is also subjected to various types of abiotic stress and biotic stress which influence on the crop performance either directly or indirectly. Abiotic stresses include low nutrient availability, floods, high salinity, heavy metals etc. while biotic factors like insects, fungi, bacteria, virus and nematodes have been reported to cause damage to the rice crop in the world.

Rice diseases have always had a notable influence on rice supply. The major rice diseases that often cause high losses are rice blast, sheath blight, bacterial leaf blight and Tungro virus diseases especially in South and Southeast Asia. Among them bacterial diseases are most destructive causing yield loss upto 15-90 per cent (Zafar *et al.*, 2014).

Many bacterial diseases are known to infect rice *viz.*, Bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*), Bacterial leaf streak (*Xanthomonas oryzae* pv. *oryzicola*), Bacterial panicle blight (*Burkholderia glumae*), Bacterial brown stripe *Acidovorax avenae* subsp. *avenae*) etc. out of these,

bacterial leaf blight of paddy caused by *Xanthomonas oryzae* pv. *oryzae* is a destructive pathosystem of rice in all of tropical and temperate rice growing regions due to its high epidemic potential (Mew, 1987).

The disease occurs at the seedling stage, vegetative and reproductive stages. Infected seedlings get killed by blighting within two or three weeks of being infected while adult plants survive through diminished rice yield loss upto 75 per cent depending on the weather, location and rice cultivar used (Ou, 1985).

For the restriction of the pathogen, resistant genes are being identified and transferred to generate disease resistant cultivars (Peng, 2015). But these new high yielding cultivars are found susceptible in varying degrees. The very usual method of chemical control of phytopathogens has led to increased concerns over environmental contamination. In order to resolve pathogen control, more eco-friendly and sustainable approach is required. The utilization of beneficial microbes considered as potential alternate and environmental friendly method to protect plants from pathogens.

Bacterial endophytes in recent times have been under focus as biocontrol agents, as they carry additional benefits in relation to rhizospheric microbes (Hallmann *et al.*, 2001). Bacterial endophytes enter from the rhizospheric region at the site of wound, cut or opening and colonize in different parts of the plants like roots, stem, leaf, flowers and fruits (Gray and Smith 2005; Compant *et al.*, 2005 a,b).

Mechanisms by which endophytes enhance plant growth promotion are categorized as direct and indirect (Longet *al.*, 2008). Direct mechanisms include nitrogen fixation, phosphate solubilization, iron chelation etc. while indirect modes include pathogen restriction by competition for macro and micro nutrients, siderophore production, antibiotic production, induced systemic resistance, secretion of lytic enzymes and secondary metabolite production.

Endophytes are able to produce a wide range of bioactive compounds with superior biosynthetic capabilities for someone due to their presumable gene recombination with the host while residing and reproducing inside the healthy plant tissues (Li *et al.*, 2005). Searching of new antimicrobial compounds is important to overcome the difficulties related to pathogen resistance (Petersen *et al.*, 2004). Thus, endophytic microorganisms have emerged as an alternative source for the production of new antimicrobial agents such as peroxidases, polyphenol oxidases, phenylalanine ammonia lyase and phenolic compounds to inhibit plant pathogenic agents and consequently enhance plant growth (Gaiero *et al.*, 2013). Bacterial endophytes have been shown to prevent disease development through endophyte-mediated de novo synthesis of novel compounds and antifungal metabolites.

Defense related enzymes such as peroxidases, polyphenol oxidase, phenylalanine ammonia lyase, chitinase and β -1,3glucanase are linked to induction of resistance in plants (Gajanayaka *et al.*, 2014; Prasanth and De costa 2015). The increase in the production phenolics against pathogens and insects is indicated by the increased PAL activity in the affected tissues (Bi and Felton, 1995). Polyphenol oxidase catalyzes the oxidation of phenolics to free radicals which react with biological molecules creating an unfavourable environment for the growth and development of the pathogen (Jockusch, 1966). Increase in the activities of defense related enzymes by PGPR inoculation reported in wheat, cucumber and groundnut seedlings (Liang *et al.*, 2011; Mathivannan *et al.*, 2014; Hassan *et al.*, 2015).

Exploration of potential endophytes can pave the way for rich source of novel metabolites with plethora of uses in management of various diseases. Endophytic strain selection is important step as most of the endophytes are host specific. In addition it is major importance to target the disease and the host on which it is applicable. Finally the mechanism of antagonism and interaction with plant and pathogen should be arrived at by using endophytic organisms both under laboratory and field conditions.

Based on the need of identification of potential endophyte with antagonistic efficacy and level of expression of defense related enzymes for the restriction of BLB pathogen in rice, the present investigation was carried out with the following objectives.

OBJECTIVES:

1. Isolation and evaluation of endophytic bacteria against Bacterial Leaf Blight of Rice.
2. Estimation of defense related enzymes in plants inoculated with potential endophytic bacteria against BLB pathogen in Rice.
3. Evaluation of plant growth promoting characters and disease reduction in Rice treated with potential bacterial endophyte under field conditions.

Chapter – II

Review of Literature

Chapter – II

REVIEW OF LITERATURE

The literature collected on “Studies on biochemical changes in paddy on inoculation with endophyte against Bacterial Leaf Blight (*Xanthomonas oryzae* pv. *oryzae*)” was presented in this chapter. Bacterial leaf blight is an endemic disease that affects rice cultivars in most tropical Asian countries. A severely affected rice field usually loses about 50 per cent of its total yield (Ezuka and Kaku, 2000). The disease is characterized by grayish-white lesions on leaf veins, indicating that it is a vascular disease.

2.1 OCCURRENCE AND DISTRIBUTION BACTERIAL LEAF BLIGHT (BLB) DISEASE OF RICE

Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* is one of the most important and oldest known diseases of rice. The disease was first recognized by the farmers from Fukuokka area of Japan in 1884-1885 (Ishiyama, 1922) and the study of the disease commenced in Japan in 1901. The onset of bacterial leaf blight was further observed from several neighboring countries of Japan, i.e., Malaysia and Indonesia (Reitsma and Schure, 1950), Korea (Takeuchi, 1930), Taiwan (Hashioka, 1951) and Cambodia (Nishiyana, 1977) as a Kresek disease. Crop losses of 10-20 per cent in moderate conditions or severe losses of up to 50 per cent in highly conducive conditions have been recorded in several Asian and Southeast Asian countries (Ou, 1985). Globally, BLB incidence has been reported from different parts of Asia, Northern Australia, Africa, and the United States. In the United States of America, the disease was first time reported by Lozano (1977) while in Northern Australia, the disease was also found to occur in many cultivars of rice (Aldrick *et al.*, 1973). In India it was first reported from Koloba district of Maharashtra by Srinivasan *et al.* (1959). The disease was considered to be of minor

importance until it broke out in an epidemic form in Shahabad district of Bihar in 1963 (Srivastava *et al.*, 1967). In India, BLB disease has been observed in most important rice-growing states like Andhra Pradesh, Bihar, Haryana, Kerala, Orissa, Punjab and Uttar Pradesh.

2.1.1 Economic Impact

Bacterial leaf blight of rice had been a most serious issue in Southeast Asia especially from the promotion of high yielding dwarf varieties (Seneviratne, 1962; Fekain, 1971). The disease in its severe form is known to result in yield loss ranges from 74-81 per cent in susceptible cultivar (Shivalingaiah *et al.*, 2013). In Japan, yield losses recorded ranged between 25-35 per cent, often increasing up to 60 per cent (Ou, 1985) while in Philippines and Indonesia, losses were recorded as 1.08 per cent to 24.50 per cent (Exconde, 1973), while in India and Bangladesh, the heavy yield losses have been reported 12-32 per cent (ShahJahan, 1992). Similarly, in East India, reported losses of the yield were 7-62 per cent and 82 per cent at same genetic resources (Srivastava *et al.*, 1967; Singh *et al.*, 1980 and Srivastav and Kapoor, 1982).

In India millions of hectares was severely infected with the disease, causing yield loss up to 40 per cent (Pascuzzi and McCouch, 2007). The disease became prominent in the 1960s, when new high yielding cultivars were first developed and introduced yield loss ranging up to 26 per cent has been reported on susceptible rice cultivars. It is particularly destructive in Asian countries (Adhikari *et al.*, 1995; Ghasemie *et al.*, 2008). Yield losses due to this disease corresponds to the plant growth stages, infection at booting stages does not affect yield but results in poor quality and high proportion of broken kernels (Anonymous, 2009). Recent studies in West African countries such as Burkina Faso, Niger and Mali revealed the occurrence of bacterial leaf blight causing significant crop damages (Basso *et al.*, 2011).

2.2 SYMPTOMATOLOGY

The pathogenic bacterium invades the rice plants through the water pores using the fresh wounds of 24 hours (Mukko, 1957). The pores for water percolation are present on leaf edges of the top of leaf. So, lesions normally start at leaf margins on the upper part near the tip area. Firstly, small water-soaked lesions appear which later turned to yellowish white color expanding from the equal sides in a square shape to produce elongated circular to quite uneven lesions. Lesions edges were adjoining the healthy areas on the leaves and showing the most characteristic symptom of the disease i.e. the wavy margins, which can be clearly seen on the leaf blade. The lesions normally start on one or both margins of leaves or can be observed on the fresh infected leaf veins under humid conditions. The environment played a key role for the appearance of the symptoms, development of the disease and the appearance of the symptoms in the field. The disease could be characterized mainly into two distinct phases; leaf blight phase and the “Kresek phase” while the later is the destructive one for the epidemic of disease (Reddy and Ou, 1976; Ou, 1985).

2.2.1 Kresek Phase

The word “Kresek” is derived by the Vernacular term in Java, meaning “the sound of dead leaves” stroked with one another (Wakimoto, 1969). Kresek stage of the disease was firstly accounted in Indonesia considering a different disease of rice in the mid twenty centuries (Reitsma and Schure, 1950). Later, after a long time of consecutive studies, the pathogen of the distinct disease was identified and named (Mizukami, 1956). The “Kresek” phase of bacterial leaf blight disease was characterized mainly by the systemic infection due to this phase. The symptoms of disease usually appear 1-2 weeks after transplantation of the nursery into the main field. Under severe conditions leaves of the rice plant become grayish green to whitish and

suddenly withered. Sometimes “Kresek” phase of the disease happens on mature plants (Watanabe, 1975; Goto, 1992). Mew *et al.* (1979) reported that the lesion length on the leaf blade gradually lessens with the gradual increase in the age of the plant by the inoculation of 40, 60 and 80 days after sowing of the nursery.

2.2.2 Leaf Blight Phase

Leaf blight phase of the disease becomes visible very early in the temperate regions of the rice growing countries with the initial symptoms on the leaf blades and after the tillering the phase gain its peak position. The symptoms usually initiated at lower parts in plants and gradually proceeds towards to the upper areas of the plants (Cha, *et al.*, 1982; Goto, 1992). The severe symptoms of the disease could be observed on vulnerable genotypes when grown under the impact of extreme nitrogen fertilization. The upper portion of leaf or total area of the leaf blade turned pale yellow before drying up due to the severity of the disease (Mizukami and Wakimoto, 1969). In severe attack of the disease yellow to white stripes appeared inside the margins of the leaf blades which later on turned to pale yellow and become necrotic (Ou, 1985).

2.2.3 Etiology

BLB is favoured by warm temperatures (25 to 30 °C), high humidity, rain and deep water. The disease is more prevalent in wetland areas where these conditions often occur. Severe winds enough to cause wounds and excess nitrogen also favour the disease. The severity of the disease is in part dependent on the virulence of the bacterial isolates present. Bacterial blight is severe in susceptible rice varieties under high nitrogen fertilization (Anonymous, 2010). The bacterium can be disseminated by irrigation water, rain splashing or windblown rain,

plant-to plant contact, by trimming tools used in transplanting and wounds caused by handling plants during transplanting (Mew, 1992).

2.3 THE PATHOGEN

2.3.1 Discovery

Bacterial Blight was first characterized in the Fukuoka Prefecture of Japan in 1884. It was originally believed to be presence of acidic soil (Tagami and Mizukami, 1962). In 1909, masses of bacteria were isolated from the turbid dew drops of infected rice leaves, and the disease was reproduced by inoculating healthy leaves with these dew drops. Shortly thereafter it's etiology as a bacterial disease was established and the causal agent was isolated and classified as *Bacillus oryzae* (Bokura, 1911). The bacterium was renamed *Pseudomonas oryzae* and later *Xanthomonas oryzae* (Ishiyama, 1922). In 1978, it was reclassified as *X. campestris* pv. *oryzae* (Dye, 1978).

2.3.2 Morphology of Pathogen

X. oryzae is a rod-shaped, round end, Gram-negative. Individual cells vary in length from approximately 0.7 μm to 2.0 μm and in width from 0.4 μm to 0.7 μm . Cells are motile by means of a single polar flagellum. Colonies on solid media containing glucose are round, convex, mucoid and yellow in colour due to the production of the pigment xanthomonadin, characteristic of the genus (Bradbury, 1984).

Colonies of *X. oryzae* pv. *oryzae* on NA are circular, entire, smooth, convex, opaque, and pale yellow at first, straw yellow colour later. Colonies reach 1–2 mm after 5–7 days and their survival on solid media is short. Colony formation from a single cell is poor and frequently fails to grow in many media. On potato sucrose agar growth is faster, reaching 2 mm in 3–4 days, honey yellow and longer lived. Colonies of pathogens on NBY are pale yellow, circular, raised and

muroid; on Peptone Sucrose Agar, colonies are pale yellow, muroid and shiny; on Growth factor agar, colonies are very small, yellow and shiny (Agarwal *et al.*, 1989; Sakthivel *et al.*, 2001). On Modified XOS agar medium (mXOS), colonies have a characteristic rose pink colour, muroid, raised and glistening after 3–5 days (Di *et al.*, 1991; Gnanamanickam *et al.*, 1994).

2.3.3 Physiology of Pathogen

Yoshimura *et al.* (1970) described the ultrastructure of the bacterium which possess a cell wall that encircles the cytoplasmic covering and nuclear stuff of fibrillar emergence. The cytoplasm contains polysomes, ribosomes and different types of food granules. The outer surface of the bacterium is enclosed by viscous capsule like substance, which is composed of hetero polysacchrides, help the bacterium to protect from host toxins and unfavorable environmental conditions (Leigh and Coplin, 1992).

X. oryzae cells produce copious capsular Extracellular polysaccharide (EPS). This EPS is important in the formation of droplets or strands of bacterial exudate from infected leaves, providing protection from dessication and aiding in wind and rain borne dispersal of bacteria (Ou, 1972; Swings *et al.*, 1990).

The cells carry out aerobic respiratory metabolism and are non fermentative. The pathogen shows oxidase-negative (or weakly positive) and catalase-positive reactions. The major means of glucose metabolism is the Entner–Doudoroff pathway. Acid is produced from mono and disaccharides. A weakly buffered medium acid is produced from many carbohydrates but not from rhamnose, inulin, adonitol, dulcitol, inositol, or salicin, and rarely from sorbitol.

Xanthomonas oryzae inject effector proteins into plant host cells to elicit disease via a type-III (T3) secretion system (White *et al.*, 2009).

These proteins can confer pathogenicity and/or dictate host specificity (Jacques *et al.*, 2016).

2.3.4 Mode of Infection

Xoo enters the rice leaf typically through hydathodes at the leaf tip and leaf margin (Ou, 1985). Cells on the leaf surface may become suspended in guttation fluid as it exudes at night and enter the plant by swimming or passively as the fluid is withdrawn into the leaf in the morning (Curtis, 1943). Bacteria multiply in the intercellular spaces of the underlying epitheme, then enter and spread into the plant through the xylem (Noda and Kaku, 1999). *Xoo* may also gain access to the xylem through wounds or openings caused by emerging roots at the base of the leaf sheath (Ou, 1985). Within the xylem, *Xoo* presumably interacts with xylem parenchyma cells (Hilaire *et al.*, 2001). The pathogen moves vertically through primary veins of leaf but also progresses laterally through commissural veins. Within a few days of entry, bacterial cells and EPS fill the xylem vessels and bacterial cells come out as ooze from hydathodes, forming beads or strands of exudates on the leaf surface, a characteristic sign of the disease and a source of secondary inoculum (Mew *et al.*, 1993).

2.3.5 Survival and Transmission of the Pathogen

X. oryzae pv. *oryzae* survives primarily in rice stubble and on weed hosts, notably *Leersia oryzoides*, *Zizania latifolia*, *Leptochloa chinensis*, *L. panicea* and *Cyperus rotundus*. In Australia, the bacterium is known to survive in wild *Oryza* species (*O. rufipogon* and *O. australiensis*). *Xoo* can also survive for short periods on infected seed and in soil, but these have not been demonstrated to be important sources of inoculum. In tropical areas, the bacterium may also survive in irrigation water and perennial weeds (Mew, 1992).

Southern cutgrass (*Leersia hexandra* Swartz) is a common grass found in the southern United States, South America, Africa, and Asia and acting as collateral host (Guo and Ge, 2005). Interestingly, this and other grasses in this genus, such as *Leersia sayanuka*, *Leersia oryzoides*, and *Leersia japonica* are susceptible to *Xoo* (Noda and Yamamoto, 2008) and can serve as reservoirs for inoculums. Recent genome investigations of *Leersia perrieri*, a cutgrass found in Madagascar as source of inoculum (Copetti *et al.*, 2015; dos Santos *et al.*, 2017).

2.4 PATHOGENICITY TEST

Clipping method of artificial inoculation helps in establishing pathogenic interaction of *Xoo* with the host because the bacterial cells might have get deposited directly on the cut ends of xylem vessels, where the pathogen manifested itself in the host systematically and caused the lesion development (Kauffman *et al.*, 1973)

Leaf clipping and spray inoculation methods are available for inoculations of *Xoo* (Kauffman *et al.*, 1973; Cottyn *et al.*, 1994).

Ghasemie *et al.* (2008) assessed 18 isolates of *Xanthomonas oryzae* pv *oryzae*. All isolates found pathogenic on rice cultivar ‘khazar’ in which symptoms observed after 14 days of inoculation.

Chithrashree *et al.* (2011) confirmed pathogenicity by using leaf clipping method on IR-64 rice cultivar with 48 hr *Xoo* culture. All the ten *Xoo* isolates positive in production of typical BLB symptoms at 14 days after inoculation thus confirming the pathogenicity of the *Xoo* isolate.

Shankara *et al.* (2016) conducted pathogenicity test with paddy variety ‘BPT-5204’ in glasshouse conditions and artificially clip inoculated to 45 days old seedlings with bacterial suspension at a

concentration of 10^8 Cfu/ml and observed for the development of symptoms and re isolated the pathogen.

Chandraprakash *et al.* (2018) conducted pathogenicity test on 11 isolates of *X. oryzae* pv. *oryzae* on susceptible paddy cultivar, ADT 39 by clip inoculation method. Among all isolates the isolate Xoo M1 was found virulent.

2.5 ENDOPHYTIC BACTERIA:

Plants are constantly involved in interactions with a wide range of bacteria. These plant-associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere and (epiphytes) inside the plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissues, having been destructively isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Kobayashi and Palumbo, 2000). Several bacterial endophytes have been shown to support plant growth and increase nutrient uptake by providing phytohormones (Kang *et al.*, 2007) and biologically fixing nitrogen (Jha and Kumar, 2007).

2.5.1 Definition

Endophytic microorganisms grow within the healthy tissues of living plants during all or part of their life cycle without causing harmful effects on the host (Hallmann *et al.* 1997; Sturz *et al.* 2000 and Ray *et al.* 2017). These microorganisms are often isolated from internal plant tissues. Endophytes either remain localized to their points of entry or spread to other parts of the plant (Hallmann *et al.* 1997). They occupy the interior of cells, intercellular spaces or the vascular system of various plant species (Hallmann *et al.*, 1997; Sturz *et al.* 2000; Rosenblueth and Martínez-Romero, 2006).

2.5.2 Diversity and Populations of Endophytic Microorganisms

Endophytes comprise a large but little explored share of fungal diversity (Perottoab *et al.*, 2013). In general, endophytic populations are at low densities compared to rhizosphere populations (Rosenblueth and Martínez-Romero, 2006). Endophytes, colonizing the same host plant, are not limited to a single species, but they can include several genera and species. The density of endophyte populations varied mainly depending on microbial species, host genotypes, development stage of host plant, colonized tissues and environmental conditions (Tan *et al.* 2003; Ryan *et al.* 2008).

Endophytes harbour all plants. Based on published information reports (Mundt and Hinkle, 1976) more than 200 genera of bacteria have been reported as endophytes from surface sterilized plant tissue.

Different plant organs are associated with different endophytic bacterial communities in terms of diversity and composition. The number of bacterial cells within leaf phyllosphere (10^6 – 10^9 bacterial Cells/ g plant tissue) and root endophytic environments reaches 10^4 – 10^8 per gram of root tissues (Bulgarelli *et al.*, 2013). The work of de Oliveira Costa *et al.* (2012) on the leaf microbiome of common bean plants (*Phaseolus vulgaris*) showed that Proteobacteria, Actinobacteria and Firmicutes are the dominant groups, which was revealed by a culture-based analysis and also reported that the density of endophytic populations varied from 4.5×10^2 to 2.8×10^3 Cfu/g of fresh weight.

The most widely studied endophytic bacteria belong to three major groups namely Actinobacteria, Proteobacteria and Firmicutes including the genera of *Azoarcus* (Krause *et al.*, 2011), *Acetobacter* (renamed *Gluconobacter*) (Bertalan *et al.*, 2009), *Bacillus* (Deng *et al.*, 2011), *Enterobacter* (Taghavi *et al.*, 2010), *Burkholderia* (Weilharter *et al.*, 2011), *Herbaspirillum* (Pedrosa *et al.*, 2011), *Pseudomonas* (Taghavi

et al., 2009), *Serratia* (Taghavi *et al.*, 2009), *Stenotrophomonas* (Ryan *et al.*, 2009), *Alcaligenes* (Castro *et al.*, 2014) and *Streptomyces* (Suzuki *et al.*, 2005).

Endophytic population density is continuously being evaluated in many of the plant species, which vary either with the genotype or weather conditions (Lamb *et al.*, 1996; Hallmann *et al.*, 1997; Chi *et al.*, 2005).

The abundance of endospheric microbes (10^3 - 10^6 Cf/g) is recorded in rice, cotton, corn but lower than ectospheric microbes (10^6 - 10^8 Cf/g). The diversity of the endophytic microbial density declines from roots towards the upper parts such as stem, leaves etc. (Lamb *et al.*, 1996) and there is a wide variation in the population density of endophytes (8.91×10^1 to 7.24×10^6 Cf/g) between cultivated rice (sabita, swarna, swarna subi) and wild rice (*Oryza eichingeri*). The endophytic population density was also varied from cultivar to cultivar (NLR-34449: 4.4×10^6 to 7.65×10^6 and MTU-1010: 2.4×10^6 to 7.2×10^6) which was reported by Kavitha *et al.* (2019).

Studies performed on the endosphere microbiome of different plants, using high-throughput amplicon sequencing, have revealed that host plant species, growing season (Shen and Fulthorpe, 2015; Ding and Melcher, 2016), genotype (Marques *et al.*, 2015; Rodriguez-Blanco *et al.*, 2015), plant organ type, host plant nutrient status (Hameed *et al.*, 2015), developmental stage (Ren *et al.*, 2015a; Yu *et al.*, 2015; de Almeida Lopes *et al.*, 2016), geographical location, soil type, cultivation practice (Edwards *et al.*, 2015) and fertilization (Rodriguez-Blanco *et al.*, 2015) are the observed factors that significantly influence the plant endosphere microbiome.

Ren *et al.* (2015b) demonstrated that leaf endophytic bacteria appear to be more vulnerable to climate change than soil bacterial

communities. The community structure of endophytic bacteria in rice leaves was influenced by elevated CO₂ levels at the tillering and filling stages, but not during maturity, and this influence also correlated with N fertilization levels (Ren *et al.*, 2015a).

The microbiome in the leaf endosphere is significantly less diverse than microbiomes in the rhizosphere and bulk soil (Liu *et al.*, 2017)

2.5.3 Colonization of Endophytic Bacteria

Endophytic bacteria colonize an ecological niche similar to that of plant pathogens, especially vascular wilt pathogens. Exploiting an additional microbial habitat for biocontrol efficacy and increase consistency in performance, since the endophytic agent could avoid unfavourable conditions prevailing in the soil environment by entering and localizing in the intercellular spaces of the epidermal cells of root tissues.

Various endophytic bacterial strains colonize different plant tissues. Some strains primarily colonize plant roots, while others colonize roots, stem, leaves, flowers and fruits.

Bacterial endophytes are capable of colonizing different seed parts including the embryo. These endophytes likely mobilize and grow in the developing seedlings during germination and early seedling growth (Nelson, 2017). As seedlings emerge and plant growth begins, interactions between the roots and the soil microbiome commence. Plant exudates act as fuel for microbial activities in the rhizosphere, which facilitate the attachment and entry of bacteria into the plant roots. Eventually, certain endophytes initiate colonization of tissues beyond the roots such as the stems and leaves, and ultimately throughout the plant endosphere. Some bacterial endophytes also colonize flowers and seeds,

and most likely get transferred vertically from the maternal endophyte community into the offspring. (Compant *et al.*, 2010; Mitter *et al.*, 2017)

2.5.4 Endophytic Bacteria as a Biocontrol Agent

Endophytic bacteria inhabit plant internal tissues in a similar niche as phytopathogens, and they may compete with bacterial pathogens as biocontrol agents (Berg *et al.*, 2005; Hallmann *et al.*, 1997). Inoculation of plants with beneficial endophytes can inhibit disease symptoms caused by viral, insect, fungal and bacterial pathogens (Berg and Hallmann, 2006; Sturz *et al.*, 2000). The beneficial effects derived from endophytic bacteria are similar to those from rhizosphere bacteria (Ryan *et al.*, 2008). The Bt toxin synthesized by *B. thuringiensis* is currently one of the most effective, commercially available bio-insecticides (Jeong *et al.*, 2016). *Streptomyces* spp., *Pseudomonas viridiflava*, *Serratia marcescens* and *Paenibacillus polymyxa* are endophytic bacteria that produce active metabolites with antimicrobial and antifungal activities (Guan *et al.*, 2005; Li *et al.*, 2005).

Endophytic bacteria have shown significant control of diseases such as *Fusarium vasinfectum* in cotton (Van Buren *et al.*, 1993), *Verticillium albo-atrum*, *Rhizoctonia solani* and *Clavibacter michiganensis* subsp. *sepedonicum* in potato (Nowak *et al.*, 1995), *Sclerotium rolfsii* in bean (Pleban *et al.*, 1995), *Rhizoctonia solani*, *Pythium myriotylum* and *Gauemannomyces graminis* in rice (Mukhopadhyay *et al.*, 1996) and *Fusarium moniliformae* in maize (Hinton and Bacon, 1995).

The development of biocontrol strategies using endophytes is an emerging area in crop protection to reduce the damage caused by plant pathogens in economically important crops. Similarly, endophytic bacterial isolates of *Bacillus* (39 %), *Pseudomonas* (27.6 %), *Corynebacterium* (16.7 %), *Actinomyces* (11.1 %) and *Staphylococcus*

(5.6 %) isolated in solid media from *Jacaranda decurrens* (a medicinal plant) were reported (Carrim *et al.*, 2006). In addition to biological control, endophytic bacteria improved plant growth in different crops like potato (Sturz *et al.*, 1995), rice (Hurek *et al.*, 1994), maize (Hinton and Bacon, 1995), cotton (Chen *et al.*, 1995), beans (Pleban *et al.*, 1995), coconut (Rajendran *et al.*, 2007).

Hallmann *et al.* (1997) speculated that the observed plant growth promotion in different crops might have been caused by enhanced plant mineral uptake and improved plant water relationships associated with the colonization of endophytic strains. Some strains of *Pseudomonas*, *Enterobacter*, *Staphylococcus* produce plant growth regulators such as ethylene, auxins or cytokinins and have, therefore, been considered as beneficial micro organism involved in plant growth and development. In addition to a direct mechanism for growth promotion, plant growth promotion is also thought to be colonization due to the suppression of deleterious microflora by introduced endophyte (Kloepper *et al.*, 1991; Leifert *et al.*, 1994). The beneficial effects of bacterial endophytes, however, vary and appear to operate through similar mechanisms as described for PGPR (Kloepper *et al.*, 1991; Hoflich *et al.*, 1994). However, because of the different habitats colonized, endophytes offer another tool for developing biological control strategies. By integrating the use of bacterial endophytes with rhizosphere antagonists, a holistic biological control system could be developed that works against the pathogens.

Endophytic bacteria can not only promote plant growth and act as biocontrol agents, but also produce nature products like lipopeptides hydrogen cyanide, 2,4-diacetylphloroglucinol etc. to control plant diseases (Guan *et al.*, 2005) reducing disease severity (Senthilkumar *et al.*, 2007). *Bacillus* species are among the most common bacteria found to colonize plants endophytically (Lilley *et al.*, 1996; Mahaffee and

Kloepper, 1997) and it is likely that their endophytic ability could play a role in the biocontrol of vascular plant pathogens. The endophytic *Bacillus* spp. CY22 isolated from balloon flower produced iturin A with antifungal activity against *Rhizoctonia solani*, *Pythium ultimum* and *Fusarium oxysporum* (Cho *et al.*, 2003).

2.6 IN VITRO EVALUATION OF ENDOPHYTIC BACTERIA AGAINST BACTERIAL LEAF BLIGHT PATHOGEN.

Biocontrol of phytopathogens is essentially based on three mechanisms of action including competition for nutrients and sites of infection, antibiosis and induction of systemic resistance (ISR) in the plant (Malfanova *et al.*, 2013) which involved several antimicrobial metabolites and/or elicitors.

Antibiosis has been widely used by endophytes against phytopathogenic agents (Sessitsch *et al.*, 2004; Nandhini *et al.*, 2012 and Vethavalli and Sudha, 2012). The effect occurs through the release of antibiotics, hydrolytic enzymes, and/or other antimicrobial metabolites synthesized by these microorganisms in the area of their interaction with target pathogens.

Haustuti *et al.* (2012) assessed the effectiveness of ten *Streptomyces* spp. isolates in suppressing BLB disease *in planta* and *in vitro*. Among them AB131-1 gave highest plant height and LBR02 gave the highest inhibition activity against *Xoo* followed by AB131-1 and AB131-2.

Nagendran *et al.* (2013) isolated 40 bacterial endophyte strains from rice crop and tested against bacterial leaf blight. Among all strains, *Bacillus subtilis* var. *amyloliquefaciens* (FZB 24), EPB 9, EPB 10, EPCO 29 and EPCO 78 recorded a significantly higher inhibition zone over control under *in vitro*.

Van Hop *et al.* (2014) screened 2690 actinomycetes strains. Among all, seventeen actinomycetes strains were found to be capable of inhibiting all 10 major races of *Xoo*. One strain VN08- A-12 selectively inhibited all 10 *Xoo* races *in vitro* varied from 0.2 to 2.6 cm.

Jambhulkar *et al.* (2014) isolated Fluorescent pseudomonads from twenty rhizospheric soil samples, four isolates were found to inhibit growth of *Xoo*. All the four isolates were identified as *Pseudomonas fluorescens*. Their efficacy to inhibit *Xoo* varied widely and RRb-11 was found very effective for control of *Xoo*.

El-shak *et al.* (2015) assessed the antagonistic efficacy of five endophytic strains (A1, A2, A3, A13, and A15) and two rhizospheric *Bacilli* (D29 and H8) against BLB *in vitro* and *in vivo*. All seven strains showed high potential antagonistic activity against *X. oryzae* pv. *oryzae* with inhibition diameter of 28.5, 26, 13.33, 22, 17.33, 24 and 26 mm respectively.

Khoa *et al.* (2016) isolated and identified antagonistic soil bacteria to be used for biological control of the Bacterial leaf blight (*Xanthomonas oryzae* pv. *oryzae*). Among the 830 isolates, CT-66, CT-78, CT-88 exhibited strong antagonistic effects against the pathogen.

Azman *et al.* (2017) screened 93 endophytic bacterial strains against bacterial leaf blight pathogen. Among them, 16 isolates showed positive antagonistic activity indicated by the inhibition zone from 3.3 to 15.0 mm. UPMC 10 strain showed the highest potential by forming inhibition zone of 15.0 mm. SR12-1 strain showed lowest potential with 3.3 mm diameter of inhibition zone.

Yousefi *et al.* (2018) screened 39 endophytic bacterial isolates against *Xoo* strain, 21 bacterial isolates exhibited the most potent antagonistic activity against the *Xoo*. The maximum inhibitory activity was recorded for OS59 with an inhibition zone of 32.6 mm, whereas the

isolates OS58, OS43, OS20 and OS40 could produce inhibition zones of 32.3 mm, 26.6 mm, 18 mm and 16.7 mm, respectively.

Abdallah *et al.* (2019) studied effects and mechanism of *Paenibacillus polymyxa* Sx3 on growth promotion and suppression of bacterial leaf blight of rice and the results from plate assay indicated that Sx3 inhibited the growth of 20 strains of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) under *in vitro*.

Kavitha *et al.* (2020) isolated and screened 45 endophytic bacterial isolates against BLB pathogen the zone of inhibition was ranged from 0.0 to 16.8 mm. Isolate EMP-5 and EBK-3 showed highest antagonistic activity with inhibition zone of 16.8 mm and 16.6 mm, respectively. This was followed by EBA-5 (15.9 mm) isolate.

2.7 PLANT DEFENSE RELATED ENZYMES

The mechanisms of plant growth promotion by endophyte include production of growth hormones *e.g.*, Indole Acetic Acid (IAA), solubilization of inorganic phosphate (Panhwar *et al.*, 2014), fixation of atmospheric nitrogen, ACC deaminase activity (Chen *et al.*, 2013) and zinc solubilization. They can also be involved in promoting plant health by suppressing phytopathogens using various mechanisms *i.e.* competition, production of siderophores, antagonism and induced systemic resistance (Gomez *et al.*, 2014). PGPR-induced systemic resistance activates the plant's latent defense that leads to the activation of multiple defense-related compounds/enzymes at sites distant from the pathogen attack (Vanitha and Umesh, 2011).

Peroxidase is one of the fast responding defenses related enzyme against plant pathogens which are involved in lignification, suberification, polymerization of hydroxyl proline-rich glycoproteins, regulation of cell wall elongation, wound healing, and resistance against pathogens in plants (Maksimov *et al.*, 2014). Polyphenol oxidase is

important in the initial stage of plant defense where membrane damage causes release of phenols such as chlorogenic acid. PPO catalyzes the oxidation of phenolics to free radicals that can react with biological molecules, thus creating an unfavorable environment for pathogen development (Jockusch *et al.*, 1966). Phenylalanine ammonia-lyase (PAL) is the primary enzyme in the phenyl propanoid metabolism and plays a significant role in the synthesis of several defense-related secondary compounds such as phenols and lignin (Tahsili *et al.*, 2014).

Induced systemic resistance (ISR) in the plant is largely activated by root colonizing bacteria (Kloepper *et al.*, 2004; Van Loon *et al.*, 2006; Van Wees *et al.*, 2008; Pieterse *et al.*, 2009). ISR is mainly dependent on the signaling pathways of jasmonic acid and/or ethylene rather than salicylic acid (Pieterse *et al.*, 2009). However, some ISR inducers seem to activate the dependent pathway of salicylic acid which indicates that multiple signaling pathways may be cooperated when the ISR mechanism is triggered (Niu *et al.*, 2011).

Induction of synthesis of defense-related proteins such as peroxidases, chitinases, and β -1, 3-glucanases was demonstrated by Fishal *et al.* (2010) *Pseudomonas* sp. and *Burkholderia* sp. used as biocontrol agents against *F. oxysporum* f. sp. *cubense*. In addition, a significant interaction between the antifungal potential of *P. fluorescens* towards *R. solani* and its ability to produce β -1,3 glucanase, salicylic acid, and hydrogen cyanide acid was noted (Nagaraj kumar *et al.*, 2004). Among the most commonly tested elicitors, salicylic acid plays an important role in the expression of both local resistance, controlled by major genes, and ISR developed after an initial pathogen attack (Hammerschmidt and Smith-Becker, 2000).

ISR in plants can be associated with increase in activity of chitinase, β -1-3-glucanases, peroxidase and other pathogenesis related protein accumulation of phytoalexin, lignin, callose and hydroxyproline

rich glycoproteins. ISR also involves the induction of lytic enzymes, phenolics and phenylalanine ammonia-lyase (PAL) (Meena *et al.*, 2000).

Plant pathogens produce a large number of pectolytic and cellulolytic enzymes that degrade components of plant cell being the main virulence factor (Sutic and Sinclair, 2000). The interaction between the pathogen and host plant induces some changes in cell metabolism, primarily in the enzyme activities, including peroxidase and polyphenol oxidase (Salari *et al.*, 2012; Ngadze *et al.*, 2012).

Peroxidase, oxidizes phenolics to highly toxic quinones hence it has been assigned a role in disease resistance (Vidhyasekaran, 1988). PAL is the first enzyme of Phenyl propanoid metabolism in higher plants and has been suggested to play a significant role in regulating the accumulation of phenolics (Massala *et al.*, 1980).

PO and PPO mainly catalyze the oxidation of phenolic substances through a PPO-PO-H₂O₂ system, whose reaction products are highly reactive and toxic to pathogens, and are supposed to impart resistance to host (Tayal *et al.*, 1984). Enzymatic oxidation of phenols is usually associated with the disintegration of host cells and darkening of tissues indicating the significance of oxidative enzymes (Ranganathan and Vidhyasekharan, 1982).

Benhamou *et al.* (2000) reported that the endophytic bacterium *Serratia plymuthica* showed raised levels of phenolics in cucumber roots, affording protection against *Pythium ultimum*.

Biocontrol strains stimulate the activities of defense enzymes PO, PPO and PAL in plants that could be involved in the synthesis of phytoalexins (Chen *et al.*, 2000; Van Loon and Bakker, 2005).

Increased PO and PAL activity was reported by Ramamoorthy *et al.*, (2002) in tomato infected by *Fusarium oxysporum* and *Pythium* upon seed treatment with *P. fluorescence*. The enzyme PAL catalyses the

conversion of phenylalanine to trans-cinnamic acid intermediates in the synthesis of salicylic acid (Ryals *et al.*, 1996).

Similarly, Rajendran *et al.* (2006) reported that the activities of PO, PPO and PAL were found to be higher after two days of challenge inoculation after that it was declined in the cotton plants treated with endophytic *Bacillus* EPCO 16 and EPCO 102 upon challenge inoculation with *X. axonopodis* pv. *malvacearum*

Chithrashree *et al.* (2011) evaluated seven rhizospheric *Bacillus* spp. for growth promotion and induced systemic resistance in rice against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Seed treatment with suspension of strains SE34 and GB03 followed by challenge inoculations with *Xoo* resulted in the increased accumulation of phenylalanine ammonia-lyase, polyphenol oxidase and peroxidases compared to untreated control seedlings.

Nagendran *et al.* (2013) reported about the PO and PPO activity in the combination treatment of seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @500g/ha + foliar spray @ 500 g/ha with *B. subtilis* (FZB 24) on challenging with *Xoo* at 4 days after inoculation compared to untreated plants in control. The increased activity of PO and PPO was observed only upto the third day of *Xoo* inoculation in untreated control plants and afterwards drastic reduction.

Yasmin *et al.* (2016) observed increased activity of defense related enzymes including phenylalanine ammonia-lyase and polyphenol oxidase, and peroxidase in plants inoculated with *Pseudomonas* sp. Rh323 and P-solubilizing bacteria in response to *Xoo*.

Verma *et al.* (2016) conducted a study to determine in expression of peroxidases, polyphenol oxidase and cellulose activities against seed borne bacterial blight and leaf spot diseases in pea caused by *Pseudomonas syringae* pv. *lisi* and *Xanthomonas lisi* respectively. A

significant higher enzymatic activity of peroxidase, polyphenol oxidase and cellulase were found in infected seeds as compared to healthy seeds due to infection of bacterial pathogens.

Mishra *et al.* (2018) conducted experiments on defense mechanism adopted by *W. somnifera* under *A. alternata* stress. Significantly, the highest phenylalanine ammonia lyase (PAL) activity was observed in *B. amylo liquefaciens* and *P. fluorescens* treated plants (1.68- to 2.35-fold) after 24 hapi, and this activity was slightly reduced at 72 hapi (1.61- to 2.15-fold) compared to uninfected plants. The maximum PPO activity (2.03-fold) was observed to be higher at 72 hapi in the combination treated plants than in the infected control.

Rasul *et al.* (2019) observed increase in the activity of defense related enzymes namely phenylalanine ammonia lyase (PAL: 5.165 activity as μ mole cinnamic acid g⁻¹. f.wt.), peroxidase (POD: 2.4 activity g⁻¹. f.wt.) and polyphenol oxidase (PPO: 65 activity g⁻¹. f.wt.) in response to inoculation of P-solubilizing antagonistic bacteria to suppress rice bacterial leaf blight.

2.8 MOLECULAR CHARACTERIZATION OF ENDOPHYTES BY 16S rDNA:

16S Ribosomal RNA (16S rRNA) sequencing is widely and most popularly used in microbiological studies to identify the diversities in prokaryotic organisms as well as other. The advantages of using ribosomal RNA in molecular techniques are as ribosomes and ribosomal RNA are present in all cells and RNA genes are highly conserved in nature. So, 16S rRNA gene sequencing has been established as the “gold standard” for identification and taxonomic classification of bacterial species. Woese and Fox (1977) proposed the use of ribosomal RNA genes for molecular taxonomic research (Pace, 1997; Woese and Fox, 1977). Molecular characterization using 16S rRNA have been shown to be reliable and effective (Woese, 1987).

16S rRNA is a type of RNA that plays a major role in synthesis of protein. As the mechanism of protein synthesis doesn't vary much from one organism to another, the RNA that assists the protein production also doesn't vary much in evolution. The variations that occur are in predictable locations. It is observed that the nucleotide sequences of some portions of the 16S ribosomal deoxy ribonucleic acid (rDNA) are highly conserved. 16S rRNA identifies organisms by comparing certain locations on a 16S rRNA molecule with a database of previously identified organisms whose 16S rRNA mark is known. 16S rRNA is located in the major rRNA contains very well-conserved regions among biological species, which makes the comparison of 16S rRNA sequences possible in studies of molecular evolution. 16S rRNA sequences also enable the identification of microorganisms because the 16S rRNA contains variable sequences that change according to different species. More than one 16S rRNA sequences may exist in a single bacterium. It is considered to be fast and better alternative to other methods of bacterial identification. Along with its use identifying the bacteria, 16S sequencing can also be used to re-categorize the bacteria into new species (Kim and Chun, 2014).

Molecular biological methods using 16S ribosomal ribonucleic acid (rRNA) gene sequences are commonly used for identifying and classifying bacteria.

Chung *et al.* (2015) isolated 15 endophytic bacterial isolates from roots of rice plants, based on 16s rDNA sequencing these bacteria found to be closely related to *Paenibacillus jamilae*, *Bacillus methylotrophicus*, *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus simplex* and *Bacillus daliensis*. YC7007 and YC7010 which showed high antagonistic activity *in vitro* against rice pathogens represent a novel species of genus bacillus for which the name *Bacillus oryzicola* was proposed.

El-shakh *et al.* (2015) assessed the efficacy five endophytic strains (A1, A2, A3, A13 and A15) and 2 *Bacilli* (D29 and H8) against bacterial leaf blight of rice. Based on 16S rRNA gene sequence, the strains were assigned isolates A1, A3 and A13 as *Bacillus amyloliquefaciens* while isolates A2 and A15 as *Bacillus methylotrophicus* and *B. subtilis* respectively.

Banik *et al.* (2016) isolated 35 endophytic bacterial isolates from four rice genotypes, based on 16S rDNA sequencing divided them into five groups belonging to five classes- alpha (*Ancylobacter*, *Azorhizobium*, *Azospirillum*, *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Novosphingobium* spp.), beta (*Burkholderia* spp.), gamma (*Acinetobacter*, *Aeromonas*, *Azotobacter*, *Enterobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas* spp.) *Proteobacteria*, *Bacilli* (*Bacillus*, *Paenibacillus* spp.) and *Actinobacteria* (*Microbacterium* spp.).

Wilasinee *et al.* (2016) isolated 126 endophytic bacteria from rice roots and stems. Based on their partial 16S rRNA gene sequences, they were characterized as members of phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*. Three novel bacterial isolates characterized *Pedobacter*, *Sphingomonas* and *Paenibacillus*.

Yousefi *et al.* (2018) isolated 39 bacterial isolates among them, identified the four most antagonist bacterial isolates OS23, OS40, OS52 and OS53 belonged to *Bacillus* sp., *Bacillus subtilis*, *Enterobacter* sp. and *Pseudomonas putida* respectively through 16s rDNA sequencing.

Kavitha *et al.* (2020) isolated 45 endophytic bacterial isolates among which one isolate was found to be potential and identified as *Enterobacter asburiae* through 16S rRNA sequencing.

2.9 EVALUATION OF PLANT GROWTH PROMOTING CHARACTERS AND DISEASE REDUCTION IN RICE TREATED WITH POTENTIAL BACTERIAL ENDOPHYTE UNDER FIELD CONDITIONS.

Biological control of plant pathogens by antagonistic microorganisms is known to be a cheap, effective and eco-friendly method for the management of crop diseases (Cook and Baker, 1983). The use of biological control agents as an alternative to fungicides has been increasing rapidly in the present day agriculture due to the deleterious effects of chemical pesticides. Members of the genus *Pseudomonas* and *Trichoderma* have long been known for their potential to reduce the plant disease caused by fungal and bacterial pathogens (Pant and Mukhopadhyay, 2001). The bacterial antagonists have the twin advantage of faster multiplication and higher rhizosphere competence.

The use of biocontrol agents to bring about bacterial leaf blight (BLB) suppression remains to be explored in detail. However, use of antagonistic bacteria like *Bacillus* spp. for suppression of BLB of rice has been documented (Vasudevan, 2002).

Jeyalakshmi *et al.* (2010) found that the combination of seed treatment, soil application and foliar spray with *P. fluorescens* resulted in minimum disease incidence of bacterial leaf blight with maximum yield in comparison with the chemical treatment and control.

Hastuti *et al.* (2012) concluded that plants inoculated with AB131 endophytic *Streptomyces* spp. recorded significantly highest plant height and produced higher tiller number than control plants ($P < 0.05$) under greenhouse conditions.

Nagendran *et al.* (2013) treated rice plots with *Bacillus subtilis* var. *amyloliquefaciens* (FZB 24) through seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar application @

500g/ha strain registered a significantly lower intensity of bacterial leaf blight (2.80 %) due to expression of defense related enzymes like peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and phenols compared to untreated control plots (19.82 %).

Chung *et al.* (2015) isolated novel endophytic strain YC7007 from roots of rice and showed its suppression activity against bacterial leaf blight with disease reduction by 70.5 per cent and increased shoot and root length by 1.1-2.9 fold at seedling, tillering and booting stages with high germination rates and tillering capacity.

Yasmin *et al.* (2016) observed increased activity of defense related enzymes including phenylalanine ammonia-lyase, polyphenol oxidase, and peroxidase enzyme in plant inoculated with *Pseudomonas sp.* Rh323 which significantly improved plant health in terms of reduced diseased leaf area (80 %).

Patil *et al.* (2017) evaluated the efficacy of different commercially available antibacterial chemicals and bio-agents against the bacterial leaf blight of rice. Treatment streptomycin + copper oxychloride treated plots showed the lowest per cent disease incidence of (22.33 %) and highest grain yield (56.49 q ha⁻¹) the highest disease incidence was recorded in control (55.53 %). Lowest grain yield was obtained in *Bacillus subtilis* (41.33 q ha⁻¹).

Yasmin *et al.* (2017) conducted field experiment on efficacy of strain BRp3 against *Xoo*. The inoculation with the strain BRp3 showed 51 per cent grain yield, 55 per cent straw yield as compared to untreated control under field conditions.

Abdallah *et al.* (2019) assayed the antagonist activity of against *Xoo* under *in vivo* conditions. The results indicated that Sx3 showed 83.26 per cent of disease inhibition with increased shoot length, root

length, fresh weight and dry weight (44.76 %, 40.61 %, 62.93 % and 100 % respectively) as compared to the pathogen control.

Kavitha *et al.* (2020) reported that combination of seed treatment with EMP5 isolate @ 10^9 cells ml^{-1} + foliar application of EMP-5 isolate @ 10^9 cells ml^{-1} showed highest reduction in per cent disease index (4.92 %) with increased plant growth promoting ability in terms of increased plant height (80.8) cm increased no. of effective tillers (97.4 %), highest grain yield (5340 kg ha^{-1}) and straw yield (6013 kg ha^{-1}) over the untreated control.

Chapter – III

Material and Methods

Chapter – III

MATERIAL AND METHODS

This chapter includes all the materials used and methods adopted in the present study on “Studies on biochemical changes in paddy on inoculation with endophyte against Bacterial Leaf Blight (*Xanthomonas oryzae* pv. *oryzae*)”.

3.1 LOCATION OF WORK:

The laboratory experiments pertaining to the present research work were conducted during the year 2019-2020 in the Department of Plant Pathology, S.V. Agricultural College, Tirupati and Agricultural Research Station, Nellore.

3.2 MATERIALS

3.2.1 Glassware

Glassware made of borosil were used throughout the present investigation. These include petri plates (90 mm diameter), test tubes (15 ml, 27 ml), conical flasks (250 and 500 ml), beakers (100, 500 and 1000 ml) and measuring cylinders (10, 50, 100 ml).

3.2.1.1 Cleaning of Glassware

The glassware were first cleaned with detergent (labolin) followed by rinsing with tap water then placed them in cleaning solution for 24 h, rinsed with distilled water for 3-4 times and air dried. The cleaning solution contains 60 g of Potassium dichromate ($K_2Cr_2O_7$) and 60 ml of concentrated Sulphuric acid (H_2SO_4) dissolved in 1000 ml distilled water.

3.2.2 Chemicals:

Chemicals used in the present study were of Analytical Reagent (AR) and Guaranteed Reagent (GR) grades of standard mark. The pH of the media was adjusted by using either 0.1N HCl or 0.1N NaOH.

3.2.3 Equipments:

Hot air oven and autoclave were used for sterilization of glassware and culture media preparation respectively. BOD incubator was used for incubating cultures at different temperatures. Weighments were done on a single pan electronic balance with a sensitivity of 0.001g. The cultures were preserved at 4°C in refrigerator. Phase contrast microscope (Olympus cx 41, UK) was used for microscopic studies.

Other tools which were used in the present investigation for various purposes include inoculation needle, inoculation loop, cork borer, slides, cover slips, parafilm, scissors, sterile scalpel, pipettes (200 µl,1000 µl), aluminium foil, etc.

3.2.4 Sterilization:

Petri plates used for the present investigation were kept in sterilization tins and were sterilized in the hot air oven at 160 °C for 90 minutes. The surface of laminar air flow (LAF) was sterilized by wiping with cotton dipped in 70 per cent ethyl alcohol. Inoculation loop, L shaped glass rod, cork borer, forceps and scalpel were sterilized by dipping in alcohol and heating to red hot.

Pestle and motor were cleaned and wiped with ethyl alcohol and sterilized in an autoclave. The culture media and distilled water were sterilized in the autoclave at 121°C for 15 minutes.

3.3 CULTURE MEDIA USED:

3.3.1 Nutrient agar (NA):

Composition:

Ingredients	g/l
Peptone	5 g
Beef extract	3 g
Agar-agar	15 g
Distilled water	1000 ml
pH	6.9 - 7.2

Dissolve required quantities of peptone and beef extract in 500 ml of sterile distilled water in a beaker. 15 g of agar was melted separately in 500 ml of distilled water and mixed with nutrient broth. Final volume of the medium was adjusted to one litre by adding distilled water. The pH of the medium was adjusted by adding either 0.1N HCl or NaOH and the contents were dispensed in 250 ml conical flasks at the rate of 150 ml per flask. The flasks containing media were sterilized in an autoclave at 121°C for 15 min.

3.3.2 Nutrient Broth

Composition:

Ingredients	g/l
Peptone	5 g
Beef extract	3 g
Distilled water	1000 ml
pH	7.0

Five grams of peptone and three grams of beef extract were added to 800 ml of distilled water and then boiled. pH of the medium was adjusted to 7.0 by either adding 0.1N HCl or 0.1N NaOH and the volume was made up to 1000 ml with distilled water and the contents were dispensed in 250 ml conical flask at the rate of 150 ml per flask. The flasks were sterilized in an autoclave at 121°C for 15 min.

3.3.3 Tryptone Soya Agar:

Ingredients	g/l
Tryptone	15 g
Soya peptone	5 g
Sodium chloride	5 g
Agar	15 g
Distilled water	1000 ml
pH	7.3±0.2

Dissolve required quantities of Tryptone, soya peptone and Sodium chloride in 500 ml of water and boiled. Then 15 g of agar was melted separately in 500 ml distilled water and mixed with tryptone soya broth. Final volume of the medium was adjusted to one litre by adding distilled water. The contents were dispensed in 250 ml conical flasks at the rate of 150 ml per flask. The flasks containing media were sterilized in an autoclave at 121°C for 15 min.

3.3.4 Preparation of Nutrient Agar (NA) Slants:

Nutrient Agar slants were prepared by transferring 5ml of molten NA medium into test tubes. The tubes were plugged with non-absorbent cotton and sterilized in an autoclave at 121°C for 15 min. After sterilization, the tubes were removed from the autoclave in hot condition (i.e. approximately 40°C) and kept in slanting position to solidify. After solidification, the slants were kept in refrigerator for further use.

3.3.5 Preparation of disinfectant solutions:

3.3.5.1 Sodium hypochlorite solution (1%)

One per cent Sodium hypochlorite solution was prepared from 10 per cent stock solution by transferring 10 ml of Sodium hypochlorite from stock solution into 90 ml of distilled water. This solution was used for surface sterilization of test material to eradicate the surface borne saprophytic organisms.

3.4 LABORATORY TECHNIQUES

The general laboratory techniques described by Dhingra and Sinclair (1995), Rangaswami and Mahadevan (1999) and Aneja, (2003) were followed.

3.5 COLLECTION AND ISOLATION OF LEAF SAMPLES:

3.5.1 Collection of Rice Bacterial Leaf Blight Sample:

The samples were collected from major rice growing areas of Chittoor district of Andhra Pradesh during *kharif* 2019. Sample showing typical symptoms of bacterial leaf blight disease were collected for isolation of pathogen.

3.5.2 Collection of Healthy Leaf Sample for Isolation of Endophytic Bacteria

Leaves from healthy plants were collected and kept in ice box and were brought to the laboratory and used for further studies of isolation of endophytic bacteria.

Samples were documented in the following format:

S. No.	District	Mandal	Village	No. of Cfu's × 10⁶/g of leaf	Variety	GPS

3.5.3 Isolation of bacterial leaf blight Pathogen from diseased sample:

Infected samples were confirmed by observing bacterial ooze from cut ends by diffusion method. The infected leaves were excised with sterile scalpel across the yellow lesion and placed in a test tube filled with sterilized clean water. Yellowish bacterial mass can be seen oozing out from the cut ends of the leaf bits. After 30-40 min. the entire water in the test tube becomes yellowish and turbid.

X. oryzae pv. *oryzae*, the causal agent of bacterial leaf blight was isolated from the diseased plants of rice. The leaf surface was sterilized with one per cent Sodium hypochlorite for three minutes and then washed with sterile distilled water. Leaf bits of infected leaves after drying on sterile

blotting paper were transferred onto nutrient agar (NA) medium and incubated at 28°C for 72 h (Jabeen *et al.*, 2012). The emerging colonies which are round, convex, mucoid and yellow in colour were sub-cultured onto NA plates for pure culture. The culture was maintained on NA slant at 4°C in refrigerator for short term use and in 20 per cent glycerol at -20°C for long term preservation.

3.5.4 Pathogenicity test:

Artificial inoculation of the pathogenic bacterium was carried out to prove the pathogenicity using leaf clip inoculation technique under glass house condition.

Pathogenicity test was conducted on cultivar NLR-34449 with 48 h old bacterial culture. Prior to inoculation the plants were thoroughly washed with sterilized water and bacterial suspension was clip inoculated to 45 days old plants by excising 2-3 cm of rice leaves with a sterilized scissors which was immersed in the bacterial suspension (10^8 cells ml⁻¹) prepared in a Nutrient broth. Control plants inoculated with sterile broth were also maintained. Soon after inoculation the plants were covered with polythene bag for a period of 72 h for providing humid conditions. Plants were observed for symptoms after 72 h up to 14 days. The pathogen was re-isolated from artificially inoculated plants. Individual bacterial colonies which were found similar to the original culture were maintained to carry out further studies.

3.5.5 Isolation and Purification of Endophytic Bacteria:

For isolation of endophytes, 2g of leaves were collected from healthy plants. The disinfection and isolation was performed according to Araujo *et al.* (2002). Briefly, the leaves were disinfected superficially by following the protocol as 70 per cent ethanol for one min, Sodium hypochlorite (2.5%) for four min, ethanol for 30 sec and finally three rinses in sterile distilled water (SDW). To confirm the disinfection protocol, measured quantity of 0.1ml aliquots from the final rinse of sterile distilled water was plated in 10 per cent TSA (Tryptone Soya Agar) medium and the plates were examined for growth

after incubation at 28°C for 72 h. Samples were discarded, if growth was detected in the sterile check within 48 h.

Samples were triturated in 0.02M Phosphate buffer (pH 7.0) in sterile pestle and the mortar. The triturate was serially diluted in sterile distilled water. Six dilutions (10^{-1} to 10^{-6}) were made by transferring one ml of the suspension to successive sterile water columns. From last two series of the dilution, 0.1 ml was taken and plated on Tryptic Soya Agar (TSA) and incubated at 28°C for 1 to 12 days. The no. of colony forming units were counted according to the following formula.

$$\text{No. of Cfu/g} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{volume plated}}$$

3.6 IN VITRO EVALUATION OF ENDOPHYTIC BACTERIAL ISOLATES AGAINST *X. oryzae* pv. *oryzae*:

Cell suspension of *Xoo* was prepared in nutrient broth to a concentration of 10^8 Cfu/ml. One ml of the bacterial cell suspension (*Xoo*) was mixed with 19 ml of Nutrient Agar (NA) medium and poured onto the sterile petri plates. After solidification, four wells were formed with the help of 6 mm diameter cork borer at one cm away from either side of the petri dish and 10 μ l of each endophytic bacterial isolate was poured in the well. The inoculated plates were incubated at 28 ± 2 °C for 48 h after which the diameter of the inhibition zone was measured (Salah *et al.*, 2010). The level of *X. oryzae* pv. *oryzae* growth inhibition was determined by measuring the difference between the clear zone formed around the well and expressed in terms of millimeters (mm).

The Endophytic bacterial isolates which have recorded more than 10.0 mm zone of inhibition were used for further studies.

3.7 ESTIMATION OF DEFENSE ENZYMES IN PLANTS INOCULATED WITH EFFECTIVE ANTAGONISTIC ENDOPHYTIC BACTERIA AGAINST BLB PATHOGEN IN RICE.

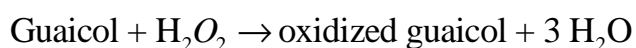
Effective antagonistic endophytic bacteria to suppress BLB was evaluated in pot culture experiments under greenhouse conditions. Rice seeds (cv. NLR-34449) were surface sterilized with two per cent Sodium hypochlorite for 30 sec, rinsed in sterile distilled water and dried overnight. The seeds were dipped in broth cultures of endophytic bacteria for 45 minutes and directly sown in pots to raise nursery. On 21st day of sowing, seedlings were dipped endophytic bacterial suspension. On 23rd day of sowing the leaves were inoculated with bacterial leaf blight pathogen by clip inoculation method (Kauffman *et al.*, 1973). Foliar spraying of endophyte suspension was done after 24 h of clip inoculation.

Enzyme assay was carried after 24h of foliar spray of endophytic bacteria on rice plants. 4-5 leaves were randomly harvested and cut into small pieces. The leaf bits were grounded into fine powder and processed immediately for an enzyme assay. The activity of peroxidase (PO), polyphenol oxidases (PPO) and phenylalanine ammonia lyase (PAL) was determined by spectrophotometric measurement.

3.7.1 Peroxidases

Peroxidases includes in its widest sense a group of specific enzymes such as NADP-peroxidase, fatty acid peroxidase etc. as well as a group of very non-specific enzymes from different sources which are simply known as PO. PO catalyses the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, hydroquinones etc.

Principle: Guaiacol is used as substrate for the assay of peroxidises



The oxidized dehydrogenated guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product is a measure of the PO activity and can be assayed spectrophotometrically at 436nm (Putter, 1974; Malik, 1980).

Chemicals used:

Monobasic sodium phosphate-(NaH_2PO_4)

Dibasic sodium phosphate-(Na_2HPO_4)

Guaicol solution

Hydrogen peroxide solution

Preparation of reagents:

Phosphate buffer, 0.1M (pH: 7.0)

Solution A: Monobasic sodium phosphate-(NaH_2PO_4)

Solution B: Dibasic sodium phosphate-(Na_2HPO_4)

- Add solution A of 39 ml to solution B of 61ml and make up to a total volume of 200 ml with distilled water
- 20 mM Guaicol solution
- Hydrogen peroxide solution (0.042%= 12.3mM): Dilute 0.14ml of H_2O_2 to 100ml of distilled water

Extraction

- Fresh sample of 1g was grinded with 3 ml of Sodium phosphate buffer (pH 7.0) in pre cold mortar using pestle at 4°C.
- Then the sample was centrifuged at 18,000 rpm for 15min at 5°C
- Collect the supernatant

Estimation

- Buffer solution of 3ml, 0.05 ml of Guaicol, 0.03 ml of Hydrogen peroxide and 1ml of enzyme extract were taken into a test tube and the resultant was mixed well.
- Preparation of solution without enzyme extract serves as a blank to calibrate the spectrophotometer.
- Then read absorbance (OD) at 436 nm by UV-VIS spectrophotometer using kinetics method. The enzyme activity was expressed as OD/min/g.

3.7.2 Phenylalanine ammonia lyase:

Chemicals used

Tris HCl

β -mercaptoethanol

Phenylalanine

Hydrochloric acid

Ethyl acetate

Extraction and Estimation:

Apical segments of rice leaves about 3 cm was harvested and washed thoroughly with deionized water. A 50 mg quantity of washed leaf tissue was then homogenized with a mortar and pestle under chilled conditions in 0.65 ml of 50mM Tris-HCl buffer (pH 8.8) containing 15mM of β -mercaptoethanol. The homogenate was centrifuged at 10,000 g for 20 min at 4°C, and the supernatant was retained as the enzyme extract. 0.1 ml volume of the enzyme extract along with 1ml of the extraction buffer, 0.5 ml of 10 mM Phenylalanine and 0.4 ml of deionized water, was incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.5 ml of 6M HCl and the product was extracted with 15 ml of ethyl acetate using a rotary evaporator. The solid residue was suspended in 3 ml of 0.05M NaOH and the Cinnamic-

acid concentration was measured spectrophotometrically for the absorbance at 290 nm. One unit of PAL activity was defined as 1 μ mol. of Cinnamic acid produced per minute (Ross and Sederoff, 1992).

3.7.3 Polyphenol oxidase:

Chemicals used

Monobasic sodium phosphate-(NaH_2PO_4)

Dibasic sodium phosphate- (Na_2HPO_4)

Catechol

Preparation of reagents:

Phosphate buffer, 0.1M

Solution A: Monobasic sodium phosphate - (NaH_2PO_4)

Solution B: Dibasic sodium phosphate - (Na_2HPO_4)

- Add solution A of 39 ml to solution B of 61ml and make up to a total volume of 200ml with distilled water.
- Dissolve 1.14 g of Catechol in 100 ml of water to give 0.1M Catechol solution.

Extraction:

The sample of 1 g was homogenized in 2ml of 0.1M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged at 20,000 rpm for 15min. The supernatant served as enzyme source (Mayer *et al.*, 1965).

Estimation:

Polyphenol oxidase activity was determined as given the reaction mixture consisted of 1.5 ml of 0.1M sodium phosphate buffer (pH 7.0) and 200 μ l of the enzyme extract. To start the reaction, 200 μ l of 0.1M Catechol was added and the activity was expressed as change in absorbance at 495 nm at 30sec intervals for 3 min. The enzyme activity was expressed as OD/min/g.

3.8 MOLECULAR CHARACTERIZATION OF POTENTIAL ENDOPHYTIC BACTERIA BY 16S rDNA:

3.8.1 Selection of Effective Antagonistic Endophytic Bacterial Isolates:

The effective antagonistic bacterial isolates having more than 10mm zone of inhibition against *X. oryzae* pv. *oryzae* were selected for molecular characterization. Effective antagonistic endophytic bacterial isolates were selected and grown on nutrient agar at 28±2°C for overnight for DNA extraction.

3.8.2 Isolation of DNA:

A loop full of 24 h growth of effective antagonistic endophytic isolates were transferred into eppendorf tubes which contain Nutrient broth. Cells were collected by centrifuge at 10,000 rpm for three min and supernatant was discarded to collect pellet and 200 µl of lysis buffer was added to the pellet and resuspended it. Then it was incubated at 37°C for 15 min. Proteinase K of 10 µl (10mg/ml) was added to it and vortexed it and added seven µl of 20 per cent Sodium dodecyl sulphate (SDS). Kept it in water bath at 60°C for 1 h. The tubes were inverted for every 15 min and then centrifuged at 10,000 rpm for 5 min. at 20°C. The supernatant was taken and discarded the pellet and added equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) to it. The tubes were inverted for 5 min and centrifuged at 10,000 rpm for 5 min. Supernatant was taken and added 0.1 volume of 3M Sodium acetate and two volumes of ice cold iso propanol. Then these tubes were incubated for 2 h to overnight for precipitation. Then centrifuged at 13,000 rpm for 15 min at 4°C. Pellets were washed with 70 per cent ethanol at 13,000 rpm for 10 min. Ethanol was discarded and tubes were air dried. Then dissolved in TE buffer (Maniatis *et al.*, 1982).

3.8.3 Qualitative and Quantitative Verification of DNA from Different Effective Antagonistic Endophytic Bacterial Isolates:

DNA samples (5 µl) from each isolate mixed with 4 µl of 1X loading dye and were loaded on the wells of the 1 per cent agarose gel along with 5 µl of DNA marker in order to verify the quality of DNA. Alternatively, the quality and quantity of DNA was also verified by Nanodrop spectrophotometer.

3.8.4 Amplification of 16S rDNA from Antagonistic Endophytic Bacterial Isolates.

The bacterial genomic DNA isolated from the individual endophytic bacterial culture was selected for amplification of their 16S ribosomal gene. The 16S rDNA from effective antagonistic endophytic bacterial isolates have been amplified by using 27F and 1492R primers.

27F- 5¹ AGAGTTTGATCACTGGCTCAG-3¹

1492R- 5¹TACGGACTTACCTTGTTACGACTT-3¹

As a part of this, PCR technique has been standardized and the following components were used for the amplification of 16S rDNA.

The PCR reaction mixture for 25 µl volume was as follows:

10X Reaction buffer	: 2.5 µl
Mgcl ₂	: 2.0 µl
dNTP's	: 0.5 µl
27F(10 µm)	: 1.0 µl
1492R(10µm)	: 1.0 µl
Taq polymerase	: 0.2 µl
Nuclease free water	: 14.8 µl
Template DNA	: 3 µl

The 16S rDNA amplification was carried under the following Conditions:

Stage-I: Initial denaturation at 94°C for 4 min.

Stage-II: Denaturation at 94°C for 30 sec

Annealing at 53.5°C for 40 sec and

Extension at 72°C for 1 min.

Number of cycles: 30

Stage-III: final extension at 72°C for 10 min.

The aliquots were checked for amplification of the expected size (approximately 1400 bp) on 0.8 percent agarose gel and visualised in Gel documentation system.

3.8.5 Buffers used for Gel Electrophoresis:

Composition of 10Xbuffer TBE buffer:

Tris base	:	54.0 g
Boric acid	:	27.5 g
EDTA	:	4.65 g
Distilled water	:	500 ml
pH	:	8.0

Preparation:

Each chemical was dissolved in separate beakers using distilled water and all were mixed finally. The pH was adjusted to 8.0 by using 0.1N HCl or NaOH and volume was made up to 500 ml and sterilized by autoclaving at 121°C for 15 min.

Composition of loading dye (10X):

Glycerol	:	5 ml
10 X TBE	:	1 ml
Bromo phenol blue(saturated)	:	1 ml
Xylene cyanol (10%)	:	1 ml
Double distilled water	:	10 ml

Preparation:

Contents were mixed well and divided into one ml aliquot, sterilized and stored at -20°C for further use.

3.8.6 Preparation of Gels:

Gel plates (13 x 14 cm) were washed thoroughly with cleaning solution followed by distilled water and dried. The two open sides of the plates were sealed with cellophane tape. Gel solution was prepared by mixing 0.8 g of agarose in 100 ml of 1X TBE buffer (0.8% gel) in a conical flask and boiled in an oven until a clear solution was obtained and 4 μ l of ethidium bromide (10mg μ l⁻¹) was added. The solution was poured on to the sealed plate, inserted the suitable comb and allowed to polymerize.

3.8.7 Loading and Running of Gels:

The inserted comb was gently removed from the gel after polymerization. The gel plate was placed in horizontal apparatus and fixed with 1X TBE buffer. The samples were loaded in the wells with help of micro pipettes. After loading, the electrophoretic unit was connected to power pack with a regulated electric power supply of 100V. At the end of run, the gel was carefully removed and analyzed.

The isolate which recorded highest zone of inhibition under *in vitro* and highest level of expression of defense related enzymes were selected for further studies.

3.9 EVALUATION OF POTENTIAL ENDOPHYTIC BACTERIAL ISOLATES AGAINST BACTERIAL LEAF BLIGHT IN RICE *IN VIVO*.

3.9.1 Field Studies

The experiment was conducted at Agricultural Research station, Nellore. Seeds were surface sterilized with two per cent Sodium hypochlorite for 30 sec, rinsed in sterile distilled water and dried overnight. For treatment-T₁ the seeds were soaked in potential endophytic bacterial suspension (EYK-3) which was grown 48 h in nutrient broth, for three h and air dried then proceeded to nursery sowings.

On the day of transplanting the untreated seedlings were uprooted and dipped in 48 h old potential endophytic bacterial suspension (EYK-3) for 3 to 4 h which serves as treatment-T₂. The treated seedlings were uprooted and dipped in EYK-3 bacterial suspension for 3 to 4 h which serves as T₃.

Pathogen of 48 h old culture (10^9 Cfu/ml) which was grown in nutrient broth was inoculated on 23 days after transplanting to leaves of rice plants through clip inoculation method (Kauffman *et al.*, 1973).

Foliar spraying of Streptomycin sulphate @ 100ppm + Copper Oxchloride 0.3 per cent at 35 days after transplanting (T₄). Foliar spraying of potential endophytic bacterial suspension (EYK-3) to rice plants at 35 days after transplanting (T₅) and also within 10 days interval i.e., at 45 days after transplanting (T₆) (Table 3.1)

Table 3.1. Details of the treatments for the field evaluation of the potential bacterial endophyte against BLB pathogen.

Treatment No.	Treatment details
T ₁	Seed treatment with potential endophytic bacteria EYK-3 @10 ⁹ Cfu /ml
T ₂	Seedling dip with potential endophytic bacteria EYK-3 @10 ⁹ Cfu /ml
T ₃	T ₁ +T ₂
T ₄	Foliar spraying of Streptomycin sulphate @ 100ppm + Copper Oxychloride 3g/litre.
T ₅	Foliar spray with potential endophytic bacteria EYK-3 @10 ⁹ Cfu /ml at 35 DAT
T ₆	Foliar spray with potential endophytic bacteria EYK-3 @10 ⁹ Cfu /ml at 45 DAT
T ₇	Control

3.9.2 Layout of the Field

The nursery was sown with cultivar NLR-34449 during *rabi* 2019-2020 on 16/11/2019 and transplanting was done on 16/12/2019. The layout was laid out in Randomized Block Design with seven treatments and three replications. Total field area was 315 m² with 21 plots each individual plot size was 5 x 3 m with 15 cm spacing from plant to plant and 15 cm spacing from row to row.

3.9.3 Mass Multiplication of Bacterial Leaf Blight Pathogen (*X. oryzae* pv. *oryzae*)

Bacterial leaf blight pathogen, bacterium was mass multiplied in nutrient broth. Loopful of bacterial culture was added to the medium and incubated in a rotary shaker for 48 hours at room temperatures (25±2°C) then used for clip inoculation.

3.9.4 Mass Multiplication of Potential Endophytic Bacteria.

Potential endophytic bacterium was mass multiplied in nutrient broth. Loopful of bacterial culture was added to the medium and incubated in a rotary shaker for 48 h at room temperature (25±2°C).

3.10 BACTERIAL LEAF BLIGHT DISEASE ASSESSMENT:

3.10.1 Per cent Disease Index (PDI):

Three replications were maintained for each treatment in RBD under glasshouse. The effectiveness of the treatments on the intensity of BLB was observed fifteen days after pathogen inoculation, with a 0-9 scale of the Standard Evaluation System (1923) for rice (IRRI) and the per cent disease index was calculated using the formulae of McKinney (1923)

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

3.11 PLANT GROWTH PROMOTING TRAITS:

3.11.1 Yield Parameters

1. Number of tillers per plant

The total number of tillers were counted at 75 DAS

2. Number of productive tillers

3. Grain yield

3.12 STATISTICAL ANALYSIS:

Complete Randomized Design (CRD) was used for laboratory studies, where as Randomized Block Design (RBD) for field studies. The data recorded in the experiments were subjected to statistical analysis using SPSS statistics version 20 software and online OP STAT software.

Chapter – IV

Results & Discussion

Chapter – IV

RESULTS AND DISCUSSION

The present investigation, “Studies on biochemical changes in paddy on inoculation with endophyte against Bacterial Leaf Blight (*Xanthomonas oryzae* pv. *oryzae*)” was carried out to evaluate the antagonistic efficacy and expression of defense related enzymes by the endophytic bacteria against Bacterial Leaf Blight (BLB) pathogen, *Xanthomonas oryzae* pv. *Oryzae* under *in vitro*. Further the potential isolate of the endophytic bacteria was assessed for the disease suppression and plant growth promotion characteristics against BLB under field conditions. The data was statistically analysed using OP STAT.

4.1 COLLECTION OF INFECTED SAMPLES AND ISOLATION OF BACTERIAL LEAF BLIGHT (BLB) PATHOGEN

4.1.1 Collection of the Bacterial Leaf Blight Sample

Leaf samples with water soaked to yellowish orange lesions along the leaf blade and possessing yellow bacterial ooze similar to dew droplets were collected in the early morning hours from wetland farm, S.V. Agricultural college, Tirupati (Plate 1a and 1b). The collected samples were brought to the laboratory and used for the isolation of the pathogen.

4.1.2 Isolation and Identification of *Xanthomonas oryzae* pv. *oryzae*

The collected samples were further confirmed by observing bacterial ooze from cut ends of the leaf blades by diffusion method as mentioned in Material and Methods 3.5.3.

The bacterial leaf blight pathogen *X. oryzae* pv. *oryzae* was isolated on Nutrient agar (NA) medium from the collected leaf samples. Infected leaf bits were inoculated and kept for incubation at 28±2°C for 72 h. Single well separated, pin head sized, light yellow, mucoid, round and raised colonies

with smooth margins were carefully picked up and further purified on NA medium (Plate 1c). In the gram staining, the isolate was stained as gram negative with rod shaped structures (Plate 2). The isolate was maintained on NA slant at 4°C in refrigerator for short term use and in 20 per cent glycerol at -20°C for long term preservation.

The results were in accordance with reports of Jabeen *et al.* (2012) who isolated *X. oryzae* pv. *oryzae* on Yeast dextrose carbonate agar (YDCA) medium with yellow, smooth, circular, convex and viscous bacterial colonies.

Shankara *et al.* (2016) isolated *X. oryzae* pv. *oryzae* by streak plate method on modified Wakimoto's medium with well separated, yellow, mucoid colonies of the bacterium while Shaheen *et al.* (2019) isolated *X. oryzae* pv. *oryzae* on Luria Bertani Agar (LBA) with yellow, convex, mucoid and shiny texture colonies.

The colonies of *X. oryzae* pv. *oryzae* isolated from the collected diseased leaf samples showed yellow, circular, smooth convex and viscous round bacterial colonies on NA medium which were similar to the characteristics presented by Jonit *et al.* (2016) and Kavitha *et al.* (2020).



a. BLB infected Paddy crop

b. Symptom of Rice BLB



Plate 1. Isolation and Purification of BLB Pathogen of Rice (*Xanthomonas oryzae* pv. *oryzae*).

4.1.3 Proving Pathogenicity of the Isolate

The BLB pathogen isolate was evaluated for its pathogenicity by clip inoculation method (Kaufmann *et al.* 1973) on susceptible cultivar NLR-34449. The clip inoculation was carried out on healthy leaves of 45 days old plants with pathogenic bacterial suspension (10^8 cells ml⁻¹). The inoculated leaves were observed for the initiation of lesions from 72 h after post inoculation and complete blighting of leaves after 15 days of inoculation.

The symptoms on the inoculated leaves initiated as small water soaked stripes from the clipped ends of the leaves and slowly expanded in length and breadth leading to yellowish lesions with wavy margins. Blighting of the leaf proceeded from clipped ends towards the base of the leaf followed by drying of the leaf margins with the progress of the disease (Plate 3).

Later, the pathogen was re-isolated from the infected portion of the leaf and obtained yellowish mucoid round colonies which were similar in morphological and cultural characteristics of the original isolate there by proving the Koch's postulates.

The results were in correlation with Akhtar *et al.* (2009) who studied on different methods of inoculation of BLB pathogen for pathogenicity *viz.*, clip inoculation, pin prick inoculation and brush methods. Among the three methods of inoculation, distinct lesions were obtained in clip inoculation method.

Similarly, Chung *et al.* (2015) carried out clipping bioassay for bacterial leaf blight pathogen using scissors dipped in *Xoo* suspensions.

The clip inoculation bioassay for bacterial leaf blight pathogen with scissors dipped in *Xo* suspension on 30-45 days seedlings resulted in the characteristic symptoms of small water soaked stripes at initial stages leading to yellowish lesions with wavy margins and later blighting of the leaf was

observed after 15 days post inoculation (Chithrashree *et al.*, 2011; Shankara *et al.*, 2016; Chandraprakash *et al.*, 2018).

4.2 ISOLATION AND PURIFICATION OF ENDOPHYTIC BACTERIA FROM COLLECTED HEALTHY LEAVES

4.2.1 Isolation of Bacterial Endophytes from Collected Healthy Leaf Samples

Leaf samples were collected from healthy rice plants for isolation of endophytic bacteria from four mandals of the Chittoor district during *kharif* 2019. From each mandal three villages were selected based on the cropping area and occurrence of the disease. List of the villages along with the GPS coordinates are shown in the Table 4.1 and Plate 4.

The disinfection and isolation procedures were performed according to Araujo *et al.* (2002) as mentioned in Material and Methods 3.5.5 (Plate 5).

The endophytic bacterial density in the healthy leaf samples collected from four mandals is presented in terms of Cfu/g of leaf (Table 4.1). The results revealed that the mean population density of the bacterial endophytes varied with the cultivar and stage of the crop at time of sample collection.

Among the samples collected from 12 villages spread across four mandals of the Chittoor district, highest mean population density of 5.5×10^6 Cfu/g was observed in the sample collected at maximum tillering stage in cultivar MTU-1010 from Isugathageli village of Yerpedu mandal. Subsequent highest density of 5.3×10^6 Cfu/g of leaf was observed in the sample collected at maximum tillering stage in RNR-15048 cultivar from Pudipatla village of Chandragiri mandal. The mean population density of the bacterial endophytes in the samples collected at panicle initiation stage and milky stage ranged between 1.3×10^6 /g to 4.1×10^6 /g of leaf which was lower than the bacterial density in the samples collected at maximum tillering stage (4.8×10^6 /g to 5.3×10^6 /g) of the crop irrespective of the cultivars.

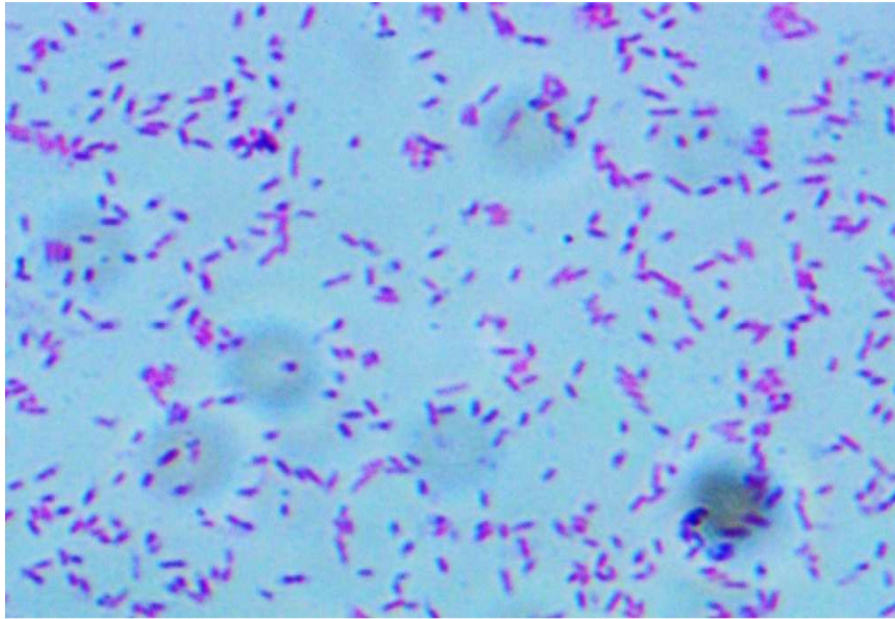


Plate 2. Microscopic view of *Xanthomonas oryzae* pv. *oryzae*.

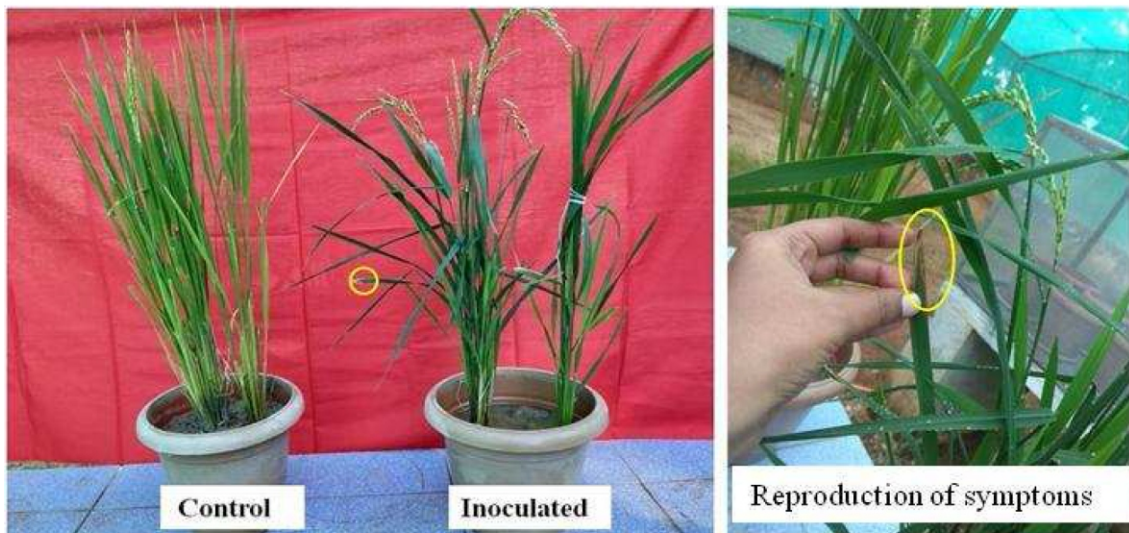


Plate 3. Pathogenicity test for *Xanthomonas oryzae* pv. *oryzae*.



Plate 4. Collection of healthy rice leaf samples from different mandals of Chittoor district for endophytic bacterial isolation.

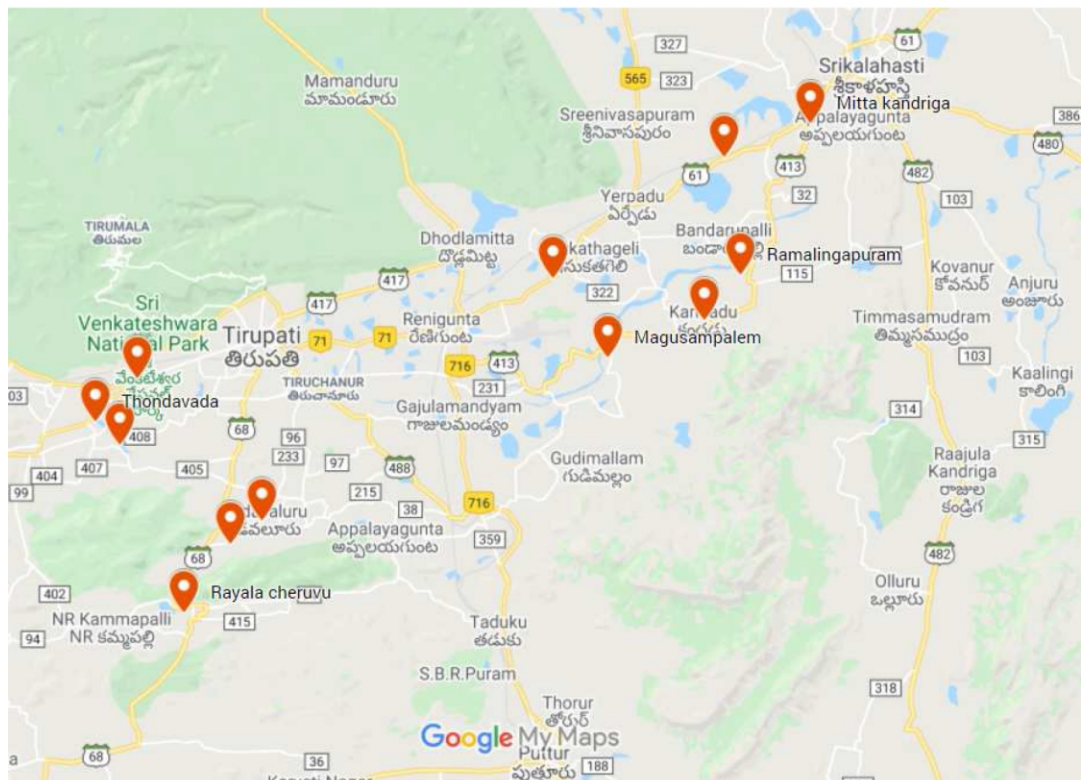


Plate 5. Location of villages of sample collected from Chittoor district.

Endophytic population density is continuously being evaluated in many of the plant species, which vary either with the genotype or weather conditions (Lamb *et al.*, 1996; Hallmann *et al.*, 1997; Chi *et al.*, 2005).

The abundance of the endospheric microbes (10^3 - 10^6 Cfu/g) is recorded in rice, cotton, corn but lower than the ectospheric microbes (10^6 - 10^8 Cfu/g). The diversity of the endophytic microbial density declines from roots towards the upper parts such as stem, leaves *etc.* (Lamb *et al.*, 1996) and there is a wide variation in the population density of endophytes (8.91×10^1 to 7.24×10^6 Cfu/g) between cultivated rice (sabita, swarna, swarna subi) and wild rice (*Oryza eichingeri*).

de Oliveria costa *et al.* (2012) reported that the density of endophytic populations isolated from the leaves of the common bean (*Phaseolus vulgaris*) varied from 4.5×10^2 to 2.8×10^3 Cfu/g of fresh weight.

In the present investigation also there is wide variation in the mean population density of the endophytic bacteria among the cultivars (MTU-1010; 2.3×10^6 to 5.5×10^6), (BPT-5204; 1.3×10^6 to 4.8×10^6) and (ADT-37; 2.9×10^6 to 3.8×10^6) and stage of the crop (1.3×10^6 to 5.5×10^6) at the time of sample collection. Within the cultivated rice there is wide variation in the endophytic population density from cultivar to cultivar (NLR-34449: 4.4×10^6 to 7.65×10^6 and MTU-1010: 2.4×10^6 to 7.2×10^6) as reported by Kavitha *et al.* (2019). The observations support the premise that endophyte diversity is highly depended on host, genotype, soil and environmental conditions (Pillay and Nowak, 1997; Tan *et al.*, 2003)

4.2.2 Purification of Endophytic Bacteria.

Morphologically distinct bacterial colonies which were differ in size, shape and colour were selected and repeatedly streaked over NA in order to achieve bacterial isolates.

The isolated endophytic bacteria were designed in such a way that the first letter denoting as endophyte, second letter representing mandal and third letter representing village of sample collection. Numbers were given serially.

The extract of healthy leaf samples from the Chandragiri mandal on plating resulted in the mean bacterial density varying from 2.9 to 5.3×10^6 /g of leaf. Pudipatla village samples recorded highest mean bacterial density of 5.3×10^6 /g of leaf followed by samples of Shanambatla with mean bacterial density of 3.8×10^6 /g of leaf.

A total of seven isolates were selected and purified from the bacterial colonies obtained on NA medium from the leaf samples collected in Pudipatla, Thondavada and Shanambatla villages of Chandragiri mandal. seven isolates, two isolates were isolated from the samples of Pudipatla village (ECP-1 and ECP-2) from cultivar RNR-15048 at maximum tillering stage. Three isolates were isolated from samples of Thondavada village from ADT-37 cultivar at milky stage (ECT-3, ECT-4 and ECT-5) and two isolates (ECS-6 and ECS-7) from samples of Shanambatla village from cultivar ADT-37 at milky stage (Table 4.1).

Similarly, from the samples of the Srikalahasthi mandal, the mean bacterial density varied from 3.3×10^6 /g to 4.6×10^6 /g. Among the samples, Magusampalem village sample recorded highest mean bacterial density of 4.1×10^6 /g of leaf followed by isolates of Ramalingapuram with a mean bacterial density of 3.6×10^6 /g of leaf.

Similarly, seven isolates were selected and purified from the bacterial colonies obtained on NA medium from the extract of healthy leaf samples collected in Magusampalem, Ramalingapuram and Mitta kandriga of Srikalahasthi mandal. Among them, two isolates were isolated from the samples of Magusampalem village (ESM-1 and ESM-2) from cultivar MTU 1010 at milky stage. Three isolates were isolated from samples of Ramalingapuram village from MTU-1010 cultivar at milky stage (ESR-3,

ESR-4 and ESR-5) and two isolates (ESM-6 and ESM-7) from samples of Mitta kandriga village from cultivar MTU-1010 at milky stage (Table 4.1).

The extract of the healthy leaf samples from the Yerpedu mandal, the mean bacterial density resulted in varying from $1.3 \times 10^6/g$ to $5.5 \times 10^6/g$. In the samples, Isukathageli village recorded highest mean a bacterial density of $5.5 \times 10^6 /g$ of leaf followed by samples of Kapuguneri with bacterial density of $3.6 \times 10^6 /g$ of leaf.

A total of eight isolates were selected and purified from the bacterial colonies obtained on NA medium from the leaf samples collected in Kandadu, Isukathageli and Kapuguneri villages of Yerpedu mandal. Among them three isolates were isolated from the samples of Kandadu village (EYK-1, EYK-2 and EYK-3) from cultivar MTU-1010 at panicle initiation stage. Two isolates were isolated from samples of Isukathageli village from MTU-1010 cultivar at maximum tillering stage (EYI-4 and EYI-5) and three isolates (EYK-6, EYK-7 and EYK-8) from samples of Kapuguneri village from cultivar BPT-5204 at milky stage (Table 4.1).

Similarly, the extract of the leaf samples from Ramachandra puram mandal, the mean bacterial density resulted in varying from $1.3 \times 10^6/g$ to $4.8 \times 10^6/g$. Among the samples, Nadavaluru village recorded highest mean bacterial density of $4.8 \times 10^6 /g$ of leaf followed by samples of Rayala cheruvu with bacterial density of $1.8 \times 10^6/g$ of leaf.

Seven isolates were selected and purified from the bacterial colonies obtained on NA medium from the leaf samples collected in Kamma kandriga, Nadavaluru and Rayala cheruvu villages of Ramachandra puram mandal. Among them, two isolates were isolated from the samples of Kamma kandriga village at milky stage of BPT-5204 cultivar (ERK-1 and ERK-2). ERN-3 and ERN-4 were isolated from Nadavaluru village at maximum tillering stage of BPT 5204. Isolates ERR-5, ERR-6 and ERR-7 and were isolated from BPT 5204 of Rayala cheruvu village at panicle initiation stage.

A total of 29 endophytic bacterial isolates obtained from 12 healthy leaf samples collected from the cultivars *viz.*, ADT-37, MTU-1010, BPT-5204 and RNR-15048 which are popularly cultivated in Chittoor district (Plate 6).

There is an increased interest in the isolation of endophytes from the associated crop plants of as they colonize plant tissue same as that of plant pathogens thereby acting as biocontrol agents. Keeping in view of the diversity of the plant species and significance of their functionalities, several workers have isolated endophytes associated with the crop plants for the management of plant diseases. Similar reports were obtained by Nagendran *et al.* (2013) who isolated 26 endophytic bacterial isolates from different field crop plants *viz.*, rice, greengram, cotton, redgram and ragi, *etc.* on Trypton soya agar (TSA) medium while Chung *et al.* (2015) isolated 250 endophytic bacterial strains from the rice roots on TSA medium. El-shak *et al.* (2015) isolated five endophytic strains from rice seeds of two cultivars *viz.*, Katy and MH86 on Luria Bertani agar (LBA) medium using dilution plate technique. Yousefi *et al.* (2018) screened eight cultivars of rice and isolated sixty-three endophytic bacteria from different parts of the plants including leaves and roots. Similarly, 45 endophytic bacterial isolates were isolated from healthy leaf samples on TSA medium from three cultivars of rice *viz.*, MTU-1010, NLR-34449 and BPT-5204 grown in Chittoor and Nellore districts (Kavitha *et al.*, 2020).

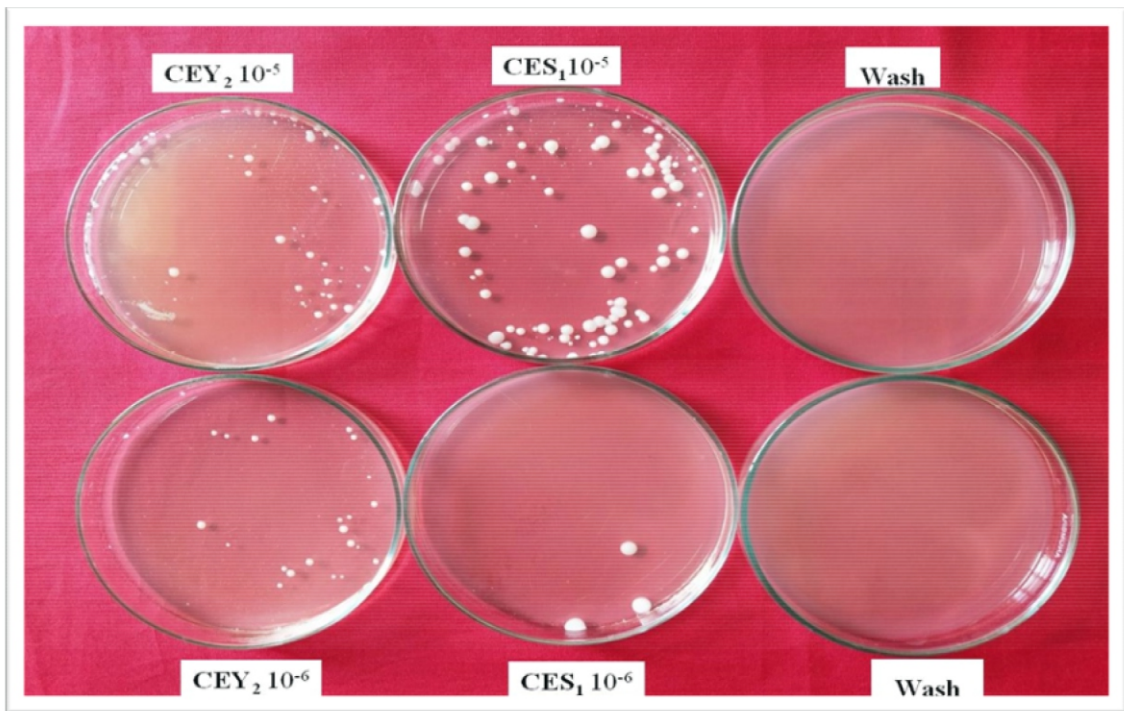


Plate 6. Isolation of endophytic bacteria from paddy leaves by serial dilution method.

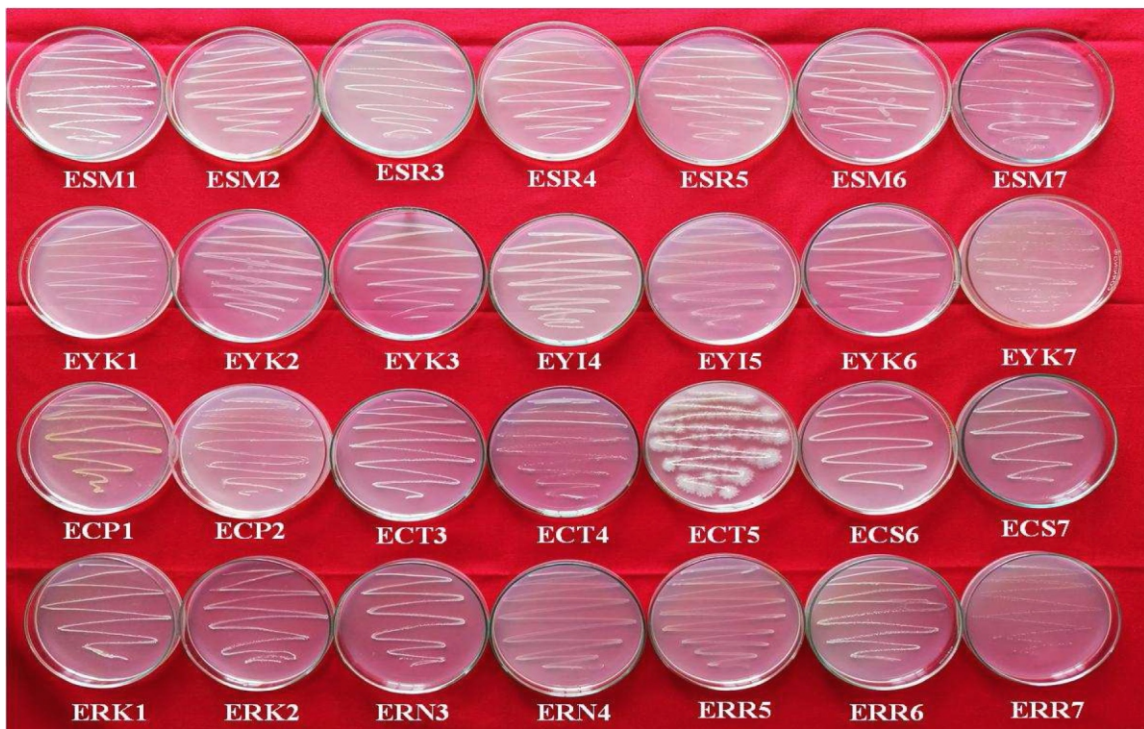


Plate 7. Pure cultures of endophytic bacterial isolates.

Table 4.1. List of endophytic bacterial isolates collected from four mandals of Chittoor District.

S. No	Mandal	Village	GPS Coordinates	Variety	Stage of the crop	*Population density (10 ⁶ /g of leaf)	No. of isolates selected
1	Chandragiri	Pudipatta	N 13°36'29.08528" E 79°21'34.25508"	RNR-15048	Maximum tillering	5.3 ^{ab}	ECP-1, ECP-2,
		Thondavada	N 13°35'15.58896" E 79°19'44.22684"	ADT-37	Milky stage	2.9 ^{ef}	ECT-3, ECT4, ECT5,
		Shanambatta	N 13°35'43.73952" E 79°20'30.49116"	ADT-37	Milky stage	3.8 ^{cd}	ECS6, ECS7,
2	Srikalahasti	Magusampalem	N 13°41'53.20716" E 79°39'28.39608"	MTU-1010	Milky stage	4.1 ^c	ESM-1, ESM-2
		Ramalingapuram	N 13°40'0.68162" E 79°39'1.65348"	MTU-1010	Milky stage	3.6 ^{cd}	ESR-3, ESR-4, ESR5
		Mitta kandriga	N 13°43'50.07324" E 79°40'36.25932"	MTU-1010	Milky stage	3.3 ^{de}	ESM-6, ESM-7
3	Yerpedu	Kandadu	N 13°38'8.19024" E 79°37'17.9274"	MTU-1010	Panicle initiation	2.3 ^{fe}	EYK1, EYK2, EYK-3
		Isukathageli	N 13°42'0.39924" E 79°39'41.80644"	MTU-1010	Maximum tillering	5.5 ^a	EY14, EY15
		Kapuguneri	N 13°41'51.24984" E 79°38'59.49492"	BPT-5204	Panicle initiation	3.6 ^{cd}	EYK6, EYK7, EY8
4	Ramachandra puram	Kamma kandriga	N 13°32'15.02592" E 79°24'14.24448"	BPT-5204	Milky stage	1.3 ^h	ERK1, ERK2
		Nadavaluru	N 13°32'48.38676" E 79°25'14.96648"	BPT-5204	Maximum tillering	4.8 ^b	ERN3, ERN4
		Rayala cheruvu	N 13°30'22.32036" E 79°22'53.94828"	BPT-5204	Panicle initiation	1.9 ^{gh}	ERR5, ERR6, ERR7
					C.D	0.71	
					C.V %	11.94	

*Mean of three replications

**Means in a column followed by same super script letters are not significantly different according to DMRT.

4.3 *IN VITRO* EVALUATION OF ENDOPHYTIC BACTERIA AGAINST *X. oryzae pv. oryzae*

The aim of the investigation is to evaluate the antagonistic efficacy of the endophytic bacterial isolates, isolated from 12 villages across four mandals of the Chittoor district against *X. oryzae pv. oryzae*. Antagonistic efficacy was assayed under *invitro* by using Agar well diffusion method as mentioned in Material and Methods 3.6.

Twenty-nine endophytic bacterial isolates were screened against *X. oryzae pv. oryzae* under *in vitro*. Among them, 26 isolates showed antagonistic efficacy while three isolates *viz.*, ESM-6, EYI-4 and ERN-4 showed zero inhibition in the screening. The inhibition diameter among the 29 isolates varied from 0.0 to 14.5 mm. Highest inhibition was recorded in the isolate EYK-3 (14.5 mm) where as other endophytic bacterial isolates ECP-1, ESR-5 and ECT-3 recorded inhibition zones of 12.3, 11.43 and 10.53 mm respectively (Table 4.2, Plate 7).

Similar results were obtained by Nagendran *et al.* (2013) who tested 40 endophytic bacterial isolates for their antagonistic efficacy against *Xoo*. Among all isolates, isolates FZB 24, EPB 10, EPB 9, EPCO 78 and EPCO 29 showed maximum diameter of inhibition zone of 20.0 mm. Van Hop *et al.* (2014) screened 167 isolates against *Xoo* races using agar-piece method and found 17 strains were found to inhibit all 10 *Xoo* races.

El-shak *et al.* (2015) assessed the antagonistic efficacy of five endophytic strains (A1, A2, A3, A13, and A15) and two rhizospheric *Bacilli* (D29 and H8) against BLB *invitro* and *invivo*. All the strains showed high antagonistic efficacy against *X. oryzae pv. oryzae* with inhibition diameter of 28.5, 26, 13.33, 22.0, 17.33, 24.0 and 26.0 mm respectively

Yousefi *et al.* (2018) screened 39 endophytic bacterial isolates against *Xoo* strains and among them 21 isolates exhibited antagonistic activity against *Xoo*. Isolate OS59 recorded highest inhibitory activity with zone of 32.67mm.

OS58, OS43, OS20 and OS40 produced inhibition zones of 32.33 mm, 26.65 mm, 18 mm and 16.7mm, respectively. The antibacterial activity of *P. polymyxa* Sx3 against 20 strains of *Xoo* by agar diffusion technique showed inhibition diameter ranging from 0.33 cm to 0.57 cm after 24 h of incubation (Abdallah *et al.*, 2019).

Kavitha *et al.* (2020) screened 45 bacterial isolates against BLB pathogen with zone of inhibition ranging from 0.0 to 16.8 mm. Among the 45 isolates, isolate EMP-5 and EBK-3 showed highest antagonistic activity with inhibition zone of 16.8 mm and 16.6 mm respectively.

The isolates which recorded inhibition zone of more than 10.0 mm *viz.*, EYK-3, ECP-1, ESR5 and ECT-3 were consequently subjected to gram staining and found that EYK-3, ECP-1 as gram negative while ESR-5 and ECT-3 as gram positive. In shape EYK-3, ESR5 and ECT-3 appeared as short rods while ECP-1 as spirillum (Plate 8).

Further the four endophytic isolates with inhibition zone of more than 10.0 mm *viz.*, EYK-3, ECP-1, ESR-5 and ECT-3 were tested for the production of defense related enzymes through artificial inoculation on rice plants to identify the potential endophyte for field studies.

Table 4.2. Bio-efficacy of endophytic bacterial isolates against BLB pathogen (*X. oryzae* pv. *oryzae*) under *in vitro*.

S. No.	Name of the isolate	Inhibition zone of antagonistic activity (mm)
1	ESM-1	7.76 ^f
2	ESM-2	4.63 ^{jk}
3	ESR-3	7.46 ^f
4	ESR-4	4.43 ^k
5	ESR-5	11.43^c
6	ESM-6	0.00 ⁿ
7	ESM-7	6.33 ^g
8	EYK-1	4.30 ^k
9	EYK-2	5.60 ^h
10	EYK-3	14.50^a
11	EYI-4	0.00 ⁿ
12	EYI-5	6.20 ^g
13	EYK-6	4.40 ^k
14	EYK-7	2.06 ^m
15	ECP-1	12.30^b
16	ECP-2	5.33 ^{hi}
17	ECT-3	10.53^d
18	ECT-4	3.26 ^l
19	ECT-5	8.33 ^e
20	ECS-6	7.26 ^h
21	ECS-7	3.30 ^l
22	ERK-1	5.03 ^{ij}
23	ERK-2	4.20 ^k
24	ERN-3	2.06 ^m
25	ERN-4	0.00 ⁿ
26	ERR-5	5.30 ^{hi}
27	ERR-6	4.20 ^k
28	ERR-7	2.10 ^m
29	ERR-8	6.16 ^g
30	Control	0.00 ⁿ
C.D.		0.84
C.V%		9.68

*Mean of three replications

**Means in a column followed by same super script letters are not significantly different according to DMRT

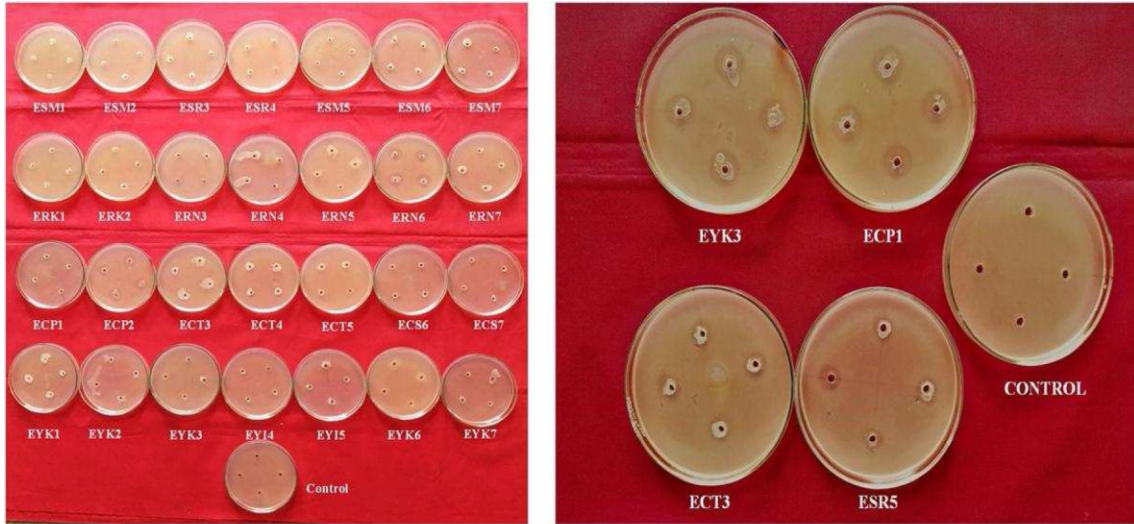


Plate 8. Bio-efficacy of endophytic bacterial isolates against BLB pathogen (*X. oryzae* pv. *oryzae*) by agar well diffusion method.

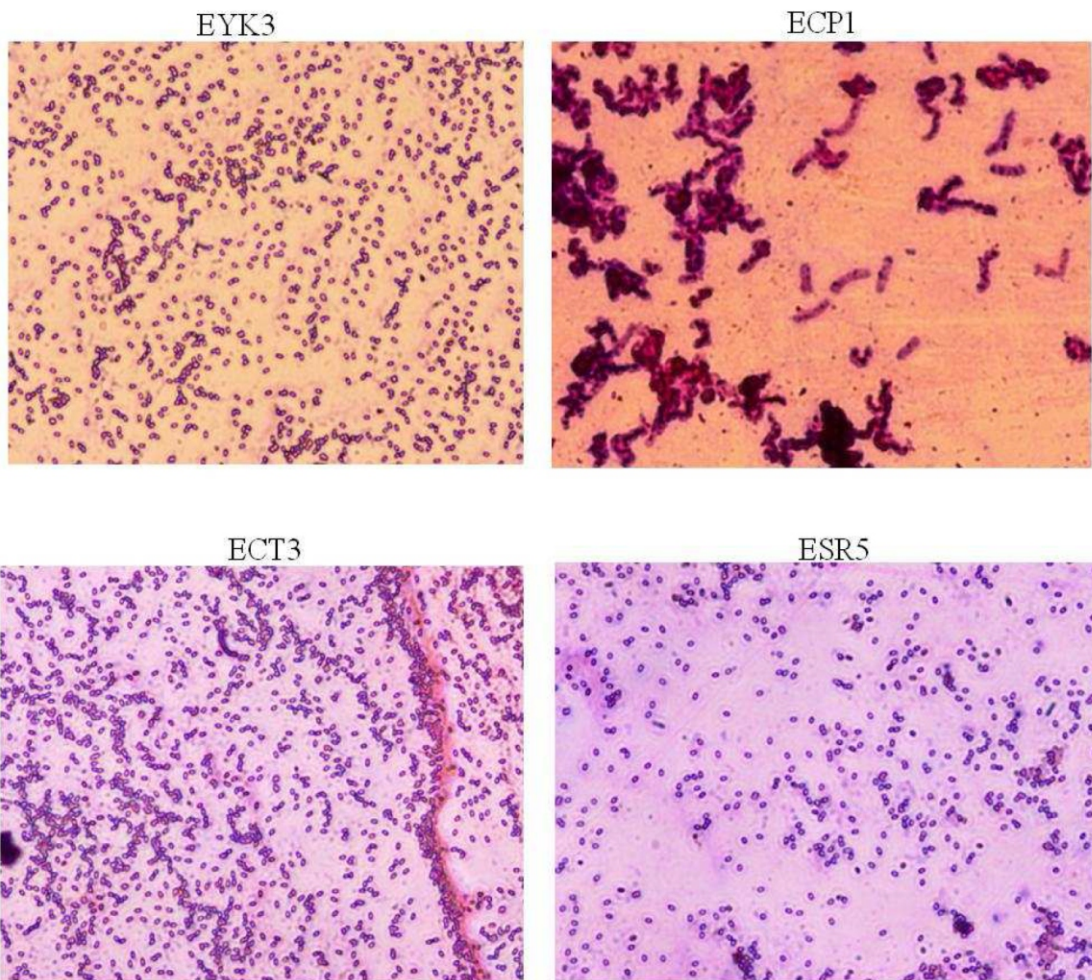


Plate 9. Microscopic view of effective endophytic bacterial isolates after gram staining.

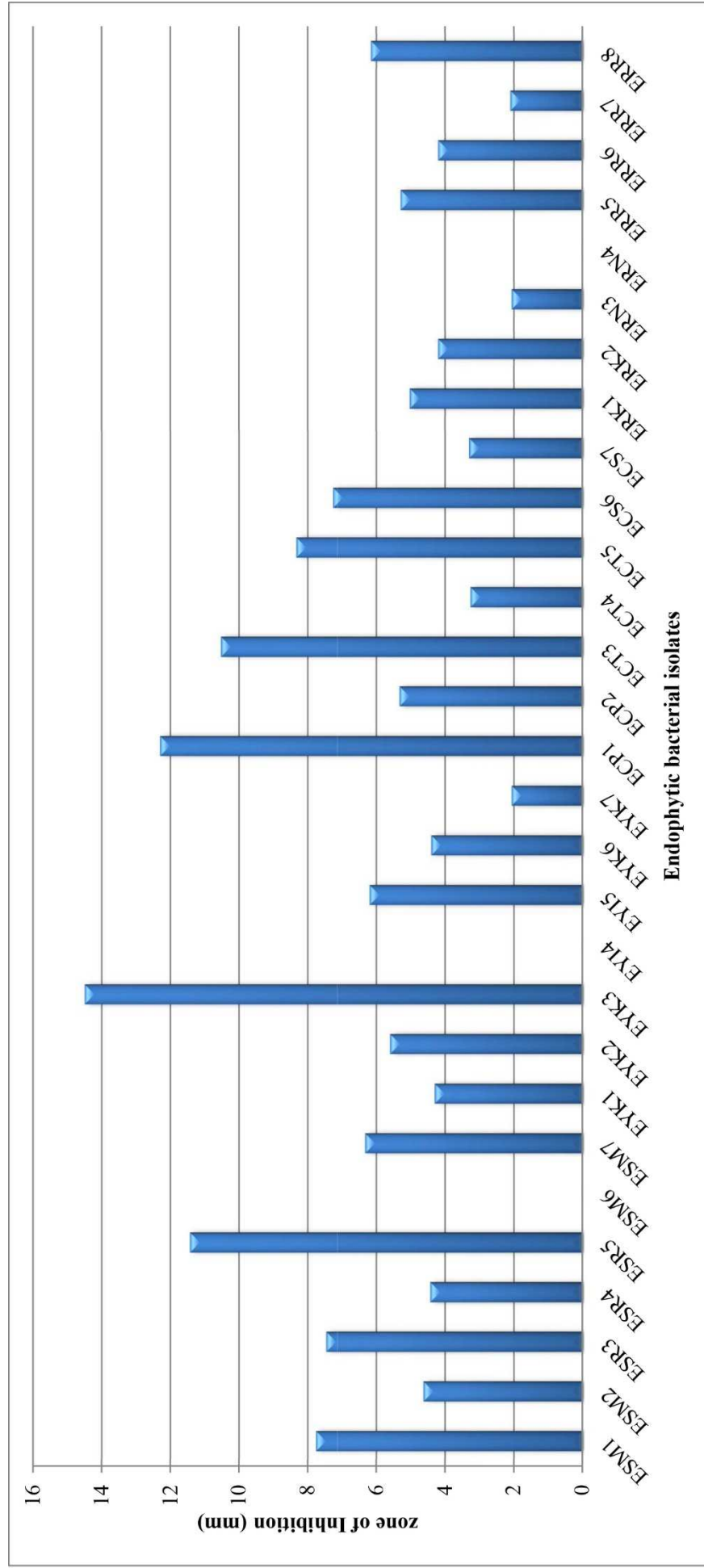


Fig. 4.1. Bio-efficacy of endophytic bacterial isolates against BLB pathogen (*X. oryzae* pv. *oryzae*) under *in vitro*.

4.4 ESTIMATION OF DEFENSE RELATED ENZYMES INOCULATED WITH EFFECTIVE ANTAGONISTIC ENDOPHYTIC BACTERIAL ISOLATES AGAINST BLB PATHOGEN IN RICE.

The experiment was conducted under greenhouse conditions. The procedure of disinfection of seeds, sowing, transplanting and inoculation of each of antagonistic endophytic bacterial isolates and *Xoo* was done as described in 3.7 (Plate 9). The leaves were collected and used for assessment of activity of different defense related enzymes.

The activity of Peroxidase (PO), Polyphenol oxidase (PPO) and Phenylalanine ammonia lyase (PAL) were expressed in terms of $\Delta A_{470}/\text{min/g}$, $\Delta A_{280}/\text{min/g}$ and $\mu\text{mol cinnamic acid}/\text{min/g}$ respectively.

The activity of peroxidase started to increase from 24 h post pathogen challenging and reached maximum after 72 h in all the rice plants inoculated with each of the effective antagonistic endophytic bacterial isolates *viz.*, EYK-3, ECP-1, ECT-3 and ESR-5 individually. The activity of peroxidase was maximum at 72 h after challenging and thereafter declined from 96 h onwards while in control the meagre increase in PO activity declined after 72 h post inoculation. Among the four effective antagonistic endophytic bacterial isolates, EYK-3 showed highest peroxidase activity at 72 h (Table 4.3 and Fig. 4.2).

Similarly, the activity of polyphenol oxidase started to increase from 24 h post pathogen challenging and reached peak after 72 h in all rice plants inoculated with each of the effective antagonistic bacterial endophytes *viz.*, EYK-3, ECP-1, ECT-3 and ESR-5 individually while in control there was increase in the activity upto 48 h and thereafter declined from 72 h. Polyphenol oxidase activity was maximum 72 h after challenging and thereafter declined from 96 h onwards. EYK-3 showed highest polyphenol oxidase activity at 72 h post inoculation (Table 4.3 and Fig. 4.3).

The activity of phenylalanine ammonia lyase (PAL) increased with pathogen challenging from 24 h in plants inoculated with EYK-3, ECP-1 isolates and reached peak after 72 h while in ECT-3, ESR-5 and in control the peak activity was observed upto 48 h and which declined from 72 h onwards. Among the four effective antagonistic bacterial endophytes, EYK-3 recorded highest PAL activity (10.48 μmol cinnamic acid/min/g leaves) (Table 4.3 and Fig. 4.4).

The results clearly demonstrated the increased synthesis of defense enzymes like PO, PPO and PAL in plants inoculated with effective antagonistic endophytic bacterial isolates *viz.*, EYK-3, ECP-1, ECT-3 and ESR-5 after challenge inoculation with *Xoo* pathogen. The increased defense related enzyme activities in the endophytic bacteria treated rice plants play either a direct or an indirect role in the suppression of pathogen growth.

The accumulation of plant defense related enzymes (PO, PPO and PAL) in plant tissues is related with the plant defense response and induced resistance by endophytic bacteria (Liang *et al.*, 2011) and it is well established fact that the appropriate stimuli or signals are needed to induce defense genes (Saikia *et al.*, 2016).

Increased PO and PAL activity was reported by Ramamoorthy *et al.*, (2002) in tomato infected by *Fusarium oxysporum* and *Pythium* upon seed treatment with *P. fluorescence*. The enzyme PAL catalyzes the conversion of phenylalanine to trans-cinnamic acid intermediates in the synthesis of salicylic acid (Ryals *et al.*, 1996).

The results were also in correlation with the studies of Chithrashree *et al.* (2011) who treated rice seed with suspension of rhizobacterial strains SE34 and GB03 followed by challenge inoculations with *Xoo* increased accumulation of phenylalanine ammonia-lyase, polyphenol oxidase and peroxidases compared to untreated control seedlings.

Nagendran *et al.* (2013) reported about the PO and PPO activity in the combination treatment of seed treatment @ 4g/kg + of seedling dip @ 4g/l +soil application @500g/ha + foliar spray @ 500 g/ha with *B. Subtilis* (FZB 24) on challenging with *Xoo* at 4 days after inoculation compared to untreated plants in control. The increased activity of PO and PPO was observed only upto the third day of *Xoo* inoculation in untreated control plants and afterwards drastic reduction. Similar trend was observed in our results.

Similarly, increased activity of defense related enzymes including Phenylalanine ammonia-lyase and Polyphenol oxidase, and Peroxidase was observed in plant inoculated with *Pseudomonas sp.* Rh323 and P-solubilizing bacteria in response to *Xoo*. (Yasmin *et al.*,2016; Rasul *et al.*,2019).

Among the four effective antagonistic endophytic bacteria, EYK-3 endophytic bacterial isolate showed highest level of expression of defense related enzymes along with antagonistic efficacy.



Seedling dip with effective antagonistic bacteria



clip inoculation with *Xoo*

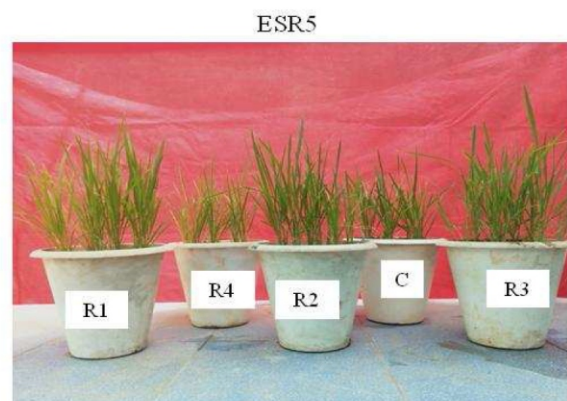
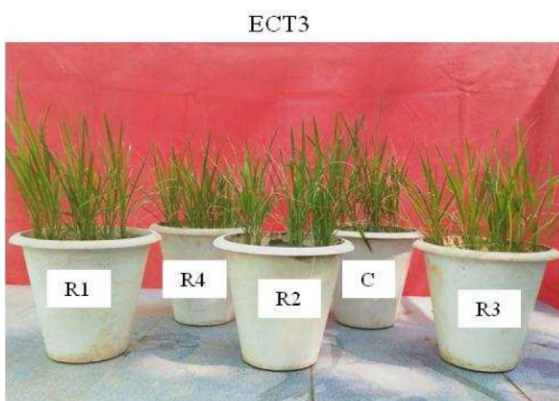
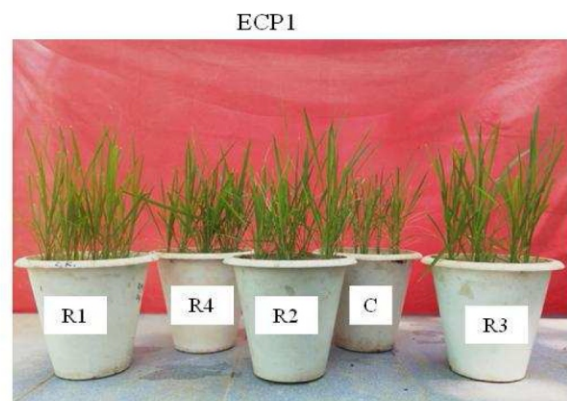
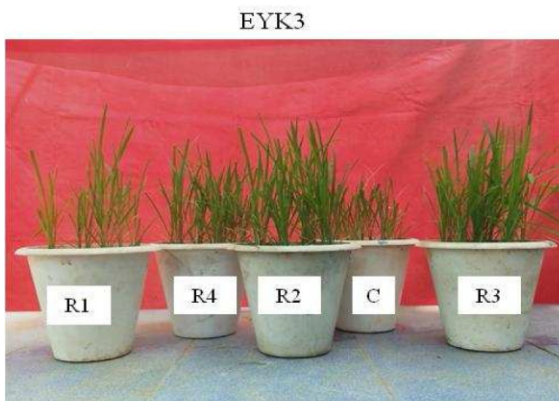


Plate 10. Estimation of defense related enzymes in rice plants inoculated with effective antagonistic endophytic bacteria

Table 4.3. Expression levels of Peroxidase, Polyphenol oxidase and Phenylalanine Ammonia Lyase activity in rice plants treated with effective antagonistic endophytic bacteria and challenged with *X. oryzae* pv. *Oryzae*

Endophytic bacterial isolates	Peroxidase activity (PO) (OD/min/g)					Polyphenol oxidase activity (PPO) (OD/min/g)					Phenylalanine ammonia lyase activity (PAL) (μmol cinnamic acid/min/g)				
	Hours after inoculation					Hours after inoculation					Hours after inoculation				
	24 h	48 h	72 h	96 h		24 h	48 h	72 h	96 h		24 h	48 h	72 h	96 h	
EYK-3	0.71	1.33	1.51	0.58		2.15	3.52	5.11	3.18		5.30	8.56	10.48	7.27	
ECP-1	0.66	0.83	0.91	0.50		1.56	2.43	3.08	2.36		4.68	5.48	7.35	4.46	
ECT-3	0.41	0.51	0.61	0.40		1.15	1.97	2.26	1.00		3.85	4.26	3.76	3.37	
ESR5	0.50	0.59	0.61	0.30		1.23	1.26	2.48	1.58		4.51	5.85	4.83	5.18	
Control	0.20	0.30	0.31	0.12		0.9	1.27	0.92	0.86		1.68	2.20	1.55	1.47	
C.D	0.032					0.056					0.067				

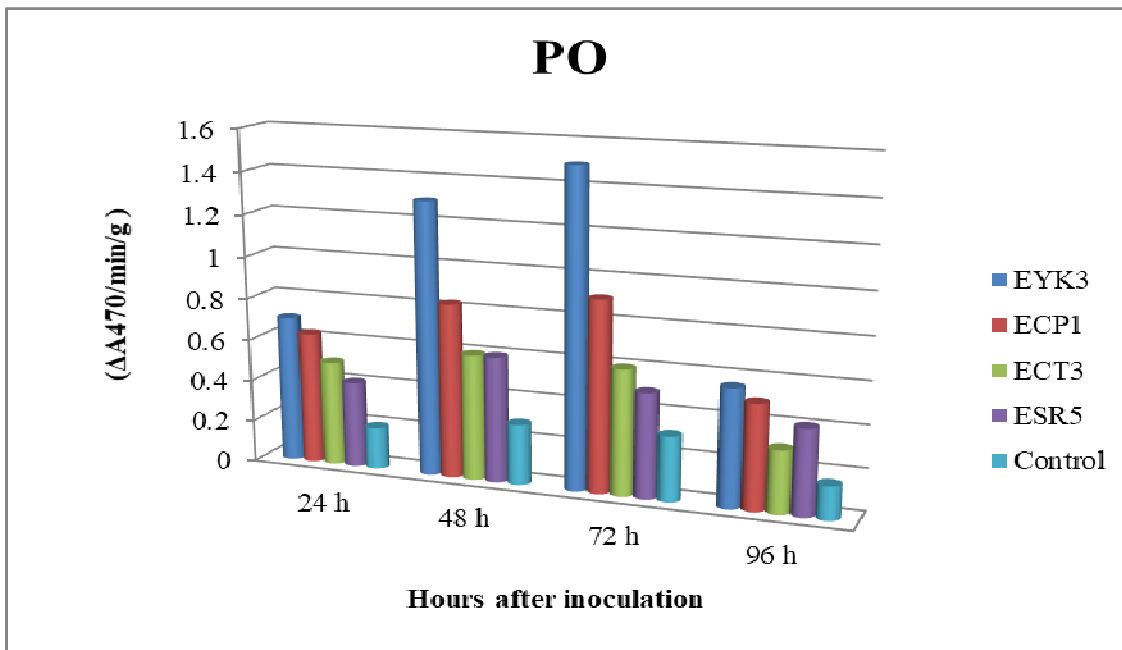


Fig. 4.2. Expression levels of PO activity in rice plants treated with effective antagonistic endophytic bacterial isolates and inoculated with *Xoo*.

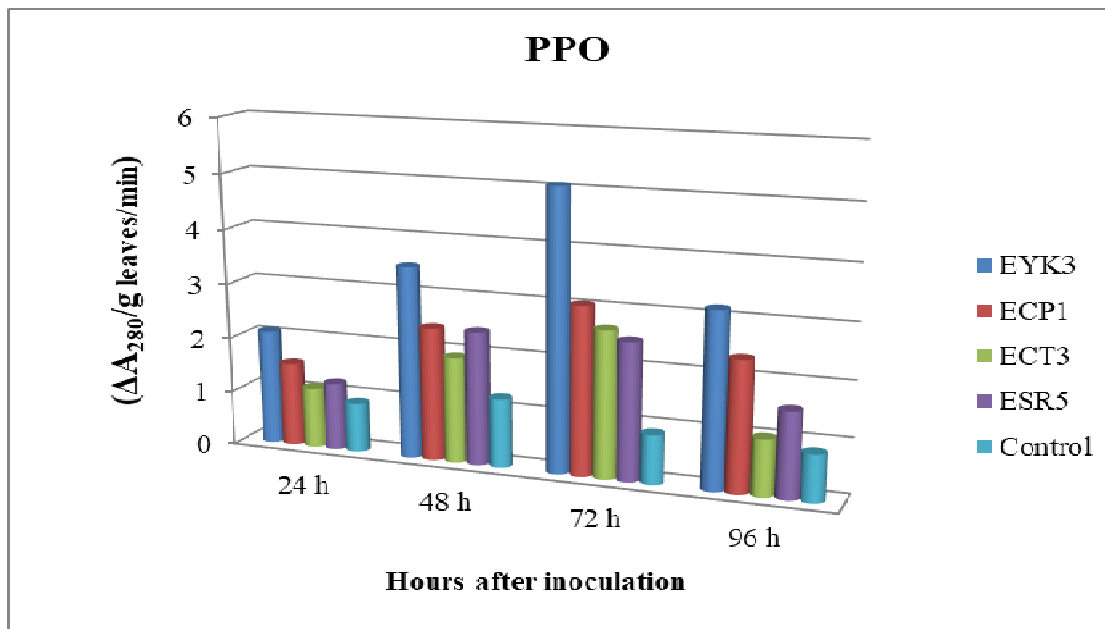


Fig. 4.3. Expression levels of PPO activity in rice plants treated with effective antagonistic endophytic bacterial isolates and inoculated with *Xoo*.

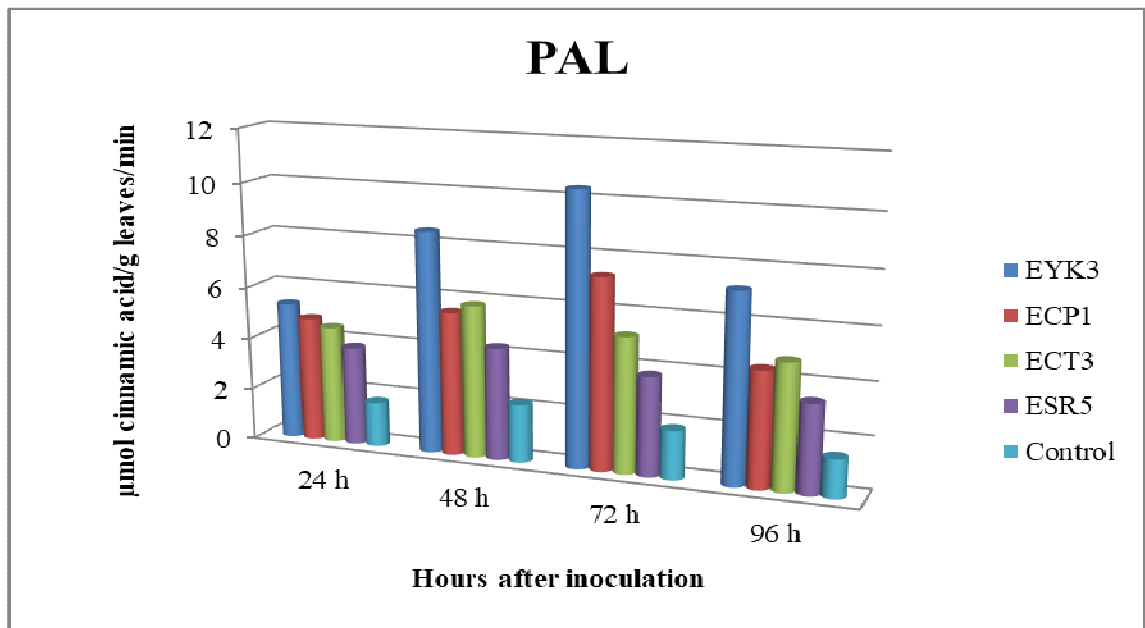


Fig. 4.4. Expression levels of PAL activity in rice plants treated with effective antagonistic endophytic bacterial isolates and inoculated with *Xoo*.

4.6 MOLECULAR CHARACTERIZATION OF EFFECTIVE ANTAGONISTIC ENDOPHYTIC BACTERIAL ISOLATES BY 16S rDNA:

The antagonistic bacterial isolates *viz.*, EYK-3, ECP-1, ECT-3 and ESR-5 which recorded inhibition zone of more than 10.0 mm against *X. oryzae* pv. *oryzae* under *invitro* were considered as effective antagonistic isolates and selected for molecular characterization.

4.6.1 Isolation of DNA:

The extraction of genomic DNA from the potential endophytic bacterial isolates was done as per the procedure mentioned in 3.8.2. The quality of genomic DNA was analysed by running 2µl of each sample on 1 per cent agarose gel and quantity of DNA was arrived at by using Nanodrop spectrophotometer (Table 4.6). The agarose gel analysis and Nanodrop spectrophotometer readings confirmed the quality and quantity of DNA as pure. The concentration of genomic DNA was adjusted to 100 ng/µl for the 16S rDNA amplification.

Table 4.4. Quantitative verification of genomic DNA of effective antagonistic endophytic bacterial isolates by spectrophotometer

S. No.	Endophytic bacterial isolate	A_(260/280)	Conc. of the genomic DNA (ng/µl).
1	EYK-3	1.82	1718.6
2	ECP-1	1.88	1106.8
3	ECT-3	1.85	1314.9
4	ESR-5	1.90	2284.5

4.6.2 Amplification of 16S rDNA from Effective Endophytic Bacterial Isolates.

16S rDNA specific target primers *viz.*, 27F and 1492R were used for PCR amplification of 16S rRNA region of effective antagonistic bacterial isolates. Both primers produced amplicon having size of approximately 1400 bp observed on 0.8 per cent Agarose gel (Plate 10). This indicated that the effective endophytic isolates were bacteria and belongs to the kingdom of prokaryotes.

16S ribosomal RNA sequences have been used extensively in the classification and identification of *bacteria*. The comparison of almost complete 16S rRNA gene sequences has been widely used to establish taxonomic relationships between prokaryotic strains. 16S rRNA region identifies organisms by comparing certain locations on a 16S rRNA molecule with a database of previously identified organisms whose 16S rRNA mark is known. It is considered to be fast and better alternative to other methods of bacterial identification (Kim and Chun, 2014).

The results were in accordance with the reports of Chung *et al.* (2015) who identified two endophytic strains YC7007 and YC7010 with the strongest antagonistic activity using a polyphasic approach. The 16S rRNA gene sequence of the two strains was a continuous stretch of 1513 base pairs, which aligned identically. Similarly, El-shakh *et al.* (2015) carried out 16S rRNA gene sequence analysis using primers 16S f and 16S r for five endophytic isolates (A1, A2, A3, A13 and A15). Isolates A1, A3 and A13 belong to the *B. amyloliquefaciens* strain 1A (KF112077), strain HS6-7 (JN661697) and strain L04 (JN700138), respectively. The isolates A2 and A15 were identified as *B. methylotropicus* strain Hk8-14 (JF899259) and *B. subtilis* strain F321122 (EF423590) respectively.

Similar reports were obtained by Banik *et al.* (2016) extracted the genomic DNA and genes were amplified using universal primers namely 27F

and 1492R. Phylogenetic analysis based on 16S rDNA sequencing divided them into five groups belonging to five classes- alpha, beta, gamma, Proteobacteria and Actinobacteria. Wilasinee *et al.* (2016) identified the potential antagonistic bacteria using 16S rDNA.

Yousefi *et al.* (2018) extracted DNA from four most efficient antagonistic endophytes (OS52, OS40, OS23 and OS53) isolates were amplified using universal 16S rDNA primers (P1 and P6) and a PCR product size of 1500 bp was obtained. It was shown that the isolates OS23, OS40, OS52 and OS53 belonged to *Bacillus* sp., *B. subtilis*, *Enterobacter* sp. and *Pseudomonas putida*, respectively.

Kavitha *et al.* (2020) extracted genomic DNA from 12 endophytic bacterial isolates and amplified the DNA using 16S rDNA specific target primers 27F and 1492R, a amplicon size of approximately 1400 bp were formed and identified the potential endophytic bacteria EMP-5 as *Enterobacter asburiae*.

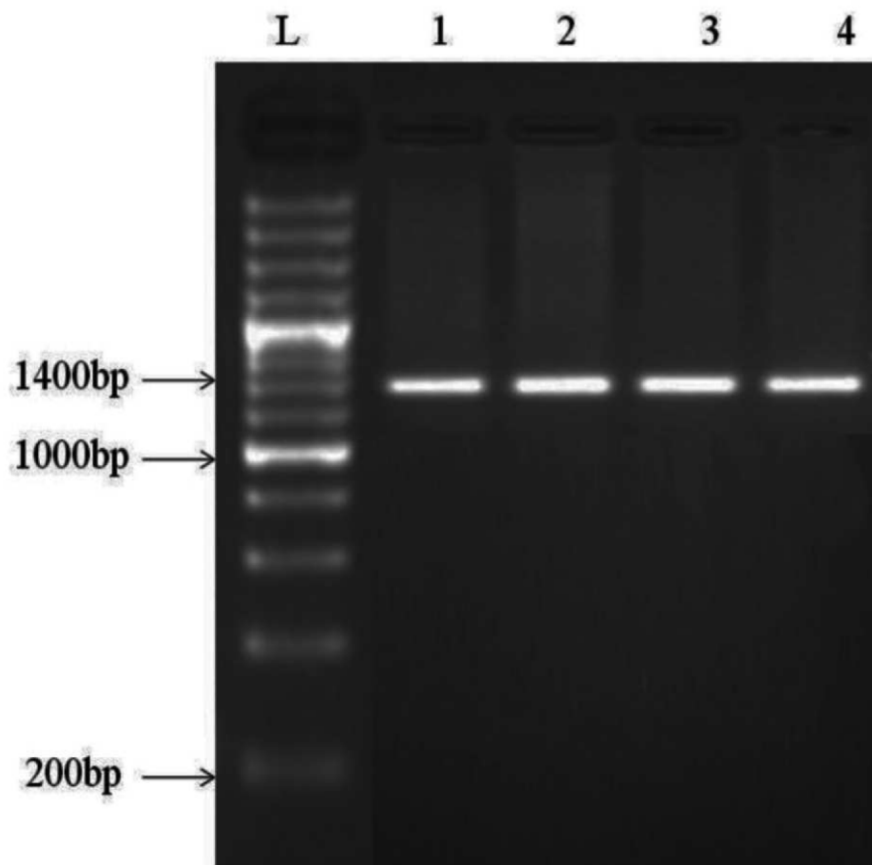


Plate 11. PCR amplication product obtained by 16S rDNA

L: Ladder

1: EYK-3

2: ECP-1

3: ESR-5

4: ECT-3

4.7 EVALUATION OF PLANT GROWTH PROMOTING CHARACTERS AND DISEASE REDUCTION IN RICE TREATED WITH POTENTIAL BACTERIAL ENDOPHYTE (EYK-3) UNDER FIELD CONDITIONS.

Among the four effective antagonistic endophytic bacterial isolate, EYK-3 showed highest antagonistic activity along with increased expression of defense related enzymes on challenging with *Xoo* pathogen. Hence EYK-3 was selected for evaluation of its efficacy in the management of bacterial leaf blight rice along with plant growth promoting characteristics under field conditions.

The experiment was conducted with cultivar NLR- 34449 during *rabi* 2019-20 at Agricultural Research Station, Nellore. The experiment was laid out in a Randomized Block Design (Plate 12).

4.7.1 Effect on Per cent Disease Index

Among all the treatments imposed in the management of BLB under field conditions, treatment T₃ (Seed treatment with EYK-3 @ 10⁹ Cfu/ml + Seedling root dip with EYK-3 @10⁹ Cfu/ml) recorded lowest percent disease index (7.3 %) and significantly superior over all other treatments including untreated control. In other treatments the per cent disease index varied from 10.7 to 17.3 per cent and 24.5 in untreated control (Plate 13).

4.7.2 Effect on Plant Height

Among all the treatments highest plant height (85.3cm) was recorded in T₃ (Seed treatment with EYK-3 @ 10⁹ Cfu/ml + Seedling root dip with EYK-3 @10⁹ Cfu/ml) which was significantly superior over all other treatments including untreated control. In other treatments plant height ranged from 82.0 to 77.1 cm while in control it was 73.4 cm (Table 4.5 and Fig. 4.6).

The application of potential endophytic bacteria EYK-3 during the early stages of the crop i.e., in the treatments T1, T2 and T3 resulted in the

increased plant height of 80.4, 82.0 and 85.3 respectively probably implying the contribution of the endophytic bacteria to the plant growth promotion.

4.7.3 Effect on No. of Tillers

Among all the treatments, T₃ (Seed treatment with EYK-3 @ 10⁹ Cfu/ml + Seedling root dip with EYK-3 @10⁹ Cfu/ml) recorded highest mean tiller number of 24.3 which is on par with T₂(23.0). Treatments T₁, T₄, T₅ and T₆ recorded tiller number of 21.5, 19.4, 20.0 and 19.49 respectively. Lowest tiller number was recorded in untreated control T₇(17.0) (Table 4.5 and Fig. 4.7).

4.7.4 Effect on Productive Tillers

Among all the treatments, highest no. of productive tillers (22.3) was recorded highest in treatment T₃ (Seed treatment with EYK-3 @ 10⁹ Cfu/ml + Seedling root dip with EYK-3 @10⁹ Cfu/ml) 22.3 which is on par with T₂19.93. Lowest productive tiller number (15.2) was recorded in untreated control T₇. Treatments T₁, T₄, T₅ and T₆ recorded productive tiller number of 19.7, 17.2, 17.3 and 16.6 respectively (Table 4.5. and Fig. 4.8.).

4.7.5 Effect on Grain Yield

The grain yield in the treatments varied from 4859 Kg/ha to 6373 Kg ha⁻¹. Among all the treatments T₃ (Seed treatment with EYK-3 @ 10⁹ Cfu/ml + Seedling root dip with EYK-3 @10⁹ Cfu/ml) recorded highest grain yield of 6373Kg ha⁻¹ which was significantly superior over all other treatments including untreated control. The yields in treatments T₁ (Seed treatment with EYK-3 @ 10⁹ Cfu /ml) (5781 Kgha⁻¹), T₂ (Seedling root dip with EYK-3 @10⁹ Cfu/ml) (5853 Kgha⁻¹), T₄ (Foliar spraying of Streptomycin sulphate @ 100ppm + COC 0.3%) and T₅ were on par with each other (5782, 5853 and 5560 Kgha⁻¹ respectively) and significantly superior over T₆ and control (Table 4.5., Fig. 4.9. and Plate 14).

In this present investigation lowest PDI of the BLB disease was recorded in treatment T₃ (Seed treatment with EYK-3 @ 10⁹ Cfu/ml + Seedling root dip with EYK-3 @10⁹ Cfu/ml) which indicates that seed bacterization with potential endophytic bacteria EYK-3 resulted in effective colonization of endophytic bacteria contributing to the induction of resistance, number of tillers and plant height.

Further the activation and accumulation of defense related enzymes viz., peroxidases, polyphenol oxidases and phenylalanine ammonia lyase at the sited infection might have resulted in the avoidance of the colonization of pathogen. The reduction in the disease and the increased biomass might have resulted in recording of the highest yield (6373 Kgha⁻¹) in the treatment.

Biocontrol agents having plant growth promoting traits may be used in addressing the complicated and integrative phenomena of plant disease suppression and growth promotion through plant augmentation (Yasmin *et al.*, 2016).

Jeyalakshmi *et al.* (2010) found that the combination of seed treatment, soil application and foliar spray with *P. Fluorescens* resulted in the minimum disease incidence of bacterial leaf blight with maximum yield in comparison with the chemical treatment and control. Hastuti *et al.* (2012) reported that plants inoculated with endophytic *Streptomyces* spp. were significantly taller and produced higher tiller number than control plants.

Nagendran *et al.* (2013) treated rice plots with *Bacillus subtilis* var. *amyloliquefaciens* (FZB 24) through seed treatment @ 4g/kg + seedling dip @ 4g/l + soil application @ 500g/ha + foliar application @ 500g/ha strain registered a significantly lower intensity of bacterial leaf blight (2.80 %) due to expression of defense related enzymes like peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and phenols compared to untreated control plots (19.82%). Yasmin *et al.* (2016) observed increased activity of defense related enzymes including phenylalanine ammonia-lyase, polyphenol oxidase, and

peroxidase enzyme in plant inoculated with *Pseudomonas sp.* Rh323 which significantly improved plant health in terms of reduced diseased leaf area (80 %).

Yasmin *et al.*, (2017) reported that BRp3 inoculated rice plants showed consistent suppression of BLB pathogen due to production of secondary metabolites (siderophores, rhamnolipids etc.) and also due to induction of defense related enzymes. The inoculation resulted in increased grain yield and straw yield by 51 per cent and 55 per cent respectively under field conditions.

Kavitha *et al.* (2020) reported that combination of seed treatment with EMP5 isolate @ 10^9 cells ml⁻¹ + foliar application of EMP-5 isolate @ 10^9 cells ml⁻¹ showed highest reduction in disease index (4.92 %) with increased plant growth promoting ability in terms of plant height (80.8) cm increased no. of effective tillers (97.4 %), grain yield (5340 kgha⁻¹) and straw yield (6013 kgha⁻¹) over the untreated control.

The isolate used under field experiment EYK-3 was found effective in growth promotion and reduction of disease. The isolate showed significant potential to suppress BLB with the induction of disease resistance in rice coupled with rice growth promotion. This strain may have the potential to be used as bio-inoculant in rice production.

Table 4.5. Evaluation of Plant Growth Promoting characters and disease reduction in rice treated with potential endophytic bacterial (EYK-3) under field conditions.

S. No	T. No.	Treatment details	*Per cent disease index (PDI)	*Plant height (cm)	*No. of tillers	*No. of productive tillers	*Yield (Kg ha ⁻¹)
1	T1	Seed treatment with potential endophytic bacteria EYK-3 @10 ⁹ Cfu /ml	11.8 ^c (20.0)	80.4 ^b	21.5 ^{bc}	19.7 ^{bc}	5782 ^b
2	T2	Seedling dip with potential endophytic bacteria EYK-3 @10 ⁹ Cfu /ml	10.7 ^{bc} (19.0)	82.0 ^b	23.0 ^{ab}	19.9 ^{ab}	5853 ^b
3	T3	T ₁ +T ₂	7.3 ^a (15.7)	85.3 ^a	24.3 ^a	22.3 ^a	6373 ^a
4	T4	Foliar spraying of Streptomycin sulphate @ 100 ppm + Copper oxy Chloride 0.3%	8.8 ^{ab} (17.2)	76.5 ^d	19.4 ^c	17.2 ^{bc}	5710 ^b
5	T5	Foliar spray with potential endophytic bacteria EYK-3 @ 10 ⁹ Cfu /ml at 35 DAT	14.9 ^d (22.7)	79.6 ^{bc}	20.0 ^c	17.3 ^{bc}	5560 ^{bc}
6	T6	Foliar spray with potential endophytic bacteria EYK-3 @ 10 ⁹ Cfu /ml at 45 DAT	17.3 ^e (24.6)	77.1 ^{cd}	19.4 ^c	16.6 ^{bc}	5214 ^{cd}
7	T7	Control	24.5 ^f (29.6)	73.4 ^e	17.0 ^d	15.2 ^c	4859 ^d
	CD (0.05)		2.1	2.9	2.2	2.73	489.3
	CV %		8.7	2.1	6.0	7.94	4.84

*Mean of three replications

**Means in a column followed by same super script letters are not significantly different according to DMRT

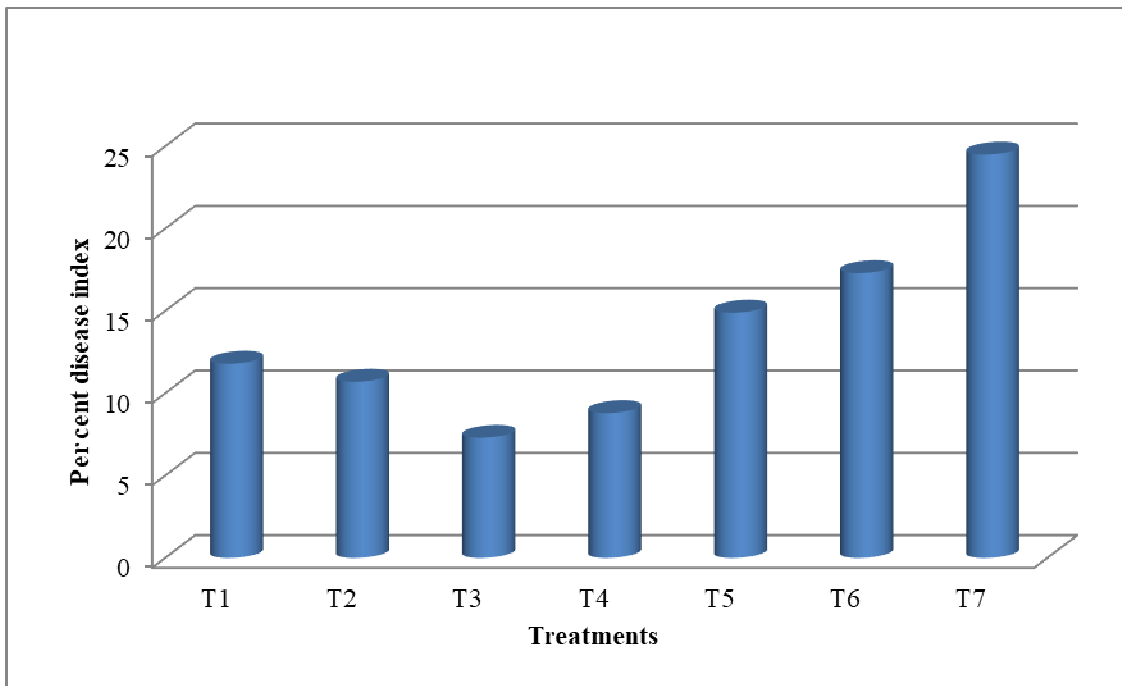


Fig. 4.5. Effect of potential endophytic bacterial isolate (EYK-3) on incidence of BLB in rice under field conditions.

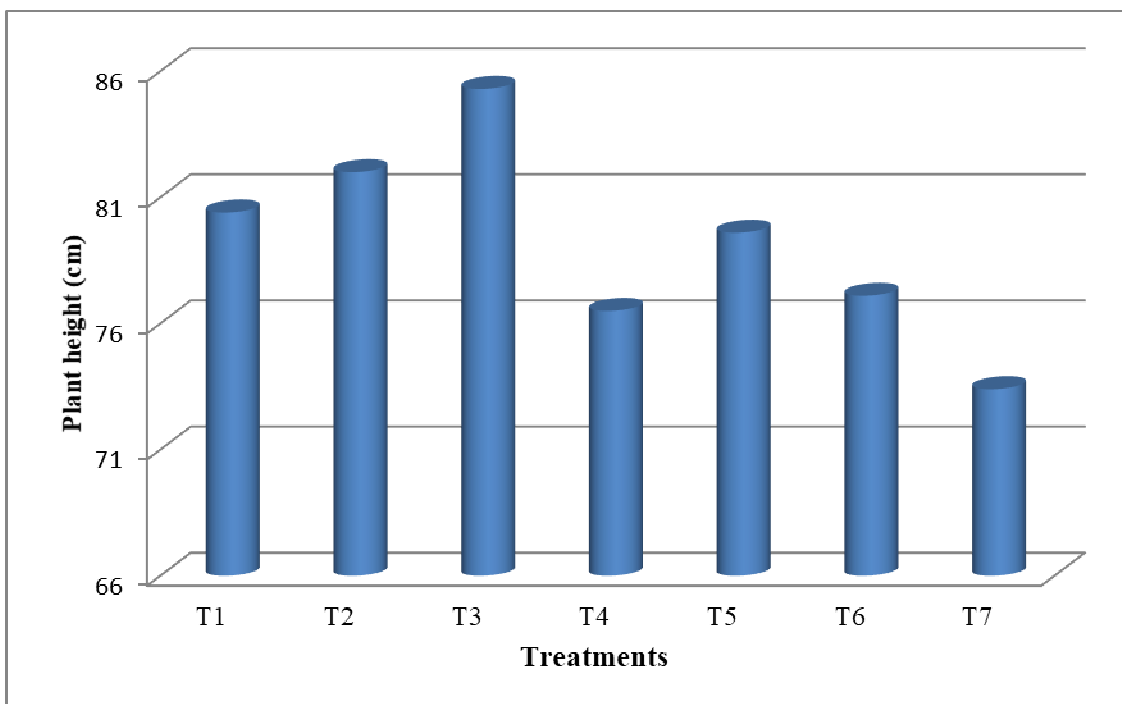


Fig. 4.6. Effect of potential endophytic bacterial isolate (EYK-3) on plant height under field conditions.

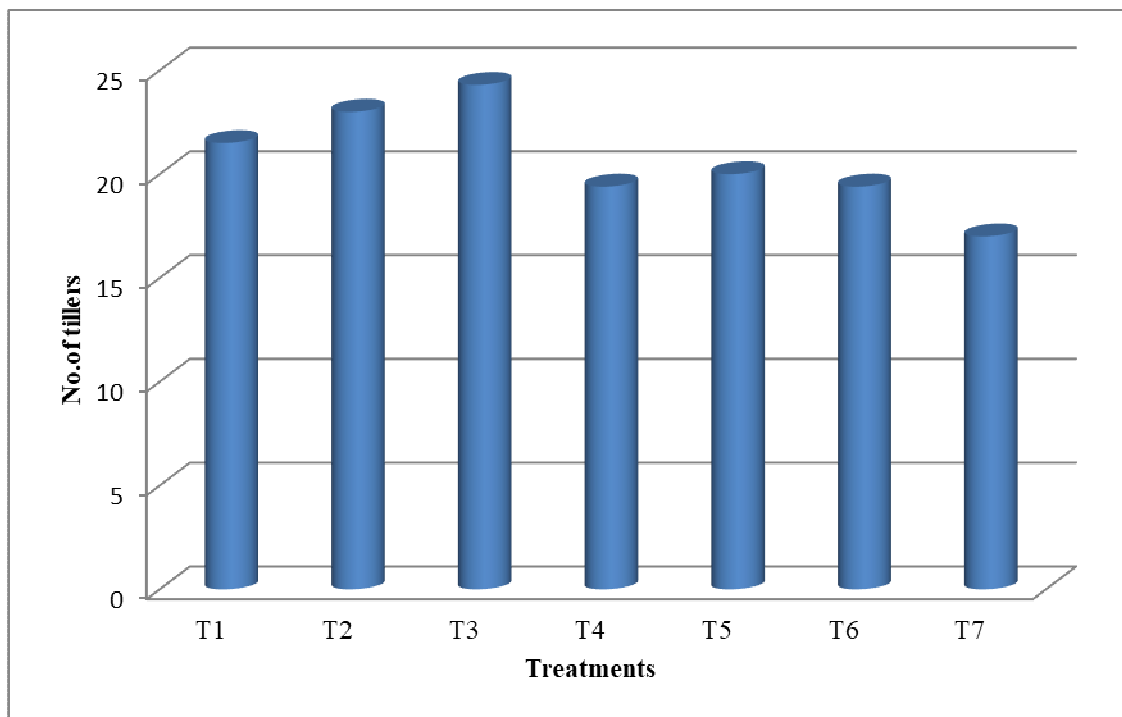


Fig. 4.7. Effect of potential endophytic bacterial isolate (EYK-3) on tiller number under field conditions.

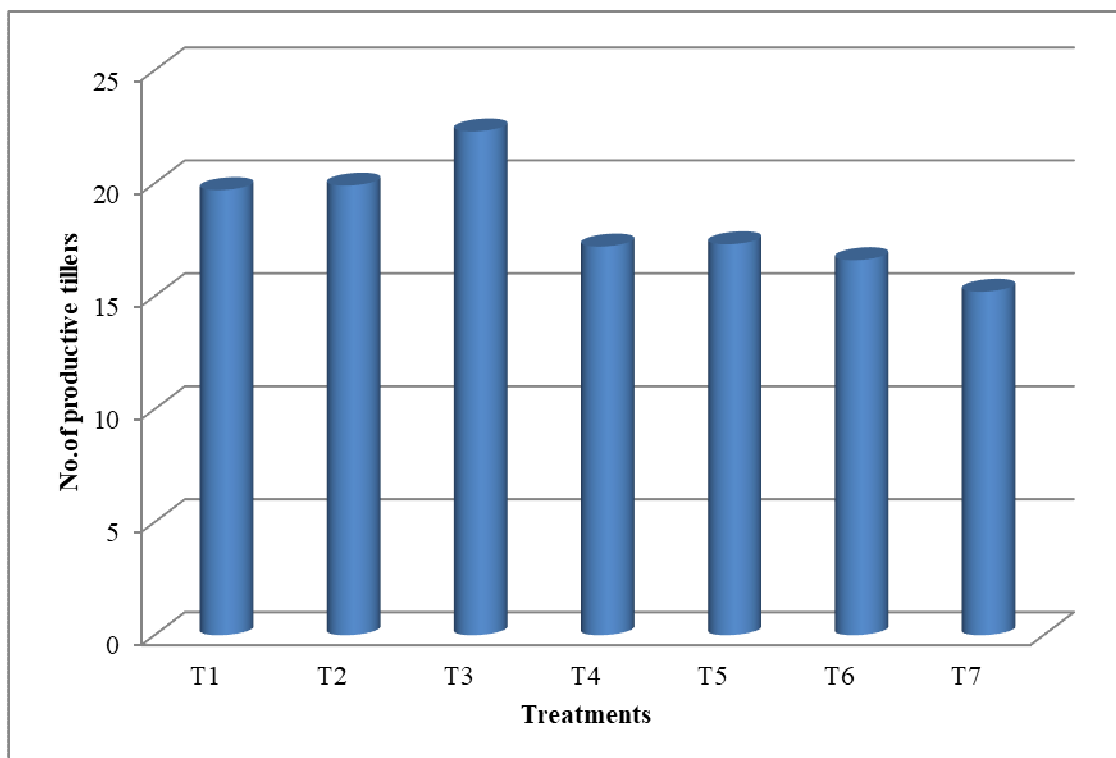


Fig. 4.8. Effect of potential endophytic bacterial isolate (EYK-3) on productive tiller number under field conditions.

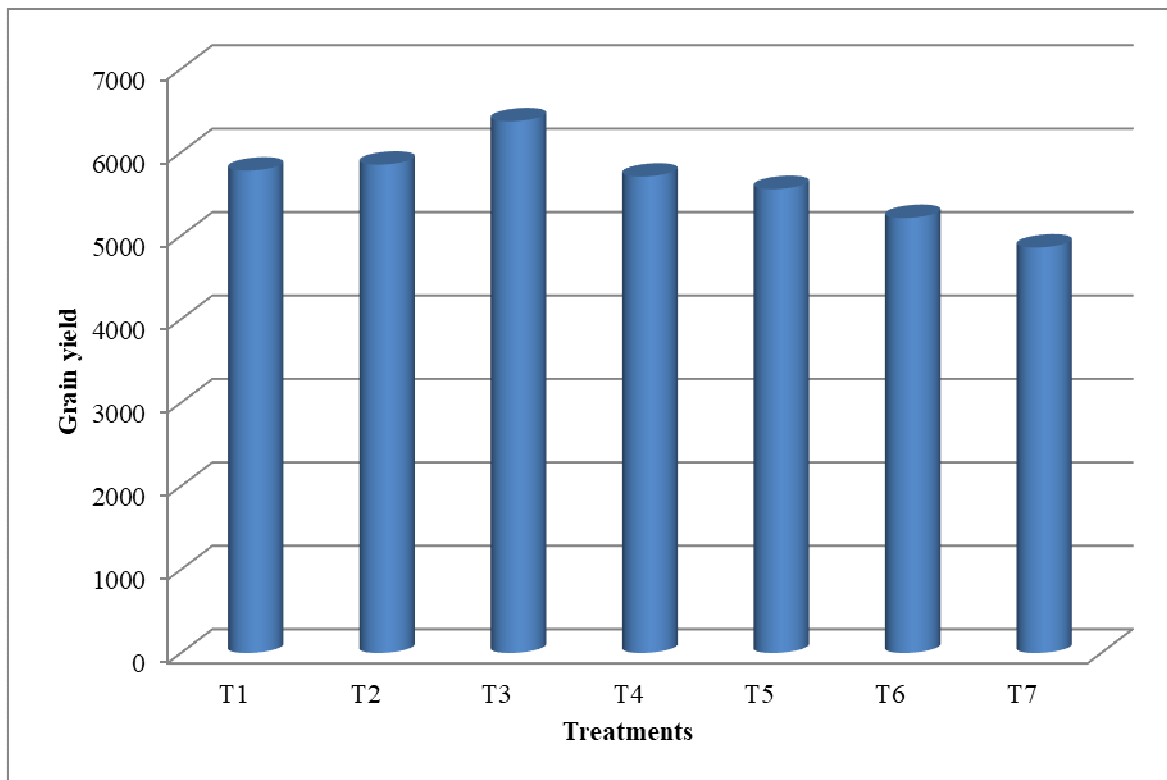


Fig. 4.9. Effect of potential endophytic bacterial isolate (EYK-3) on grain yield under field conditions.



Plate12. View of experimental site of the field of Rice during *rabi* 2019-20



Plate 13. Severity of bacterial leaf blight of rice in different treatments.



Mass multiplication of *Xoo* on NB



Mass multiplication of (EYK-3) on NB



Seed bacterization with EYK-3



Seedling dip with EYK-3



Spraying of EYK-3 in Rice field



Symptom development in BLB inoculated plant

Plate 14. Management of BLB of rice by potential endophytic bacteria EYK-3 under field conditions.

Chapter – V

Summary & Conclusions

Chapter - V

SUMMARY AND CONCLUSIONS

Rice (*Oryza sativa* L.) is life for most of the Asians with more than three billion people around the world consume rice every day. Paddy crop is cultivated over a range of environments which is subjected to various types of abiotic stress and biotic stress that ultimately influence the performance of the crop. Rice diseases have always had a notable influence on rice supply. Among the rice diseases, bacterial leaf blight is the most destructive disease which cause yield loss upto 15- 90 per cent.

Bacterial Leaf Blight (BLB) pathogen was isolated from diseased leaf sample on Nutrient agar (NA) medium which produced single well separated, pin head sized, light yellow, mucoid, round and raised colonies with smooth margins.

Pathogenicity for *X. oryzae* pv. *oryzae* was established by clip inoculation method on rice cultivar NLR-34449. The pathogen was re-isolated from the inoculated plant, proving the Koch's postulates.

Healthy leaf samples were collected for isolation of endophytic bacteria from four cultivars viz., MTU-1010, BPT-5204, ADT-37 and RNR-15048 which were popularly grown in Chittoor district. There is a wide variation in the mean population density of the endophytic bacteria among the cultivars (MTU-1010; 2.3×10^6 to 5.5×10^6), (BPT-5204; 1.3×10^6 to 4.8×10^6), (RNR-15048; 5.3×10^6) and (ADT-37; 2.9×10^6 to 3.8×10^6) and also the stage of the crop (1.3×10^6 to 5.5×10^6) at the time of sample collection.

A total of 29 endophytic bacterial isolates were obtained from healthy leaf samples collected from 12 villages spread across four mandals of Chittoor district. Among 29 endophytic bacterial isolates which were screened against *X. oryzae* pv. *oryzae* under *in vitro* 26 isolates showed antagonistic efficacy

while three isolates *viz.*, ESM-6, EYI-4 and ERN-4 showed zero inhibition in the screening. The inhibition diameter among the 29 isolates varied from 0.0 to 14.5 mm and EYK-3 (14.5 mm) recorded highest inhibition while other endophytic bacterial isolates ECP-1, ESR-5 and ECT-3 recorded inhibition zones of 12.3, 11.43 and 10.53 mm respectively.

The isolates which recorded inhibition zone of more than 10.0 mm *viz.*, EYK-3, ECP-1, ESR5 and ECT-3 were consequently subjected to gram staining and found that EYK-3, ECP-1 as gram negative while ESR-5 and ECT-3 as gram positive. In shape EYK-3, ESR5 and ECT-3 appeared as short rods while ECP-1 as spirillum.

Further the four endophytic isolates with inhibition zone of more than 10.0 mm *viz.*, EYK-3, ECP-1, ESR-5 and ECT-3 were tested for the production of defense related enzymes through artificial inoculation on rice plants to identify the potential endophyte for field studies.

The activity of Peroxidase (PO) and Polyphenol oxidase (PPO) started to increase from 24 h after post pathogen inoculation and was maximum at 72 h after challenging with *Xoo* and thereafter declined from 96 h onwards while in control the meagre increase in PO activity was there upto 72 h and declined after 72 h post inoculation. Among the four effective antagonistic endophytic bacterial isolates, EYK-3 showed highest levels of expression of peroxidase and polyphenol oxidase activity at 72 h.

The activity of Phenylalanine ammonia lyase (PAL) increased with pathogen challenging from 24 h in plants inoculated with EYK-3, ECP-1 isolates and reached peak after 72 h while in ECT-3, ESR-5 and in control the peak activity was observed upto 48 h and which declined from 72 h onwards. Among the four effective antagonistic bacterial endophytes, EYK-3 recorded highest PAL activity (10.48 μ mol. cinnamic acid/min/g leaves).

The increased levels of expression of defense related enzymes in the endophytic bacteria treated rice plants play either a direct or an indirect role in the suppression of pathogen growth.

The genomic DNA was extracted from four isolates *viz.*, EYK-3, ECP-1, ECT-3 and ESR-5 and 16S rDNA specific target primers *viz.*, 27F and 1492R were used for PCR amplification of 16S rRNA region of effective bacterial isolates. Both primers produced amplicon having size of approximately 1400 bp observed on 0.8 per cent agarose gel. This indicated that the effective endophytic isolates were bacteria and belongs to the kingdom of prokaryotes.

Among the four effective antagonistic endophytic bacterial isolate, EYK-3 showed highest antagonistic activity along with increased levels of expression of defense related enzymes on challenging with *Xoo* pathogen. Hence EYK-3 was selected for evaluation of its efficacy in the management of BLB of rice along with plant growth promoting characteristics under field conditions.

Among all the treatments imposed in the management of BLB under field conditions, treatment T₃ (Seed treatment with EYK-3 @ 10⁹ CfU /ml + Seedling root dip with EYK-3 @ 10⁹ CfU /ml) recorded lowest per cent disease index (7.3 %) and in other treatments the per cent disease index varied from 10.7 to 17.3 while it was 24.5 in untreated control.

Similarly the treatment T₃ (Seed treatment with EYK-3 @ 10⁹ CfU /ml + Seedling root dip with EYK-3 @ 10⁹ CfU /ml) recorded highest plant height (85.3 cm), No. of tillers (24.3), No. of productive tillers (22.3) and grain yield (6373 Kg ha⁻¹) whereas lowest plant height (73.4 cm), No. of tillers (17.0), No. of productive tillers (15.2) and grain yield (4859 Kg ha⁻¹) were recorded in untreated control T₇.

Biocontrol agents having plant growth promoting traits may be used in addressing the complicated and integrative phenomena of plant disease suppression and growth promotion through plant augmentation (Yasmin *et al.*,

2016). The results strongly make a point that endophytic bacterial isolate (EYK-3) could be efficiently used for the management of bacterial leaf blight and as well as growth promotion of the plant.

Future line of work

- Research need to be carried out on the colonization of the endophytic bacterial isolate in different parts of the plants and the traits associated with the colonization process.
- Population dynamics of the endophytic bacterial isolate need to be studied for logically arriving at the required density of the endophyte to culminate the growth of the pathogen. This helps in the optimization of the dosage for effective control of the disease.
- Formulation of the identified endophytic bacterial isolate using appropriate carrier material will pave the way for method of application in the effective disease management.

Literature Cited

LITERATURE CITED

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