Characterization of endophytic bacterial metabolites and their usage for biocontrol of *Colletotrichum falcatum* causing Red Rot in Sugarcane crop

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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2020


I BESTOW THIS WORK
TO
"My Family"

CERTIFICATE

This is to certify that the thesis entitled "**Characterization of endophytic bacterial metabolites and their usage for biocontrol of *Colletotrichum falcatum* causing Red Rot in Sugarcane crop**" submitted by "**Ms. Beenu Shastri**" is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university. The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (PhD) regulations - 1999 as amended in 2008/2010/2013 and it is fit for submission and evaluation for the award for the degree of Doctor of Philosophy of the University.

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DECLARATION

I, **Beenu Shastri**, hereby declare that the thesis work entitled "**Characterization of endophytic bacterial metabolites and their usage for biocontrol of *Colletotrichum falcatum* causing Red Rot in Sugarcane crop**" is my own work carried out under the guidance of **Prof. Rajesh Kumar, Head,** Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, (A Central University) Vidya Vihar, Raebareli Road, Lucknow. The matter embodied in this thesis is written by me and has not been submitted to any other university for the fulfilment of the requirement of any other Degree or Diploma.

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"I find that the harder I work, the more luck I seem to have."

— Thomas Jefferson

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Beenu Shastri

Beenu Shastri

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LIST OF ABBREVIATIONS

2,4 DAPG	:	2,4- Diacetylphloroglucinol
BLAST	:	Basic local alignment search tool
BNF	:	Biological Nitrogen Fixation
CAS	:	Chrome Azurol S
cfu	:	Colony forming Unit
cm	:	Centimeter
DAP	:	Days After Planting
DMRT	:	Duncan Multiple Range Test
DNSA	:	Dinitrosalicyclic Acid
E.C.	:	Enzyme classification
FTIR	:	Fourier transform-infrared
GC-MS	:	Gas chromatography-mass spectroscopy
Glc-NAc	:	N-Acetyl D- Glucosamine
HAQ	:	4 hydroxy-2-alkylquinolines
IAA	:	Indole Acetic acid
IISR	:	Indian Institute of Sugarcane Research
ISR	:	Induced Systemic Resistance
KB	:	King's B
LB	:	Luria Bertani
LC-MS/MS	:	Lquid Chromotography – mass spectrometry
LPs	:	Lipopeptides
MEGA	:	Molecular evolutionary genetics analysis
mm	:	Milimetre
MRVP	:	Methyl red voges proskauer's
MSM	:	Minimal Salt Medium
NA	:	Nutrient Agar
NCBI	:	National council for biotechnological information
ND	:	Not detected
NIST	:	National institute of standards and technology
OD	:	Optical density
PAGE	:	Polyacrylamide gel electrophoresis

PAL	:	Phenylalanine ammonia lyase
PCA	:	Phenanzine 1- carboxylic acid
PCR	:	Polymerase chain reaction
PDA	:	Potato Dextrose Agar
PGP	:	Plant Growth Promoting
PGPB	:	Plant Growth Promoting Bacteria
PGPR's	:	Plant Growth Promoting Rhizobacteria
PHA	:	1-hydroxy phenanzine
PO	:	Peroxidases
PPO	:	Polyphenol oxidases
PS	:	Phosphate Solubilization
PSU	:	Percentage Siderophore Unit
rpm	:	Revolution per minute
rRNA	:	Ribosomal Ribonucleic acid
RT	:	Retention time
SD	:	Standard deviation
SDS	:	Sodium dodecyl sulfate
SE	:	Standard Error
SEM	:	Scanning Electron Microscopy
SI	:	Solubilization Index
UV	:	Ultra violet
VOC's	:	Volatile Organic Compound(s)

LIST OF SYMBOLS

%	:	Percentage
λ	:	Wavelength
~	:	Approximately equal
<	:	Less than
=	:	Equal
>	:	Greater than
±	:	Plus - minus
≥	:	Greater than or equal to
°C	:	Degree Celsius
µg	:	Microgram
µg/ml	:	Microgram per milliliter
µl	:	Microliter
µmole	:	Micromole
3′	:	Three prime
5′	:	Five prime
bp	:	Base pair
Fig	:	Figure
g	:	Gram
g/L	:	gram per liter
h	:	Hour
Kbp	:	Kilo base pair
kDa	:	Kilo dalton
L	:	Liter
M	:	Molar
m/z	:	Mass-to-charge ratio
mg/g	:	Milligram per gram
mgL^{-1}	:	Milligram per liter
min	:	Minute
mL	:	Milliliter
mm	:	Millimeter
mM	:	Milimolar
mmol	:	Millimole

N	:	Normality
nm	:	Nanometer
P	:	Phosphorous
pH	:	Power of hydrogen
s	:	Second
U/ml	:	Unit per milliliter
v	:	Volume
v/v	:	Volume by volume
w/v	:	Weight by volume

ABSTRACT

Red rot is one of the threatening disease of sugarcane and its management practices requires attention of eco-warrior to effectively control the disease through cost-effective biological agent. Endopyhtic bacteria has turned out to be most effective when comes to management of disease. Therefore, present study dealt with the isolation and characterization of endophytic bacteria (beyond N-fixer diazotroph) from the sugarcane plant. Further, these endophytic were tested for their *in vitro* inhibition against the red rot pathogen (*Colletotrichum falcatum*). Screening of antagonistic endophytic bacteria for various PGP traits and the presence of various lytic enzymes (mainly chitinase and β-1,3 glucanase) and diffusible and volatile antibiotics were performed. On the basis of presence of various biocontrol traits alongwith plant growth promoting traits six bacterial isolates for 16S rRNA gene sequencing and were identified as *Bacillus cereus* (S8), *B. aryabhattai* (S12), *B. subtilis* (S17), *B. licheniformis* (S26), *B. paramycoides* (R5), and *Pseudomonas aeruginosa* (BS-4). Further, enzyme activity, SDS-PAGE, zymogram study and molecular screening for gene of chitinase and β-1,3 glucanase were detected and confirmed for the selected isolates. Also, analytical technique such as FTIR (for functional group), LC-MS/MS (detected the possibility for the presence of Surfactin, Bacillomycin, Fengycin, Iturin, Phenazine, Pyoluteorin etc. in various selected isolates) and GC-MS (detected the presence of volatile antibiotic) were conducted. Based on the molecular and analytical technique studies, strain *B. paramycoides* (R5), *B. aryabhattai* (S12), *Pseudomonas aeruginosa* (BS-4) and mixture of *B. paramycoides* + *B. aryabhattai* (R5+S12) were selected to check the bioefficacy of these isolates against red rot (through different trials) under *in vivo* study. *In vivo* study demonstrated the individual strain R5 and BS-4 were highly effective in controlling the disease as compared to mixture strain. Besides disease assessment, various growth parameter was also evaluated and found that endophytic bacterially treated plant showed promotion in plant growth and suppression in red rot disease over control plants.

In a nutshell endophytic bacteria contains various metabolites which helped in controlling the red rot disease.

Keywords: *Bacillus* spp., *Colletotrichum falcatum*, Endophytic bacteria, PGP and antifungal traits, *Pseudomonas sp.*

Chapter 1

Introduction

Chapter 1

INTRODUCTION

1.1. Introduction:

India is known for its high species richness as well as agro- biodiversity covering an area of 2.4% of the total land biomass and supports 17% world's human population. Agriculture plays a pivotal role in India's economy and 58.6% of the population is involved in the agriculture and related sector. With the advent of Indian green revolution in the sixties of 20th century; new innovative and advanced technologies were adopted in the agricultural domain for improvement of the crop production which not only escalated the production of agriculture but also their yield in the developing country. Of all the agricultural crops grown; majority (approx. 70%) of them are grasses such as rice, wheat, maize, sugarcane and sorghum. Sugarcane (*Saccharum* species hybrid) is a tall tropical perennial grass belonging to the family of Poaceae. It is a paragon of plant kingdom with high efficiency of hoarding solar energy and transforming them into sucrose (Khan et al. 2019).

The sugarcane crop provides a treasure trove of valuable products right from the intact canes to the crushing of crop. The primary economic product of sugarcane is sugar which has become the part and parcel of human's diet. The sugarcane crop is principal sugar crop in the world and accounts for over 75% of sugar production and 100% of that in India; hence continues to be the sole source of sugar in the country (Srivastava and Lal 2019). Besides sugar, a primary product; the crop's utility is amplifying after considering their widening scope of its agro-industrial uses and its by-product, particularly in co-generation of electricity through bagasse and

production of ethanol for blending in the petrol-driven vehicle. Also, the crop is utilized in the manufacturing of jaggery and khandsari, along with the other commercially important by-products like bagasse, molasses, filter-cake, wax etc. (Sanghera and Jamwal, 2019). In fact, in many areas sugarcane tops also serve as fodder for cattle, stubbles and roots as organic manure and leaf trash as fuel and compost. Thus, sugarcane has established itself as an important cash crop and opened up new opportunity for agro-processing industry.

The Sugar industry is the second-largest agro-processing industry next to cotton in the country and play a prominent role in uplifting the socio-economic status of rural people by offering them a better source of income and employment opportunities directly and indirectly. The cultivation of commercialized crop is practiced in many parts of the world, including 110 countries of tropical and sub-tropical region, thus, holding an esteemed position in the agrarian economy of India. Brazil has become the numero-uno in the world, covering the maximum acreage and production of sugarcane. India ranks seconds after Brazil with respect to cane acreage and cane production (Duttamajumder 2008). The area under sugarcane (2018-2019) is hovering around 5.04 million hectares with the production of approximately 411 million tonnes of cane with an average productivity of 81.5 tonnes per hectare of sugarcane (ISMA 2019). It is grown in both tropical and sub-tropical regions of India. In sub-tropical region of country, it is mainly cultivated in Uttar Pradesh, Uttarakhand, Haryana, and Punjab.

However, sugarcane production does not correspond with the cultivated area in India. Several factors including the high cost of cultivation, inadequate irrigation facilities, breakdown of resistance of varieties to diseases and other natural calamities, etc., are responsible for low sugarcane productivity (Lal 2019). However,

the major constraint in the successful crop production is the occurrence of several deadly diseases which not only affects its yield but also quality. Globally, it has been reported that plant disease causes nearly 13% of losses in crop production which is equivalent to $220 billion every year (Kandel et al. 2017a,b). It has been also estimated that sugarcane diseases alone reduce the crop yield by 20% every year (Lal 2019). Disease not only affects the reduction in the crop yield and sugar production but also causes deterioration in the juice quality of infected cane. Generally, fungi, bacteria, virus and phytoplasmas are mainly responsible for causing serious threat to sugarcane cultivation (Viswanathan and Rao 2011). However, phytopathogenic fungi are the most common causes of a wide range of diseases occurring in economically important crops (Mehnaz et al. 2013). The sugarcane is a vegetatively propagated crop with serious drawback of concomitant propagation of many diseases (Lal 2019). It is highly vulnerable to diseases right from the seed/sett stages till the harvest of crop which leads to reduction in cane yield and sucrose recovery and finally leads to heavy economic losses. Red rot, wilt, smut and sett rot are the main fungal diseases causing significant loss to sugarcane production.

Red rot is the key menance of sugarcane in India. The fungal pathogen *Colletotrichum falcatum* Went is responsible for causing devastating red rot disease in the standing crop. The pathogen can attack any part of the plant; be it a stalk, leaf , bud or root. Generally, red rot is sett-borne disease; but the spores of the pathogen *C. falcatum* persist in the soil in the dormant stage and can grow on decayed host plant parts as active saprophytes (Patel 2019).

Red rot is highly devastating disease of sugarcane, which reduces yield as well as quality of the juice. Epidemic outbreaks of this disease have been very common ever

since its occurrence in India since the 1890s which is a major constraint for crop cultivation in South Asia. The disease is responsible for the the elimination of many commercial varieties in India in the earlier decades (Hassan et al. 2010; Viswanathan 2010) involving the failure of important commercial varieties like Co 312, Co 419, Co 658, Co 997, Co 1148, Co 6304, CoC 671, CoJ 64, CoS 8436, CoSe 95422, BO 11, BO 17, BO 54 (Viswanathan 2010, 2012). The disease is distributed throughout the country except in the states of Maharashtra and Karnataka in India (Viswanathan 2010).

The Indian state of Uttar Pradesh has the largest acreage for cultivation and therefore, disease is spreading at par scale in the state. Previously, numerous methods including chemical fungicides have been employed for combating the disease; however, such methods failed as synthetic fungicides are resistant to degradation and their residue gets accumulated in the soil affecting the health of other beneficial microorganisms and their environment (Jeyarajan and Nakkeeran 2000; Patel 2019). Another important approach against disease was developed by using new resistant varieties of crop against the pathogen; however, this method also did not worked satisfactorily due to the recurrent emergence of new race of *C. falcatum* pathogen against such varieties.Thus, a wide range of sugarcane varieties have become susceptible to red rot (Viswanathan et al. 2003). Consequently, sustaining and enhancing the growth and yield of sugarcane have become a major focus of research. Different approaches are needed to be focussed for investigation in various agro-climatic regions which can improve its productivity as well as provide protection against fungal pathogen. Hence, there is an emergent requirement for adopting alternative eco-friendly strategies for the management of this diseases. The use of organically and biologically safe practices have drawn the attention of

various eco-warriors, researchers, and scientists for manoeuvring such methods which are safe and eco-friendly. Use of bio-agents gained attention in last two decades and still is in infancy, for the the management of diseases because of their non-hazardous nature, easy biodegradability and non- bioaccumulation (Harman et al. 2004; Lal 2004, Ben Slama et al 2019). Microorganisms are the biological agents which have gained their importance in agriculture for their usage in improving the health status of crop. Plant growth promoting rhizobacteria's (PGPR's) are a specialized group of microorganisms which are intensely associated with the rhizosphere zone of plants and enhance the crop growth and its productivity. They exert a beneficial effect on the crop by forming a symbiotic association with the host plant either in a direct or indirect manner. Certain genera belonging to the genus *Pseudomonas, Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Rhizobium, Erwinia, Mycobacterium, Mesorhizobium, Flavobacterium, Serratia* etc. comprise the special member of the PGPR's family that helps to raise the growth of plants against biotic and abiotic stresses. In the recent era, certain beneficiary micro-organisms and their metabolites have been found to play a pivotal role in management of the phytopathogen to a great extent. Certain rhizopsheric micro-organisms enter into the plant system, colonizing the root cortex region and stalks and function as endophytes (Brader et al. 2014).

Endophytes are defined as plant-associated microorganisms that are intensely colonized inside the tissue of host plants without causing any substantial harmful effect on the plants. Endophytic bacteria reside in the inter-cellular spaces of the cell walls and xylem vessels of almost every part of plant ranging from tissue of roots to stem, leaf, flower, fruit and seed (Hallmann et al. 1997). Studies have reported for their intra-cellular colonization in the cytosol and their survival in the apoplast of

plant cells (Cocking et al. 2006; Thomas and Sekhar 2014; White et al. 2014; Koskimaki et al. 2015). Plants interact with these communities of endophytic microorganisms to a great extent as they are in close ecological niche with them; thus, helps in improving the well-being of crop by increasing their growth and yield and also in disease management. Nowadays, endophytes have become a hotspot due to the abundant secondary metabolites, plant growth promoting (PGP) activities and plant protection role. Therefore, endophytic bacteria have been a major area of interest due to their unexploited source of natural products which enhances plant growth besides providing the resistance against phytopathogens. Thus, endophytes are considered as potential tool for agriculture by serving a crucial role in plant health and disease management (Azevedo et al. 2000).

Bacterial endophytes offer several ecological benefits to plant over rhizopsheric/phyllospheric bacteria since they reside in close vicinity of host within a protected environment; which provides the opportunity to communicate and interact directly with the host tissue (Ali et al. 2012; Coutinho et al. 2015). Thus, they play an indispensable role in sustaining the well-being of plants; as they can fortify the plant against biotic and abiotic stress and also help in intensifying the growth and yield of crop. Likewise, plant growth-promoting rhizobacteria (PGPR); endophytes also possess the traits for plant growth promotion (PGP) in a direct as well as in indirect manner. Direct PGP mechanisms of endophytes include biological nitrogen fixation (BNF) and mineral solubilization (P, Fe), as well as the production of phytohormones (auxins, cytokinin and gibberellin), while indirect mechanisms include arresting the growth of phytopathogens facilitated by antibiotics, competition for nutrients and niches, or the induction of an induced systemic

resistance (ISR) response (Rosenblueth and Martínez- Romero 2006; Compant et al. 2010; Mei and Flinn 2010; Santoyo et al. 2016; Orozco-Mosqueda et al. 2018).

Numerous endophytic bacteria have been reported from multiple plants; however, *Bacillus* and *Pseudomonas* are well-characterized species associated with plants in large concentration. They have been found to be the most effective in disease management, enhancing nutrient uptake and productivity in several crops. They can employ a diverse array of mechanisms to inhibit their target pathogen(s). These include mechanisms such as mycoparasitism, antibiosis and competition as well as indirect actions such as induction of systemic resistance (ISR) in plant and growth promotion.

Sugarcane is an ideal crop for the use of endophytes. as it is vegetatively propagated crop and help to maintain their population to sustain their productivity and yield because the crop continues in the same field for 2 to 3 years by the way of ratoon(s). Monoculture of sugarcane also helps the multiplication of endophytes without interruption. Similarly, endophytic bacteria have also been reported from numerous variety of plants including sugar beet (Dent et al. 2004), prairie plants, agronomic crops (Zinniel et al. 2002), potato varieties (Sessitsch et al. 2002), tomato (Abdallah 2017), corn (Szilagyi-Zecchin et al. 2014), wheat (Germida and Siciliano, 2001), and rice (Sun et al. 2008); but the functional status of various endophytes is yet to be explored. In order to explore the potential endophytes, different approaches need to be justified for the isolation of diverse range of endophytic communities from various economically, taxonomically and metabolically distinct plants.

Earlier, most of the research on endophytic bacteria has been focused on diazotrophs (the N- fixer), such as *Gluconacetobacter diazotrophicus*, *Herbaspirillum* spp

(Baldani et al. 1986; Cavalcante and Döbereiner 1988) and *Azospirillum amazonense* (Reis et al. 2000). However, only a limited study has been conducted on the growth promoting properties of endophytic bacteria other than diazotrophs isolated from sugarcane. It is also known that the population of diazotrophs seems to be low in Indian sugarcane; when compared with another group of bacterial population (Suman et al. 2001). Therefore, it is necessary to uncover new plant growth-promoting endophytes and explore their potency with diversifying role in influencing plant growth and inhibit the pathogen. Thus, the present study deals with the identification and characterization of diverse putative bacterial endophytes with novel antifungal and plant growth-promoting traits isolated from root and stalks of different sugarcane varieties. Understanding the diversity of endophyte associated with the plants and their role in plant development is necessary step for increasing the crop production, and sustaining agro-ecosystems (Germida et al. 1998; Sturz et al. 1999).

There has been a considerable interest in screening the endophyte for bioactive compound (Kumar and Hyde 2004; Ben Slama et al. 2019). A large number of compounds (secondary metabolites) have been extracted, isolated and characterized from endophytic microbes (Tan and Zou 2001; Strobel et al. 2004; Ben Slama et al. 2019). Hence, the present study which aims to target the endophytic bacterial community and their metabolites as discussed above for biocontrol of *C. falcatum* causing red rot in sugarcane crop is being proposed with the following objectives:

1.2 Objectives:

1. Isolation and characterization (morphological, biochemical and molecular) of endophytic bacteria from sugarcane crop.

2. Characterization of isolates for PGPR properties (IAA, Siderophore, Phosphate solubilisation, etc.) and secondary metabolites (chitinase, β-1, 3 glucanase, antibiotic etc.).

3. Molecular characterization and quantitative assessment of secondary metabolites (chitinase, β-1, 3 glucanse, antibiotic etc).

4. To test the biocontrol efficacy of characterized endophytic isolates against *Colletotrichum falcatum* causing red rot in sugarcane *in vivo* condition.

1.3 Organization of the thesis:

The four objectives listed above are discussed in this thesis as separate chapter. Therefore, the report of the present thesis have been categorized majorly into eight different chapters. Apart from current chapter (Chapter 1) on 'Introduction', there are seven more chapter namely Review of Literature as Chapter-2, First Objective as Chapter-3, Second Objective as Chapter-4, Third Objective as Chapter-5, Fourth objective as Chapter-6, Summary as Chapter-7 and lastly, Conclusion and Future Prospective will be the Chapter-8.The intent and content of each of these chapters is being presented below in a concise form.

Second chapter on '*Review of Literature*' presents first the general features of Sugarcane crop, their cultivation and economic importance. The various biotic and abiotic stresses are also discussed and concern of sugarcane crop against Red Rot disease is discussed. The various treatments methods is discussed with more elaborative discussion on endophytic bacteria. In the next section of review of Literature; various role of endophytic bacteria in a direct way in growth promotion and indirect way in protecting the crop against disease is discussed; with special

emphasis on various metabolites and their mechanisms as a potential role in relation to biocontrol.

Third chapter on 'Isolation and characterization (morphological, biochemical and molecular) of endophytic bacteria from sugarcane crop" which is first objective is discussed. The detailed description of this chapter includes the brief introduction on the relevant area associated with isolation of endophytic bacteria, various methods and approaches used for the isolation and characterization of endophytic bacteria from sugarcane crop with proper sterilization technique. As thesis's main idea is centered on biological control of red rot disease of sugarcane. Therefore, isolated endophytic bacteria were screened for dual-culture antagonism of isolated against red rot fungal pathogen. The results findings include the various characterized antagonistic endophytic bacteria with detailed discussion related to objective findings.

Fourth chapter on 'Characterization of isolates for PGPR properties (IAA, Siderophore, Phosphate solubilisation, etc.) and secondary metabolites (chitinase, β-1, 3 glucanase, antibiotic etc.)" deals with *in vitro* screening of selected antagonistic endophytic bacteria for various plant growth promoting, other secondary metabolites (volatile and diffusible antibiotic) as well as presence of lytic enzyme. This chapter filtered the criteria for selecting the isolates with maximum antifungal and plant growth promoting traits that can be further exploited for future application.

Fifth chapter on 'Molecular characterization and quantitative assessment of secondary metabolites (chitinase, β-1, 3 glucanse, antibiotic etc).*'* has been devised keeping in mind the fulfilment of the objectives and scope of the present study as continuation of Chapter-4. This chapter dicusses the confirmation of various lytic

enzyme possessed by the selected endophytic bacteria with the help of molecular approach and further exploration of various other secondary metabolites of selected endophytic bacteria using the various analytical techniques such as FTIR, LC-MS/MS and GC-MS. Thus, exploring the various metabolites which indicates that certain metabolites are responsible for causing suppression of fungal pathogen by isolates under *in vitro* as well as under *in vivo* conditions

Sixth chapter on 'To test the biocontrol efficacy of characterized endophytic isolates against *Colletotrichum falcatum* causing red rot in sugarcane *in vivo* condition*'* is presented. This chapter deals with checking the efficacy of the selected endophytic bacteria possessing maximum traits (as biofertlizer and biocontrol) against red rot fungus under *in vivo/situ* condition also by carrying the pot experiment in four different methods. The mechanism due to ISR and by certain metabolites are responsible for controlling the sugarcane plant against disease is discussed. The various other defense responses by sugarcane plants are also reported when it is attacked by the pathogen after inoculating with various endophytic bacteria as reponse of systemic resistance against pathogen.

Seventh Chapter is on *"Summary"* which briefly describes all the main findings and outcome of the thesis as per the objectives.

Last and Eighth chapter is on *"Conclusion and Future Prospective"*which represents the conclusion drawn from this study, limitation and recommendation of further research regarding the current study.

Chapter 2

Review of Literature

2.1 Sugarcane crop

2.1.1 History of Sugarcane:

Sugarcane species are perennial true grasses of genus *Saccharum* (Cheavegatti-Gianotto et al. 2011). The plant of height ranging 2-6 meters contains stout, jointed, fibrous stalks rich in sucrose. Being a member of family Poaceae (Aitken et al. 2014), it imposes economic importance similar to their family members of forage crops as wheat, rice, maize, and sorghum (Shewry and Hey 2015). Sugarcane is native to 'temperate to tropical' regions of earth (Cheavegatti-Gianotto et al. 2011). Origin of Sugarcane is estimated to be in New Guinea in about 6000 BC, from where it was migrated to other parts of world as Asia, India etc. since 1000 BC. In 600-1400 AD, it reached to the Mediterranean. Further, by 715 AD it was distributed into other countries like Syria, Cyprus, and Spain. The crop was further spread by Portuguese, British and French West Indies.

The sugarcane is a native of India and is known from Vedic times. Sugarcane belong to the genus Saccharum originated from Sanskrit word 'sarkara' or 'sakkara' meaning sugar; which get transformed into 'sukkar' in Arabic and 'Sakharon' in Greek. It is tropical perennial grass and is believed to have originated in Indo-Burma (now Myanmar)-southern China region extending to Java (now Indonesia). The mature cane stalk stored the sugar in the parenchymatous tissue of the the internode portion. The stalk portion between the two internode is generally referred as 'node' which act as a vehicle of vegetative propagation due to the possession of bud and root

primordia. At the time of harvesting, mature cane stalk usually contain about 20-30 internode for extraction and crushing purpose. The vegetative propagation is commercially used cultivation practice for this crop; and sugarcane is planted with stalk cutting containing bud and root primordia called as 'sett'. Usually the sett may contain one, two, three or more buds but generally, a three-budded sett is preferred over other for large-scale planting. The cane stalk used for seed purpose is termed as 'seed cane'. The another important feature of this crop is that it is a ratoon or stubble crop which act as sprout and allows them to initiate another cycle of crop growth after the harvest. The sugarcane seed (a product of sexual reproduction) are importantly used by genetic breeders for giving rise to new variability and improving the status of sugarcane crop. The concept of using seed of sugarcane get arised again in the late 1880s in Java that transformed the overlook profile of sugarcane in the world. Thus, presently sugarcane industry is wholly dependent on hybrid cane varieties. Traditionally, *S. officinarum* and *S. spontaneum* have been recognized in the genus Saccharum as cultivated species and wild species respectively. The four other varieties namely; *S. barberi, S. sinense, S. robustum, S. edule* are generally considerd as natural hybrids of *S. officinarum* and *S. spontaneum.* In the present scenario, commercial species of sugarcane is designated as '*Saccharum spp*. Hybrid' due to their heterozygity, complexity in polyploidy, and hybrid nature exploiting chromosomes/gene complements from diverse but related genera and species.

2.1.2 Economic importance:

India exported sugar of one cultivar for about 250 years to west countries with the name of 'Creole' or "Cana Criolla". Now as days, Sugarcane *(Saccharum spp.)* is one of the highest economically important crops in the world. Across the world, it is cultivated on millions of hectare to produce thousand million metric tons crop.

Taxonomically sugarcane belongs to: Kingdom: Plantae, Division: Magnoliophyta, Class: Liliopsida, Order: Poales, Family: Poaceae and Genus: *Saccharum*. Genus *Saccharum* represents an enriched diverse collection of multiple species. One of the important points is that each of the species can further cross breed to each other and can produce stable diversity. Majorly Genus *Saccharum* represents six species (Cheavegatti-Gianotto et al. 2011)viz:

Table 2.1 Different species of genus *Saccharum* with their characteristic.

***Saccharum* Species**	**Remark**
S. spontaneum (Meng et al. 2019)	Wild species which are mostly found in Indonesia and Asia
S. robustum (Al-Janabi et al. 1994)	Wild species which are mostly found in Indonesia and Asia
S. officinarum (Cheavegatti-Gianotto et al. 2011)	Cultivated species. Sugar producing. It is considered as derivative of *Saccharum robustum* (D'Hont et al. 1993). The clones which are currently cultivated are derivatives of *S. officinarum* and *S. spontaneum* obtained by inter-specific hybridization during 20th century (D'Hont et al. 1998).
S. barberi (Dong et al. 2018)	Cultivated species
S. sinense (Song et al. 2016)	
S.edule (Cheavegatti-Gianotto et al. 2011)	Cultivated species. It is served as vegetable due to its aborted inflorescence (Irvine, 1999)

2.1.3 Crop Description and Climate:

Sugarcane was originated in Asia (New Guinea). Most of the commercial sugarcane is cultivated between 35°N and S of the equator. Sugarcane (*Saccharum officinarum*) is

cultivated at about 13 million hactare on commercial world ground. It produces approximately 1254.8 million ton/year cane or 55 million ton/year sucrose. (FAOSTAT, 2001). Optimum temperature for sprouting and growth is 32-38°C and 22-30°C respectively. Optimum growth is achieved with mean daily temperatures between 22 and 30°C. Sugarcane has high nitrogen and potassium needs and relatively low phosphate requirements, or 100 to 200 kg/ha N, 20 to 90 kg/ha P and 125 to 160 kg/ha K for a yield of 100 ton/ha cane.

2.1.4 Yield:

Sugar yield depends on cane quality in terms of tonnage and sugar content. Cane should be harvested at suitable moment of optimum recoverable sugar per area. Cane tonnage at harvest varies between 50 and 150 ton/ha, which depends on length of growing period and weather. Cane yields can vary greatly. Good yield in the humid tropics ranges between 70-100 ton/ha, while in the dry tropics and subtropics it ranges between 110-150 ton/ha cane. The water utilization for harvested yield with 80% moisture is 5-8 kg/m^3, while for sucrose containing without moisture ranges between 0.6-1.0 kg/m^3. With maturity, vegetative growth reduces and sugar content increases greatly. Sugar content at harvest ranges between 10-12% of fresh weight.

2.1.5 Usage:

Besides being a cash crop, sugarcane plant is also serves as food, source of energy and feedstock for industry. Because of efficient photosynthetic mechanism it can also fix an amount of green biomass, along with high capacity of CO_2 fixation than moderate climate zone woods (Ferreira et al. 2017). Being as a good carrier of metabolic energy, it is also used for animal feeding. In addition to this, more than 50 commercial products and by-products are widely used in alcohol synthesis, fertilizer

and livestock feed (Bušić et al. 2018). Besides being an affluent solar energy reservoir, its bagasse is burnt as fuel for production of steam and electricity. Bagasse is extensively used for the production of pulp and paper (Rezende et al. 2011).

2.2 Abiotic and biotic stresses:

Plants are sessile organism and have necessarily evolved various strategies to cope with changing and sometimes extreme environments while meeting the resources demands to grow and complete their life cycle. Sugarcane is a unique crop with the ability to accumulate high levels of sugar and is a commercial viable source of biomass for bioelectricity and second generation bioethanol (Tapia and Simone 2019). The abiotic stresses that plant is exposed to include: drought, heat, salinity, ultraviolet light, flooding, gaseous pollution, freezing and heavy metals (He et al. 2018). In general, biotic stress can be understood as stress / damage pounded to an organism by other organisms belonging to micro or macro biota (Pandey et al. 2017). These organisms may be bacteria, fungi, virus, insects etc. The types and extent of biotic stresses are cumulative representation of environment of organism as well as the capacity of host to resist stresses. Since biotic stresses and plant yield are directly linked with economic decisions, therefore biotic stresses are the major concern of agricultural research, since it causes huge economic losses to cash crops (Pandey et al. 2017). Biotic stress also impacts horticultural plant health and natural habitats ecology (Mittler 2006). It also has dramatic changes in the host recipient. Conclusively, plants are exposed to many biotic and abiotic stress factors, such as drought, high salinity or pathogens, which reduce the yield of the cultivated plants or affect the quality of the harvested products. Although there are many kinds of biotic stress, the majority of the plant diseases are caused by the fungi. Following is the list of selected diseases causing biotic stress:

2.2.1 Bacterial Diseases:

Ratoon stunting (*Clavibacter xyli* subsp *xyli*) (Brumbleyet al. 2006), Leaf scald (*Xanthomonas albilineans*) (Rott et al.1995), Gumming (*Xanthomonascampestris*) (Peros and Lombard 1992), Mottled stripe (*Pseudomonas rubrisubalbicans*) (Olivares et al. 1997), Red stripe (*Pseudomonas avenae*) (Shan et al. 2017).

2.2.2 Fungal Diseases:

Black rot (*Ceratocystis adiposa*) (Hubert et al. 2014), Black stripe (*Cercospora atrofiliformis*), Brown spot (*Cercospora longipes*) (Hsien, 1980), Downy mildew (*Peronosclerospora sacchari*) (Malein 1993), Eye spot (*Bipolaris sacchari*), Leaf blast (*Didymosphenia taiwanensis*), Leaf blight (*s*), Leaf scorch (*Stagonospora sacchari*), Pineapple disease (*Ceratocystis paradoxa*) (Hubert et al. 2014), Pokkah boeing (*Gibberella fujikuroi*) (Hsuan et al. 2011), Red leaf spot (*Dimeriella sacchari*), Red rot (*Glomerella tucumanensis*), Red spot of leaf (*Mycovellosiella vaginae*), Shoot rot (*Rhizoctonia solani*), Ring spot (*Leptosphaeria sacchari*), Root rots (*Marasmius sacchari*), Schizophyllum rot (*Schizophyllum commune*), Sclerophthora disease (*Sclerophthora macrospora*), Seedling blight (*Alternaria alternata*), Sheath rot (*Cytospora sacchari*), Yellow spot (*Mycovellosiella koepkei*) (Aljanabi et al. 2007).

2.2.3 Nematode parasitic diseases:

Lesion (*Pratylenchus sp*.), Root-knot (*Meloidogyne sp*.) (Ye et al. 2019), Spiral (*Helicotylenchus sp*.) (Yan et al. 2017).

2.2.4 Viral Disease:

Chlorotic streak (Virus putative) (Shepherd et al. 2010), Dwarf (Sugarcane dwarf virus) (Kannan et al. 2018), Fiji disease (Sugarcane Fiji disease virus) (Dhileepan and

Croft, 2003), Mosaic (Sugarcane mosaic virus), Streak (Maize streak virus) (Shepherd et al. 2010).

2.3 Red rot disease of sugarcane:

Red rot disease of sugarcane is a chronic and widely distributed fungal disease that is known to cause devastating reduction in crop yield (Mohanraj et al. 2003). It was described by Went in 1893 (Sharma and Tamta 2015). It is caused by *Colletotrichum falcatum.* Red rot disease is originally named as red smut in 1893. After causing severe losses by virulent form of red rot in India and Nigeria in 1932-1942 and 1951 respectively, the disease has been recognized as of international importance.

C. falcatum is causal agent of sugarcane red rot (Bukhari et al. 2012). Conidial stage of red rot fungus as *C. falcatum* was firstly described by Went in 1893 which belongs to division Eumycota, subdivision Deuteromycotina, class Coelomycetes, order Sphaeropsidales and family Sphaeropsidaceae. Early detection of red rot is a difficult task. First symptom is seen on leaf with cease of plant growth. Leaves become discolored, leading to the withering of the entire tip at the initial stage. With time, infection is visualized as dark reddish area on the leaf midrib which elongates rapidly. Older lesions bear dull grey color with accumulation of conidial powdery mass of conidia. The pathogens enter the host tissue and rapidly produces septate mycelium (inter and intracellular) which changes the colour of host protoplasm. A gummy dark red material is ooze out, which connect the intercellular spaces of the cells. The typical red rot appearance comes due to absorption of soluble red pigment by cell walls. Finally, the hyphae of the fungus produce chlamydospores, which can sustain in soil for longer time. In favorable environmental condition, small black dot like bodies appear at the surface of host. These bodies are called conidia. The conidia are

20-38 µm long and 5-7 µm wide sickle shaped large oil globule, arranged in rounded or elongated acervuli. These are found at conidiophores (Patel et al. 2019).

Primarly, infected cane setts carry the infection to the field. If setts are infected and sufficient inoculum is present, it causes both pre and post emergence death of the sprouts during April to June. Once the crop cycle get completed; pathogen get established itself in the soil, it makes an entry to the plants from soil and finally extends its way to the stalk by the various way (Patel 2019). Although the cycle of *C. falcatum* gets completed on leaf; however, infection on leaves may not affect overall yield to a great extent but the attack on the stalk causing serious damage to the crop and its yield. Expression of the disease may vary depending upon nature of infection and prevailing environmental conditions. Typical symptoms in standing crops are seen during monsoon season. External stalk symptoms includes the discolouration of the rind by the appearance of brown stripes externally and internally causes the reddening of internal tissues with the prevalence of white spots. The reddening of the cane is probably due to the accumulation of phenolic compound, as pathogen converts the sucrose of cane into alcohol by the help of invertase enzyme, along with the sourly alcoholic smell. Similarly, early symptoms on leaf during pre-monsoon period are expressed as dark brown coloured continuous lesions with heavy sporulation on both the surfaces of the mid-rib of unfolded leaf spindle and old leaf sheaths. As the lesion size gets increase with age, ashy gray centre appears on the mid-rib of the leaf alongwith elongated red lesions.

Due to high diversity in virulence, novel physiological races are reported from different parts of the world. Each pathotypes are identified on the basis of parameters related with morphology, physiology and host reaction. For example, some strains for sugarcane red rot are characterized by hydrolytic and polyphenol oxidase enzymes

(Srinivasan, 1969). Besides these, different virulent pathotypes of *C. falcatum* are also being differentiated by molecular techniques based diagnostic kit etc (Narayanasamy, 2008; Malathi and Viswanathan, 2012.). Besides sugarcane, other hosts as sorghum, Johnson grass, common grass *(Liptochloa flliformis)* and *Miscanthus* also bear some stages of *C. falcatum*. But the role of alternate hosts is negligibly explained (Patel et al. 2019).

Pathogen spreading may be due to many reasons as: *C. falcatum* can produce acervuli by growing saprophytically in soil. The active stages of pathogen lie for 3-4 months, which is sufficient to carry over the disease to the new crop. Secondary infection is also possible with acervuli through insects, water and wind. Chlamydospores and perithecial stages are also suitable for long term survival of pathogen in soil. Beyond these, sugarcane setts are the chief source of spread of live disease, those give rise to infected shoots appear in the field. Conidial penetration into injured tissue of host bears multiple reasons of infection through ploughing, hoeing and earthing up. If we see the disease cycle, it can be observed that during growth of sugarcane, midrib infection is main inoculums for dissemination of disease. Spores are spread by wind, rain water etc. Direct mycelia extension from setts to canes is most common way of spread. Red rot fungus is not reported for any other disease.

Extent of damage caused by red rot can be seen as: killing of photosynthesis area, death of new tissue generating tops of plant, reduction of sucrose quantity and reduction in field stands. Due to it high end of damage, multiple aspects of disease control had been applied.

2.4 Management practices of red rot disease:

Various strategies have already been adopted as management practices of red rot. The important practices belong to (i) agronomic and cultural measures, (ii) heat therapy, (iii) chemical control and (iv) use of resistant varieties.

2.4.1 Agronomic and cultural measure:

Red rot is minimized by crop rotation. Mono cultivation supports in building up of inoculums for disease development. Therefore crop rotation is encouraged. As agronomy practices, diseased parts of plant are burnt. Sanitation of the field and development of hygienic cultivation conditions are of important agronomy practices. Therefore, the practice of cultivation of same variety is discouraged.

2.4.2 Heat therapy:

Heat therapy is related with elimination of diseased inoculums. Here to eradicate red rot inoculums from seed material, sugarcane sett is treated with 52°C hot water for 18 minute or moist hot air.

2.4.3 Resistant varieties

Many red rot resistance varieties as *Saccharum spontaneum* are used as the best mean to control disease. The chief source of resistance varieties are various sugarcane breeding institutes worldwide.

2.4.4. Chemical control:

Initially fungicide chemicals are used to eradicate the red rot disease. These chemicals are dedicated to the specific stage of pathogen life cycle. For example: Aretan and Agallol were used for eradication of superficial inoculums. Thiophanate methyl is

specific against the red rot pathogen *C. falcatum*. In general, fungicides are not used for sett treatment. Repeated use of fungicide causes resistance in pathogen. Considering all the above mentioned constraints, disease control by use of chemicals is not adoptive. Therefore non-chemical method is implemented for long term treatment of sugarcane red rot. Non-chemical method adopts microorganisms and other natural components for disease control. Natural organism may include bacteria, fungi, insects, nematodes, rodents and weeds. These biological methods exploit natural organisms for fighting against red rot disease.

2.4.5 Biocontrol agents:

An ideal biocontrol agent can be featured with (i) sustainability in soil for longer time, (ii) accessibility to pathogen, (iii) safe to health, (iv) economical mass multiplication and (v) capable to interfere with the life cycle of pathogens. Although various organisms can be employed for biocontrol, but preferential are those organisms which directly influence growth and survival of host plant. Such organisms are plant growth promoting fungi (PGPF) and plant growth promoting rhizobacteria (PGPR). In addition to antagonistic activity, rhizobacteria produces phyto-hormone which helps in plant growth. Rhizobacteria also mobilizes insoluble nutrients from soil. List of endophytes related with sugarcane has been tabulated in Table 2.2.

2.5 Plant growth-promoting rhizobacteria (PGPR): Their potential as biocontrol agents

2.5.1 Plant Growth Promoting Rhizobacteria (PGPR):

Rhizosphere is nutrient rich narrow zone of soil of the root system (Beneduzi et al. 2012). A high density of microorganisms is found to be colonizing in rhizopsheric

zone. Reason behind it is accumulation of amino acids and sugars as plant exudates. This accumulation becomes rich source of energy for bacterial growth. Depending on the effects on plant growth, these rhizospheric bacteria may be classified as beneficial, deleterious or neutral to plant (Dobbelaere et al.2003). Beneficial free-living soil bacteria are usually promotes the plant growth (Kloepper et al. 1989). Out of diversified bacteria as PGPR, majority are *Bacillus* sp. and *Pseudomonas* sp.(Podile and Kishore 2006). PGPRs affect plant growth in two ways: (i) by direct promotion and (ii) indirect promotion. In direct benefit, plant utilizes compounds synthesized by bacteria. While in indirect benefit, plant is prevented by deleterious effects of biotic stress. Here PGPR acts as biocontrol agents. These biocontrol agents either produce antagonistic substances or induce resistance to pathogens (Glick, 1995).

2.5.2 PGPR as biocontrol agent:

Biocontrol agents are those rhizobacteria that reduce the incidence or severity of plant diseases (Beattie 2007). While those exhibit antagonistic activity are antagonists. The bacterial antagonistic activities can be highlighted as: (1) synthesis of hydrolytic enzymes, such as chitinases, glucanases, proteases, and lipases, that can lyse pathogenic fungal cells (Neeraja et al. 2010; Maksimov et al. 2011.) (2) competition for nutrients and suitable colonization of niches at the root surface (Kamilova et al. 2005), (3) regulation of plant ethylene levels through the ACC-deaminase enzyme, which can act to modulate the level of ethylene in a plant in response to stress imposed by the infection (Bernard et al. 1997; Van Loon 2007), and (4) production of siderophores and antibiotics. **Induction of resistance (ISR and SAR) in host:** Resistance in host has been classified assystemic acquired resistance (SAR) and 'Induced Systemic Resistance' (ISR). ISR was first reported in 1991 by Wei *et al* (Wei 1996) in two plants carnation and cucumber. Carnation developed ISR against

F. oxysporum f. sp. dianthi by rhizobacteria named *P. fluorescens* strain WCS417r; while cucumber developed ISR against *Colletotrichum orbiculare*. In this way non-pathogenic rhizobacteria performed as biocontrol agents. Rhizobacteria-mediated ISR is similar to pathogen induced systemic acquired resistance (SAR) in that both provide resistance to the uninfected part of plant (Van Wees et al. 1997; Van Loon 2007). But ISR and SAR act through two different signaling processes. SAR is induced via salicylic acid (SA) and ISR through jasmonic acid (JA) and ethylene (ET) signaling (van Loon et al. 1998). ISR provide less significant protection than SAR. But combined effect of ISR and SAR is more protective against diseases (Van Wees et al. 1997). SAR and ISR can be differentiated by expression of PR-1 gene, which is a molecular marker for existence of SAR mechanism (Van Loon 2007). This molecular differentiation was shown in *Arabidopsis* by *P. syringae* (Pieterse et al. 1996) and *P. fluorescens* and *P. putida* (Van Wees et al. 1997). Impact of ISR response of certain rhizobacteria was seen in several species of plants (van Loon et al. 1998). Some interaction specificity was also observed between rhizobacteria and plants (Van Loon 2007).

2.6 Endophytes

Definition:

Bacteria that live and thrive inside their host plant are called endophytic bacteria. Such living associations among members ecosystem are found in natural environments. Microorganisms can develop efficient beneficial associations with plants (Lodewyckx et al. 2002; Ding et al. 2011). Such association provides benefits to host plants for tolerating biotic and abiotic stresses (Pandey et al. 2017).

Occurrence:

Endophytes are a subclass of rhizobacteria, which acquire specialized ability to invade the host plant (McDowell and Dangl 2000). Endophytes share traits for growth of host plant found in rhizobacteria. Endophytes provide additional beneficial effects to host plant. Such effects exacerbate under the stressed conditions (Cheng et al. 2018).

Origin:

Endophyte term was first coined by De Barry (1866). Earlier, endophytes were described in 1926, as specific growth stage of bacteria (Katznelson et al. 1948). Now endophytes are described as the bacteria isolated from plant tissues and who do not impose harm to host.

Rhizospheric root have been established as the main entry point of the potential endophytes from soil and provide a base camp for colonization of other plant organs. Therefore, another theory states that endophytic bacteria can be considered as a subset of rhizosphere/root-associated bacteria which get enter inside the plant either by "active pathway" through wound after secreting some cell-wall degrading enzyme or by "passive pathway" through a natural opening such as root hair and epidermal conjunction; thus, colonize the internal system of plants and function as endophyte (Sharrock 1991; Kluepfel 1993; Hallmann et al. 1997; Germida et al. 1998; Marquez-Santacruz et al. 2010). Structure of the endophytic community is defined by abiotic and biotic factors such as environmental conditions, microbe–microbe interactions, and plant–microbe interactions (Ryan et al. 2008). Diverse effects of endophytic bacteria on plant health and growth have been well documented. The endophytes aid nutrient availability and uptake, enhance stress tolerance, and provide disease resistance (Hamilton et al. 2012; Ryan et al. 2008).

Life style: Ecological niche of these bacteria are plant endosphere as well as soil, which represents biphasic life cycle (Pandey et al. 2017).

2.7 Direct and Indirect mechanisms for growth promotion:

Host plant avails direct or indirect benefits of association with endophytes. Direct benefits include: nutrition acquisition and modulation of growth hormones. Indirect benefits include: production of antibiotic and lytic enzymes which discourges the phytopathogens and priming the defense mechanism which protects the host plant from future attack of pathogens.

2.7.1 Nutrient acquisition is required due to the scarcity of necessary nutrient compounds. Endophytic increases the quantity of limited nutrients as: nitrogen, iron, and phosphorus (Glick 1995).

2.7.1.1 Nitrogen availability*:* Endophytes express nitrogenase activity which finally concludes into supply of fixed nitrogen to host plant (Olivares et al. 1997). It has been observed that endospheric endophytes fix nitrogen more efficiently than rhizosphere microorganisms. This efficient capacity of nitrogen fixation comes due to high rate of fixation. *Paenibacillus* strain P22 is found in tree, which contributes to nitrogen pool of plant (Dong et al. 2018).

2.7.1.2 Phosphorus availability*:* Soil phosphorus is insoluble, so plants can't use it directely. Endophytic bacteria solubilize precipitated phosphates through various mechanisms (acidification, chelation, and ion exchange) to make the phosphorus available to plant. Endophytes secrete acid phosphatase which mineralizes organic phosphorus. Endophytes also has role in adsorbtion of soluble phosphates (Podile and Kishore 2006).

2.7.1.3 Iron availability*:* Iron is important for various physiological processes. It occurs in insoluble ferric forms (carbonates, hydroxides, oxides and phosphates), which makes iron unavailable to the plant. Endophytes produce siderophores which perform iron chelation to make iron available to plant by chelate degradation or ligand exchange (Patel et al. 2019).

2.7.2 Phytohormone production and modulation:

Endophytes produce growth regulating phytohormones, which affects nutrient accumulation and metabolism due to endophytic colonization (Maksimov et al. 2011). Five major plant hormones which govern plant-endophyte interaction include: abscisic acid, cytokinins, ethylene, gibberellins and indole-3-acetic acid (IAA).

2.7.2.1 Modulating plant IAA level*:* IAA affects numerous physiological processes of host including: cell-cell interaction, regulation of plant growth, and elicitation of defense systems. Endophytes improve biomass and surface area by origination of lateral roots through IAA production. Lower amount of IAA induces growth of root, while overproduction caused shorter root (Patel et al. 2019).

2.7.2.2 Control of ethylene levels*:* Ethylene controls the responses against the act of stresses (abiotic and biotic). It controls processes like nodulation and initiation of root, leaf senescence and abscission of leaf, elongation of cells, ripening and auxin transport. Ethylene production increases with elevation of stress. Endophytic bacteria hydrolyse 1-aminocyclopropane-1-carboxylate (ACC), which is a precursor of ethylene. Endophytes can cleave ACC into α-ketobutyrate and ammonia, and provide a source of nitrogen. In this way, hydrolysis of ACC release plant stress motivates the improvement of plant growth under challenged condition (Kim and An 2002; Ferreira et al. 2017).

2.7.2.3 Production of plant cytokinins and gibberellins*:* Endophytic bacteria can produce cytokinins and gibberellins. Like ethylene, Gibberellin also alleviates stress. More research is required to elucidate the role of endobacterial produced gibberellins and cytokinins in relation of bacteria and plant (Cheng et al. 2018).

2.7.3 Indirect growth promotion by suppression of phytopathogens:

Suppression of pathogen by endophytes indirectly supports the plant growth. Endophytes produce antagonizing substances. These substances perform in multiple ways like: (i) inhibition of pathogens by antibiotics, (ii) kills the microorganism by toxins, (iii) removes micro elements for growth inhibition e.g. siderophore, lytic activity by hydrolytic enzymes, negative induction by volatile compounds (Podile and Kishore 2006). Indirect effect by endophyte can target both bacterial and fungal pathogens (Lodewyckx et al. 2002). *Actinobacteria, Bacillus, Paenibacillus, Pseudomonas* and *Serratia* are the commonly described endophyte groups expressing indirect impact on host plants. Endophytes produce cell-wall targeting enzymes (chitinase, proteases and glucanases) for antifungal activity (Maksimov et al. 2011). Plant beneficial endophytes use induced systemic resistance (ISR) mechanism to protect host from phytopathogens. Endophytes (e.g. *Bacillus, Pseudomonas* and *Serratia*) initialize ISR using salicylic acid, jasmonic acid and ethylene using interconnected signaling pathway (Beneduzi et al. 2012).

2.8 Multi-niche adaptation to endophytic lifestyle:

Endophytes need to adapt multi-niche plant-associated lifestyle, in which different metabolites are required for survival. It is evidenced that comparatively higher number of metabolite-encoding gene clusters were found in pseudomonads (*P. syringae* and *P. fluorescens*) than free living *P. putida* strains. Beyond this, single

niche colonizing endophytes produces low number of metabolites (Kamilova et al. 2005; Meziane et al. 2005; Malathi and Viswanathan 2012).

2.9 Colonization

Endophytic bacteria are a subset of rhizospheric bacteria set. In other words, endophytic bacteria are special class of rhizobacteria having ability to invade in plant tissue after foundation of rhizospheric bacterial population. Endophytic bacteria live within the plant tissue therefore show added advantage over growth promoting rhizobacteria. Endophytes show symbiotic relation by exerting direct benefit as return for supply of nutrients. Endophytic colonization to plants is a process of establishment of communication between bacteria and host plant. This communication is regulated by colonization traits, which include compounds provided by endophytes into root. This colonization process by endophytes occurs in similar pattern as rhizobacteria. However, endophytes use multiple factors (environmental and genetic) to enter into endosphere of plant. After entering into root, they infect adjacent tissues followed by aerial parts of plant (Van Wees et al. 1997; De Boeret al. 2005; Podile and Kishore 2006; Li et al. 2012).

2.9.1 Rhizospheric colonization:

The colonization process is a competitive task to occupy space and get nutrients. Those rhizobacteria which get succeeded to impose beneficial impact on plant growth, become successful in colonization into rhizosphere. Motility and production of polysaccharides are important bacterial traits which are required for colonization. Colonization process involves sequential steps. After inoculation into soil, bacterial cells attach to root surface and form string of linked bacteria, which progressively develop into biofilm or micro-colonies. Rhizosphere population covers 10^7-109 cfu/g

of rhizospheric area. To impose the beneficial effects on host, endophytes must compete with other rhizospheric bacteria. This is the reason colonization does not occur in uniform manner. It depends on distribution and density of bacteria into rhizoshere. This non-uniform colonization also includes other factors which control colonization as: pattern of exudation, attachment to surface, motility, growth rate, antagonistic substances and availability of nutrients. During the colonization process, endophytes show metabolical adaptation according to the nutrients into host (Harman et al. 2004).

2.9.2 Root colonization:

After being establish into rhizosphere, endophytes proceed to enter inside the plant root. First step to enter into root is adhesion to cell surface, which is supported by polysaccharides, mobility by pili and bacterial adhesins. Endophytes follow their own specific colonization pattern as well as colonization site. Root penetration process may be passive or active. Passive penetration uses already existing cracks to enter into host, while active penetration exploit dedicated machinery with lipopolysaccharides, flagella, pili, etc. (Podile and Kishore, 2006).

2.9.3 Systemic colonization:

After penetration inside roots, endophytes spread themselves into other systemic tissues of stem and leaf etc. But the beneficial effect to host plant may differ from root colonization. Due to the physiological constraints, very few bacteria can establish systemic colonization into aerial parts of host. Therefore, the systemic colonizing endophytes were found to be more adaptive niche. Intercellular migration requires cell-wall degrading enzymes (Weller et al. 2012). However, long pores of xylem allow endophytes without any cellular damage. To reach at final sink of leaf tissue,

endophytes perform in similar pattern as phytopathogenic bacteria by entering through leaf stomata (Van Wees et al. 1997).

2.10 Endophytes as a source of secondary metabolites:

Bacteria and fungus both have been reported for production of metabolites as Endophytes. Endophytes bacteria being colonize into rhizosphere, also detected inside stems, leaves and inside of reproductive organs of plant. Endophytes favor their niche due to adaptation capability towards host plant. Adaption also involves competition for resource-rich and predator-rich environment and arsenal of metabolites for defense purpose. Obligate endophytic bacteria face lesser competition due to production of specific metabolites which can be used for interaction with the host as well as in support to host. Many facultative colonizers compete in the rhizosphere before entering the plant and therefore are well-equipped with arsenal of metabolites. This makes them good in communication and defense. Many high capacity rhizobacteria switches from root surface to inside tissue to survive as endophytes. *Bacillus* and *Pseudomonas* are the good examples of such endophytes. Such endophytes are characterized with metabolites as lipopeptides, which are efficient in antibiosis as well as inducing defense mechanisms in plant. *Streptomyces* sp. HKI0595 was found with multicyclic indolosesquiterpenes in mangrove tree *Kandelia candel*. *Streptosporangium oxazolinicum* K07-0450T produces spoxazomicins A-C in orchids. Endophytes of Chinese medicinal plants are reported for *Pseudomonas aeruginosa* generated siderophores and uncharacterized metabolites with gene cluster with NRPS (non-ribosomal peptide synthases) and PKS (polyketide synthases). Endophytes contribute in two ways: (i) by influencing growth and stress tolerance by single bacteria; and (ii) metabolite production in combination with associated bacteria. Single bacteria based influence is wide spread in aromatic and medicinal

plants. For example, *Pseudonocardia* sp. (YIM 63111) enhances production of artemisinin. While in association example, flavor of strawberries are the result of furanoids along with plant-associated methylobacteria. Similarly, in South African Rubiaceae, polyamine pavettamine is produced by bilateral biosynthesis.

2.11 Metabolites for communication between plant and bacteria:

To fulfill nutrient uptake, many endophytic bacteria interact with plants through secondary metabolites. Involvement of siderophore by *Herbaspirillum seropedicae* for attraction of iron is the example for it. Besides this, metabolites are also reported for bio-film formation, as virulence factors and interfering hormone signaling in plants; although these features of metabolites, don't provide clear demarcation between endophytes and pathogens.

2.12 Genome reduction:

Although genome reduction is property of intracellular pathogens, but it was also observed with obligate endophytes than free living relatives. *Candidatus Burkholderia kirkii* is the best example of reduced genome endophyte. It protects its host plant *Psychotria kirkii* from pathogens and herbivores.

2.13 Why discovery of novel biocontrol agent (endophyte) is tough? :

Indications for possibility of wide range of metabolites were high but methods to dig out the un-described metabolites are unexplored. Reason behind this challenge comes because of requirement of identification of metabolites has constraint of natural niche as well as specific pathogenic circumstances. While under *in-vitro* condition, only partial realization of metabolic potential is possible. But now with genomic

revolution, endophytic bacterial genomes are now being used to explore the existence of novel metabolic compounds.

Table 2.2. Some common endophytic bacterial genera in reference of their agronomic host of colonization (adapted from Afzal et al. 2019)

Plant	**Endophytic bacterial genera**
Alfalfa	*Bacillus, Erwinia, Microbacterium, Pseudomonas, Salmonella*
Banana	*Azospirillum, Burkholderia, Citrobacter, Herbaspirillum, Klebsiella*
Black pepper	*Arthrobacter, Bacillus, Curtobacterium, Micrococcus, Pseudomonas, Serratia*
Canola	*Acidovorax, Agrobacterium, Aureobacterium, Bacillus, Chryseobacterium, Cytophaga, Flavobacterium, Micrococcus, Pseudomonas, Rathayibacter,*
Carrot	*Agrobacterium, Bacillus, Klebsiella, Pseudomonas, Rhizobium, Salmonella, Staphylococcus*
Clover	*Agrobacterium, Bacillus, Methylobacterium, Pseudomonas, Rhizobium*
Cotton	*Bacillus, Burkholderia, Clavibacter, Erwinia, Phyllobacterium, Pseudomonas*
Cucumber	*Agrobacterium, Bacillus, Burkholderia, Chryseobacterium, Clavibacter, Curtobacterium, Enterobacter, Micrococcus, Paenibacillus, Phyllobacterium, Pseudomonas, Serratia, Stenotrophomonas*
Grapevine	*Comamonas, Enterobacter, Klebsiella, MoraxellaPantoea, Pseudomonas, Rahnella, Rhodococcus, Staphylococcus, Xanthomonas*
Maize	*Achromobacter, Agrobacterium, Arthrobacter, Bacillus, Burkholderia, Corynebacterium, Curtobacterium, Enterobacter, Erwinia, Herbaspirillum,MicrobacteriumMicrococcus, Paenibacillus, Phyllobacterium, Pseudomonas, Rhizobium, Serratia*
Pineapple	*Azospirillum, Burkholderia*
Potato	*Acidovorax, Acinetobacter, Actinomyces, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Capnocytophaga, Chryseobacterium, Comamonas, Corynebacterium, Curtobacterium, Enterobacter, Erwinia, Klebsiella, Leuconostoc, Methylobacterium, Micrococcus, Paenibacillus, Pantoea, Pseudomonas, Psychrobacter, Serratia,*

	Shewanella, Sphinogomonas, Stenotrophomonas, Streptomyces, Vibrio, Xanthomonas
Radish	*Proteobacteria, Salmonella*
Red clover	*Acidovorax, Agrobacterium, Arthobacter, Bacillus, Bordetella, Cellulomonas, Comamonas, Curtobacterium, Escherichia, Klebsiella, Methylobacterium, Micrococcus, Pantoea, Pasteurella, Phyllobacterium, Pseudomonas, Psychrobacter, Rhizobium, Serratia, Sphingomonas, Variovorax, Xanthomonas*
Rice (wild and cultivated)	*Agrobacterium, Azoarcus, Azorhizobium, Azospirillum, Bacillus, Bradyrhizobium, Burkholderia, Chromobacterium, Enterobacter, Herbaspirillum, Ideonella, Klebsiella, Micrococcus, Pantoea, Pseudomonas, Rhizobium, Serratia, Stenotrophomonas*
Soybean	*Erwinia, Agrobacterium, Pseudomonas, Klebsiella, Enterobacter, Pantoea, Bacillus*
Sugar cane	*Acetobacter, Gluconacetobacter, Herbaspirillum, Klebsiella*
Tomato	*Brevibacillus, Escherichia, Pseudomonas, Salmonella*
Wheat	*Bacillus, Burkholderia, Flavobacterium, Klebsiella, Microbispora, Micrococcus, Micromonospora, Mycobacterium, Nacardiodes, Rathayibacter, Streptomyces*

2.14 Metabolites:

Pathogens attack on host plant as a biotic stress. The plant defense system generates secondary metabolite to fight against pathogen attack (Grayer and Kokubun 2001). The major secondary metabolites belong to chemical classes of phenols, glucosides, glucosinolates, terpenes, non-protein amino acids, sesquiterpenoids and sterols (Bennett 1994).

2.14.1 Phenolic metabolites

Phenolic metabolites are a group of compounds constituted naturally in the plants. Phenolics are essential part of plant defense system. This essence of defense is due to

presence of 'OH' groups in structure, which creates hydrogen binding with target enzymes and significantly increases the antimicrobial activity (Puupponen-Pimiä et al. 2001). Phenolics also contribute into blocking of cell wall structures and causing spore dormancy (Nesci et al. 2007). Biosynthesis of Aflatoxin in fungus is interrupted by some of phenolic compounds as acetosyringone, syringaldehyde, and sinpinic acid.

2.14.2 Plant peptides

Plant peptides are the weapon for natural immunity (Farrokhi et al. 2008). Plant peptides show sequence homology with toxin produced by insects, honey bees, scorpions, and spider venoms. These peptides show antifungal (Hevien and Snakin) and antibacterial (cyclotides) activity. In this way these peptides in crops creates disease resistance by their antimicrobial activity.

2.14.3 Phytoalexins

Phytoalexins are the metabolites of low molecular weight and produced in response to biotic as well as stress (Kuc 1995). More than 350 phytoalexins from 30 plant families belongs to chemical family of isoprenes, flavonoids and polyacetylenes (Goodman et al.1986). Flavonoids generated due to stress are called "induced compounds", flavonoids which accomplish during normal development of plant are called "preformed flavonoids". All the Phytoalexins have common linear substrate geranylgeranyl –diphosphate which are further processed for cyclization. Phytoalexins show antifungal activity. As arvelexin and 1-methoxyspirobrassinin, acts against *Sclerotinia sclerotiorum* and *Rhizoctonia solani* in leaves of *Erucastrum gallicum* (Pedras and Ahiahonu 2004). Similarly 6,7-dimethoxycoumarin causes resistance against the mycotoxigenic fungi *Aspergillus parasiticus* and *Fusarium verticillioides* in *Citrus sinensis*. It also causes reduced production of aflatoxins

(Mohanlall and Odhav 2006). A biphenyl phytoalexin causes resistant against *Venturia inaequalis* (Hrazdina et al. 1997). Similarly flavonoids were observed to be elicited due to powdery mildew in cucumber (McNally et al. 2003).

2.15 Mechanisms involved in biocontrol:

When endophytic microorganism coexists with pathogen within the host, then a type of competitive attitude is generated within the system of these three components. This behavior turns into a form of biocontrol as cumulative result of competition. This competition can be for nutrients, niche, both for nutrient and niche, parasitism, damaging activities, killing behavior and development of resistance.

2.15.1 Antibiosis:

Beyond the growth promotion, endophytes also provide protection from pathogenic attack to host plants. This protection is favored by production of low concentration of a range of low molecular weight metabolites. Antibiosis was first reported for *Streptomyces* sp. colonized at *Rhizoctonia solani* and *Verticillium alboatrum* (Zeilinger et al. 1999). Several reports provide evidences for reinforcement of antibiosis against soil borne pathogenic bacteria. The widely used antibiotics against phytopathogens include: kanosamine, 2, 4-diacetyl phloroglucinol, Phenazine-1 - carboxylic acid, Phenazine-1-carboxamide, Pyrrolnitrin, Zwittermicin, kurskatin, kanosamine, butyrolactones, oligomycin A, oomycin A, mycosubtilin, herbicohn, bacillomycin D, fengycin, surfactin, and iturin A etc. (Podile and Kishore 2006).

i) 2,4 diacetylphloroglucinol (DAPG): 2,4-diacetylphloroglucinol (2,4-DAPG) is a polyketide phenolic compound, and has been characterized for broad spectrum antibiotic ability including performance against multidrug resistant

microorganisms. DAPG is synthesized by colonized (rhizospheric and endospheric) fluorescent pseudomonads for biocontrol activity in many agricultural crops as wheat, maize etc. (Weller et al. 2012).

ii) ***Pyoluteorin***: Pyoluteorin (PLT) is a chlorinated polyketide produced by *Pseudomonas spp*. It's antibacterial and antifungal activities is well known against *Pythium* sp. It is also perform regulation of production of secondary metabolites by rhizosphere bacteria (Kilani-Feki et al. 2010).

iii) ***Pyrrolnitrin***: Pyrrolnitrin is a chlorinated phenylpyrrole, which perform against phytopathogenic fungal activity. It is produced by *Pseudomonas* sp., *Burkholderia* sp., *Enterobacter* sp., *Myxococcus* sp. and *Serratia* sp. Pyrrolnitrin (PRN) has diffusion resistance capacity, which makes it long persistence in the soil. It is known to control *R. solani* from seedling disease caused of *P. fluorescens* BL915 (mutant strains). It shows antifungal activity against basidiomycetes, deuteromycetes and ascomycetes (Pawar et al. 2019).

iv) ***Phenanzine***: Phenazines (Phz) are a tricyclic ring and nitrogen containing compounds. Phz show wide spectrum antifungal activity. Phz is produces by *Pseudomonas, Streptomyces, Nocardia, Sorangium, Brevihacteriu, Pantoea and Burkholderia* species. Phenazine derivatives (pyocyanine, phenazine-1-carboxamide and phenazine1-carboxylic acid) are also known for their efficient antifungal activity. Single endophyte (e.g. *P. aeruginosa*) can simultaneously produce multiple derivatives of Phz (Chin-A-Woeng et al. 2003; Kilani-Feki et al. 2010).

v) ***Surfactin***: Surfactin is a cyclic lipoheptapeptide. It performs as biotensides (reducing the surface tension of water), antibacterial, antitumoral, and antiviral

agent. As antibiotic activity it alters the cell membrane by interacting with divalent cations. It is interacts with phospholipids and behaves like detergent to disrupt the cell (Jasim et al. 2016 a, b).

vi) Iturins: Similar to surfactin, Iturins are also cyclic lipopeptides, but it differs in its lipidic part by containing β-amino fatty acid. Important examples of iturin group members are iturin A-E, bacillomycin D, F, L, and Mycosubtilin. Iturin performs as strong antifungal metabolite (Jasim et al. 2016 a,b).

vii) Zwittermicin A: Zwittermicin A is a novel metabolite from several *Bacillus* sp. It is known for antifungal, insecticidal (against *B. thuringiensis*) activities (Kevany et al. 2009).

2.15.2 Competition:

i) Competition for niche: For example: Ice nucleation protein favors bacteria to promote nucleation of ice at the cell surface, which causes frost damage in host. *Pseudomonas syringae* is an example to create leaf frost damage. It is reported that mutant (-ve nucleation protein) rule over the pathogenic wild type strain in completion of niche (Lindow, 1983; Chin-A-Woeng et al. 2003).

ii) Competition for energy source: Capability to exploit energy sources, in competition of other microorganisms, is an important aspect of any biocontrol agents in rhizosphere. Biocontrol agents bear superior capacity to utilize organic acids for survival in rhizopheric soil (Goddard et al. 2001). Similarly, utilization of inorganic compounds in competition of pathogen is another potential aspect to survive as biocontrol agent. As inorganic nutrient constituent, iron is essential for fungal growth. Many rhizobacteria releases iron chelating named siderophores, which favors

biocontrol bacteria in an iron competitive environment (Whipps 2001). Iron is transported into cell as Ferric-siderophore complexes bound to the surface receptors on organisms. PGPR as biocontrol agent has specific capacity to draw iron from the heterologous siderophores of their competitors (Whipps 2001; Lodewyckx et al. 2002).

2.15.3 Parasitism:

Parasitism is behavior of attainment of growth by feeding on other microorganism. Biocontrol agents also bear this capability. *Stephanoma phaeospora* (Hoch 1978), *Gliocephaiis hyaline* (Jacobs et al. 2005) and *Trichoderma* (Steyaert et al. 2003) are known for mycoparasitism, where microorganism feed on fungi. Fungal parasites adopt biotrophic strategy to obtain nutrients from host (Barnett and Binder 1973). For example, *Trichoderma* wrap around host fungus and uses hydrolyzing enzymes to penetrate the cell wall (Zeilinger et al. 1999). It also blocks re-synthesis of host cell wall. Similarly, bacterial mycophagy is also reported (De Boer et al. 2005). For example *Paenibacillus* sp. in mycorrhizosphere of sorghum bicolor target the fungus *Fusarium oxysporum* (Budi et al. 2000). Fungicidal activities have been reported for a variety of taxonomically defined actinomycetes, p-proteobacteria, bacilli and myxobactcria (De Boer et al. 2005).

2.15.4 Mechanical damage of cell:

Degradation of cell wall as mechanism for biocontrol is supported by many evidences. Degradation causing lytic enzymes is produced by endophytic microorganisms, which depolymerise the cell infrastructure building components as chitin, cellulose and hemicellulose etc. β -1, 3-glucanase, cellulases, chitinases and proteases are some of the hydrolytic enzymes produced by biocontrol agents (Chin-A-

Woeng et al. 2003). For example, *P. anomala* releases β -1, 3-glucanase to acts against *Botrytis cinerea* on apples. This enzyme is also reported to inhibit the pathogen *in vitro* (Grevesse et al. 2003). Furthermore, biocontrol capability of *Lysobacter* is associated with β-1, 3-glucanase (Palumbo et al. 2005). This enzyme from *Trichoderma* sp. inhibits pathogens (*R. solani, B. cinerea* and *Phytophthora citrophthora*) in synergistic cooperation with antibiotics and chitinases (Howell 2003; Harman et al. 2004). Similarly, β-1, 3 linkages breaking lytic enzyme Glucanase indirectly suppress pathogen. Such lytic behavior also supports in induction of plant host defense. Oligosaccharides are another example of inducer from lysis of cell wall.

2.15.5 Inhibition of cytochrome C oxidase and metallo enzymes:

Many biocontrol agents inhibit cytochrome C oxidase and metallo enzymes by cyanogenesis process. In this process microorganism produces cyanide by oxidation of glycine amino acid through HCN synthase enzyme. Many studies stand in supporting of cyanogenesis for biocontrol. *P. fluorescens* suppresses *Thielaviopsis basicola* which causes root rot in tobacco (Voisard et al. 1989; Laville et al. 1998). Similarly *P. putida* suppresses the wheat pathogens. Cyanides are absorbed through surface and acts in support of metal ions (Rennert and Mansfeldt 2002).

2.16 Host response:

Induction of resistance in plant, due to biotic / abiotic stresses, is a state of enhanced level of defense mechanism. Such resistance has been proven for many organisms as fungi, viruses, bacteria, nematodes etc (van Loon et al. 1998; McDowell and Dangl 2000; Vallad Walling 2000 and Goodman 2004). Resistance is classified as Systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Bakker et al. 2003; Vallad and Goodman 2004). This classification is based on signal transduction

pathway and nature of elicitor (Chin-A-Woeng et al. 2003; Vallad and Goodman 2004). Studies had proved that plant induced resistance is usually non-specific to pathogen; therefore resistance based biocontrol acts at wide spectrum of plant. **Systemic acquired resistance (SAR)**: it is sustainable type of resistance induced by chemical or biological elicitor and develops at a distal site from infection. Biological inducers may be microbes, which chemical inducers may include salicylic acid, 2,6-dichloro-isonicotinic acid (INA) or benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH). **Induced systemic resistance (ISR)** is stimulated by non-pathogenic rhizosphere microbes. It is suitable for biocontrol of both soil borne as well as aerial plant diseases (Kloepper et al. 1989). Unlike SAR, no accumulation of pathogenic elicitator or salicylic acid is involved. ISR mechanism pathways are regulated by jasmonate and ethylene (Yan et al. 2002). For example: *Pseudomonas fluorescence* releases 2,4 diacetylphloroglucinol to induce ISR in arabidopsis (Weller et al. 2012); *P. putida* releases siderophores and lipo-polysaccharide to generate ISR in arabidopsis, bean and tomato (Meziane et al. 2005); *Bacillus amyloliquefaciens* and *Bacillus subtilis* strains releases volatile compound 2, 3-butane diol to trigger ISR in Arabidopsis (Ryu et al. 2004); Third type of mechanism for ISR induction was found by 3-amino butyric acid (BABA), which remain active when other elicidators like salicylic acid, methyl jasmonate, and ethylene become fail to induce ISR (Zhang et al. 2001) and a new type of elicitor named N-acylhomoserine lactones may follow some alternative mechanism, it is reported from Gram(-ve) bacteria (Schuhegger et al. 2006). *H. seropedicae* colonises into root of rice (Olivares et al. 1997). As response to endophyte colonization, plant respond by biosynthesis of phytosiderophores, and upregulation of S-adenosylmethionine synthetase, methylthioribose kinase and acireductone dioxygenase. Development of siderophore, is the response of plant in

deficiency of iron. Enhancement of antioxidant activity is another impact of production of siderophores and hydrolytic enzymes (Afzal et al. 2019).

2.16.1 Small molecules involved in host response:

i) Salicylic acid (a component of phenyl propanoid pathway) is plant messenger, which come in existance as respose of resistance due to colonization of endophytes (Beneduzi et al. 2012). Colonization may cause mechanical damage of cell. Plant response for any such damage due to endophytes is represneted by jasmonic acid (a product of lipoxygenase pathway) (van Loon et al. 1998; Kloepper et al.2004; Beneduzi et al. 2012). As a part of defense response, host plant inform about endophyte colonization to the neighboring plants by communicating through release of ethylene (gaseous hormone) from leaf tissue. Response by volatile components were also observed in Arabidopsis, *Glycine max*, and C6 plants (Podile and Kishore, 2006). Besides these, proteinase inhibitors, polyphenol oxidases, and phytoalexin are also observed as deference response by plant(Kuc, 1995; Pedras and Ahiahonu, 2004).

As ISR response; phenols, peroxidase (POX), phenylalanine ammonia-lyase (PAL), polyphenoloxidase (PPO), β−1, 3-glucanase and chitinase are expressed. They work as first line of defense during response of ISR due to endophytic colonization (Srinivasan 1969; Hrazdina et al. 1997; Zeilinger et al. 1999; Grevesse et al. 2003).

ii) Phenylalanine:Phenylalanine ammonia-lyase (PAL) is a marker biochemical which is produced as response of Induced systemic resistance (ISR). PAL imposes beneficial effect of enhancing crop productivity by increasing protein content. PAL and lignin is used in sysnthesis of phytoalexins and phenolic compounds. This synthesis is catalysed by enzymes peroxidase (POX), polyphenol oxidase (PPO).

iii) POX (Peroxidases): Peroxidases (POX) are another marker biochemcals which came in existence due to ISR response of plant. POX is also a defense gene product which catalyses synthesis of phenolics and phytoalexins for biosynthesis of lignin. Role of POX and chitinases in ISR response have been described. Co-synergistic effect of improvement of Salicylic acid on *Penicillium resedanum* was also observed in abiotic stress like osmotic stress (Srinivasan 1969; Hrazdina et al. 1997; Zeilinger et al. 1999; Grevesse et al. 2003).

iv) Polyphenyl Oxidase (PPO): Similar to PAL and PO, elevated level PPO is also an marker biochemical as response of plant ISR. It also perform as defense gene product which elicitate the for disease suppression. PPO has ability to oxidise phenols into quinones. PPO is responsible to reinforncement of total enviornment of cell barriers by production of phenolic compounds during endophytic colonization (Srinivasan 1969; Hrazdina et al. 1997; Zeilinger et al. 1999; Grevesse et al. 2003).

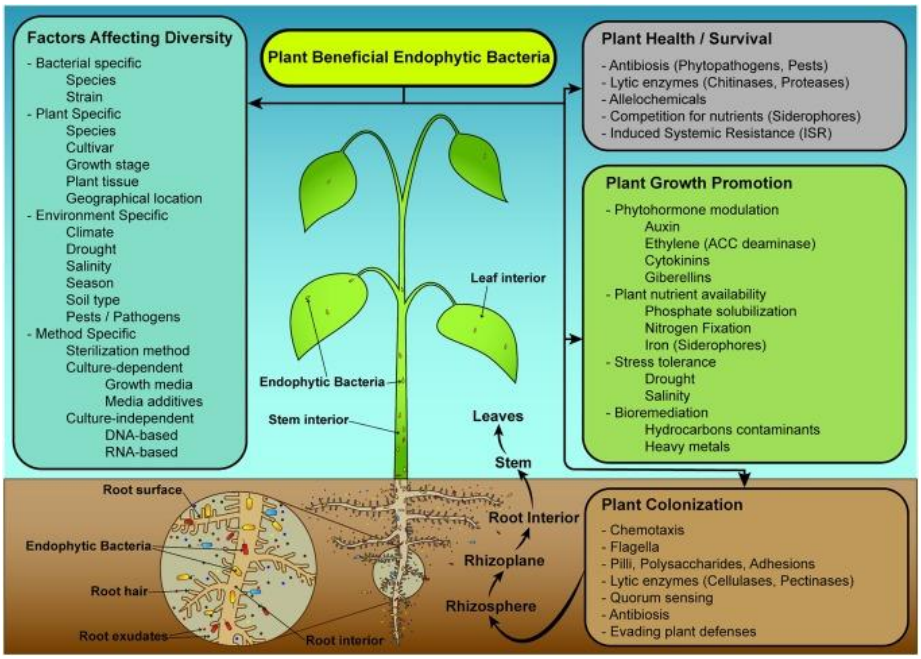


Figure 2.1 Applications of endophytes (adapted from Afzal et al. 2019)

Table 2.3 List of Plant Growth Promoting bacteria reported from different part of the sugarcane crop. (adapted from Mehnaz 2011)

PGP Bacteria	**Source**	**PGP Bacteria**	**Source**
Acinetobacter baumanii	Apoplast	*Bacillus spp.*	Root
Agrobacterium tumefaciens	Stem	*B. cereus*	Apoplast
Azospirillum sp.	Root	*B. pumilus*	Apoplast
A. brasilense	Root	*B. subtilis*	Apoplast, Root
A. lipoferum	Root	*Beijerinckia sp.*	Root
A. amazonense	Roots, stem	*B. fluminensis*	Root
Azotobacter chroococum	Roots	*B. indica*	Root
A. vinelandii	Root	*Brevibacillus sp.*	Stem, leaves
Burkholderia spp.	Stem, leaves	*G. saccharii*	Leaf
B. ambifaria	Root	*Herbaspirillum seorpedaceae*	Stem, leaf
B. cepacia	Root, stem	*H. rubrisubulbicans*	Leaf
B. cenocepacia	Roots, stem	*Klebsiella spp.*	Stem
B. fungorum/graminis	Root	*K. oxytoca*	Root, stem
B. gladioli	Root	*K. pneumoniae*	Root, stem
B. plantarii/glumae	Stem	*K. variicola*	Stem
B. sacchari	Root	*Kocuria kristinae*	Apoplast
B. silvatlantica	Root	*Lactococcus lactis subsp. lactis*	Leaf
B. tropica	Root	*Microbacterium oleivorans*	Apoplast
B. unamae	Stem	*M. testaceum*	Stem
B. vietnamiensis	Stem	*Micrococcus luteus*	Apoplast
Caulobacter crescentus	Root	*Ochrobactrum intermedium*	Root
Citrobacter sp.	Root	*Paenibacillus*	Root

PGP Bacteria	**Source**
Comamonas testosteroni	Apoplast
Curtobacterium sp.	Stem
Delftia acidovorans	Stem, leaves
Derxia gummosa	Root
Enterobacter sp.	Root
E. aerogenes	Stem
E. cloacae	Root
E. oryzae	Stem
Erwinia cypripedii	Apoplast
E. herbicola	Stem
Gluconacetobacter diazotrophicus	Root, stem,
S. epidermidis	Apoplast
S. saprophyticus	Apoplast
Stenotrophomonas maltophilia	Root
S. pavanii	Stem
Xanthomonas spp.	Stem
X. campestris	Apoplast
X. oryzae	Apoplast
Zymomonas sp.	Stem

PGP Bacteria	**Source**
azotofixans	
P. polymyxa	Root, stem
Pannonibacter phragmitetus	Root
Pantoea sp.	Stem, leaf
P. ananatis	Stem
P. herbicola	Root, stem, leaf
P. stewartii	Stem
Pseudomonas spp.	Root
P. aeruginosa	Stem
P. aurantiaca	Stem
P. fluorescence	Root, stem
P. putida	Root , stem
P. reactans	Stem
Rahnella aquatilis	Root
Rhizobium sp.	Root
R. rhizogenes	Apoplast
Saccharibacillus sacchari	Apoplast
Serratia spp.	Stem
Staphylococcus sp.	Stem, leaves

Chapter 3

Isolation and characterization (morphological, biochemical and molecular) of endophytic bacteria from sugarcane crop.

Content of Chapter 3

Isolation and characterization (morphological, biochemical and molecular) of endophytic bacteria from sugarcane crop.

3.1 Introduction:

The fungal pathogen *Colletotrichum falcatum* is the major cause of red rot disease and infection in the sugarcane plant (Patel et al. 2019). Presently, such diseases are proliferating at pace in sugarcane growing states of India causing a loss in the economy of the country (Viswanathan 2017). Injudicious use of chemicals for its management results in the accumulation of toxicants in the environment and therefore a sustainable approach is required (De Silva et al. 2019) that will not only tackle the menace of *Colletotrichum falcatum* but also control other pests and promote plant growth and productivity and is environment-friendly. Nowadays, exploiting antagonistic endophytes as biocontrol agents are in favour of the management of various plant diseases resulting in minimal impact on the ecosystem. The term endophyte was first introduced by De Bary (1866) and since then its significant role came into the provision in agricultural era. Endophytic bacteria can be defined as non-pathogenic bacteria that colonize the internal tissue of the living plants without causing any negative impact on the host and can be only isolated after surface disinfection of plant tissue (Kado 1992; Hall-man et al. 1997; Kobayashi 2000; Yan et al. 2018). They play an indispensable role in sustaining the well-being of plants; as they can fortify the plant against biotic and abiotic stress and also help in intensifying the growth and yields of the crop (Kumar et al. 2016; Hazarika et al. 2019).

Existing studies demonstrated various bacterial genera including *Pseudomonas, Enterobacter, Burkholderia, Ochrobactrum, Gluconacetobacter* and *Bacillus* that

have been found to be associated with sugarcane rhizosphere with the ability to suppress the *C. falcatum* (Chen and Zhou 2009; Hassan et al. 2010; Katiyar et al. 2017). Still, exploration and selection of efficient antagonistic endophytes from the sugarcane plant are in nascent stage though work has been carried out in the last two decades but the literature available is very scanty on the existence of endophytic bacteria in association with sugarcane plant (Viswanathan et al. 2003; Quecine et al. 2012). Several studies (Hazarika et al. 2019; Shen et al. 2019; Yan et al. 2018) have reported that endophytes establish relations by interacting not only with the host plant but also with the plant pathogen; thus, inducing the plant growth. Various findings have demonstrated that plants inoculated with endophytes result in suppression of disease development due to their interaction with the pathogen. Therefore, the development of effective biological control against *C. falcatum* requires the screening and evaluation of native potential antagonistic bacteria capable of reducing red rot under *in vitro* conditions. As most of the studies are based on N-fixer diazotrophs; therefore, it is necessary to uncover beyond diazotrophs endophytes with multiple roles in stress tolerance and with this background the current chapter encompasses the isolation and *in vitro* screening of endophytic bacteria from sugarcane having antagonistic action against the red rot fungal pathogen.

3.2 Materials and Methods

3.2.1 Sample collection:

Healthy clumps of sugarcane variety (shoot and root) *viz.* Co 1148, CoS 767 and CoJ 64 were collected from the Indian Institute of Sugarcane Research Farm (IISR), Lucknow. All the samples were collected in a sterile plastic cover, transferred to the laboratory and processed further.

3.2.2 Isolation of endophytic bacteria:

The endophytic bacteria from **root** and **shoot** were isolated by using different nutrient-rich media and selective media amended with only 3% agar as described below:

i) Different nutrient-rich media:

Endophytic bacterial communities from the root and stalks of the sugarcane plant were assessed by culture-dependent technique. The outer surface of plants was washed properly with tap water to remove any adhered traces of soil. Further, the surface of plants was rinsed with 70% ethanol for 3 min followed by commercial bleach (4.5% NaOCl) for 4.5 min and again washed with ethanol for 30 s; and finally washed five times with sterile distilled water. After proper sterilization of the outer surface of all the 3 varieties; roots and stalks portion were aseptically stripped off using a sterile knife. The tissue cylinder from stalk was then taken off using a sterile cork-borer and cut into 1-2 cm pieces; and similarly, root tissue was also cut into 1-2 cm pieces. Henceforth, plant tissues were sterilized by vigorously shaking them in 70% ethanol for 30 s followed by 3% NaOCl for the 30s and five times rinsed with sterilized distilled water. Tissues were then blotted dry on sterilized filter paper and crushed with a sterile glass rod and finally placed in conical flasks containing 25 ml sterile distilled water. The suspension was then prepared by shaking the flask for 1 h at 150 rpm. Afterward, 1 ml of suspension was taken with the help of micropipette from the flasks (containing root tissues) and plated on 5 different media viz; Potato Dextrose agar (PDA), Luria Bertani agar (LB) and King's B agar (KB), Nutrient agar (NA) as described by Viswanathan and Samiyappan (2001), Viswanathan et al. (2003) and Jasim et al. (2014) with slight modification; and on N-free LGIP media (g/100 ml; saccharose 10, K_2HPO_4 0.02, KH_2PO_4 0.06, $MgSO_4.2H_2O$ 0.02,

$CaCl_2.2H_2O$ 0.002, $NaMO_4.2H_2O$ 0.0002, 0.5% Bromothymol blue in 0.2 M KOH, $FeCl_3.6H_2O$ 0.001, Agar 1.5, pH 7.1) for the isolation of diazotrophs (Döbereiner et al. 1995; Suman et al. 2005). Similarly, for the isolation of endophytes from the stalk tissue; 1 ml suspension from the flask containing stalk tissue was plated on all the above media and incubated at 28±2°C for 4 days in duplicates. After incubation, morphologically distinct colonies were identified by colony characters and used for further studies. To confirm complete surface sterilization and to rule out the chances of epiphytic bacteria, the final rinsing water was also spread on all the 5 medium (PDA, LB, LGIP, KB, and NA) agar plates and examined for bacterial growth. Samples are discarded and again processed for surface sterilization if there is any microbial growth in 48 hours.

ii) **3% agar plating method:**

The rinsing, sterilization, and drying occurred under the same conditions as done in the isolation of plant material on different nutrient-rich media. Samples were exhaustively rinsed with sterile water so as to remove the epiphytic microorganisms. Roots and stalk/shoots were dissected into 1 cm pieces and aseptically placed on Petri dishes containing a sterilized 3% water-agar medium. Plates were incubated at 28±2°C for 3-4 days. The bacterial growth that appeared beneath the root/ stalk tissue or around the plant tissue placed on the agar medium were considered as endophytes. The confirmation of isolates was done by streaking the same isolates on the plate containing 3% agar and sterile root and shoot extract as the only source of nutrients (Panchal and Ingle 2011). Morphologically different colonies were picked up for purification and maintained at 4°C for further studies.

3.2.3 The fungal pathogen:

C. falcatum (CF01), the causative agent of the red-rot disease was procured from IISR, Lucknow and maintained on oatmeal agar medium at 4 °C for further use.

3.2.4 *In vitro* bacteria–fungus interaction by dual- culture antagonism assay:

In vitro screening of the antagonistic effect of all the endophytic bacterial isolates was evaluated by a dual-culture antagonism assay using the relative growth of *C. falcatum* as described by Viswanathan et al. (2003). A 5-mm agar plug cut from a fully-grown fungal pathogen plate was placed at one side of the plate and test isolates were inoculated at another opposite side of the fungus. The control plate was inoculated with *C. falcatum* without test isolates. All treatments were repeated thrice and the plates were incubated at 28°±2°C for 7 days. Thereafter, the antagonistic effect of test isolates on *C. falcatum* was recorded. The radial growth of fungus mycelium was measured in control as well as in treated plates. The percentage inhibition of mycelium radial growth of *C. falcatum* was calculated with the following formula.

$$\% \text{ Inhibition} = \frac{(C-T)}{C} \times 100$$

Where, 'C' is the radial growth (in mm) of the control fungal mycelium colony and 'T' is the radial growth (in mm) of the fungal mycelium growing in presence of antagonist endophytic bacterial isolate.

3.2.5 Tolerance to sucrose concentration:

Isolated endophytic bacteria were also checked for sucrose tolerance levels. They were grown in soft agar medium containing only sucrose as a source of carbon in a different concentration ranging from 3%, 5%, 7%, 9%, 11%, 13%, 15%, 17%, 19%, and incubated at 28±2°C for 72 h. The growth of bacterial culture after 72 h on the plates amended with different concentrations of sucrose was considered a positive result for sucrose tolerance level.

3.2.6 Characterization of isolates:

Different colonies of endophytes (selected after antagonism dual culture test) obtained on different isolation media were identified on the basis of morphological and biochemical tests according to the key of Bergey's Manual of Determinative Bacteriology (Holt et al. 1994).

3.2.6.1 Morphological characterization:

The selected antagonistic endophytic bacterial strains were characterized morphologically with the help of light microscopy followed by Gram's staining. The colony morphology of endophytic bacteria was analyzed after growing them on their respective media. The motility test was also conducted for selected antagonistic endophytes on the motility agar medium.

3.2.6.1.1.1 Gram's reaction for endophytic bacterial isolates:

Sir Christian Hans Gram (1884) developed an empirical method to differentiate the bacteria into two groups named as gram-positive and gram-negative. In this method a heat fix smear of bacterial cell was stained with crystal violet (basic dye) for 1 min, followed by washing under running tap water, iodine (mordant dye) was added for 30 s to form a crystal violet iodine (CVI) complex, decolourised with ethanol (95%) and then counterstained with safranin for 30 s. The slide was observed under the oil immersion lens of an inverted light microscope. In the microscope shape and arrangement of cells were studied. The principle behind this test is if bacteria retains primary stain (crystal violet) it is regarded as Gram-positive whereas, if the bacterial cell loses the primary stain colour and is stained by counterstain safranin it is regarded as Gram-negative.

3.2.6.2 Biochemical characterization:

The biochemical tests were performed on Himedia Biochemical Test Kit and some other tests were also conducted on the respective media as per the standard method describes by Aneja (2007) and Cappuccino and Sherman (1992). The tests performed are as given below:

3.2.6.2.1 Procedure for inoculation in the Hi-media IMViC test kit

Bacterial inoculum was prepared as per the catalogue instruction and each kit was opened aseptically by sealing off the tape in the laminar flow. Each well was inoculated with 50 µl of bacterial inoculum and incubated at 30°C for 24 to 48 hours and observed for the results.

Note: Hi-media IMViC test kit was a combination of total of 12 tests for containing sterile media for Indole, Methyl red (MR), Voges Proskauer's (VP), Citrate utilization tests and 8 different carbohydrates-Glucose, Adonitol, Arabinose, Lactose, Sorbitol, Mannitol, Rhamnose, Sucrose.

Note: Though isolation was done at 28±2°C; but biochemical and other tests were performed at 30°C due to the required demand of the particular protocol (as per the particular metabolite formation at required temperature); Also, the best part is this that all the test isolates of the study were also able to grow optimally at 30 ±2°C.

Results interpretation for indole, methyl red, and VP were determined after the addition of the reagents following incubation as given below.

Indole Test: Well No. 1

- Development of reddish-pink colour within 10 s after the addition of 1-2 drops of Kovac's reagent indicates positive reaction while pale colour is indicative of a negative result.

Methyl Red Test: Well No. 2

- Methyl Red reagent (1 to 2 drops) was added after incubation and appearance of red colour indicates positive results.

Voges Proskaeur's Test: Well No. 3

- Baritt's reagent A (2 to 3 drops) and Baritt's reagent B (1-2 drops) was added and if pinkish-red colour developed within 5 to 10 min, it indicates a positive result.

Result determination: Results for all the 12 tests were determined as per the chart given below (Table 3.1).

Table 3.1: Result interpretation chart for Hi-media IMViC kit.

Test	**Reagents to be added after incubation**	**Principle**	**Original colour of the medium**	**Positive reaction**	**Negative reaction**
Indole	1-2 drops of Kovac's red reagent	Detects deamination of tryptophan	Colourless	Reddish pink	Colourless
Methyl red (MR)	1-2 drops of Methyl red reagent	Detects acid production	Colourless	Red	Yellow
Voges Proskauer's (VP)	1-2 drops of Baritt reagent A and 1-2 drops of Baritt reagent B	Detects acetoin production	Colourless	Pinkish red	Colourless/ slight copper
Citrate utilization	-	Detects capability of an organism	Green	Blue	Green

		to utilize citrate			
Glucose	-	Glucose utilization	Red	Yellow	Red
Adonitol	-	Adonitol utilization	Red	Yellow	Red
Arabinose	-	Arabinose utilization	Red	Yellow	Red
Lactose	-	Lactose utilization	Red	Yellow	Red
Sorbitol	-	Sorbitol utilization	Red	Yellow	Red
Mannitol	-	Mannitol utilization	Red	Yellow	Red
Rhamnose	-	Rhamnose utilization	Red	Yellow	Red
Sucrose	-	Sucrose utilization	Red	Yellow	Red

Some other Biochemical test

3.2.6.2.2 Oxidase test:

Oxidase Test by the disc is a rapid test for determining the oxidase-cytochrome enzyme. Oxidase reaction was carried out by touching and spreading a 10 µL of bacterial suspension over the oxidase disc (Hi-Media) placed on the sterile slides. The reaction was observed within 5-10 s at 30°C by observing the change in the colour of disc from white to deep purple/blue colour considered as a positive reaction. A change later than 10 s or no change at all is considered a negative reaction. Oxidase discs are sterile filter paper discs impregnated with N, N-dimethyl-p-phenylenediamine oxalate, ascorbic acid, and a-naphthol. These discs overcome the necessity of daily preparation of fresh reagents.

3.2.6.2.3 Urease production test:

Urea is a diamide of carbonic acid, several bacterial genera produce urease that hydrolyzes urea into ammonia and carbon dioxide. The selected endophytic bacterial isolates were tested for urease production by inoculating them onto urea supplemented agar medium followed by incubation at 30 °C for 18 to 24 h. Bacteria that produce urease; hydrolyze urea rapidly and change the colour of slant from pale orange to magenta.

3.2.6.2.4 Catalase production test:

The catalase production test was performed to detect the presence or absence of catalase enzyme. Most of the aerobes and facultative anaerobes have characteristic catalase activity. Aerobic microbes usually utilize oxygen and produce hydrogen peroxide, which is toxic to nucleic acid and many cellular proteins. Hence, this test was used to detect the presence of catalase enzymes in bacteria. A few drops of 0.3% H_2O_2 solution was poured on a bacterial colony. The presence of bubbles indicates the presence of catalase production.

3.2.6.2.5 The oxidative-fermentative (OF) test:

The oxidative-fermentative (OF) test was developed by Hugh and Leifson in 1953. They developed OF media to differentiate between oxidative bacteria (that produces acid from carbohydrates under aerobic condition only) and fermentative bacteria (that produces acid both under aerobic and anaerobic conditions). The OF test was proceeded by inoculating two tubes of OF test medium with the test organism using a straight wire by stabbing "halfway to the bottom" of the tube. One tube of each pair was covered with a 1 cm layer of sterile mineral oil or liquid paraffin (it created an

anaerobic condition in the tube by preventing diffusion of oxygen), leaving the other tube open to the air. Thereafter, both the tubes were incubated for 48 h at 30°C. The following interpretation was observed after incubation.

- If both the tube (open and covered) produced the acid, change in colour was observed from green to yellow in both the tubes, the microorganism was considered to be fermentative.

- If acid production (yellow colour) was observed only in the open tube (aerobic) and not in the oil-covered tube (anaerobic); the microorganisms were considered to be oxidative. Non-fermenting bacteria that metabolize glucose via oxidative metabolism gave an oxidative result.

- If no colour changes occurred in both the tube; the microorganism is considered to be a Non-saccharolytic bacteria.

3.2.6.3 Bacterial identification using 16sRNA sequences:

After screening through all the parameters for selecting an efficient endophyte; the potent antagonistic with tolerance to a high level of sucrose and effective growth on the isolated medium was completely identified by using the 16S rRNA sequencing technique. For this, genomic DNA was extracted and amplified by using universal primers, 27F primer (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R primer (5´-CGGTTACCTTGTTACGACTT -3´) on the agarose gel electrophoresis and the PCR product obtained were sequenced. The PCR thermal condition used: initial denaturation at 95 °C for 2 min; 35 cycles of denaturation step at 95 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 2 min; and a final extension step at 72 °C for 15 min. The obtained sequence was aligned and analyzed by using

BLASTn search tool (http://www.ncbi.nlm.nih.gov/Blast) and compared with the gene bank database with the help of the BLAST program (Altschul et al. 1997). The Neighbour-Joining method was used to identify the Bacterial and its closest neighbours (Saitou and Nei 1987) through phylogenetic tree construction. Phylogenetic analyses were conducted in MEGA6 (Tamura et al. 2013). The aligned sequence was submitted to NCBI by using the Bankit tool and accession number was obtained.

3.3 Results:

3.3.1 Sample Collection and the test pathogen:

The three different cultivars of sugarcane (Fig 3.1) were collected and washed properly to remove the soil and processed for the isolation of endophytes. The culture plate (Fig 3.2) of red rot pathogen i.e. *C. falcatum* (CF01) was also generously procured from the Department of Crop Protection, IISR-Lucknow.

Fig 3.1 The Sugarcane variety a) Co 1148; b) CoS 767; c) CoJ 64 were collected from IISR farms for the isolation of endophytic bacteria.

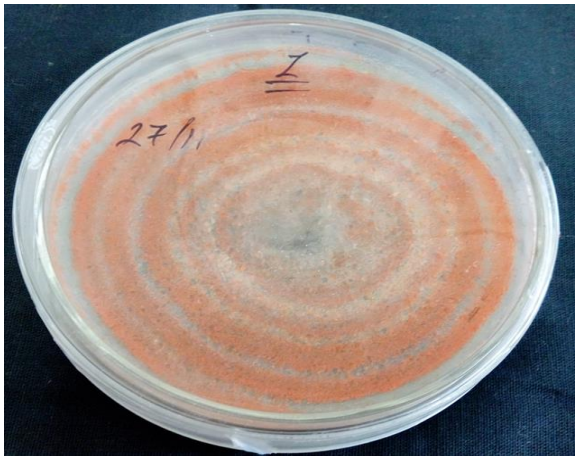


Fig 3.2 The culture plate of red rot pathogen i.e. *C. falcatum*.

3.3.2 Isolation of endophytic bacteria:

From the root and stalk tissue of sugarcane varieties (Co 1148, CoS 767, and CoJ 64) a total of 118 endophytes were isolated. The sugarcane variety Co 1148, CoS 767, and CoJ 64 yielded 59, 23, and 36 distinct endophyte bacterial colonies respectively (Table 3.2) from roots and stalk tissue. Co 1148 yielded the highest number of bacteria followed by CoJ 64 and CoS 767. As no growth appeared on the control plate, the isolates can be considered as endophytic bacteria of sugarcane (Fig 3.3). However, changes in the media onto which isolation occurred also showed varied ranges of isolated endophyte bacteria. Isolation was done on two different media i.e. different nutrient-rich media (LB, NA, PDA, KB, and LGIP media) and selective media (3% agar medium). After obtaining the results for isolation of bacteria; it was also observed that root tissue from all the cane varieties produces a higher number of distinct endophyte bacteria (Table 3.2). In a selective 3% agar medium, after 3-4 days of incubation, the growth of microorganisms was observed below the root samples and shoot sample. The microbial growth surrounding the root and shoot on the water agar medium indicated that the microorganism gained their nutrient from the root and shoot only. All the bacterial isolates (118) were then purified on the Luria Bertani

agar medium and checked for *in vitro* antagonism against *C. falcatum* on PDA medium. (NOTE: As different media was used for the isolation of isolates; however, all the isolates were able to grow on LB medium. Therefore, further culturing of isolates was performed on LB medium).

3.3.3 *In vitro* antagonism assay in dual-culture:

After isolation of endophytic bacteria, *in vitro* screening for antagonistic activity was performed on PDA plates by dual-culture assay against *C. falcatum*. A total of 118 isolates of endophytic bacteria were studied for their *in vitro* antagonistic activity. Out of them, only 29 isolated endophytes showed inhibition against red rot fungus (Fig 3.4). It was demonstrated that out of 29 isolates; a majority of strain (20) isolated from sugarcane variety Co 1148 showed inhibition against pathogen followed by variety CoJ 64 (7) and CoS 767 (2), respectively. Overall, only two strain B2 and B7 showed low *in vitro* inhibition with 47.57 ± 5.52 % and 48.57± 5.34%; while the remaining 27 endophytes showed more than 50% mycelial inhibition of fungal pathogen. Isolates S8, S11, S12 (from Co1148), BS-4 (from CoS767) and BS14, BS29 (from CoJ64) showed maximum inhibition with 82.97 ± 2.15%; 80.54 ± 2.15%; 85.00 ± 1.46%; 80.54 ± 5.68%; 81.62 ± 5.15% and 80.54 ± 2.15%, respectively (Table 3.3).

Table 3.2 Number of endophytic bacteria colonies obtained from a different cultivar of sugarcane on the respected media of isolation from a different part.

Media of Isolation	Number of morphological distinct colonies obtained from the Root	Number of morphological distinct colonies obtained from the Shoot
	From Variety Co 1148	
LB agar	15	10
NA	10	2
PDA	5	3
Kings B	5	-
LGIP	2	-
3% water agar media	5	2
Total number of colonies obtained from the variety Co 1148 (42+17= 59)	42	17
	From Variety CoS 767	
LB agar	5	3
NA	4	2
PDA	4	-
Kings B	3	-
LGIP	1	-
3% water agar media	1	-
Total number of colonies obtained from the variety CoS 767 (18+5= 23)	18	5
	From Variety CoJ 64	
LB agar	8	3
NA	5	2
PDA	3	1
Kings B	2	2
LGIP	-	-
3% water agar media	5	5
Total number of colonies obtained from the variety CoJ 64 (23+13= 36)	23	13

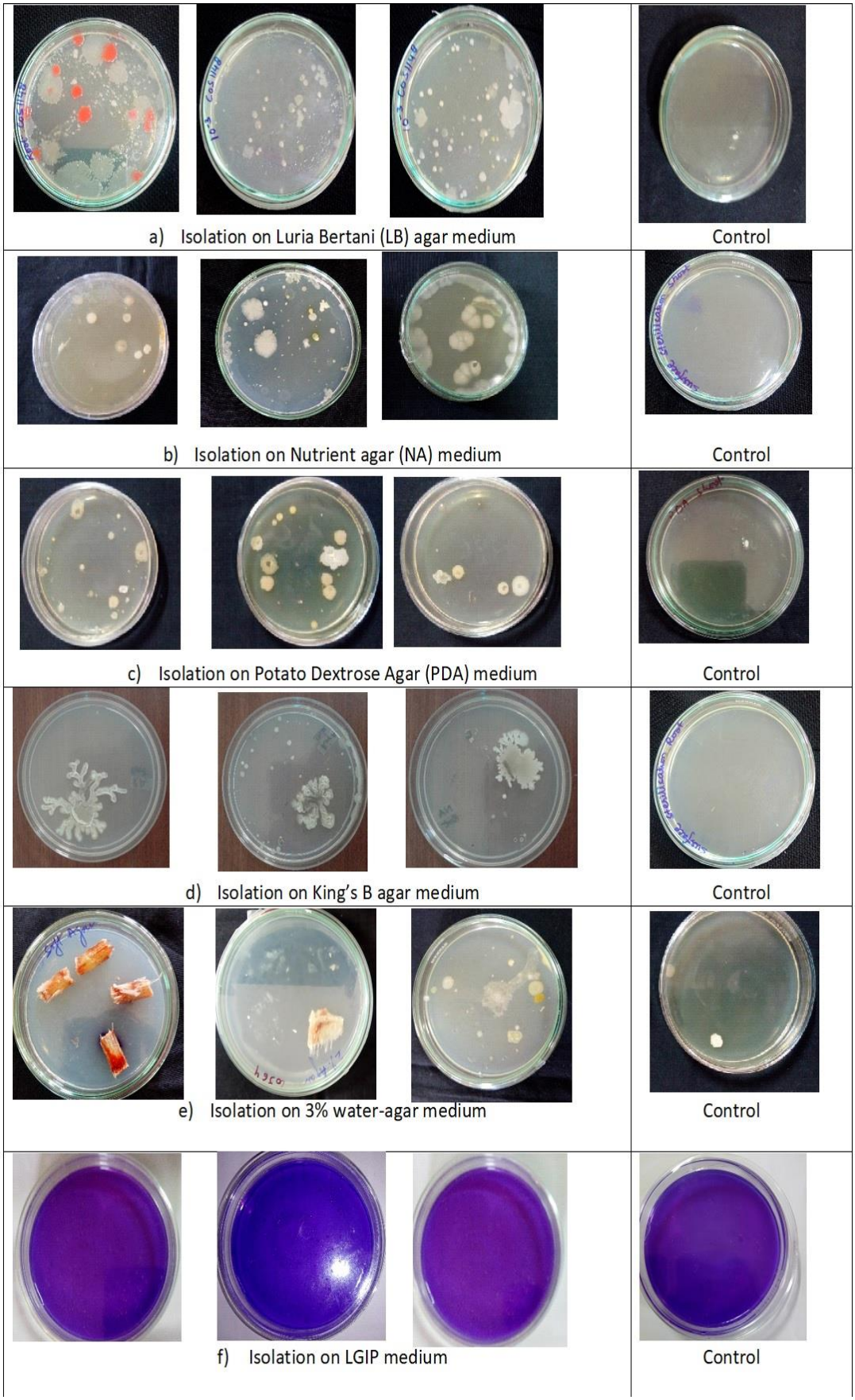


Fig 3.3 Isolation of endophytic bacteria on the six different media a) LB agar; b) NA; c) PDA; d) King's B agar; e) 3% water agar; f) LGIP.

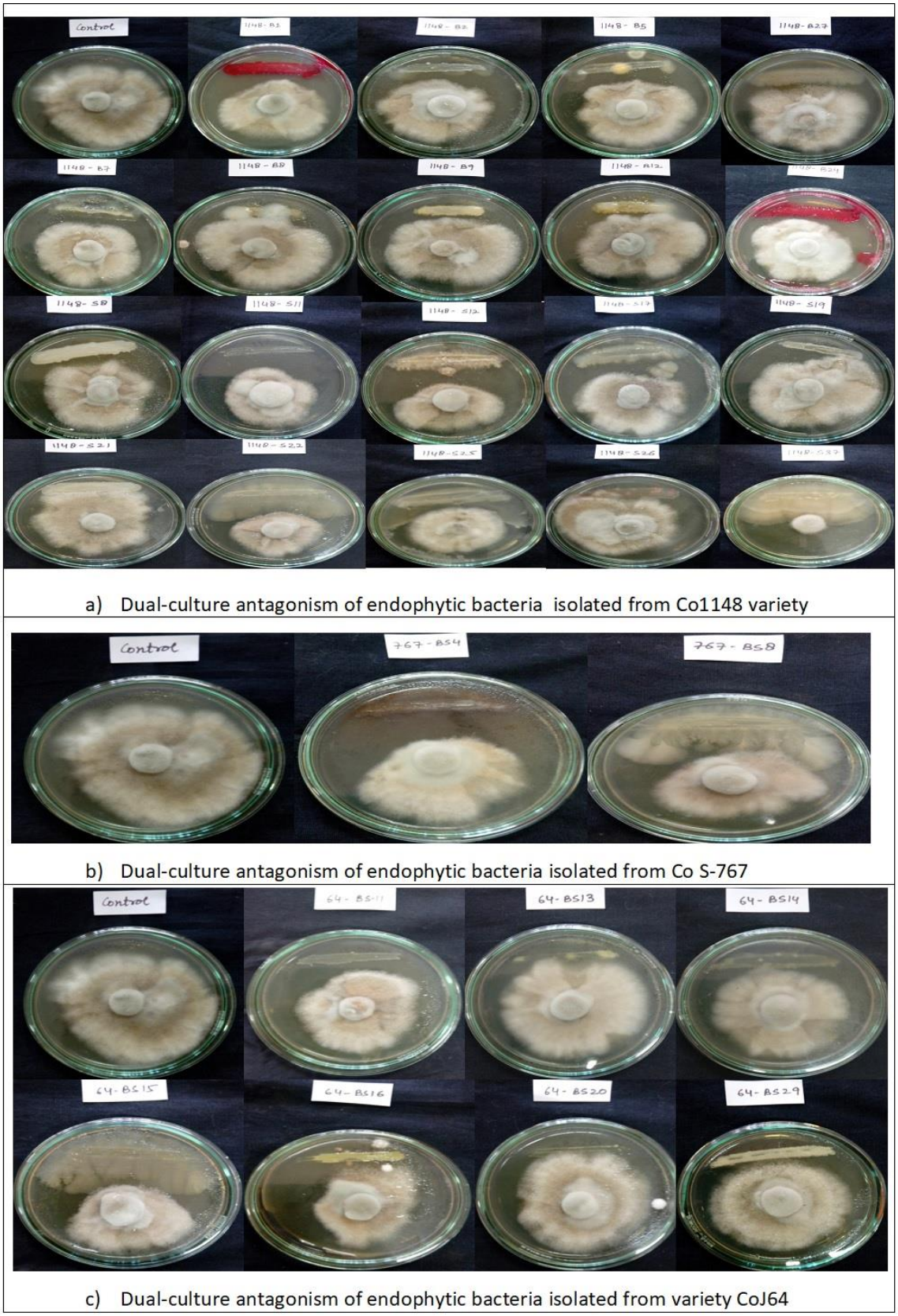


Fig 3.4 Dual-culture antagonism of endophytic bacteria isolated from the different sugarcane variety a) Co 1148; b) CoS 767; and c) CoJ 64, against *C. falcatum* on PDA medium with control (first plate with full fungal growth)**.**

Thus, a total of 29 endophytic bacteria (Fig 3.5) with strong percentage inhibition were selected for further morphological and biochemical characterization.

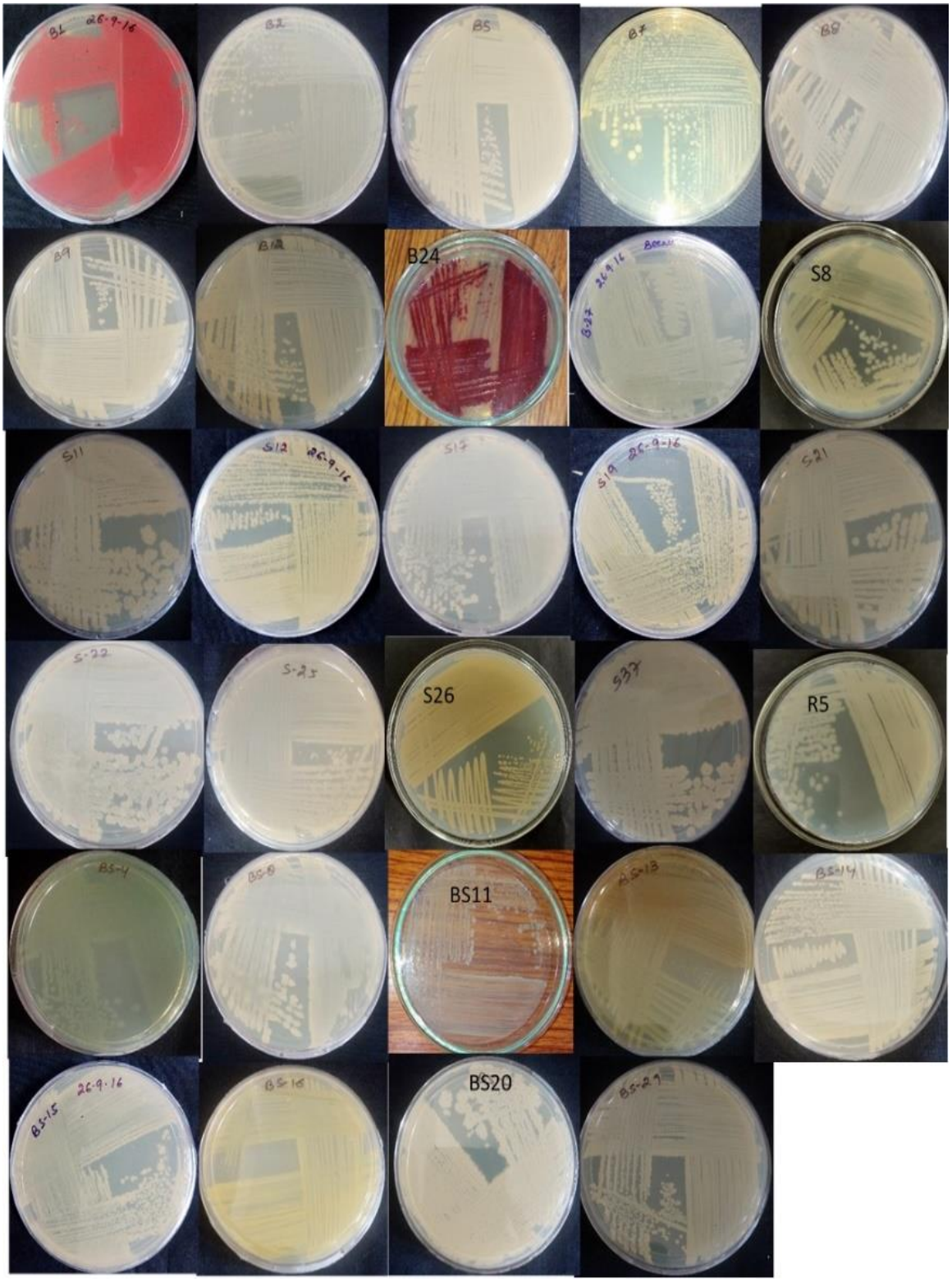


Fig 3.5 Selection of 29 endophytic bacterial isolates from different cultivar for further functional characterization.

3.3.4 Tolerance to Sucrose concentration:

All the 29 endophytic isolates that depicted antagonistic activity tolerated 11% of the sucrose level. However, when the sucrose level was further increased, only five isolates S8, S17, S12, R5, and BS-4 showed the highest tolerance level i.e. up to 19% (Table 3.3).

Table 3.3. *In vitro* antagonism of bacterial endophytes against *C. falcatum* isolated from root and shoot of three different varieties of sugarcane on different media with their incubation days along with their tolerance to sucrose level.

S. No.	Habitat Variety	Isolate Code	Plant part (Root/ Shoot)	Media of Isolation	Time at which isolates obtained after incubation (days)	Sucrose Tolerance (%)	Radial mycelial growth (mm) *C. falcatum* (7^{th} day)	Inhibition of *C. falcatum* (%)
1	Co 1148	Control				NA	41.11 ± 0.38	0.00 ± 0.00
2		B1	Root	Luria Bertani	3	13	19.78 ± 2.01	51.89 ± 4.89
3		B2	Root	Luria Bertani	3	15	21.56 ± 2.27	47.57 ± 5.52
4		B5	Root	Luria Bertani	3	13	12.78 ± 2.04	68.92 ± 4.95
5		B7	Root	Luria Bertani	3	15	21.14 ± 2.20	48.57 ± 5.34
6		B8	Root	Luria Bertani	3	11	16.24 ± 1.84	60.49 ± 4.47
7		B9	Root	Luria Bertani	3	13	14.01 ± 2.07	65.92 ± 5.04
8		B12	Shoot/ Stalk	Luria Bertani	3	15	13.41 ± 1.83	67.38 ± 4.44
9		B24	Root	Luria Bertani	3	15	9.89 ± 0.84	75.95 ± 2.04
10		B27	Root	Luria Bertani	2	11	12.62 ± 1.23	69.30 ± 3.00
11		S8	Root	Luria Bertani	2	19	7.00 ± 0.88	82.97 ± 2.15
12		S11	Root	King's B agar Medium	3	13	8.00 ± 0.88	80.54 ± 2.15
13		S12	Root	Luria Bertani	2	19	6.17 ± 0.60	85.00 ± 1.46
14		S17	Shoot/ Stalk	Luria Bertani	3	19	9.78 ± 0.96	76.22 ± 2.34
15		S19	Shoot/ Stalk	Luria Bertani	3	17	14.78 ± 0.69	64.05 ± 1.69
16		S21	Shoot/ Stalk	Luria Bertani	3	15	13.39 ± 0.25	67.43 ± 0.62
17		S22	Shoot/ Stalk	Luria Bertani	3	17	10.13 ± 0.12	75.35 ± 0.28

18		S25	Root	Potato Dextrose Agar Medium	2	13	10.78 ± 0.84	73.78 ± 2.04
19		S26	Root	Luria Bertani	2	11	9.33 ± 0.33	77.30 ± 0.81
20		S37	Shoot/ Stalk	Potato Dextrose Agar	3	11	10.33 ± 0.58	74.86 ± 1.40
21		R5	Root	Luria Bertani	2	19	8.78 ± 0.19	78.65 ± 0.47
22	CoS 767	BS-4	Root	King's B Agar Medium	2	19	8.00 ± 2.33	80.54 ± 5.68
23		BS8	Root	Nutrient Agar	2	13	9.78 ± 1.02	76.22 ± 2.48
24	CoJ 64	BS11	Root	3% Water Agar	2	11	12.44 ± 1.07	69.73 ± 2.61
25		BS13	Shoot/ Stalk	3% Water Agar	4	11	17.44 ± 1.02	57.57 ± 2.48
26		BS14	Shoot/ Stalk	3% Water Agar	3	13	7.56 ± 2.12	81.62 ± 5.15
27		BS15	Shoot/ Stalk	3% Water Agar	3	15	10.78 ± 2.14	73.78 ± 5.21
28		BS16	Shoot/ Stalk	Nutrient Agar	3	15	13.41 ± 0.75	67.38 ± 1.82
29		BS 20	Root	Luria Bertani	2	13	11.24 ± 1.81	72.65 ± 4.41
30		BS29	Root	Luria Bertani	2	11	8.00 ± 0.88	80.54 ± 2.15

All the observations were recorded by repeating experiment thrice with three replicate each and '±' values represents the standard deviation (SD); NA represents not applicable.

3.3.5 Morphological and biochemical identification of endophytes:

Twenty-nine endophytic bacterial isolates with antifungal activities were morphologically and biochemically characterized as summarized in Table 3.4 and 3.5 respectively. All the selected endophytic bacteria were distinct in their colony colour, and shape (Table 3.4). Morphological results revealed that nearly 79.31% of selected endophytes belonged to gram-positive rod-shaped and rest belonged to gram-negative rod-shaped bacteria (Fig 3.6). All the 29 isolates were found motile. The results for various biochemical tests such as catalase, urease, IMViC, oxidase, and oxidation fermentation (OF) are represented in Table 3.5. All the isolates were found positive

for catalase production, and methyl red production; and all the isolates were showing negative results for indole and VP. The oxidase test was positive for only four endophytic bacteria i.e. BS-4, BS-8, BS-11, and BS-13 only. Biochemically, sugars are very important for most of the microbe; therefore, carbohydrate utilization pattern was also recorded for all the twenty-nine isolates to check their ability to utilize and digest different carbohydrates (Table 3.5).

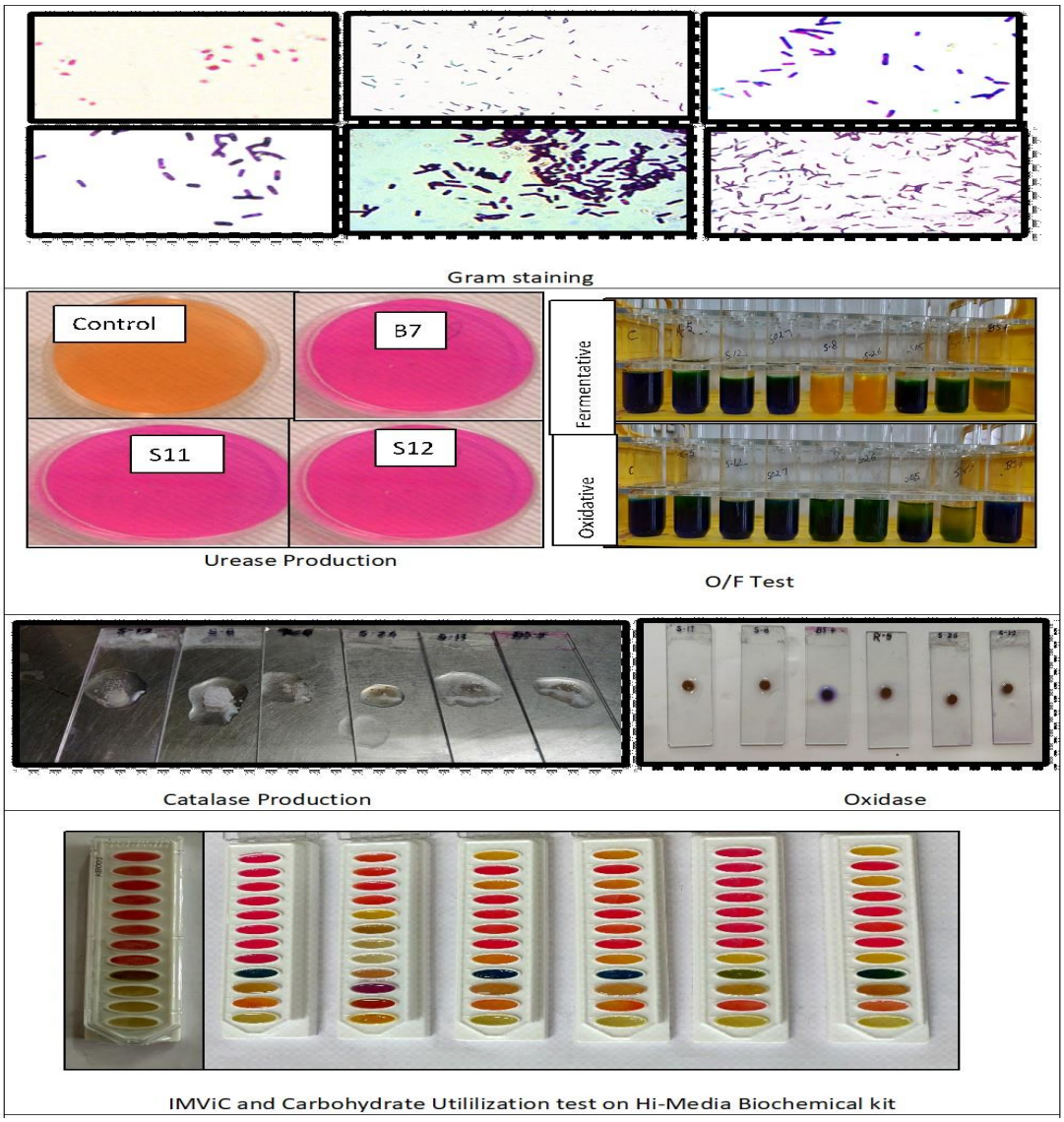


Fig 3.6 Morphological and Biochemical characterization of some of the isolated Endophytic bacteria.

Table 3.4 Summary of Morphological characterization of selected antifungal endophytes isolated from sugarcane crop.

S. No.	Morphological Characteristic			
	Isolates	**Colony Morphology**	**Gram's Staining**	**Motility**
1)	B1	Smooth, raised and red	+	+
2)	B2	Serrated, transparent and creamy white	+	+
3)	B5	Wrinkled, sticky and off white	+	+
4)	B7	Serrated, sticky and transparent	+	+
5)	B8	Wrinkled, flat and off white	+	+
6)	B9	Rough, convex and pale white	+	+
7)	B12	Wrinkled, flat and pale white	+	+
8)	B24	Serrated, raised and red	+	+
9)	B27	Smooth, sticky and light yellow	+	+
10)	S8	Irregular, opaque and white	+	+
11)	S11	Serrated, Sticky and brown	+	+
12)	S12	Undulate, raised, irregular, white	+	+
13)	S17	Irregular, wrinkle, dry	+	+
14)	S19	Serrated, sticky, beige	+	+
15)	S21	Serrated, Sticky and white	+	+
16)	S22	Serrated, sticky and yellow	+	+
17)	S25	Rough, flat and yellow	+	+
18)	S26	Rough, wrinkled, opaque, licheniform means hair-like, creamy to brown	+	+
19)	S37	Serrated, convex and yellow	+	+
20)	R5	Serrated, sticky and white	+	+
21)	BS4	Convex,shining, mucoid and fluroscent green	-	+
22)	BS8	Wrinkled, sticky and creamy	-	+
23)	BS11	Smooth, transparent and shining yellow	-	+
24)	BS13	Round, transparent and white	-	+
25)	BS14	Smooth, raised and creamy white	+	+
26)	BS15	Wrinkled, rough flat and white to cream	-	+
27)	BS16	Smooth, raised and yellow	-	+
28)	BS 20	Wavy, transparent and white	+	+
29)	BS29	Wrinkled, flat and white	+	+

In Gram's staining: '+' indicates gram-positive reaction and '-' indicates gram's negative reaction.

Table 3.5 Summary of biochemical characterization of selected antifungal endophytes isolated from sugarcane crop.

Isolates	Biochemical Characteristics															
	Citrate	**Urease**	**Indole**	**MR**	**VP**	**Catalase**	**OF**	**Oxidase**	**Glucose**	**Adonitol**	**Arabinose**	**Lactose**	**Sorbitol**	**Mannitol**	**Rhamnose**	**Sucrose**
B1	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-
B2	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-
B5	-	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+
B7	+	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-
B8	+	-	-	+	-	+	-	-	+	-	-	-	-	+	+	+
B9	-	-	-	+	-	+	-	-	+	-	-	-	-	+	+	+
B12	-	-	-	+	-	+	-	-	+	-	-	-	-	+	+	+
B24	+	-	-	+	-	+	-	-	+	+	-	-	+	+	-	+
B27	+	-	-	+	-	+	-	-	+	-	-	-	-	+	+	+
S8	+	-	-	+	-	+	F	-	+	-	-	-	-	-	-	-
S11	-	+	-	+	-	+	-	-	+	-	-	-	-	-	-	+
S12	+	+	-	+	-	+	-	-	+	-	-	-	-	+	+	+
S17	+	-	-	+	-	+	F	-	+	-	-	-	+	+	-	+
S19	-	-	-	+	-	+	-	-	+	-	-	-	-	+	-	+
S21	-	-	-	+	-	+	-	-	+	-	-	-	-	+	+	+
S22	-	-	-	+	-	+	-	-	+	+	-	-	+	+	-	+
S25	+	-	-	+	-	+	-	-	+	+	-	-	+	+	-	+
S26	+	-	-	+	-	+	F	-	+	-	-	+	+	-	-	+
S37	-	-	-	+	-	+	-	-	+	+	-	-	+	+	-	+
R5	+	-	-	+	-	+	O	-	+	-	-	-	-	+	-	+
BS4	+	-	-	+	-	+	O	+	+	-	-	-	-	+	-	-
BS8	-	-	-	+	-	+	-	+	+	+	+	+	+	+	-	+
BS11	-	-	-	+	-	+	-	+	+	+	+	+	+	+	-	+
BS13	+	-	-	+	-	+	-	+	+	+	-	-	+	+	-	+
BS14	+	-	-	+	-	+	-	-	+	-	-	-	-	+	+	+
BS15	-	-	-	+	-	+	-	-	+	+	+	+	+	+	-	+
BS16	+	-	-	+	-	+	-	-	+	-	-	-	-	+	+	+
BS 20	-	-	-	+	-	+	-	-	+	-	-	-	-	+	+	+
BS29	+	-	-	+	-	+	-	-	+	-	-	-	-	+	-	+

'+' indicates positive test and '-'indicates negative test.

In the oxidative fermenting test 'O' indicates oxidative in nature; 'F' indicates fermentative in nature.

3.3.6 Bacterial identification using 16sRNA sequences:

The most efficient endophytic bacterial isolates were completely identified by using 16S rRNA sequence analysis. Six endophytic bacteria (S8, S12, S17, S26, R5, and BS-4) based on *in vitro* antagonism and sucrose tolerance and PGPR properties (discussed in Chapter 4) were selected for molecular identification. On the basis of 16S rRNA sequencing; S8 was identified as *Bacillus cereus* with accession number (MG966498*)*, S12 was identified as *B. aryabhattai* (MH298519), S17 as *B. subtilis* (MK411294), S26 as *B. licheniformis* (MK411295), R5 as *B. paramycoides* (MN318075) and BS-4 as *Pseudomonas aeruginosa* (MG966460). The phylogenetic tree for the isolates are represented below (Fig 3.7- 3.12).

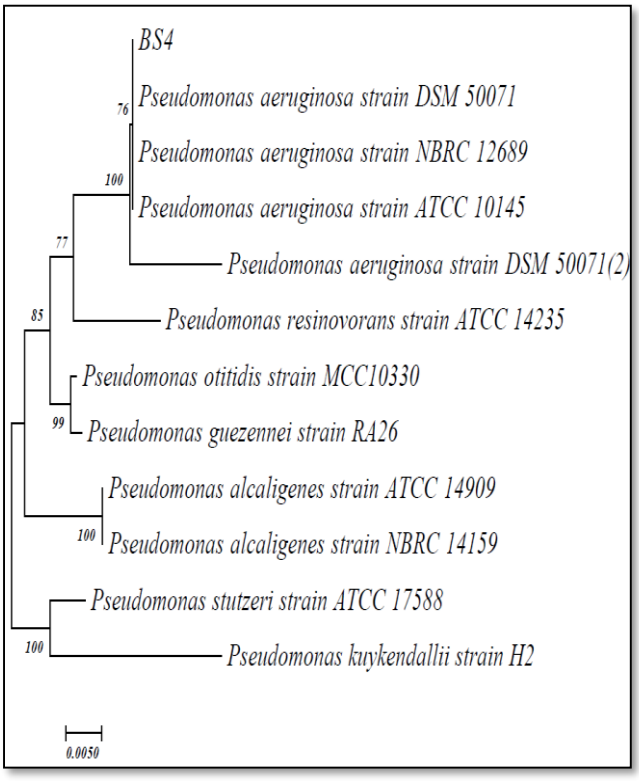


Fig 3.7 Phylogenetic tree construction for the isolate BS-4

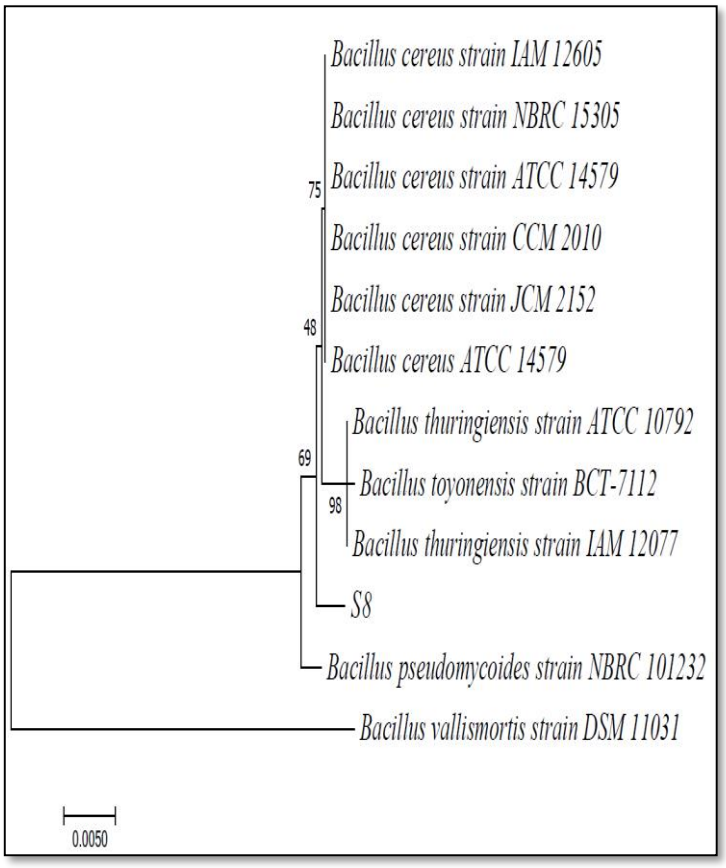


Fig 3.8 Phylogenetic tree construction for isolate S8.

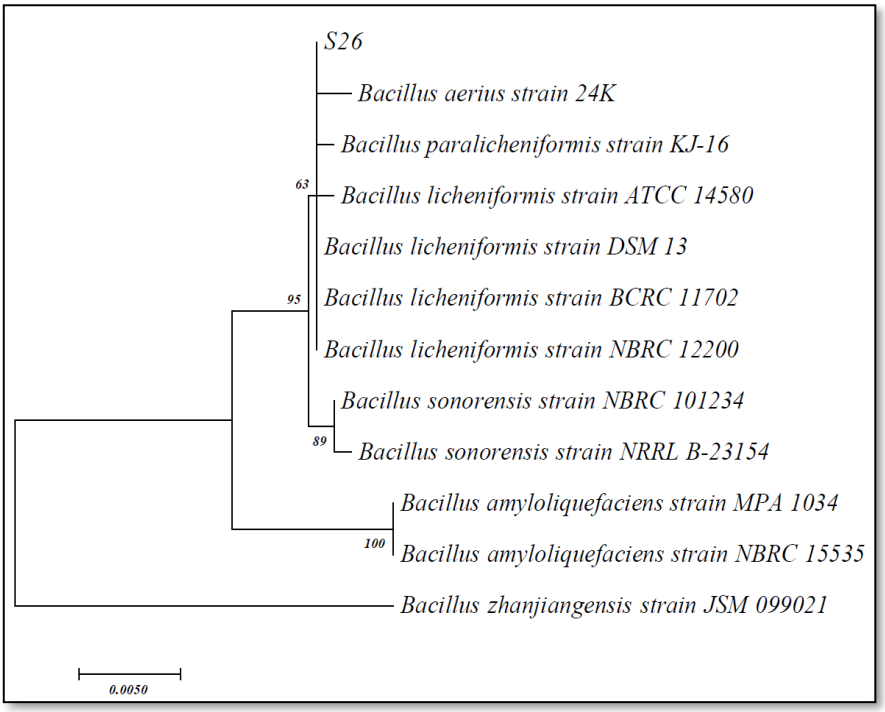


Fig 3.9 Phylogenetic tree construction for isolate S26.

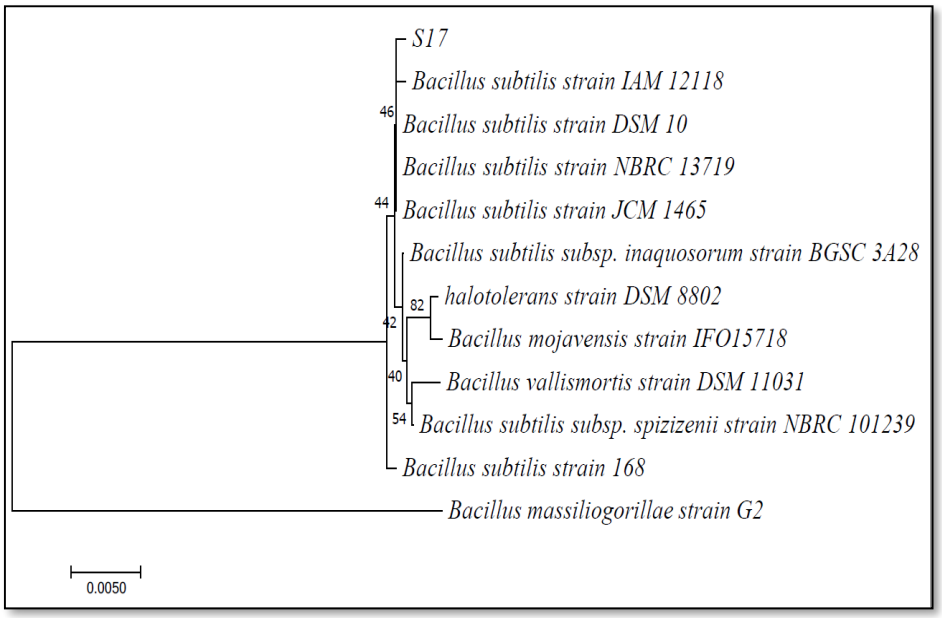


Fig 3.10 Phylogenetic tree construction for isolate S17.

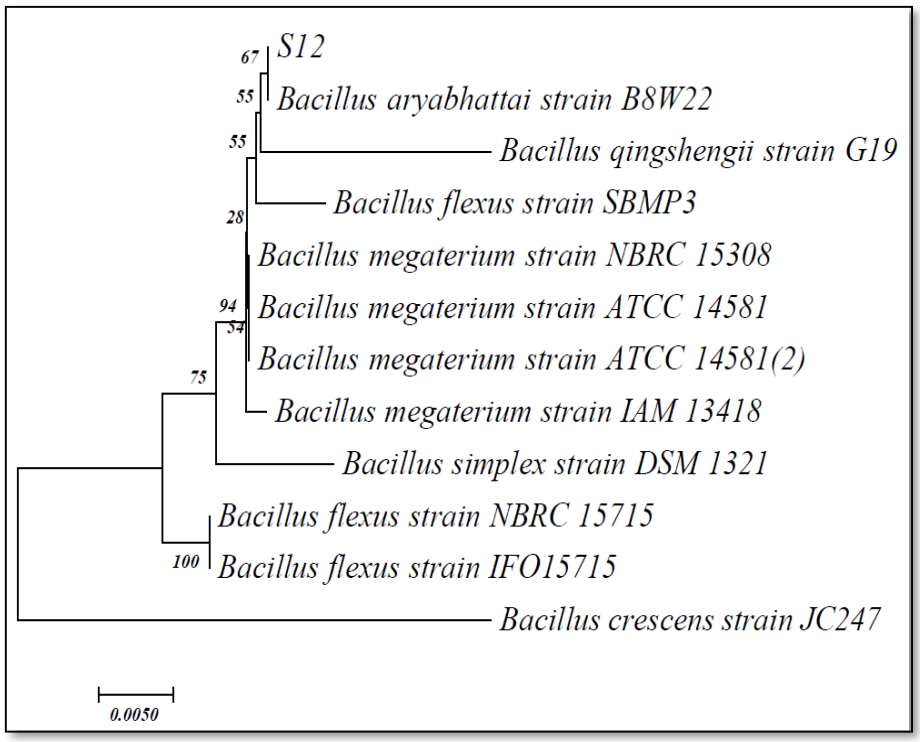


Fig 3.11 Phylogenetic tree construction for isolate S12

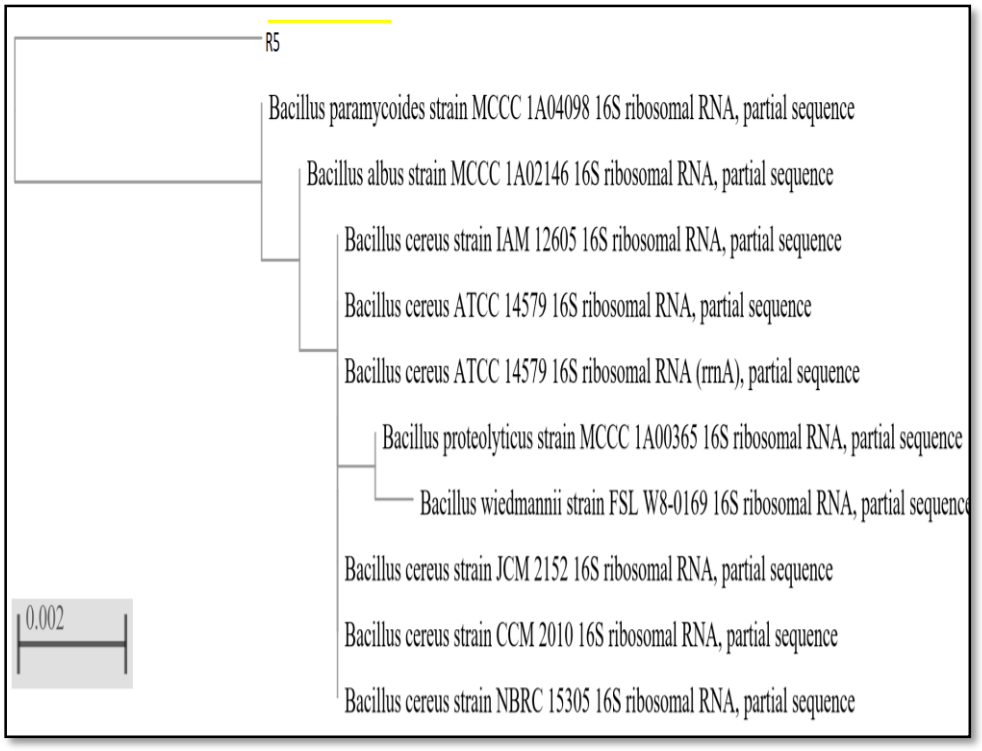


Fig 3.12 Phylogenetic tree construction for isolate R5.

3.4 Discussion:

In recent years, endophytic microorganisms are gaining interest in the field of agriculture due to their potential role in growth promotion, biocontrol and disease resistance of crop against various phytopathogen. In the present study, diverse species of bacterial endophytes were isolated from economically important cash crop sugarcane plants and investigated for various functional attributes. With the idea of getting a broad diversity of endophytes; different cultivars of sugarcane (Co 1148, CoS 767, and CoJ 64) were examined for isolation of endophytes. Red rot is one of the dreadful diseases of sugarcane and its effective management using biological safe agent i.e. by endophytes bacteria is an efficient approach. Therefore, isolated endophytes were *in vitro* screened for antibiosis and it was found that 24.57% (29/118) of the endophytic bacteria showed significant inhibitory activity against test

fungus. Based on *in vitro* inhibition properties, selective endophytes were analyzed for other functional attributes also.

Endophytic bacteria are known to be the best colonizer of plant tissue. However, effective sterilization is one of the critical and essential steps in the isolation technique. Sodium hypochlorite (NaOCl) is generally a non-toxic and easy to handle and therefore commonly used as a surface disinfectant for isolation of bacterial endophyte. Its concentration and incubation time for sterilization vary according to the plant tissue and crop variety to be sterilized. However, 0.5-5% concentration of NaOCl and 3 to 90 min of incubation time have been reported for sterilization of different crops (Munif et al. 2013). Studies also reported that using a higher concentration of hypochlorite (6%) may result in the killing of endophytic bacteria from internal plant tissue (Munif et al. 2013). Therefore, in the current study 4.5% hypochlorite was used for surface disinfection of sugarcane tissue. After surveying literature; it was also found that a combination of disinfectant can also be used for the isolation of endophytes. In this study, a double sterilization technique was followed; means inner parts of plants were sterilized with effective methods only after the disinfection of the outer surface so as to avoid the contamination of any epiphytic microorganism. In the current study, 70% ethanol followed by 4.5 % NaOCl was used for the proper sterilization of sugarcane plant tissue and isolation of endophytes. This study is also supported by Hallman et al.1997 whose finding stated that slight variation in the concentration and incubation time of surface disinfectant process greatly affects the number and type of bacteria isolated. Therefore, in the current finding for the isolation of endophytes inner tissue was sterilized with the same disinfectant but with different concentrations and time (70% ethanol for 30 s and 3% NaOCl for 30 s). The same sterilization technique was also favoured by Yan et al. (2018) and Shen et al. (2019).

Selection of media for the isolation of endophytes is also one of the deciding factors onto which a diverse cultivable group of endophytes with a high population can grow. Based on the literature survey; this study dealt with isolation on nutrient-rich media (PDA, LB, King's B, LGIP, and NA medium) as they would probably result in a higher number of the cultivable and fastidious growing population of endophytes. Besides, nutrient-rich media; one selective media (3 % agar media) was also used to check the isolation efficiency of endophytes from various varieties. As, it was a selective media and isolates growth appeared beneath the tissue of plant parts, indicating that it utilized nutrients from the plant tissue only. Also, observing the number of bacterial endophytes colonies on the media (nutrient-rich and selective media), it was found that the proportion of endophytic bacteria was higher in nutrient-rich media than the selective one. This study was also favoured by (Vartoukian et al. 2010; Pham and Kim 2012; Velázquez-Sepúlveda et al. 2012; Rohini et al. 2018). Among the various nutrient-rich media used in the present study, Luria Bertani media yielded the highest number of endophytic bacteria and higher diversity (in terms of colony morphology) followed by NA, PDA, King's B agar, and LGIP for Co 1148, CoJ 64 and CoS 767 variety. Most of the research based on endophytes of sugarcane generally centralized around N-fixer diazotrophs isolated on the LGIP medium. Although few of the endophytic bacterial colonies appeared after incubation in the LGIP medium for Co 1148 and CoS 767 variety; but none of the colonies appeared for variety CoJ 64. In the present study, the least diversity in a number of endophytes bacteria was found on LGIP medium and also, none of them showed inhibition against *C. falcatum*. Thus, current findings are in conformity as reported by Suman et al. (2001) and Magnani et al. (2010) where the prevalence of endophytic bacteria especially in relation to diazotrophs showed little diversity. Thus, it was confirmed that by using the nutrient-rich medium, a much diverse range of unexplored endophytes may get a chance to be exploited in a better way. This study was also

proven by Magnani et al. (2010) where nutrient-rich media supported the growth of many endophytes, suggesting a more complex ecology of sugarcane endophytes than previously reported. The selective 3% agar medium also yielded a sufficient number of bacterial colonies for Co 1148 and CoJ 64 varieties. The colonies appeared in agar medium under the plant tissue can be considered as true endophyte as they were developed from inside the plant and utilizing the nutrition from the plant tissue only. Since this was selective media; little diversity of endophytic bacteria was found for sugarcane variety.

This study gave an insight into the population of endophytes found in the root tissues of sugarcane which was higher than the shoot tissue. This was in correlation with findings of Zinniel et al. (2002) where the concentration of the endophytic bacteria was found more in the root than in the shoot tissue. Lamb et al. (1996) and Kruasuwan and Thamchaipenet (2016) also stated that generally, bacterial populations are more in roots and decrease in the stems and leaves. This difference in the population density of different parts may be attributed to the fact that root is the preferential site for the entrance of endophytes. This entrance is generally due to either presence of external opening like root hairs or due to wound such as root cracks that results in leakage of plant metabolites which attract large number of bacteria (Agarwhal 1987; Hallman et al. 1997; Sprent and de Faria 1998; Sørensen and Sessitsch 2007; Santoyo et al. 2016). Also, the root provides a more balanced and favourable environment for endophytes to establish inside the plant.

The extraordinary property of sucrose tolerance of all the 29 non-diazotrophs was found to be consistent with the fact that isolated bacteria were showing true endophytism as they had been isolated from high sucrose-containing host i.e. sugarcane plant tissue. Therefore, their tolerance to a high sucrose level is perhaps a natural phenomenon that might not be present in any other non-diazotrophs isolated

from other crops. Thus, the presence of this feature puts them into the 'extra-ordinary endophytes' category as this quality matches with the N-fixer diazotroph *Gluconacetobacter diazotrophicus* (isolated from sugarcane) which has certain osmotolerant mechanisms to cope with the high sucrose tolerance as pointed by Dent (2018). It is also reported that bacterial endosymbionts usually survive in an environment having high sucrose concentration (Dent 2018). The mechanisms that help to protect *G. diazotrophicus* against high sugar concentrations may also act in the same way in the endophytes isolated in the present study.

The current findings also revealed that all the isolated bacteria from sugarcane were morphologically distinct and after 16S rRNA sequential analysis of selected six potent antifungal strain against red rot pathogen confirmed the presence of diverse bacterial species i.e. *Bacillus cereus* (S8), *B. aryabhattai* (S12), *B. subtilis* (S17), *B. licheniformis* (S26), *B. paramycoides* (R5) and *Pseudomonas aeruginosa* (BS-4). *Bacillus* and *Pseudomonas* species have been previously reported as endophytes in different plant species like tomato, rhizomes of ginger, rice, turmeric and sugarcane (Magnani et al. 2010; Rashid et al. 2012; Jasim et al. 2014; Kruasuwan and Thamchaipenet 2016; Kumar et al. 2016; Rohini et al. 2018; Shen et al. 2019). The present study showed the dominance of the *Bacillus* (Gram-positive) group over another gram-negative (*Pseudomonas*). Similar results were also reported by Magnani et al. (2010) where the *Bacillus* group was predominant than the Pseudomonad group.

In the present study, various species of *Bacillus* were found to be present in sugarcane plant and many of them have been reported as endophytes in some other plant; however, their prevalence as endophytes in sugarcane plant is rare. The study of Ben Slama et al. (2019) recently reported the potential role of endophytic *Bacillus licheniformis* LMRE 36 in biocontrol of *Fusarium* sp. which is threatening the olive trees. Similarly, the *in vitro* and *in vivo* study of Nigris et al. (2018) also reported the

Bacillus licheniformis GL174 as a good biocontrol agent candidate isolated from *Vitis vinifera* cv. Glera. The strain helps the grapevine plants to cope with pathogen attacks and reduce the number of chemicals used in the vineyard. *Bacillus cereus* is again other endophytes in the list that confers resistance against a broad range of phytopathogens by producing versatile metabolites (Hong et al. 2018). *B. paramycoides* is a recently discovered novel bacteria that is further required to be explored for its various functional characteristics. The present study noticed the *in vitro* antagonistic behaviour of *B. paramycoides* strain R5 against *C. falcatum*. Further exploration of the various characteristic features of the potent strain R5 in the field of biocontrol is still to be explored especially in the sugarcane field. Recently, *B. paramycoides* has been reported as endophytes from pearl millet (Kushwaha et al. 2020) and from maize plant (Sondang et al. 2019) with multiple stress tolerance activity. However, to the best of the knowledge; this is the first study to report the *B. paramycoides* strain R5 as endophyte from the sugarcane plant. The *B. aryabhattai* strain S12 is also one of the rare species reported as endophytes from sugarcane tissue. However, their role as endophytes has been reported from the other crop such as roots of rice seedlings (Shen et al. 2019). Thus, this study would further explore the potential of *B. paramycoides strain* R5 and *B. aryabhattai* strain S12 as endophytes in sugarcane and also would provide multiple mechanisms for antagonism against red rot fungus. The antifungal property was also exhibited by endophytic symbiont *Pseudomonas aeruginosa* AL2-14B isolated from *Achyranthes aspera* L. against *Rhizoctonia solani* (Devi et al. 2017). The present findings would also elucidate the antagonistic behaviour of *P. aeruginosa* for further use as antagonistic endophytes against *C. falcatum. Bacillus subtilis* strain S17 reported in the current study was also earlier reported as endophyte from sugarcane leaves with their strong antifungal role due to the presence of lipopeptide antibiotics (Hazarika et al. 2019). Thus, the various above-cited reports support the findings of the present study by

demonstrating the multiple actions of endophytes isolated from various crop against pathogen attack. This study would also be useful in utilizing these isolates as a natural biocontrol agent and thus, reducing the use and thus minimizing the harmful impact of chemicals for our agriculture system.

3.5 Conclusion:

Endophytes have been reported from a wide variety of plants but the functional role is known only with a limited number of isolates. Sugarcane crop is associated with a diverse community of microorganisms but their full potential is yet to be explored for other functional attributes. Also, various literature available on sugarcane endophytes mainly focuses on diazotrophs. Therefore, in the present study different species of *Bacillus* and *Pseudomonas* have been isolated from the root/ stalks of different sugarcane varieties which are ubiquitous in nature and easy to isolate than diazotrophs. This study proved that these endophytes depicted potent *in vitro* antagonism against red rot fungus *C. falcatum*. Along with, inhibitory action against the pathogen, further evaluation of their PGP traits would need to be explored so that these endophytes have potential applicability in the biocontrol of red rot disease of sugarcane. Also being endophytic in nature, they reside in the same niche as with the host plant, thus communicate and protect the plant in an effective manner. Considering all these factors, this work indicated that isolates with antifungal activity must be expected to be a potential source for bioactive metabolites which would require further evaluation to develop a strong biofertilizer/biofungicides for sugarcane crop.

Chapter 4

Characterization of isolates for PGPR properties (IAA, Siderophore, Phosphate solubilisation, etc.) and secondary metabolites (chitinase, β-1, 3 glucanase, antibiotic etc.).

Contents of Chapter 4

Characterization of isolates for PGPR properties (IAA, Siderophore, Phosphate solubilisation, etc.) and secondary metabolites (chitinase, β-1, 3 glucanase, antibiotic etc.).

4.1 Introduction:

The phytopathogen attack is responsible for major losses in agricultural production and has become a severe problem due to increasing pests and climate change. The pathogen releases toxic chemicals that destroy the plant parts from the seedling stage to maturity stage. The most common approach to control the pathogen is by using the antagonistic microorganisms that serve as a biological control agent. Nowadays, realizing the toxicity associated with the chemical pesticides, farmers too have become aware and are adopting safe agricultural practices and have minimized the use of chemical pesticides but that needs to be supplemented with some good biological alternatives for them. Antagonistic microorganisms can be one of them, they secrete a plethora of secondary metabolites with antibiotic potential and plant growth promotion properties (Gao et al. 2018). Currently, endophytic microorganisms are attracting attention as a great source in the biocontrol area for agricultural crops due to untapped diverse novel metabolites that can positively affect the plant's health. The endophytic bacterial species establish a tight association with the host plant that ultimately stimulates their growth and productivity by acting as biofertilizer and biocontrol agents (Reinhold-Hurek and Hurek 2011). Likewise, plant growth-promoting rhizobacteria (PGPR); endophytes also possess the traits for plant growth promotion (PGP) in a direct as well as in an indirect manner. Direct PGP mechanisms of endophytes include biological nitrogen fixation (BNF) and mineral solubilization

(P, Fe), as well as the production of phytohormones (auxins, cytokinin, and gibberellin), production of stress reliever i.e. ACC deaminase; while indirect mechanisms include arresting the growth of phytopathogens facilitated by antibiotics, competition for nutrients and niches, or the induction of an induced systemic resistance (ISR) response (Rosenblueth and Martínez- Romero 2006; Hardoim et al. 2008; Compant et al. 2010; Mei and Flinn 2010; Devi et al. 2017; Dimkić et al. 2017; Ben Slama et al. 2019).

Various literature reports documented the manifold benefits of endophytic bacteria on plant growth and health. The endophytes aid in nutrient availability and uptake, enhance stress tolerance and provide disease resistance (Santoyo et al. 2016; Ben Slama et al. 2019). The mechanism for plant growth promotion and disease resistance properties are associated with the ability of endophytic bacteria to produce a wide range of compounds, such as indole acetic acid (IAA), phosphate solubilization, nitrogen fixation, production of hydrolytic enzymes, hydrogen cyanide (HCN), siderophores, and diffusible and volatile antibiotic production which can inhibit growth of plant pathogens and thus act as biocontrol agents (Brader et al. 2014; Santoyo et al. 2016).

The dominant group of endophytic bacteria are known from variety of crop that impart beneficial effects on the plant including species of *Bacillus* and *Pseudomonas.*

The present study also aimed to investigate the presence of broad-spectrum secondary metabolites and plant growth-promoting properties in the antagonistic endophytic bacteria isolated from different sugarcane varieties (as discussed in Chapter 3). The already isolated endophytic bacteria that showed strong antagonism against *Colletotrichum falcatum*, the red rot pathogen of sugarcane (discussed in Chapter 3)

have been used in the present objective. Thus, the present chapter would favour the screening and selection of efficient antagonistic endophytic bacteria possessing both the fungistatic as well as the presence of other growth promotory secondary metabolites that would help in controlling the red rot disease in sugarcane and also improving the health status of sugarcane crop.

4.2 Material and Methods:

4.2.1 The endophytic isolates:

The twenty-nine endophytic bacterial isolates that showed the *in vitro* antagonism effects against *C. falcatum* as discussed in Chapter-3 were used in this study for further screening of antagonistic endophytic bacteria with multiple plants growth-promoting and biocontrol traits.

4.2.2 Assay for growth-promoting abilities of isolates:

All the twenty-nine isolates were screened for indole acetic acid production, phosphate solubilization, siderophore production, HCN production, ammonia production and ACC deaminase activity by the following assays.

4.2.2.1 Indole acetic acid (IAA) production:

The ability of endophytic bacterial isolates to produce IAA was determined by the method of Gordon and Weber (1951). For qualitative determination; bacterial cultures were grown in a Luria-Bertani (LB) broth amended with tryptophan (1%) for 72 h at 28±2°C. Thereafter, cultures were centrifuged at 10,000 rpm for 20 min. 2 ml of supernatant was mixed with two drops of orthophosphoric acid and 4 ml of Salkowski reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% perchloric acid). Tubes were then incubated at room temperature in dark for 25 min. Development of pink colour

indicates the production of IAA. For the quantitative determination; the intensity of pink colour was read at 530 nm spectrophotometrically and the amount of IAA produced was extrapolated from the standard curve of pure indole-3-acetic acid.

4.2.2.2 Solubilization of organic phosphate:

The endophytic bacterial isolates were screened for phosphate solubilization on Pikovskaya agar medium as described by Slama et al. (2019). The media was inoculated with test endophytes and incubated for 96 h and the formation of halo zone around the colony due to the utilization of tricalcium phosphate present in the medium was observed. Solubilization index was calculated from the colony diameter and the total zone diameter as per the formula:

Solubilization Index (SI) = (Colony diameter + halo zone diameter) / Colony diameter

Quantitative analysis of phosphate solubilization of tricalcium phosphate in liquid medium was conducted as described by King (1932). For quantitative analysis, tricalcium phosphate amended broth was used. The isolates were inoculated in 25 ml Pikovskaya's broth and incubated for 4 days at 28±2 °C. After incubation, broth was centrifuged at 15,000 rpm for 30 min. One ml supernatant was mixed with 10 ml of chloromolibidic acid and the volume was made up to 45 ml with autoclaved distilled water. Then, 0.25 ml of cholorostannous acid was added to maintain the volume up to 50 ml with distilled water. The absorbance of the developed blue colour was read at 600 nm and the amount of soluble phosphorus was detected from the standard curve of KH_2PO_4.

4.2.2.3 Production of ammonia:

Bacterial isolates were screened for ammonia production in peptone water. Freshly grown cultures were inoculated in 5 ml peptone water in a test tube and incubated for

48 h at 28 ±2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown (++) to yellow colour (+) was regarded as a positive test for ammonia production (Cappuccino and Sherman 1992).

4.2.2.4 ACC deaminase production:

The ACC deaminase production of the endophytic bacterial isolates from sugarcane was screened using the methods described by Jasim et al. (2014). For this, the isolates were inoculated onto DF (Dworkin and Foster) salts minimal medium (potassium dihydrogen phosphate 4 g/L, disodium hydrogen phosphate 6 g/L, magnesium sulfate heptahydrate 0.2 g/L, ferrous sulfate heptahydrate 0.1 g/L, boric acid 10 mg/L, manganese (II) sulfate 10 mg/L, zinc sulphate 70 mg/L, copper (II) sulfate 50 mg/L, molybdenum (VI) oxide 10 mg/L, glucose 2 g/L, gluconic acid 2 g/L, citric acid 2 g/L, agar 12 g/L) amended with 0.2 % ammonium sulphate (w/v). The appearance of bacterial growth on this media after 2 days of incubation at 28 ±2°C was considered a positive result.

4.2.2.5 HCN production:

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Slama et al (2019). Briefly, the Luria Bertani agar medium was amended with 4.4 g glycine/L and bacteria were streaked on a modified agar plate. A Whatman filter paper no. 1 soaked in alkaline picrate solution (5% picric acid prepared in 2% sodium carbonate solution) was placed on the inner side of upper lid of the petri plate. Plates were then sealed with parafilm and incubated at 28 ±2°C for 4 days. HCN production was assessed by the colour change of yellow filter paper to brown/reddish brown.

4.2.2.6 Siderophore production:

Qualitative assay for siderophore production of all the selected antagonistic endophyte bacteria(s) was done on chrome-azurol Sulphonate agar plates (CAS agar); (Schwyn and Neilands 1987). The medium was poured on sterile Petri dishes; then each bacterial isolate was spot inoculated and incubated for 72 h at 30 ±2° C. Formation of an orange-yellow zone around the colonies were considered as positive results. This method was developed by Schwyn and Neilands, and it uses an iron-dye complex which changes colour on loss of iron, siderophores which have a higher affinity for iron as compared to dye can remove iron more efficiently, resulting in a change in colour of the dye from blue to orange.

The quantitative estimation of siderophore was done for the isolates which gave a positive result in the qualitative estimation. The siderophore production by endophytic isolates was tested by CAS-shuttle assay (Payne 1994) in which the strain was grown on Luria Bertani broth medium and incubated for 72 h at 30°C with constant shaking at 120 rpm on rotary shaking incubator. After incubation, the fermented broth was centrifuged at 10,000 rpm in a cooling centrifuge at 4^{o}C for 10 min and 0.5 ml cell-free supernatant was mixed with 0.5 ml CAS solution. The colour obtained was determined using the spectrophotometer at 630 nm absorbance after 20 min of incubation with a reference containing 0.5 ml un-inoculated broth medium and 0.5 ml CAS solution. The siderophore was expressed as percentage of siderophore units and calculated by using the formula (Payne 1994):

Percentage Siderophore Unit OR % PSU= [(Ar - As)/Ar] x 100 %

where Ar is the A_{630} nm of reference (CAS assay solution+ un-inoculated broth) and As is the A_{630} nm of the sample (CAS assay solution+ supernatant of culture).

4.2.3 Screening of lytic enzyme:

After screening of twenty-nine isolated antagonistic endophytic isolates for plant growth-promoting abilities; the isolates were further investigated for the production of various hydrolytic enzymes which together decide its major antifungal role.

4.2.3.1 Detection of chitinase enzyme:

Chitinase activity was detected by using the substrate colloidal chitin which was prepared according to the modified method as described by Hsu and Lockwood (1975). The colloidal chitin was prepared by grinding 10 g of chitin flakes to a powder and adding slowly to 100 ml of concentrated HCl with constant stirring at 4°C for 1 h. The hydrolyzed chitin was washed in distilled water until pH was 6 or 7 and the resulting colloidal chitin was stored as a paste at 4°C for further use.

Chitinase activity was detected in colloidal chitin agar media with some minor modification as described by Mitsutomi et al. (1995); Kuddus and Ahmad (2013); and Velusamy and Das (2014). The media consisted of (w/v): 0.2% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 5H_2O$, 0.05% NaCl, 0.1%NH_4Cl, 0.05% $CaCl_2$, 0.05% yeast extract 2% agar and 1% colloidal chitin. The isolates were inoculated into colloidal chitin agar and incubated for 72 h at 30°C. After incubation, plates were observed for the development of a clear zone around bacterial colonies as an indicative of chitinase production. The 0.2% congo red dye solution was poured on the agar plate after incubation so as to observe the clearance zone distinctly. Clear zones around the colonies were considered evidence of chitinase activity in a plate containing colloidal chitin.

4.2.3.2 Detection of β-1,3 glucanase enzyme:

Similarly, detection β-1,3 glucanase enzyme was done according to the modified method of Renwick et al. (1991); Özcan et al. (2013) on Luria Bertani (LB) agar amended with 0.5% (w/v) of substrate laminarin. The isolates were inoculated on the agar medium and incubated at 30°C for 72 h. After incubation, plates were stained with Congo red solution (0.2%) for 15 min and destained with 1M NaCl solution for 15 min. Clear zone around the bacterial colonies indicated the presence of β-1,3 glucanase enzyme activity.

4.2.3.3 Amylase Production:

The extracellular amylase production was verified on starch agar plates amended with 1% (w/v) soluble starch (Deb et al. 2013). The isolates were spot inoculated on the starch agar medium and incubated for 24-48 h at 30°C. After incubation at 30°C, 5 ml of 1% freshly prepared iodine solution (6 g potassium iodide (KI) was mixed with 200 ml of sterile distilled water, 3 g iodine crystals were added, and the solution volume adjusted to 1 L) was added to flood the plates and excess iodine was removed. The amylase production was confirmed by the appearance of clear zone around the colony.

4.2.3.4 Protease production:

Proteolytic activity was tested in nutrient agar plates supplemented with 1% (w/v) skim milk powder (Han et al. 2015) and presence of clear zone around endophytic bacterial colonies after 48 h of incubation was considered as positive for protease production.

4.2.3.5 Cellulase Production:

Cellulase activity was assayed on indicator plates containing 0.5% carboxymethylcellulose (CMC) agar medium (Baldan et al. 2015). All the twenty-nine isolates were inoculated onto the CMC agar medium (1.0 g KH_2PO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.5gNaCl, 0.01g $FeSO_4 \cdot 7H_2O$, 0.01g $MnSO_4 \cdot H_2O$, 0.3g NH_4NO_3,10g CMC and 15 g agar in 1 L distilled water) and plates were incubated at 30 ± 2˚C for 72 h. After incubation, plates were flooded with 0.1% Congo red for 20 min and then destained with 1M NaCl for 20 min. Regions of the agar where the hydrolysis of CMC has occurred were clear, whereas intact CMC would retain the Congo red dye (McDonald et al. 2012; Mardanova et al. 2016). After destaining procedure, plates were observed for zone formation around the test strains as an indication of cellulase production.

4.2.3.6 Pectinase Production:

Pectinase activity was detected by spotting the culture on a 1% pectin amended nutrient agar plate (Gessesse and Gashe 1997). The plates were inoculated with the test strain and incubated for 48 h at 30°C. After incubation, plates were flooded with congo red solution (0.2%) for 10 min and excess solution was removed. Thereafter, plates were observed for clear zone around test isolates which indicated the presence of pectinase production.

4.2.4 Detection of diffusible and volatile antibiotic:

For the detection of diffusible antibiotics, 100 µl of bio antagonistic bacterial suspension of all the twenty-nine isolates was uniformly spread on the PDA plate. After 24 h of incubation of plates or immediately after spreading the bacterial

suspension, 5 mm of the pathogen mycelial disc of *C. falcatum* was placed at the centre of the same PDA plate. Similarly, in control 100 µl of sterilized distilled water was spread on the PDA plates and 5 mm mycelial disc of the pathogen was placed in the centre of the plate. Plates were then sealed with parafilm and incubated for 5 days at 27 ± 2ºC.

For volatile antibiotic detection; one hundred microlitre (100 µl) of a bio-antagonistic bacterial suspension of isolates was spread on one half of the Petri dish containing LB medium, and a 5 mm disc of a five days old pure culture of *C. falcatum* was placed at the centre of another half of the Petri dish containing PDA medium. Both half plates (lower lid of petri dish) were placed face to face preventing any physical contact between the pathogen and the bacterial suspension and were tightly sealed with parafilm so as to prevent the loss of volatiles formed inside the plates and incubated at 27 ± 2 ºC for 5 days. The observation for both the diffusible and volatile antibiotics was recorded on the growth of the pathogen on 5^{th} days of incubation. Inhibition of fungal growth was measured as per the formula:

$$\% \text{ Inhibition} = \frac{(C-T)}{C} \times 100\%$$

, where 'C' is the diameter growth (in mm) of the control fungal mycelium colony and 'T' is the diameter growth (in mm) of the fungal mycelium growing in presence of antagonist endophytic bacterial isolate. The experiment was repeated thrice with three replicates (Montealegre et al. 2003; Chaurasia et al. 2005).

4.2.5 Evaluation of the effect of cell-free culture filtrate:

All the twenty-nine antagonistic isolates were grown on Luria Bertani (LB) broth medium in 100 ml conical flask at 30 ± 2 °C on a rotary shaker at 150 rpm. Culture

broth after 72 h of incubation was centrifuged at 10,000 rpm at 4 °C for 10 min, and cell-free culture filtrate was obtained by passing the supernatant through 0.22 µm pore size syringe filter (Millex-GV; Millipore). Mycelial disc of an actively growing culture of *C. falcatum* was placed in the centre of each plate containing PDA medium. Well of 5 mm was made with sterile cork borer on four end of each PDA plate in which 100 µl of the cell-free filtrate of individual strain was placed and at center 5 mm disc of *C. falcatum* was placed. Plates were incubated at 27 ± 2 °C for 7 days. Mycelial growth inhibition (%) was measured as according to the formula:

$$\% \text{ Inhibition} = \frac{(C-T)}{C} \times 100$$

, where 'C' is the radial growth (in mm) of the control fungal mycelium colony and 'T' is the radial growth (in mm) of the fungal mycelium growing in presence of antagonist endophytic bacterial isolates.

4.2.6 Ultrastructural interaction study by simple microscopy and scanning electron microscopy (SEM):

After investigating the activity of various antifungal metabolites and plant growth promoting abilities, the antagonistic mechanisms of all the twenty-nine endophytic bacterial isolates against hyphae of *C. falcatum* were observed by light microscopy and scanning electron microscopy (SEM). After a colony of endophytic bacteria contacted that of pathogen on PDA in a dual culture, interactive zones between the fungal hyphae and the endophytic bacteria were cut into 1cm pieces, which were then observed directly under a light microscope (after staining with lactophenol cotton blue) or were used for SEM study.

For SEM; a small section of agar block of fungal hyphae was cut from antagonized dual plate and was fixed in 2% glutaraldehyde for 4 h at room temperature. Subsequently, the hyphae were dehydrated in a graded series of ethanol concentrations (30%, 50%, 70%, 80%, 90%, and 100%) for 15 min each followed by drying in desiccator After that, the sample was fixed to SEM stubs using carbon tape followed by thin coating of palladium using a palladium sputter coater and directly imaged using a JEOL JSM6490LV.

4.3 Results:

All the twenty-nine antagonistic endophytic bacteria that showed strong antagonism against *C. falcatum* were screened for the presence of various plant-growth-promoting and antifungal traits and results demonstrated the presence of multiple beneficial traits in single isolates.

4.3.1 Characterization for various PGP abilities :

The selected antagonistic endophytic bacteria when checked for their PGP traits, it was observed that most of the bacteria had one or more PGP characteristics (Fig 4.1).

4.3.1.1 IAA Production:

Bacterial endophytes are an efficient producer of IAA and the detection result of IAA production of 29 endophytic bacteria with antifungal activity against *C. falcatum* revealed that 51.72% of selected antifungal endophytic bacteria demonstrated the positive confirmation to IAA production (pink colour production; Fig 4.1). The quantitative estimation of isolates for IAA production revealed that it ranged from minimum 29.99 ± 2.59 µg/ml in BS-4 to maximum 112.92 ± 4.16 µg/ml in BS14 (Table 4.1).

4.3.1.2 Phosphate Solubilization:

Phosphate solubilization screening result of 29 endophytic bacteria indicated that 93.10% of endophytic bacteria had halo zone and confirmed P-solubilization. SI (Solubilization Index) of the isolates ranged from 1.20 ± 0.03 in the isolate S22 to 2.67 ± 0.29 in the isolate S26 (Table 4.1). Only two isolates i.e. B8 and B9 were not showing any halo zone. Rest all of the endophytes were positive for P- solubilization. The quantitative estimation of available phosphate revealed that highest amount of phosphate was present in BS-4 (272.85 µgPO^{3-}_4) and lowest in B12 isolate (11.28 µgPO^3_4). Followed by BS-4; higher amount of phosphate was also detected in the isolate R5, S26, S17, S8 and so on as detailed in Table 4.1

4.3.1.3 Ammonia Production:

Twenty-nine endophytic bacteria were showing positive results for ammonia production. However, S8, S17, R5, and BS-4 showed strong ammonia production after visualizing dark yellow to brownish colour after adding Nessler's reagent as indicated by '++' in Table 4.1. Rest all the isolates showed the yellow colouration as indicated by '+' as compared to control.

4.3.1.4 ACC deaminase:

Screening results of 29 endophytic bacteria for ACC deaminase production (important PGP traits) found that only three bacterial isolates (S8, S26, and R5) depicted the production of ACC deaminase as indicated by their growth on the DF medium.

4.3.1.5 HCN production:

Besides, the above characteristic; HCN is an important PGP attribute and found that ten isolates namely B2, B5, B8, B12, S11, S17, R5, BS-4, BS8, and BS15 endophytic

bacteria were positive for HCN production as observed by change in the colour of filter paper from yellow to brown (Fig 4.1).

4.3.1.6 Siderophore Production:

The endophytic bacterial isolates were screened for siderophore production using the chrome azurol S (CAS) agar. CAS-agar plates assay demonstrated the presence of siderophore by change in colour of dye from blue to yellow-orange zones around the bacterial isolates of S8, S12, S17, S26, R5 and BS-4. Further confirmation of these isolates for their siderophore presence in liquid assay was expressed in terms of percentage siderophore unit and it ranged from 10.58 ± 4.24% PSU in S26 to maximum 93.16 ± 1.44% PSU in BS-4 (Table 4.1). The formation of yellow to orange halo around the colonies due to the chelation of iron was the indication for production of siderophore. The formation of the yellow-orange halo is as a result of the production of siderophore, which removes iron from the dye complex that changes the colour of the medium from blue to orange (Schwyn and Neilands 1987).

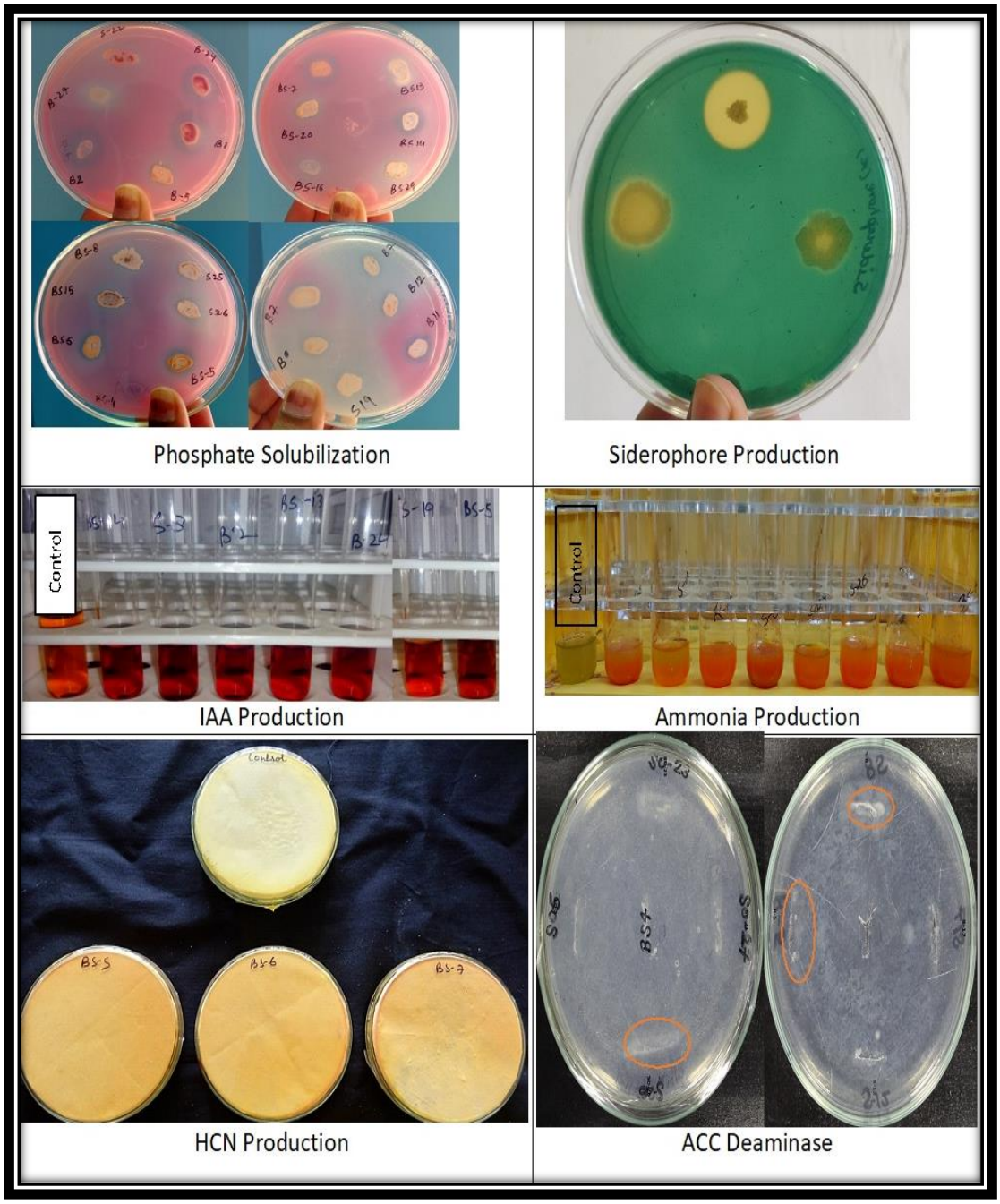


Fig 4.1 *In vitro* screening of plant growth promoting traits for isolated antagonistic endophytic bacteria.

Table 4.1 Investigation of various Plant growth-promoting attributes of antagonistic endophytes isolated from sugarcane.

PGPR attributes									
Isolates	**Solubilization Index (SI)**	**Concentration of P (μgPO_4^{3-})**	**IAA**	**IAA(µg/ml)**	**HCN**	**Ammonia**	**Siderophore**	**PSU (%)**	**ACC deaminase**
B1	1.98±0.23^{ghij}	129.70 ±23.40 ^{fg}	+	82.91±2.86 ^{f}	_	+	_	ND	_
B2	2.32±0.16^{jk}	178.19±27.77 ^{ghi}	+	85.64±1.83 ^{f}	+	+	_	ND	_
B5	1.86±0.17^{efghi}	133.83 ± 15.84^{fg}	_	ND	+	+	_	ND	_
B7	2.07±0.15^{hij}	146.98±12.82^{fg}	+	91.59±2.43 ^{g}	_	+	_	ND	_
B8	ND	ND	_	ND	+	+	_	ND	_
B9	ND	ND	+	59.52±0.55 ^{d}	_	+	_	ND	_
B12	1.56±0.29^{abcdef}	11.28±7.85^{a}	_	ND	+	+	_	ND	_
B24	2.19±0.36^{hij}	170.07±32.49 ^{gh}	+	92.97±0.43 ^{g}	_	+	_	ND	_
B27	1.45±0.23^{abcd}	45.10± 5.61^{abcd}	+	40.57±1.47 ^{b}	_	+	_	ND	_
S8	2.14±0.10^{hij}	202.51±8.07 hij	+	74.00±1.94 ^{e}	_	++	+	19.06±8.71	+
S11	1.41±0.08^{abcd}	14.09±8.31 ^{ab}	-	ND	+	+	_	ND	_
S12	1.35±0.13^{abc}	150.59±20.10 ^{fg}	+	42.01±2.77 ^{b}	_	+	+	23.89±4.19	_
S17	2.66±0.14^{k}	207.69± 20.38 ^{hij}	_	ND	+	++	+	90.65±6.21	_
S19	1.42±0.14^{abcd}	64.34± 32.21^{bcd}	+	30.65±1.50 ^{a}	_	+	_	ND	_
S21	1.80±0.26^{defgh}	63.53±16.11^{bcd}	-	ND	_	+	_	ND	_
S22	1.20±0.02^{a}	31.09±27.76^{abc}	_	ND	_	+	_	ND	_
S25	1.93±0.21^{fghij}	84.10 ± 16.13 ^{de}	_	ND	_	+	_	ND	_

PGPR attributes									
Isolates	**Solubilization Index (SI)**	**Concentration of P (μgPO_4^{3-})**	**IAA**	**IAA(µg/ml)**	**HCN**	**Ammonia**	**Siderophore**	**PSU (%)**	**ACC deaminase**
S26	2.67 ± 0.29^{k}	220.89 ± 20.09^{ij}	_	ND	_	+	+	10.58±4.24	+
S37	1.24 ± 0.14^{ab}	18.84±1.29 ab	_	ND		+	_	ND	_
R5	2.17 ± 0.36^{hij}	241.65 ± 1.77^{jk}	+	47.14 ± 1.57^{c}	+	++	+	78.25±6.32	+
BS-4	1.66 ± 0.15^{cdefg}	272.85 ± 26.57^{k}	+	29.99 ± 2.59^{a}	+	++	+	93.16±1.44	_
BS8	2.22 ± 0.12^{ij}	117.35 ± 17.41^{ef}	-	ND	+	+	_	ND	_
BS11	1.21 ± 0.15^{ab}	75.03 ± 49.05^{cde}	+	72.43 ± 0.50^{e}	_	+	_	ND	_
BS13	2.22 ± 0.24^{ij}	143.33 ± 18.53^{fg}	+	105.08 ± 1.20^{h}	_	+	_	ND	_
BS14	1.49 ± 0.22^{abcde}	63.06 ± 85.90^{bcd}	+	112.92 ± 4.16^{i}	_	+	_	ND	_
BS15	1.60 ± 0.28^{bcdefg}	29.33 ± 20.27^{abc}	_	ND	+	+	_	ND	_
BS16	1.23 ± 0.16^{ab}	14.32 ± 15.07^{ab}	_	ND	_	+	_	ND	_
BS20	1.37 ± 0.11^{abc}	12.19 ± 7.81^{a}	+	92.07 ± 2.70^{g}	_	+	_	ND	_
BS29	1.39 ± 0.20^{abc}	17.46 ± 14.55^{ab}	_	ND	_	+	_	ND	_

'+' represents positive test; '-' represent negative test; 'ND' represent Not detected

'PSU' stands for Percentage Siderophore Unit

In Solubilization Index, concentration of phosphate, and IAA(µg/ml) column means followed by same letters are not significantly different at the 5% level by DMRT.

4.3.2 Qualitative evaluation of lytic enzyme:

Petri dish-based qualitative assays revealed that endophytic bacterial isolates produced variety of lytic enzymes such as chitinase, β-1,3 glucanase, protease, amylase, pectinase, and cellulase enzymes which play an important role in lysis of fungal pathogen (Fig 4.2; Table 4.2).

4.3.2.1 Chitinase and β-1, 3 glucanase Production:

Chitinase production was observed by a halo zone around the colony after staining and destaining procedure. The isolates S8, S12, S17, S26, R5 and BS-4 demonstrated the clear halo zone around the colony. However, S8, S12, S17, R5 and BS-4 showed strong clearance zone (≥ 5 mm) and placed in '++' category while S26 was placed in '+' category (≤ 5 mm clearance zone). Similarly, S12, S17, R5 and BS-4 revealed the promising result for β-1, 3 glucanase enzyme by formation of halo zone around the colony.

4.3.2.2 Amylase Production:

Amylase production was also found to be positive for the 27 endophytic isolates out of 29. The isolates B1 and B7 did not show any amylolytic activity due to the absence of yellow zone around colonies after flooding the plate with iodine solution.

4.3.2.3 Protease Production:

A clear zone on skim milk agar was evidence for strong protease activity after 48 h of culture incubation at 30 ± 2 °C (Fig 4.2). Twenty-three isolates were showing the presence of enzyme. The isolates with halo zone 0-5 mm, 5-10 mm and 10-15 mm were categorized as '+', '++', and '+++', respectively (Table 4.2). However, no halo zone was observed for the isolates B2, B5, B7, BS16, BS20 and BS29.

4.3.2.4 Cellulase Production:

Cellulase is another important lytic enzyme responsible for degradation of cellulose. The 16 isolates out of 29 gave positive results for cellulose production (Table 4.2).

4.3.2.5 Pectinase Production:

Similarly, a clear and halo zone was also observed around the endophytic bacterial isolates of only five isolates among twenty-nine isolates *viz.* R5, BS-4, S8, S12 and S17 in pectin amended plates, thus showing positive results for pectinase production.

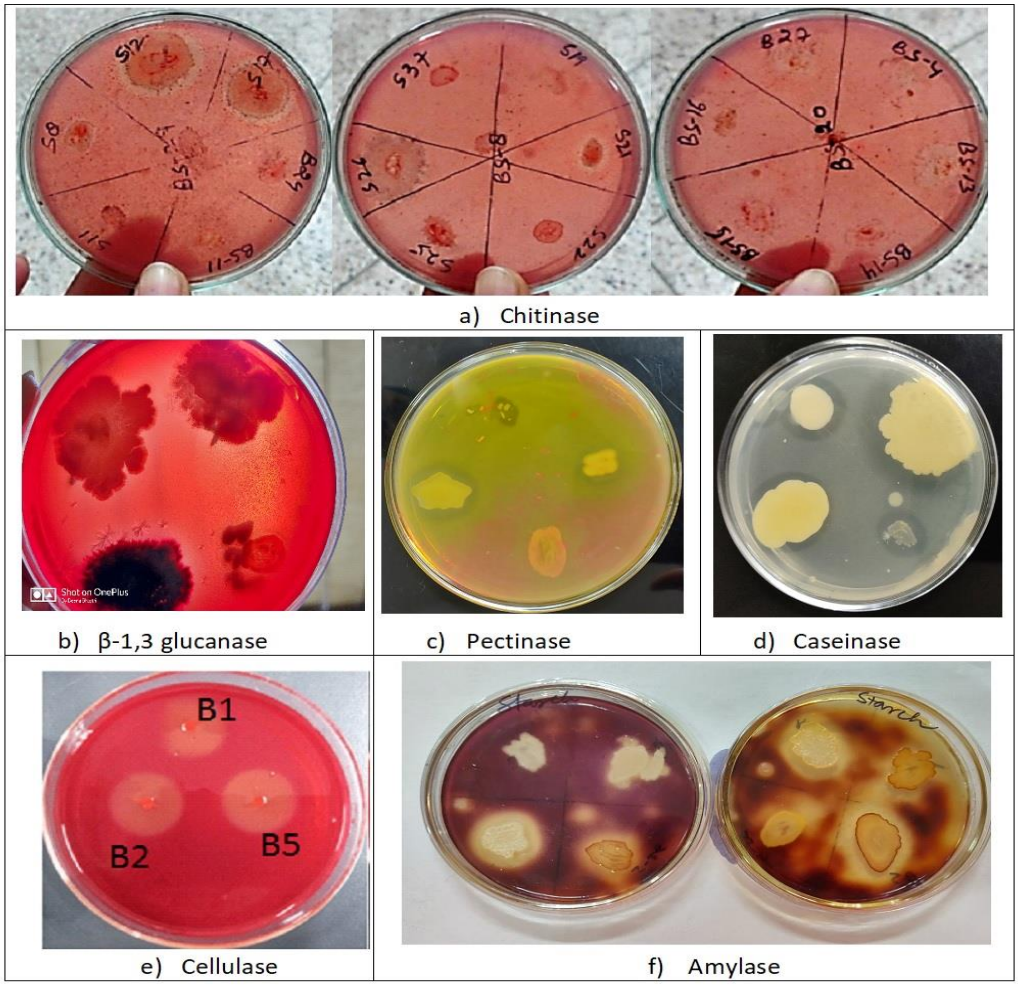


Fig 4.2 *In vitro* screening of various lytic enzyme in the antagonistic endophytic isolates.

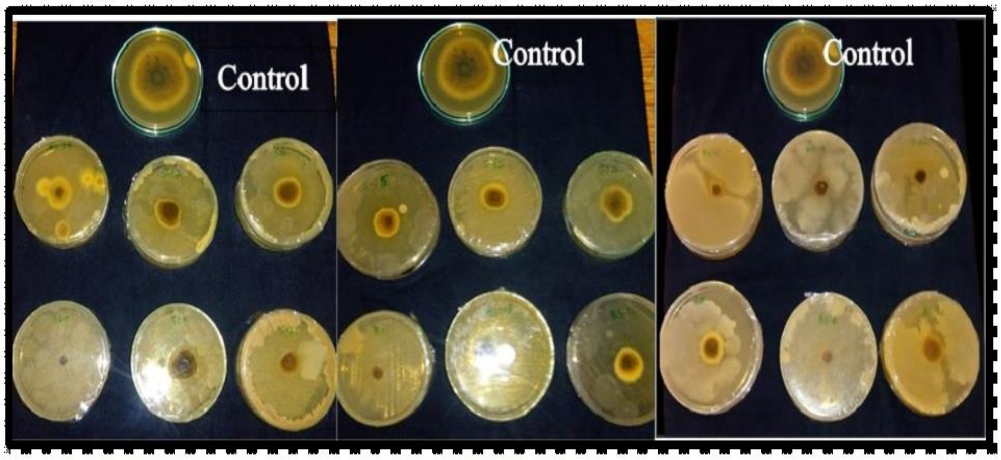
Table 4.2 *In vitro* screening of the presence of hydrolytic enzyme in the antagonistic endophytic bacterial isolates.

Isolates	Chitinase	β-1,3 glucanase	Protease	Amylase	Cellulase	Pectinase
B1	-	-	++	-	+	-
B2	-	-	-	+	+	-
B5	-	-	-	+	+	-
B7	-	-	-	-	+	-
B8	-	-	++	+	+	-
B9	-	-	++	+	+	-
B12	-	-	++	+	+	-
B24	-	-	++	+	+	-
B27	-	-	+	+	+	-
S8	++	-	+	+	+	+
S11	-	-	+	+	-	
S12	++	+	+	+	-	+
S17	++	+	+	+	-	+
S19	-	-	+	+	-	-
S21	-	-	+	+	-	-
S22	-	-	+	+	-	-
S25	-	-	+	+	-	-
S26	+	-	+	+	+	-
S37	-	-	+	+	-	-
R5	++	+	++	+	+	+++
BS-4	++	+	+++	+	+	+
BS8	-	-	+++	+	+	-
BS11	-	-	++	+	+	-
BS13	-	-	++	+	-	-
BS14	-	-	++	+	+	-
BS15	-	-	+	+	-	-
BS16	-	-	-	+	-	-
BS 20	-	-	-	+	-	-
BS29	-	-	-	+	-	-

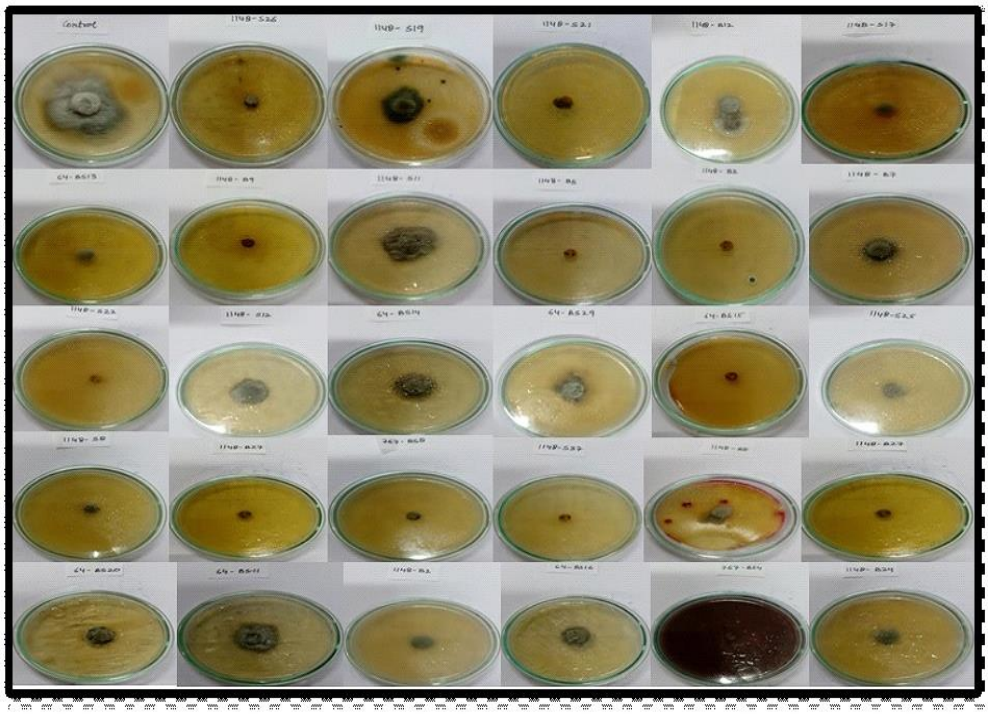
'+' represents halo zone ranging from 0-5 mm; '++' represents halo zone of 5-10 mm and '+++' represents halo zone of 10-15 mm on different respective media.

4.3.3 *In vitro* antagonism due to volatile and diffusible antibiotics:

Volatile compounds secreted by a number of bacteria play an important role in biocontrol of phytopathogen. All the 29 antagonistic endophytic bacteria produced anti-fungal volatile compound(s) (VOCs) as evident from the growth inhibition of *C. falcatum* (Fig 4.3a). Inhibition in the mycelial growth (in diameter) of fungus was significantly observed (Table 4.3) when compared to control. In addition, aerial mycelial growth was also reduced due to the effect of volatile metabolites. Raza et al. (2016) demonstrated the role of VOCs produced by *Pseudomonas fluorescens* WR-1 in biocontrol activities. Kandel et al. (2017) and Lee et al. (2017) have also recently reported that VOCs mediated antifungal activities. Similarly, all the isolates were able to inhibit the mycelial growth due to the action of its diffusible metabolites (Fig 4.3b). The observation made on 5^{th} day is summarised in Table 4.3.



a) Volatile antibiotic production



b) Diffusible antibiotic production

Fig 4.3 Production of volatile and diffusible antibiotic by endophytic isolates.

4.3.4 Evaluation of the effect of cell-free culture filtrate of isolates against *C. falcatum:*

In order to evaluate the involvement of another putative antifungal compound in their suppressive effect, the antifungal activity of cell-free supernatant from the culture of all the 29 isolates were analyzed against red rot pathogen. Results of % inhibition of

cell-free extract against *C. falcatum* signified that cell-free supernatant of the isolates BS13, BS11, S21, S37 and B9 were able to inhibit the radial mycelial growth of *C. falcatum* with less than 50% inhibition (Table 4.3). While rest all the isolates were demonstrating strong inhibition against *C. falcatum* with highest in S12 isolates with 85.95% (Table 4.3). These findings provide evidence that extracellular metabolites in the cell-free culture filtrate of isolates inhibited the growth of pathogenic fungi which prompted the further investigation of the effects caused by their metabolites on the growth of the hyphal structure. There is a greater probability that the increased antifungal activity against the pathogen fungi tested by the culture filtrate of the isolates is a consequence of the production of extracellular secondary antifungal metabolites.

4.3.5 Ultrastructural study of fungal mycelium by simple microscopy and scanning electron microscopy (SEM):

Study of fungal-antagonist (test endophyte) interaction from dual plate antagonism under simple microscopy revealed the distortion of hyphal structure in the growth of the test pathogen by all the endophytic isolates as indicated by red arrow in Fig 4.4. Further, based on the presence of all the beneficial trait in single isolate, the isolates S12, S8, S26, S17, R5 and BS-4 were selected due to the possession of maximum PGP and antifungal lytic enzyme traits for further investigation. Therefore, the antagonistic mechanisms of isolates S12, S8, S26, S17, R5 and BS-4 against hyphae of *C. falcatum* were also observed by scanning electron microscopy (SEM). These findings prompted the investigation that distortive effects (hyphal swelling, and distortion of mycelium) in pathogenic fungi, *C. falcatum* were due to the extracellular metabolites in the culture filtrates (Fig 4.5). Further, hydrolytic enzymes and secondary antifungal compounds may also be responsible for the distortion in the mycelium of fungal pathogen.

Table 4.3 *In vitro* inhibition of *Colletotrichum falcatum* by the extracellular crude metabolites and various antibiotics produced by endophytic bacterial isolates.

	Diffusible antibiotic		**Volatile antibiotic**		**Extracellular crude metabolite**	
Isolate	**Mycelial growth (diameter in mm) of *C. falcatum***	**Inhibition of *C. falcatum* (%)**	**Mycelial growth (diameter in mm) of *C. falcatum***	**Inhibition of *C. falcatum* (%)**	**Radial mycelial growth (mm) *C. falcatum***	**Inhibition of *C. falcatum* (%)**
Control	**75.33 ± 5.03**	**0.00 ± 0.00**	**70.33 ± 5.51**	**0.00 ± 0.00**	**40.33 ± 5.50**	**0.00 ± 0.00**
B1	22.33 ± 2.52	70.35 ± 3.34	13.00 ± 2.65	81.51 ± 3.76	6.00 ± 1.00	85.12 ± 2.48
B2	20.00 ± 5.00	73.45 ± 6.64	20.00 ± 2.00	71.56 ± 2.85	16.33 ± 2.08	59.50 ± 5.16
B5	21.67 ± 2.89	71.24 ± 3.83	24.33 ± 2.08	65.40 ± 2.96	11.67 ± 3.06	71.08 ± 7.58
B7	35.33 ± 2.52	53.10 ± 3.34	19.00 ± 5.57	72.98 ± 7.92	7.00 ± 2.65	82.64 ± 6.56
B8	29.00 ± 1.00	61.50 ± 1.33	26.33 ± 5.51	62.56 ± 7.83	15.00 ± 1.00	62.81 ± 2.48
B9	15.00 ± 4.36	80.09 ± 5.79	14.67 ± 4.51	79.15 ± 6.41	21.00 ± 1.00	47.93 ± 2.48
B12	42.00 ± 1.73	44.25 ± 2.30	11.33 ± 3.21	83.88 ± 4.57	15.17 ± 0.76	62.40 ± 1.89
B24	17.00 ± 2.65	77.43 ± 3.51	24.67 ± 4.93	64.93 ± 7.02	13.33 ± 0.58	66.94 ± 1.43
B27	10.00 ± 1.00	86.73 ± 1.33	24.10 ± 4.55	65.73 ± 6.47	11.67 ± 2.08	71.08 ± 5.16
S8	15.00 ± 1.00	80.09 ± 1.33	9.33 ± 2.08	86.73 ± 2.96	7.47 ± 0.55	81.49 ± 1.36
S11	39.67 ± 4.51	47.34 ± 5.99	11.67 ± 4.73	83.41 ± 6.72	8.33 ± 0.58	79.34 ± 1.43
S12	21.67 ± 5.77	71.24 ± 7.67	20.67 ± 3.06	70.62 ± 4.34	5.67 ± 2.08	85.95 ± 5.16
S17	13.67 ± 2.08	81.86 ± 2.34	18.00 ± 1.00	74.57 ± 1.45	11.90 ± 0.35	70.52 ± 0.95
S19	30.33 ± 5.51	59.73 ± 7.31	27.33 ± 3.79	61.14 ± 5.38	16.00 ± 1.00	60.33 ± 2.48

S21	13.33 ± 1.53	82.30 ± 2.03	19.63 ± 5.04	72.08 ± 7.17	25.00 ± 2.65	38.02 ± 6.56
S22	10.00 ± 1.00	86.73 ± 1.33	23.33 ± 4.73	66.82 ± 6.72	11.00 ± 1.00	72.73 ± 2.48
S25	11.00 ± 3.61	85.40 ± 4.79	24.33 ± 4.51	65.40 ± 6.41	12.33 ± 2.08	69.42 ± 5.16
S26	13.67 ± 1.53	81.86 ± 2.03	7.67 ± 2.52	89.10 ± 3.58	8.17 ± 1.04	79.75 ± 2.58
S37	15.00 ± 3.00	80.09 ± 3.98	19.00 ± 2.65	72.98 ± 3.76	22.67 ± 1.53	43.80 ± 3.78
R5	17.53 ± 1.05	76.73 ± 1.40	9.00 ± 3.61	87.20 ± 5.13	8.33 ± 2.08	79.34 ± 5.16
BS-4	11.17 ± 1.04	85.18 ± 1.39	22.33 ± 2.52	68.24 ± 3.58	10.00 ± 2.00	75.21 ± 4.96
BS8	9.83 ± 1.35	86.95 ± 1.80	13.33 ± 4.16	81.04 ± 5.92	11.67 ± 1.53	71.08 ± 3.79
BS11	29.17 ± 1.89	61.28 ± 2.51	30.67 ± 5.51	56.40 ± 7.83	34.33 ± 0.58	14.87 ± 1.43
BS13	11.83 ± 2.36	84.29 ± 3.14	17.33 ± 2.08	75.36 ± 2.96	39.33 ± 1.15	2.48 ± 2.86
BS14	22.33 ± 2.52	70.35 ± 3.34	14.00 ± 4.00	80.09 ± 5.69	14.83 ± 1.26	63.22 ± 3.12
BS15	12.33 ± 2.52	83.63 ± 3.34	14.67 ± 4.04	79.15 ± 5.74	7.33 ± 2.52	81.82 ± 6.24
BS16	12.17 ± 1.04	83.85 ± 1.38	21.00 ± 3.61	70.14 ± 5.13	13.33 ± 3.06	66.94 ± 7.58
BS 20	35.67 ± 4.04	52.65 ± 5.37	31.67 ± 5.86	54.98 ± 8.33	16.83 ± 2.25	58.26 ± 5.59
BS29	36.33 ± 3.51	51.77 ± 4.66	21.00 ± 2.65	70.14 ± 3.76	9.33 ± 8.39	76.86 ± 20.79

All the observation was recorded after repeating experiment thrice with three replicate each and '±' values represents standard deviation (SD).

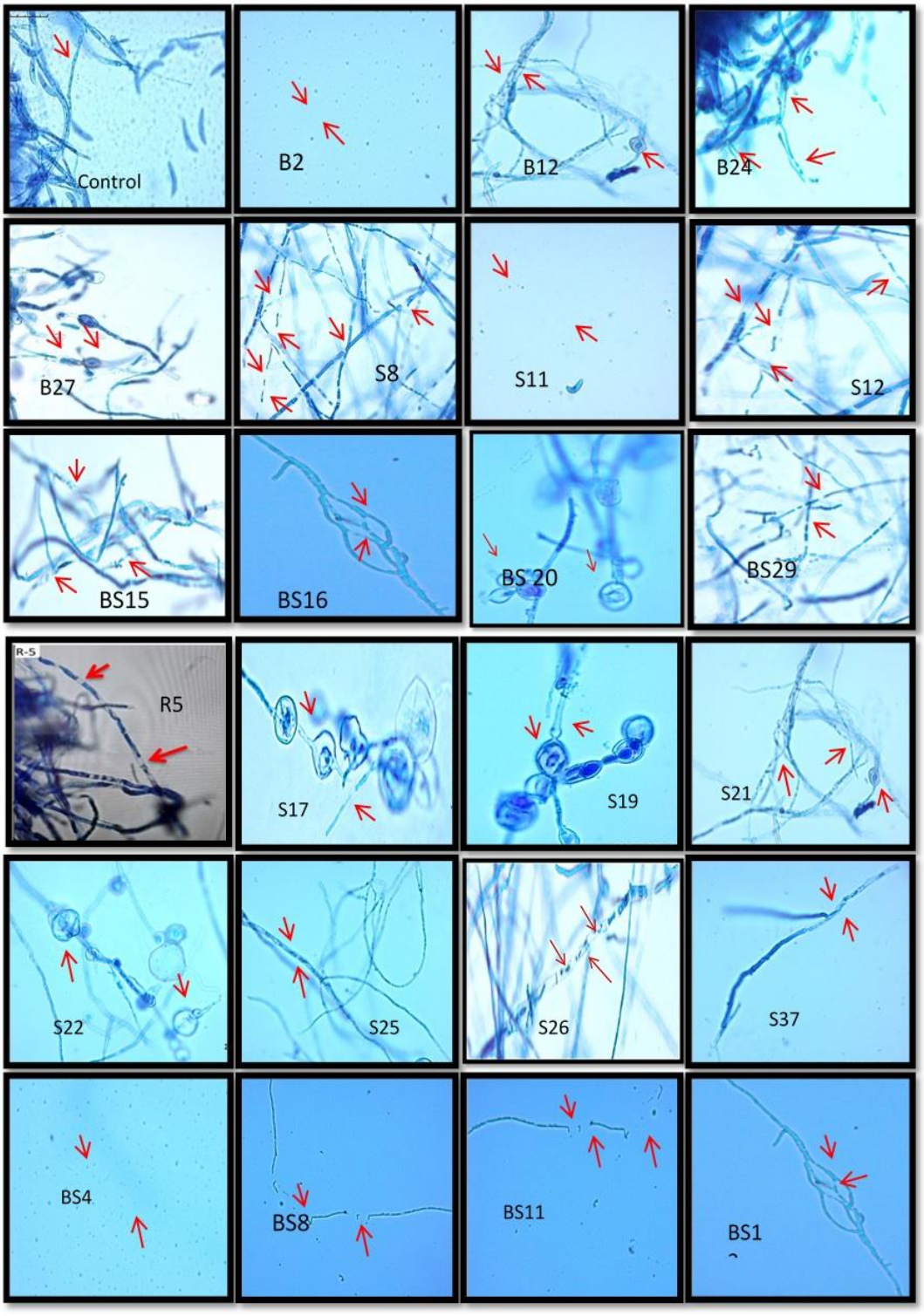


Fig 4.4 Microscopic examination of hyphae of fungal pathogen *C. falcatum* in antagonism mechanism by endophytic bacterial isolates alongwith control pic (first one with full pathogen growth) with intact hyphae.

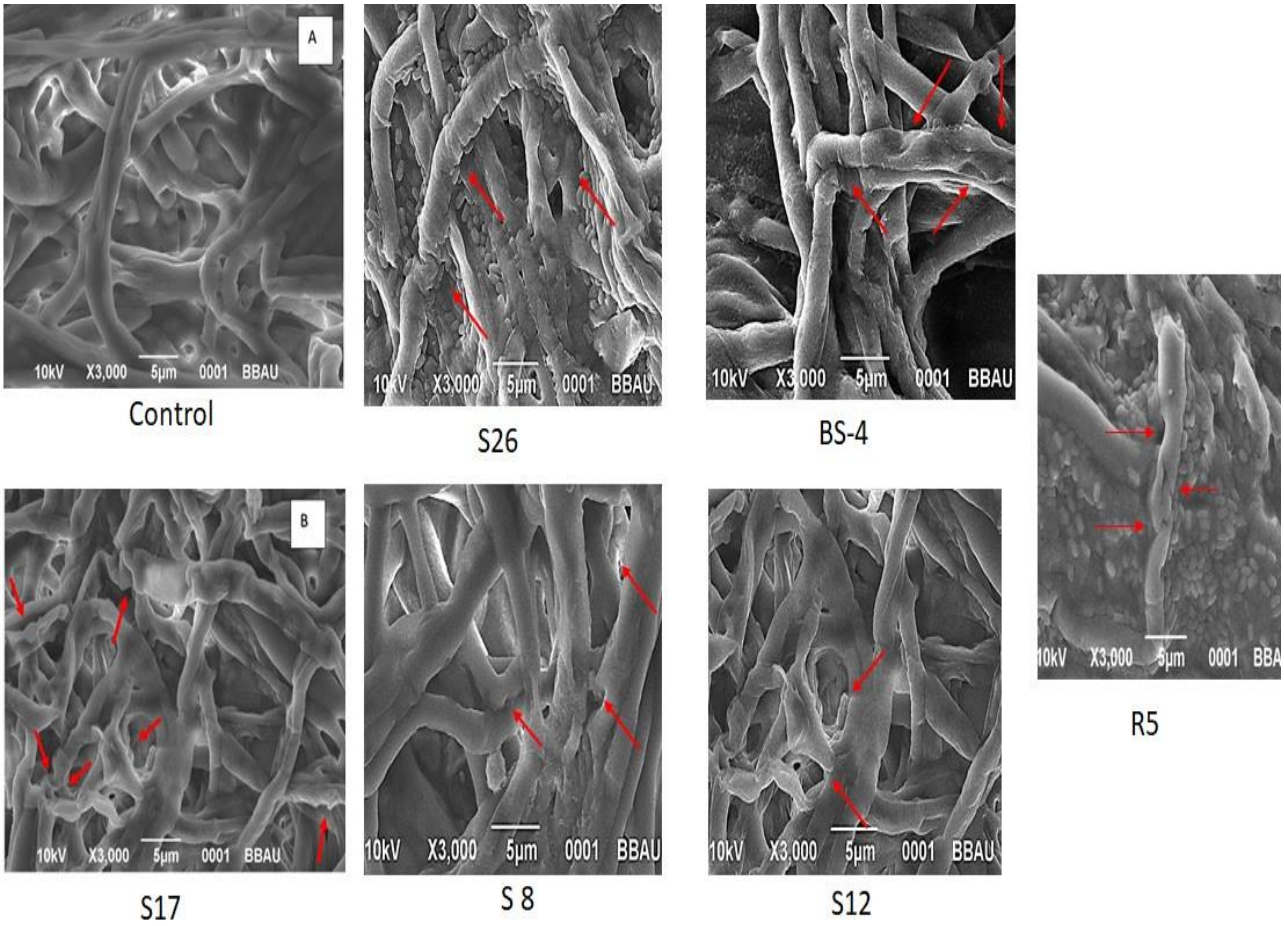


Fig 4.5 SEM analysis of fungal and endophytic bacterial interaction with Control (Full pathogen growth) without distortion.

4.4 Discussion:

Endophytic bacteria increase the fitness level of the living plant by secretion of various secondary metabolites which stimulates the growth of the plant in a direct and indirect manner. Thus, endophytic bacteria contribute in the protection and resistance to plants against invasion of pathogen. In the current study, 29 endophytic bacterial isolates from the sugarcane plants demonstrated *in vitro* inhibition against red rot pathogen (Chapter-3). This antagonism was mainly due to the production of various secondary metabolites which helps in suppressing the pathogen. Therefore, present chapter sheds light on the screening of 29 antagonistic endophytic bacteria for the

presence of various plant growth-promoting and other beneficial secondary metabolites.

Endophytes are known for their contributions in the growth promotion of plants in a direct and indirect way. Indole 3 acetic acid (IAA) production and phosphate solubilization are some of the important mechanisms for plant growth promotion as shown by the variety of PGP endophytes (Spaepen et al. 2007; Qin et al. 2015; Walitang et al. 2017; Yan et al. 2018). In the present study, the majority of the isolates showed positive results for phosphate solubilization and IAA. These results demonstrated the fact that isolated antagonistic endophytic bacteria have diverse functions in the inhibition of phytopathogens and in promoting the growth of sugarcane crop. This also suggested that these bacteria could also improve the yields of sugarcane crop by stimulating phytohormone and improving nutrient supply by solubilizing phosphate and would also protect the crop against red rot pathogen. Thus, the reason for increment in the growth of some plants is due to the colonization of endophytic bacteria (Shi et al. 2009). Indole 3 acetic acid (IAA) being an auxin can initiate cell elongation, cell division and differentiation in plants (Taghavi et al. 2009). IAA is shown to be produced by many root-associated bacteria including *Enterobacter* sp., *Pseudomonas* sp., *Bacillus* and *Azospirillium* sp. (El-Khawas and Adachi 1999; Kruasuwan and Thamchaipenet 2016; Rohini et al. 2018). This observation was also reported by Kim et al. (2011) showing that plants are colonized by high numbers of IAA-producing bacteria. Inoculation of plants with IAA producing bacteria causes changes in the structure of root by stimulating root hair formation, thereby ultimately increasing root surface area for mineral uptake and root exudation (Vessey 2003; Duca et al. 2014). The amount of IAA produced by bacteria plays an important role in plant-microbe interaction. In the current study; almost

51.72% (15 out of 29) of isolated endophytes showed IAA production. In this study, all the isolates produced although different amount of IAA, the present study filtered and selected the isolates R5, BS-4, S8, S12, S26 and S12 for future identification and application based on multiple beneficial traits. It was well demonstrated that these selected isolates demonstrated a good amount of the IAA production, but amount was found to be lower when compared with all the rest IAA producing endophytic isolates. It is also well documented from the previous study that bacteria which produce lower levels of IAA had significant influence on the root development by increasing its length and volume, which provide greater surface area for absorption of nutrients (Szilagyi-Zecchin et al. 2014). The concentration of auxin also decides its effects on plants; at low concentration it can stimulate the plant growth; however, at high concentration it was found inhibitory to the seed germination (Arshad and Frankenberger 1991). This probably is due to the reason that IAA can alter the endogenous auxin of the plant to an optimal or deleterious level (Patten and Glick 1996).

Phosphorus is an important constituent of cell and is needed by plants for growth and development, but its availability in agricultural soil's is low (Pikovskaya 1948). Endophytes are known to improve plant growth by the phosphate solubilization after releasing organic acid, polysaccharides and the presence of phosphatase enzyme (Schachtman et al. 1998). In the present study, nearly 93.10% of isolated endophytes were found to have the potential to solubilize phosphorous. Plant growth-promoting bacteria solubilize insoluble phosphates to make them available in accessible form to crop for enhancing their crop productivity. *Pseudomonas* strains are characterized as better phosphate solubilizers and IAA producers (Walitang et al. 2017). Bacteria which are intensely associated with plant have the ability to solubilize the unavailable

phosphate into soluble form to plants and increase their growth and yield. In the present study, *Pseudomonas* and *Bacillus* spp. possess multiple PGP traits such as IAA producer and phosphate solubilizer along with antifungal property. Various species of *Bacillus* are known for IAA production and isolates in the present study *viz.* *Bacillus aryabhattai* (S12), *B. paramycoides* (R5) also showed a positive result for IAA and phosphate solubilization. To the best of my knowledge, this is the first report on the isolation of such strain from sugarcane as an endophyte. Majority of the isolated bacteria belong to *Bacillus* group and it is also known that they are a valuable candidate for exploring biofertilizers. Thus, the present study significantly demonstrated the occurrence and diversity of culturable endophytic bacteria in sugarcane plant tissues exhibiting IAA and phosphorus solubilization ability. This can be utilized in future application, while applying the sett treatment with these endophytic bacteria, as this would help in maintaining and improving the health status of crop.

In addition to biofertilizer activities (IAA and phosphate solubilization); the isolated 29 bacterial isolates were tested for biocontrol activities. Siderophore production is considered as one of the potent biocontrol factor. Endophytic bacteria compete with phytopathogen by chelating iron; thus, making them unavailable to the pathogen and suppress the growth of pathogenic microorganisms. They also contribute to the indirect plant growth promotion (Ben Slama et al. 2019). In this study, siderophore production was observed only in six strains comprising *Bacillus* sp. and *Pseudomonas sp.*, similar to the previous report by Jasim et al. (2014). In the study of Zhao et al. (2018), the high correlation value (R^2= 0.93) was found between siderophore production and the fungal inhibition by endophytic bacteria which supports the fact that chelation of iron by endophytic bacteria might be a mechanism for the inhibition

of fungal growth (Miethke and Marahiel 2007; Devi et al. 2017). In the study of Yu et al. (2017), optimisation study yielded the maximum siderophore unit of 90.52% in *Bacillus sp*. PZ-1 while in the present study, without optimisation, better results were obtained as depicted in Table 4.1 by endophytic bacteria BS-4, S17 and R5. In the recent era, siderophores has gained much attention toward modern application in agriculture. This study may have a huge impact on the agricultural perspective that would encourage the more production of siderophore commercially by the isolates and utilizing them as an important metabolite for suppressing the pathogen in the application of the biological control field.

Ethylene hormone generally regulates the plant growth like any other phytohormone; but at a concentration higher than the required amount, it acts as a poison to the plant. To address this problem, some of the PGP endophytes synthesize ACC deaminase; which facilitates plant growth and their development besides IAA and phosphate solubilization mechanism (Glick 2012; Khan et al. 2016). ACC deaminase is generally responsible for the degradation of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate ethylene precursor into ammonia and α-ketobutyrate (Honma and Shimomura 1978). By decreasing ACC levels in plants, ACC deaminase-producing organisms decrease plant ethylene levels (Glick et al. 1998; Glick et al. 2007), which when present in higher concentrations can lead to plant growth inhibition or even death. In the current study, all the isolates when screened for ACC deaminase and it was found that only three isolates (S8, S26, and R5) were positive for ACC deaminase production. ACC can be utilized as a nitrogen source from the root exudates by some microorganisms (Glick et al. 2007). Previous findings stated that endophytic microbes possessing such traits can benefit the host by reducing the stress level and enhance their growth (Hardoim et al. 2008; Rohini et al. 2018).

Besides this, ACC deaminase possessing bacteria not only promotes plant growth but also helps plants in providing protection against various stresses (Glick 2014), increasing the senescence period (Ali et al. 2012), act as barrier against phytopathogen in certain plant by performing biocontrol activities (Hao et al. 2011), and favour nodulation in legume plants (Nascimento et al. 2012). Alizadeh et al. (2012) have explained the application of the ACC deaminase which has been synthesized by different genera of *Pseudomonas* in increasing the senescence of the plants. But present study demonstrated only three *Bacillus* sp. exhibiting such features. Thus, in the current study, isolates showing positive results for ACC deaminase along with other PGP traits can be the most potent candidate for promoting and protecting the sugarcane crop when applied together in the *in vivo* conditions.

Another volatile compound especially HCN and ammonia are also linked with indirect plant growth promotion and used for biocontrol mechanisms. Generally, hydrogen cyanide (HCN) blocks the cytochrome oxidase pathway of microorganisms and thus growth of microorganisms especially phytopathogen gets affected. In the present study, *P.aeruginosa* (BS-4) and *B. paramycoides* (R5) showed positive result for HCN production alongwith other PGP traits. Various findings suggested that the production of HCN by certain Fluorescent pseudomonads are believed to be involved in the suppression of root pathogens. *P. fluorescens* CHA0 was found to produce antibiotics, siderophores, and HCN (Voisard et al. 1989). HCN has been widely reported as an important biocontrol agent against pathogen; but recently, Rijavec and Lapanje (2016) suggested that HCN also play an important role in sequestration of metals and significantly result in indirect increment of nutrient availability especially phosphorus, which is advantageous for the microorganisms and their plant hosts, thus, suggesting the valuable contributions of microorganisms with HCN attributes to be

used as biological fertilizers or biocontrol to enhance crop production (Agbodjato et al. 2015; Rijavec and Lapanje 2016). Besides HCN, ammonia production by microorganisms especially plant growth-promoting bacteria (PGPB) help in the elongation of root and shoot by supplying nitrogen to the host plant; thus, results in the increment of the biomass of plant (Marques et al. 2010). Some of the PGPB strains have potential to synthesize HCN as well as ammonia and their synergetic effect on plant growth as well as modulation of plant metabolites has been observed (Singh et al. 2019). In the present study, all the isolates showed strong positive results for ammonia production. This was also favoured by Paulitz et al. (2000), where volatile ammonia has been implicated as a possible mechanism to control soil-borne pathogens.

This preliminary result suggested the fact that antagonistic role of isolated endophytic bacteria is due to the production of various secondary metabolites which are antifungal in nature. Furthermore, this chapter also revealed that various lytic enzyme such as chitinase, β-1,3 glucanase, protease, amylase, pectinase, and cellulase associated with the bacteria, may contribute to the suppression of *C. falcatum* as these enzymes are directly linked with hyper-parasitic action (Kim and Chung, 2004). Patel et al. (2019) also discussed that *Bacillus* are indefatigable members which are known for aghast production of a multitude of biologically active metabolites; which possibly controls the growth of phytopathogen. Several investigating studies provide strong evidence that strains of *Bacillus* species, including *B. subtilis* (Lee et al. 2006, 2011; Kim et al. 2012) remarkably lessen the effect of disease severity on a variety of hosts (Kloepper et al. 2004).

It is widely popularized that cell wall-degrading enzymes and secondary metabolites synthesized by the bacteria produce hindrance in the growth of other micro-organisms

(Shoda 2000). Chitinase and β-1,3 glucanase, are recognized hydrolytic enzymes which are known to be produced by a variety of bacteria and are responsible for the lysis of fungal hyphae as cell wall of fungus is chiefly made of chitin and glucan. In this study, isolate S8, S12, S17, S26, R5 and BS-4 possess the chitinase activity and , which exhibited a strong antagonism toward *C. falcatum*. Thus, in the current study; chitinase and β-1,3 glucanase enzyme support antibiosis as the sole mechanism of antagonism against red rot fungus. Similar results were also observed by Velusamy and Das (2014) where *B. subtilis* strain JD-09 exhibited strong antagonism against fungus due to chitinase activity. Mechanisms implying the plant beneficial effects comprises of the regulation of phytohormones, synthesis of antifungal compounds, cell wall degrading enzymes, and/or the attuning the physio- biochemical processes in plants (Park et al. 2013; Cho et al. 2015; Parray et al. 2015).

Another important enzyme protease, pectinase, amylase and cellulase possessed by the isolates, in particular, play a key role in the cell lysis process (Han et al. 2015). Proteases bind to the outer mannoprotein layer of the cell wall, open the protein structure, and expose inner glucan layers and chitin microfibrils. Similarly, pectinase is another important enzyme that hydrolyses pectin, a polysaccharide substrate found in almost all the cellular wall of plants. After degrading pectin, polygalacturonic acid is split into monogalacturonic acid, which is a process that softens the cell wall of the plant. Thus, isolates which possess pectin degrading ability, get easily colonized inside into the sugarcane plants and resides as endophyte bacteria; thus, confirms its endophytic behaviour (Oyeleke et al. 2012).

In this study, simple microscopy and scanning electron microscopy was performed to find out the interaction level of antagonist isolates with *C. falcatum*. The observation revealed the abnormalities leading to disruption and destruction in the fungal hyphae.

The fungal cell wall is chiefly composed of polysaccharides, proteins, and other components. They play a prime role in providing rigidity to cell and shape, act as a structural barrier, regulating ion exchange, and interactions with host-defense mechanisms (Vega and Kalkum 2011). A lytic enzyme produced by diverse antagonistic bacteria is responsible for dissolving fungal cell walls, thus, revealing the predacious nature of fungal pathogens (Xu et al. 2014). These mycelial inhibition are because of the secretion of the versatile diffusible and volatile compound by antagonistic bacteria. SEM micrograph also illustrated the distortion, denaturation, and breaking of the hyphae of phytopathogen.

4.5 Conclusion:

In the present scenario, the number of endophytic bacteria is still unexplored or under explored that needs to be exploited for the benefit of agricultural purpose. Thus, the current chapter finds the selection of potent endophytic bacterial isolate exhibiting extraordinary features of bioinoculant and biocontrol ability. This is one of the few investigation where it is reported as endophytic bacterial strains that uncover its biocontrol traits against red rot pathogen *C. falcatum* alongwith plant growth promoting traits. In conclusion, S8, S12, S17, S26, R5 and BS4 strain showed important requirements for valuable biological agents, which turn them into promising candidates to be included in biocontrol management against red rot of sugarcane caused by *C. falcatum*.

Chapter 5

Molecular characterization and quantitative assessment of secondary metabolites (chitinase, β-1, 3 glucanase, antibiotic etc).

Content of Chapter 5

Molecular characterization and quantitative assessment of secondary metabolites (chitinase, β-1, 3 glucanase, antibiotic etc).

5.1 Introduction:

In the current scenario, bioactive compounds have received much attention due to their multifarious benefits in agricultural field. Microorganisms are one of the natural producer of bioactive metabolites which are in high demands due to their eco-friendly nature. Antibiotics are also natural compounds produced by microorganisms as secondary metabolites to kill or inhibit other microorganisms (Singh et al. 2017). Various secondary metabolites from *Bacillus* and *Pseudomonas* such as non-ribosomally synthesized lipopeptides and lytic enzymes are also known for their antifungal nature. The function of lipopeptides as an antifungal has been reviewed by many researchers in the genus *Bacillus* and *Pseudomonas* (Ongena and Jacques 2008; Raaijmakers et al. 2010; Raaijmakers and Mazzola 2012). Direct analysis of metabolites has been achieved for antibiotic lipopeptides from several *Bacillus subtilis* and for pyrrolnitrin, 2,4-diacetylphloroglucinol and phenazine-1-carboxylic acid from *Pseudomonas fluorescens* strains (Raaijmakers and Mazzola 2012). Hydrolytic enzymes (chitinase and β-1,3 glucanase) are widely reported secondary metabolites able to lyse the cell wall of many fungal pathogen.

Using the bioactive natural secondary metabolites of endophytic bacteria would help in combating the dependency on chemical fungicides. Since large number of metabolites are yet to be explored from the endophytic bacteria and therefore more efforts are needed for identification of such metabolites which would pave way for

sustainable agricultural development or control of phytopathogens more effectively. Infact, the detailed study of them could lead to the identification of new signalling molecules involved in plant resistance against phytopathogens and other stresses. Thus, such compound either individually or their biosynthetic product could be easily identified. The present study is mainly based on the biocontrol of red rot disease of sugarcane by endophytic bacterial metabolites. Therefore, this chapter deals with the identification of various metabolites from the selected isolates of endophytic bacteria using analytical techniques such as Fourier-transform infrared spectroscopy (FTIR), Liquid chromatography–mass spectrometry (LC-MS/MS) and Gas chromatography–mass spectrometry (GC-MS). Also, molecular technique such as PCR amplification is also conducted for the confirmation of gene responsible for lytic enzyme.

5.2 Material and Methods:

5.2.1 Characterization of Chitinase enzyme activity:

For determination of chitinase activity, culture of isolates *viz.* R5, S8, S12, S26, S17 and BS-4 shown positive qualitative test (Chapter-4) were grown in minimal salt medium (MSM) broth (composition (g/L); Sodium chloride 0.500; Potassium dihydrogen phosphate 3.000; Magnesium sulphate 0.120; Calcium chloride dihydrate 0.013; Yeast extract 3.000; and Disodium hydrogen phosphate 6.000) with chitin and incubated for 96 h with shaking at 180 rpm at 28±2°C. After incubation, the culture was centrifuged at 10000 rpm for 10 min and supernatant obtained was used as crude enzyme. The chitinase activity was determined by incubating 1 ml of culture supernatant with 1 ml of 1% colloidal chitin prepared in a 0.05 M phosphate buffer, pH 7.0 at 30°C for 1 h. After incubation, the reaction was stopped by adding 2 ml of 1% 3, 5 dinitrosalicyclic acid (DNSA) and boiled for 15 min in a water bath and

cooled to room temp (Miller 1959; Saadoun et al. 2009) and absorbance was measured at 530 nm in UV spectrophotometer. Blank was prepared with the reaction mixture except enzyme source. The resulting monomeric N-acetyl D-glucosamine (GlcNAc) was determined using standard of N-acetyl D-glucosamine (GlcNAc) in the assay mixture for calculations. One unit (U) of enzyme was defined as the amount of enzyme required to release 1 µg of reducing sugar as N-acetyl glucosamine min^{-1}.

5.2.2 Characterization of β-l,3-glucanase activity:

β-l,3-glucanase activity was assayed colorimetrically by the laminarin- dinitrosalicylic acid method (Miller 1959; Ramada 2010) with slight modification. The four culture *viz.* R5, S12, S17 and BS-4 were positive for qualitative test (Chapter-4) were grown in MSM broth with incubation for 96 h at 28±2°C and shaking at 180 rpm. After incubation, the culture was centrifuged at 10000 rpm for 10 min and supernatant was used as crude enzyme. The reaction mixture consisted of 0.6 ml of 4% laminarin (dissolved in 0.05 M sodium phosphate buffer, pH 7) and 0.6 ml of enzyme extract. The reaction was incubated for 60 minutes at 30°C. After incubation, reaction was stopped by adding 2 ml of dinitrosalicylic reagent {prepared by adding 1 g of dinitrosalicylic acid in 20 ml of 0.5M NaOH (Solution-A) and 30 g potassium sodium tartrate added in 50ml distilled water (Solution-B); after preparing both the solution, Solution-B was poured slowly into Solution-A and volume was made upto 100 ml} and heating the tubes for 5 min on a boiling water bath and vortexed. The resulting coloured solution was diluted with 4.5 ml of distilled water, vortexed, and the amount of reducing sugar was determined by measuring its absorbance at 540 nm. All tests were performed in triplicate. One Unit (U) of β-1,3 glucanase activity was defined as the amount of enzyme that produced 1 µg of glucose per min was determined by measuring the release of reducing sugars by using laminarin (Sigma) as substrate and

glucose as standard. The blank was prepared with the reaction mixture except enzyme source.

5.2.3 Electrophoretic and Zymogram studies:

Based on the presence of chitinase enzyme activity (quantitative); isolates S8, S12, S17, BS-4 and R5 were selected for further chitinase characterization by SDS-polyacrylamide gel electrophoresis followed by zymogram analysis; and for β 1,3 Glucanase activity three isolates S12, S17 and R-5 were selected for SDS-PAGE and its Zymogram analysis based on quantitative β-1,3 Glucanase activity.

5.2.3.1 Electrophoresis and Zymogram analysis of Chitinase:

The molecular mass of crude chitinase enzyme was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10 % gel according to Laemmli (1970) method. The proteins were visualized by staining with Coomassie brilliant blue R-250. For zymogram analysis; the crude chitinase samples were mixed with loading buffer without reducing agent and heating. After electrophoresis, the gel was incubated in 100 mM sodium acetate buffer, pH 5 at 30°C for 18 h. The activated chitinase on the gel was visualized by staining with 0.1 % Congo red followed by de staining with 1N NaCl (Yamabhai et al. 2008; Pandya et al. 2014).

5.2.3.2 Electrophoresis and Zymogram analysis of β-1,3 Glucanase:

SDS-PAGE was performed using a 10% polyacrylamide gel (Laemmli 1970). Zymogram on SDS-PAGE gels was performed by a modified procedure described by Blättel et al. (2011). After SDS-PAGE; the gel was washed in 25% isopropanol in 10 mM sodium phosphate buffer, pH 6.0, to remove residual SDS for 30 min with gentle shaking. Two washing steps with 10 mM sodium phosphate buffer, pH 6.0, followed for 20 min. The gel was laid onto a gel bed, containing 0.5% laminarin and 0.01%

calcofluor white. After incubation at 30°C, enzyme activity was detected as a dark lytic zone under UV illumination.

5.2.4 Molecular Screening for chitinase and β-1,3 glucanase genes:

For screening the presence of chitinase and β-1,3 glucanase genes in the endophyte isolates, PCR based screening was performed using specific primers as listed in Table 5.1. Five endophytic bacteria (strains R5, S12, S8, S17 and BS-4) exhibiting strong *in vitro* antifungal activities were screened for the presence of chitinase and for β-1,3 glucanase (R5, S12, S17 and BS-4). For this genomic DNA was isolated and the extracted DNA was used for PCR amplification. Cycling parameters for PCR detection gene from the endophytic strains were as follows: initial denaturation 94°C for 3 min, then 35 cycles of denaturation at 94°C for 1 min, followed by annealing at 50°C for 1 min, extension for 2 minutes at 72°C and final extension for 7 minutes at 72°C.

Table 5.1 PCR primers used for amplification of chitinase and β-1,3 glucanase.

Target Gene	**Primers**	**Sequences (5'-3')**
Chitinase (Chi A)	Chitinase- Forward Chitinase- Reverse	5'- GCAAAAGCCGCAAAAG -3' 5'-GGTAAAACGGATCGGACAG-3'
β-1,3 glucanase (Glu)	β-1,3 glucanase- Forward β-1,3 glucanase-Reverse	5'-CATCATGCTCAACTACGCC -3' 5'-ATGTAAGTCTCCAACGCCC-3'

Note: Further, the isolates R5, S12, S8 and BS-4 were selected on the basis of collective information on enzymatic activity (chitinase and glucanse), presence of gene and also due to the presence of various other metabolite (as discussed in Chapter-3 and 4).

5.2.5 Fourier transform infrared radiation (FTIR) analysis of crude metabolites of Isolates:

To understand the overall chemical nature of the extracted crude metabolites, Fourier transform infrared spectroscopy (FTIR) was employed. This technique helps to explore the functional groups and the chemical bonds present in the crude extract. The isolates S8, S12, R5 and BS-4 (as maximum trait were present) were selected for FTIR analysis. The analysis was done using Thermo Scientific FTIR Spectrophotometer (Model Nicolet 6700). The sample was extracted twice with ethyl acetate and completely dried to avoid any moisture. The dried form (1mg) of extract was then mixed with sufficient quantity of potassium bromide. FTIR spectral analysis was recorded at the transmission mode from 400–4000 cm^{-1}. The spectrum was studied to interpret the chemical nature of the extracted crude metabolite (Varadavenkatesan and Murty 2013).

5.2.6 Mass Spectrometric analysis of Bioactive compound of Bacterial Endophyte using LC-MS/MS:

For the detection and identification of diffusible secondary metabolites of endophytic bacteria (S12, R5, S8 and BS-4) LC-MS/MS technique was employed. For this, the endophytic bacterial culture was inoculated in LB broth (500 ml) and incubated under controlled conditions (28 ± 2°C) with stirring (120 rpm) for 96 h. After 96 h of incubation, the culture was centrifuged at 10,000 rpm for 20 min at 4° C to obtain cell-free filtrate. The supernatants were then acidified to pH 3 using 3M HCl solution, the acidified supernatants were extracted twice with equal volume of ethyl acetate (1:1). For this, in each flask with 500 ml of acidified supernatant, 500 ml of ethyl acetate was added and shaken for 20 min in a separatory funnel (Yasmin et al. 2017). The organic layer obtained was collected in a separate beaker and the lower

layer of culture supernatant was again extracted with an equal volume of ethyl acetate. The obtained organic layer in re-extraction procedure was combined with previously extracted and collected organic layer. The combined organic layer obtained was evaporated at 40-45 °C. The residue obtained was dissolved in 5 ml LCMS grade methanol and subjected to LC-MS/MS analysis using mass spectrometer, equipped with an ESI source. The sample were filter sterilized and were injected through the direct syringe pump. The sample were scanned at both positive and negative total ion full scan modes (mass scan range m/z 200 to 1500).

5.2.7 GC-MS analysis for Volatile Compounds:

The volatile antifungal compound present in the culture supernatant of endophytic bacteria (R5, S12, S8, and BS-4) was detected by gas chromatography equipped with mass spectrometer i.e. by GC-MS analysis. For this, the culture supernatant was extracted twice using double the volume of ethyl acetate and dried in a rotary vacuum evaporator at 45°C. 100 µg of concentrated extracted sample was then taken in a separating funnel and shaken by adding 10 ml of water and ethyl acetate in the ratio of 1:4. Upper layer was collected and concentrated to 1 ml in the rotary evaporator. 50 µl N, O-Bis(trimethylsilyl) trifluoroacetamide and trimethyl chlorosilane (BSTFA+TMCS) was added and then finally 10 µl of Pyridine was also added. Samples were further heated at 60ºC for 30 minutes. To make 1 ml solution of BSTFA+TMCS, 990 µl of BSTFA and 10µl of TMCS were mixed in the ratio of 99:1 . Samples were transferred in GC vial and dried using nitrogen gas. Samples were finally dissolved in methanol before GC-MS analysis. Analytical conditions were as follows: injector temperature 260°C; oven temperature kept at 60°C, for 3 min, increased from 60°C to 250°C at a rate of 8°C min^{-1}, kept at 250°C for 2 min, increased from 250°C to 300 °C at a rate of 15°C min^{-1}, and kept at 300°C for 18 min. The analytes were injected in the split mode, Linear velocity was maintained for

Flow Control Mode; with Pressure of 73.3 kPa; Total Flow was maintained at 16.3 ml/min; Column Flow was 1.21 ml/min; Linear Velocity was 40.1 cm s^{-1}; Purge Flow was maintained at 3.0 ml min^{-1} and Split Ratio was 10.0. The mass spectra thus obtained were analysed for major antifungal compound by comparing with home-made library and related literature data and by matching their fragmentation pattern in mass spectra with those of National Institute of Standards and Technology (NIST) libraries.

5.2.8 Statistical analysis: The standard deviation (SD) of the triplicate for each measurement from their mean and the standard error (SE) of mean based on (SD) were done using Excel statistical program software (Microsoft office 2010).

5.3 Results:

5.3.1 Determination of Chitinolytic activity:

The activity of crude enzyme of six strains (R5, S12, S8, S17, S26 and BS-4) was investigated for the production of extracellular chitinase spectrophotometrically (Fig 5.1). The results revealed that all isolates demonstrated the chitinase production; however, the strain R5 exhibited the maximum chitinase production with 116 U ml^{-1}, followed by BS-4 and S17 with 84 U ml^{-1} and 79.4 U ml^{-1} respectively at pH 7.0 and temperature 30° C.

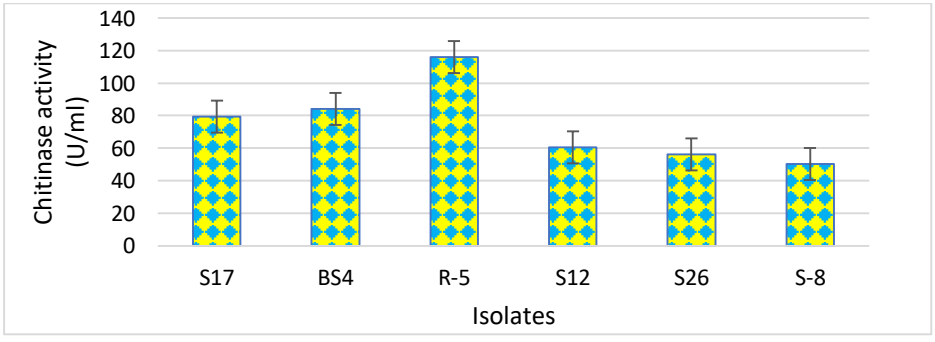


Fig 5.1 Quantitative estimation of chitinase enzymatic activity of the isolates in broth medium.

5.3.2 Determination of β-1,3 glucanase activity:

Similarly, the activity of crude enzyme of the test isolates (R5, S12, S17, and BS-4) was evaluated for β-1,3 glucanase enzyme detection and the results revealed that R5 possessed the maximum enzyme activity with 415.8 U ml^{-1} followed by S17 and S12 with 385.3 U ml^{-1} and 225.1 U ml^{-1} respectively at pH 7.0 and temperature 30° C (Fig 5.2).

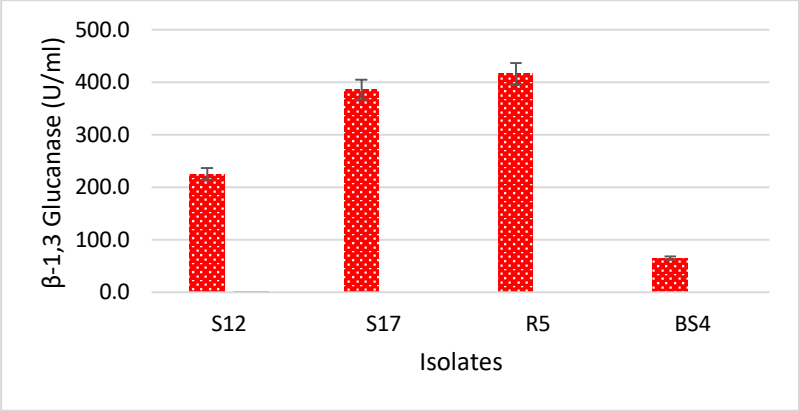


Fig 5.2 Quantitative estimation of β-1,3 glucanase enzyme activity of the isolates in broth medium.

5.3.3 Characterization of enzymatic activity by SDS and Zymogram studies:

5.3.3.1 Chitinase activity:

The molecular weight of protein present in the crude enzyme was assessed for the isolates S8, S17, S12, BS-4 and R5; it revealed various protein band profiles (Fig 5.3). Chitinolytic zymography assay used in this study revealed a high resolution of single zymographic band for all the five isolates which had molecular weight of approximately ~54 kDa (Fig 5.4) where lysis occured. However, the zymogram study of crude chitinase revealed that activity was present in all the 5 isolates and band was approximately ~54 kDa (Fig 5.4).

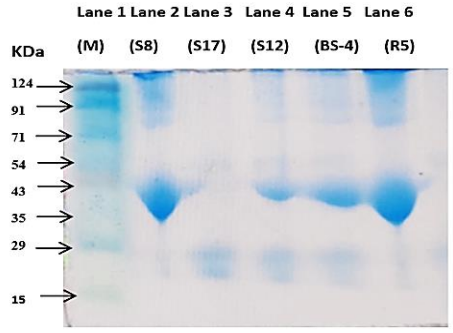


Fig 5.3 SDS-polyacrylamide gel electrophoresis (Chitinase) of the Crude enzyme samples S8, S17, S12, BS-4 and R-5. Lane 1:Marker (124 kDa-15 kDa), Lane 2: S8; Lane 3:S-17; Lane 4:S12; Lane5: BS-4; Lane 6:R-5 Crude Enzyme samples.

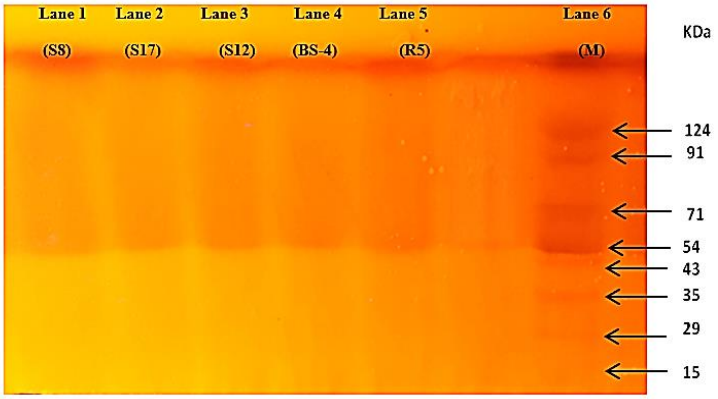


Fig 5.4 Zymogram result (Chitinase) of the Crude enzyme samples S8, S17, S12, BS-4 and R-5. Lane 1: S8; Lane 2: S-17; Lane 3: S12; Lane4:BS-4; Lane 5: R-5; Lane 6: Marker (124 kDa-15 kDa),Crude Enzyme.

5.3.3.2 β-1,3 glucanase:

On the basis of qualitative and quantitative assay, only 3 isolates *viz.* R5, S12 and S17 were selected for characterizing the enzyme activity by electrophoresis and zymogram studies. The results for SDS- PAGE analysis revealed that several protein band (Fig 5.5) were present in the crude supernatant detected after staining with Coomassie blue staining. However, single band of protein were observed as cleared zone on the agarose gel by zymography. The band of S12 corresponds to size ~ 54 kDa, molecular weight of protein band for S17 corresponds with ~60 kDa and that of R5 with ~ 59 kDa laminarin as substrate (Fig. 5.6).

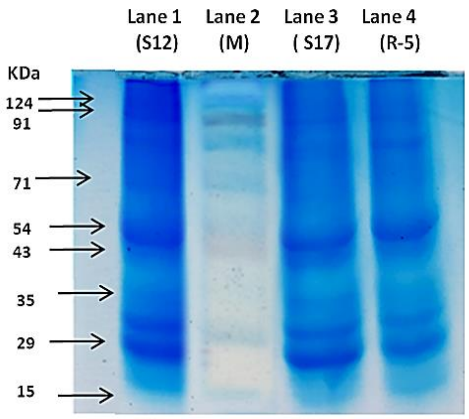


Fig 5.5 SDS-polyacrylamide gel electrophoresis (β-1,3 glucanase) of the Crude enzyme samples S12, S17 and R-5. Lane 1: S12; Lane 2: Marker (124kDa-15 kDa), Lane 3:S17 and Lane 4:R-5 Crude Enzyme samples.

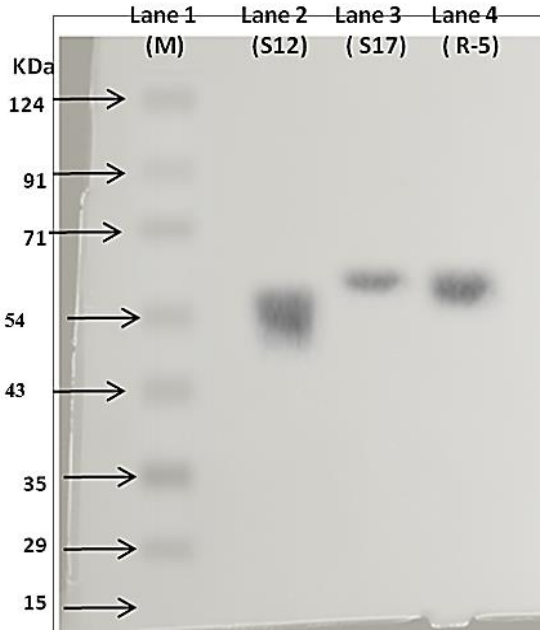


Fig 5.6 Zymogram result (β 1, 3 Glucanase) of the Crude enzyme samples S12, S17 and R-5. Lane 1: Marker (124 kDa-15 kDa); Lane 2:S-12;Lane 3:S17; Lane4:R-5 of Crude Enzyme samples.

5.3.4 Detection of chitinase and β-1,3 glucanase gene in Isolates:

Chitinase gene were detected in the four isolates namely R5, S8, S12 and BS-4 with appoximately 300 bp amplicon (Fig 5.7), while β-1,3 glucanase gene with 300 bp was found in R5, S12 and S17 isolates (Fig 5.8).

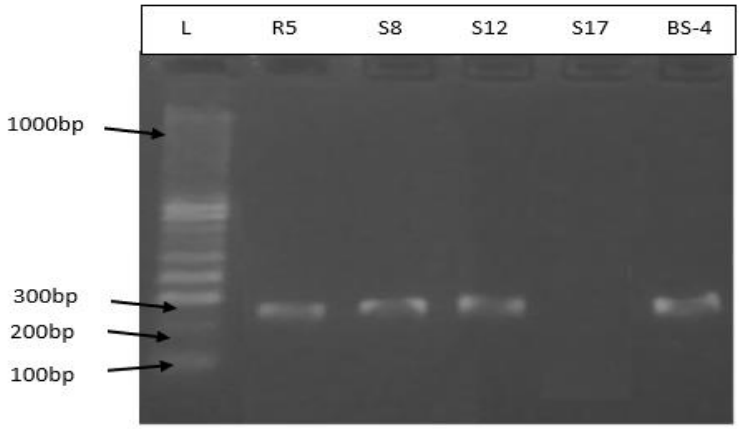


Fig 5.7 PCR based screening of chitinase gene of all the selected endophytic isolates (L- Ladder; R5, S8, S12, S17 and BS-4 are endophytic isolates).

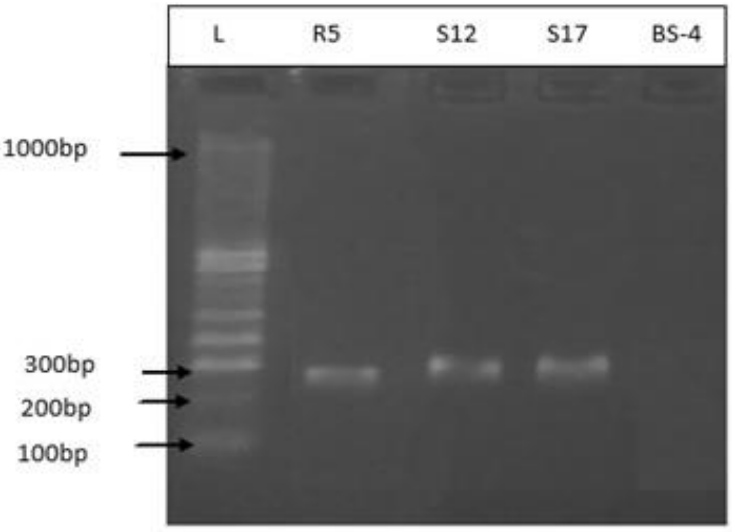


Fig 5.8 PCR based screening of β-1,3 glucanase gene of all the selected endophytic isolates (L- Ladder; R5, S12, S17 and BS-4 are endophytic isolates).

5.3.5 Fourier transform infrared radiation (FTIR) spectroscopy analysis of extracted crude supernatant:

Since the isolates BS-4, R5, S8 and S12 were considered to be the potent producer of lytic enzyme (chitinase and β-1,3 glucanase) as well as presence of multifarious plant growth promoting traits (Chapter 3 and 4) as per the result discussed above. Therefore, these isolates were carried out for further characteristics with view of an

idea to find out the more metabolites in the isolate besides, having lytic enzymatic system. Therefore, the FTIR spectra of crude supernatant produced by endophytic isolates (BS-4, R5, S8 and S12) were analyzed and it revealed the presence of different bands peaks corresponding to the following functional groups present in the molecular structure. Observed results demonstrated that FTIR spectrum of crude antifungal extracellular filtrate of isolates (BS-4, R5, S8 and S12) had characteristic peaks between 3746 - 698 cm^{-1}.

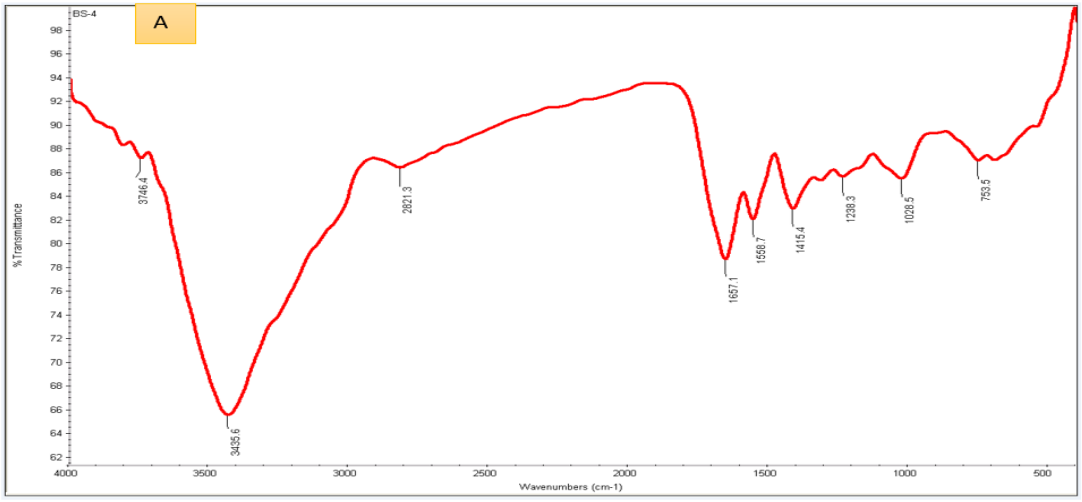
i) For BS-4; a broad absorption at 3435.6 cm^{-1} corresponding to a bounded OH stretching and N-H stretching of amine group; one sharp band at 2821 cm^{-1} due to the stretching of methyl group of alkane chain; the stretching of the carbonyl group and C=C stretching of alkene at 1657.1 cm^{-1}; band at 1558.7 and 1415 cm^{-1} representing nitro group and aromatic group respectively; band at 1238.3, 1028.5 and 753.5cm^{-1} corresponds with the stretching of C-F of alkyl halides (Fig 5.9 A).

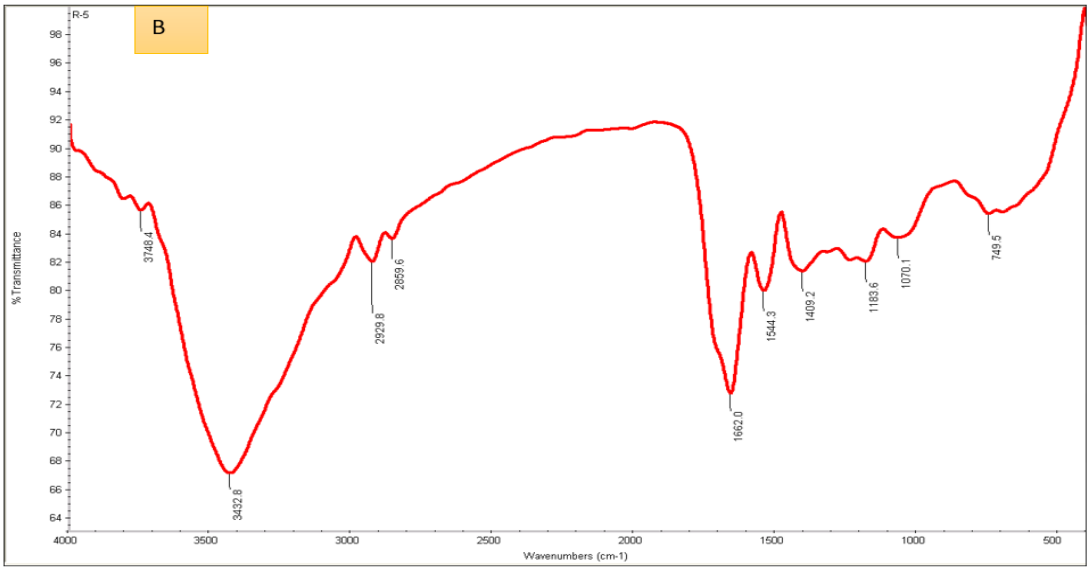
ii) For R5: The FTIR spectrum for R5 isolates detected a broad absorbance peak with wave numbers ranging from 3600 cm^{-1} to 3100 cm^{-1} with peak at 3432.8 cm^{-1} which was due to the N-H stretching vibrations, a typical characteristic of carbon containing compound with amine group. Also, the peak also corresponds with the O-H stretching of alcohol and phenol compound. The characteristic peak at 2929.8 cm^{-1}, 2859.6 cm^{-1}, 1409.2 cm^{-1} represented the aliphatic C-H stretching as indicative of aliphatic chains ($–CH_3$ and $–CH_2–$) and O-H stretch bond of carboxylic acid. A strong band was also observed at 1662.0 cm^{-1} representing the presence of C=O bonds causing CO-N stretching in the peptide bond. The peak might also correspond with the C=C stretch of alkene; and C=O stretch of ketone, aldehyde and ester. Peaks at 1409.2 cm^{-1} were also due to the aromatic group. The C–O stretching were observed at 1183.6 and 1,078 cm^{-1}. At 749.1 cm^{-1} stretch of alkyl halides were observed (Fig

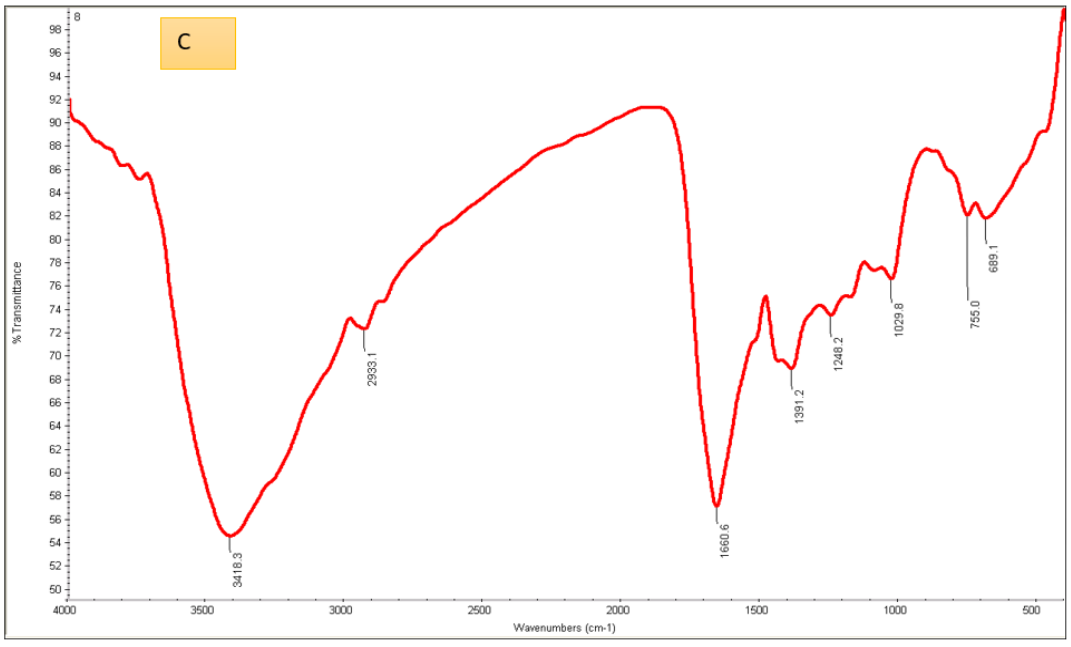
5.9 B). Most of the peaks might belong to the lipopeptide which will be confirmed in the future analytical techniques.

iii) For S8 isolates also; the peak band at 3418cm-1 corresponds with the O-H stretch of phenol/alcohol, and N-H stretch of amide; the sharp peak at 2933.1 cm^{-1} represented long chain aliphatic chain of alkane and O-H stretch of -COOH bond; The sharp peak at 1660.6 cm^{-1} corresponds with the C=O stretch of carbonyl and C=C stretch of alkene; the peak at 1391.2, 1248, and 1029.8 cm^{-1} corresponds with C-F stretch of alkyl halides (Fig 5.9 C).

iv) For S12 isolates; the band peak observed at 3426 corresponds with the O-H stretching and N-H stretching of amine; the sharp peaks at 1657 cm^{-1} demonstrated the C=O stretch of carbonyl group present, and C-C=C symmetric stretch of alkene, aromatic group and aliphatic group was confirmed at peak band of 1413.8 cm^{-1}; C-O stretch of ester, ether and acid was confirmed at 1244.7 and 1047.2 cm^{-1} (Fig 5.9-). Thus, the results revealed that antifungal nature of metabolites present in the crude supernatant as the chemical nature of metabolite might belonging to lipopeptides moieties (carbon with amino group), phenol compounds and various other group which will be confirmed in the further analysis of LC-MS/MS and GC-MS analysis as discussed in the later part in the current chapter.


A
BS-4
%Transmittance
Wavenumbers (cm-1)
3746.4
3435.6
2821.3
1657.1
1558.7
1415.4
1238.3
1028.5
793.5


B
R-5
%Transmittance
Wavenumbers (cm-1)
3748.4
3432.8
2929.8
2859.6
1662.0
1544.3
1409.2
1183.6
1070.1
749.5


C
%Transmittance
Wavenumbers (cm-1)
3418.3
2933.1
1660.6
1391.2
1248.2
1029.8
755.0
689.1

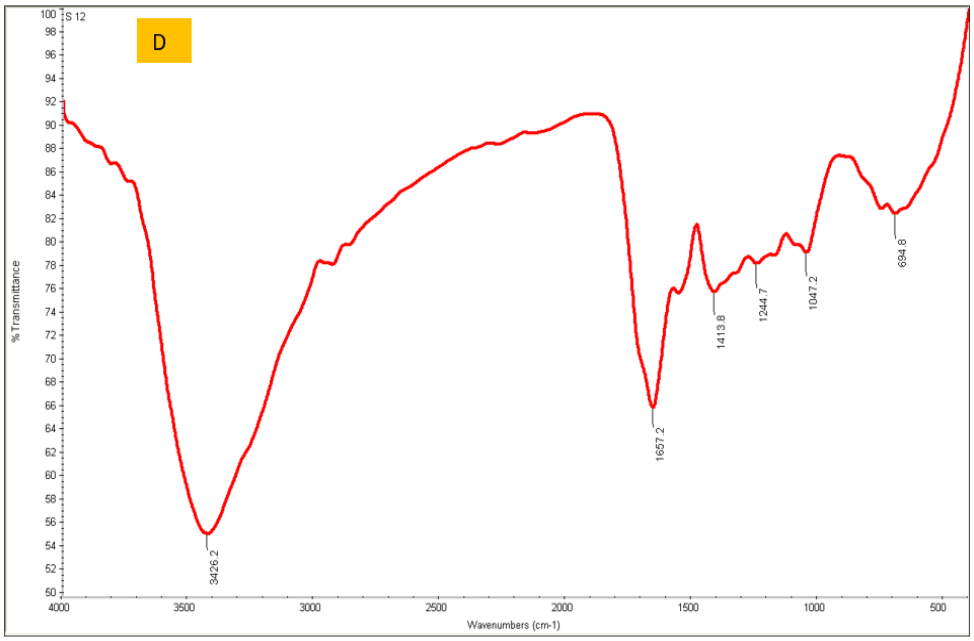


Fig 5.9 FTIR spectrums of extracted crude metabolites present in the supernatant of endophytic bacterial isolate A) BS-4, B) R5, C) S8 and D) S12

5.3.6 Identification of Antifungal compound by LC-MS/MS analysis:

Filter sterilized cell free extract of isolate BS-4, R5, S12 and S8 was studied for bioactive antifungal compound by LC-MS/MS analysis. Samples were injected using syringe and analysed at both positive as well as negative scan mode. After 96 h of bacterial growth, cell-free supernatants were extracted and analysis of crude extract of isolates BS-4 and R5 demonstrated the predominance of variety of metabolites.

i) LC-MS/MS Analysis for Isolate BS-4: The mass spectral analysis of *Pseudomonas aeruginosa* strain BS-4 revealed the presence of antifungal metabolites which might belong to 1-hydroxy phenazine having m/z at 197.1 $[M+H]^+$ (Fig 5.10), Pyocyanin having m/z at 211 $[M+H]^+$(Fig 5.11), phenazine 1- carboxylic acid (PCA) having m/z at 225 $[M+H]^+$(Fig 5.12), 2,4-Diacetylphloroglucinol (DAPG) m/z at 210 $[M+H]^+$ (Fig 5.13) and Lahorenoic acid at m/z 245 $[M+H]^+$, 267 $[M+Na]^+$ (Fig 5.14). These metabolites /compounds were found to be present in positive mode after comparing with the literature available. However, possibility of some more compound

were also found to be present in the negative mode such as prominent peak of Siderophore (pyochelin) were observed at m/z 325 $[M-H]^-$ (Fig 5.15), rhamnolipids were detected at the peak having m/z 503, $[M-H]^-$(Fig 5.16), and presence of 4 hydroxy-2-alkylquinolines (HAQ) in the range of m/z of 214 to 340 having prominent peak at 271, 298 and 340 (Fig 5.17).

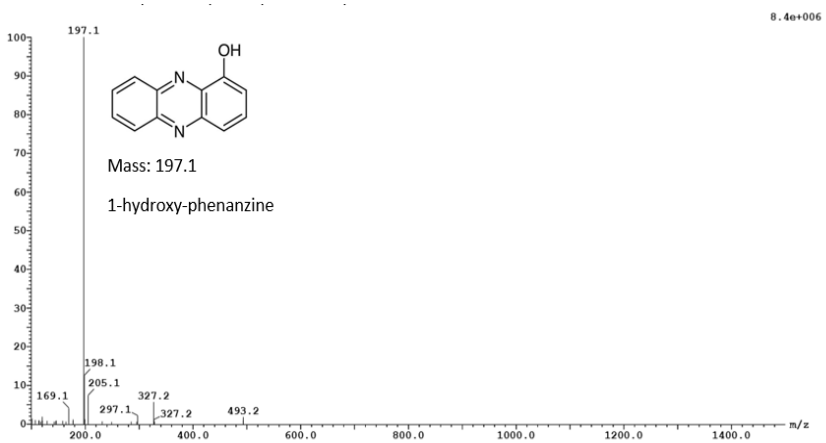


Fig 5.10 Mass spectra of LC-MS/MS based fragmentation analysis of 1-hydroxy-phenazine (m/z 197.1) of extract for isolate BS-4.

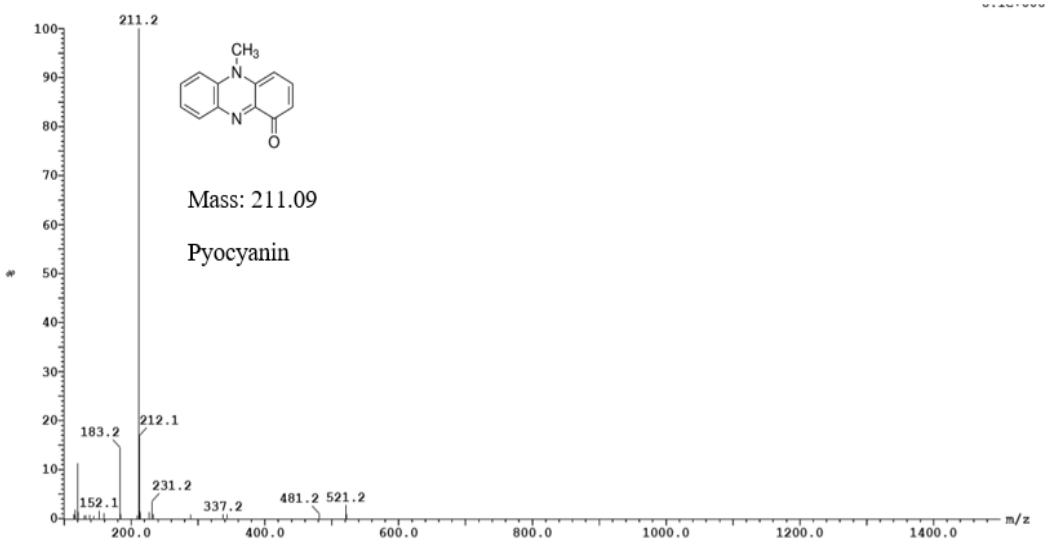


Fig 5.11 Mass spectra of LC-MS/MS based fragmentation analysis of Pyocyanin (m/z 211.09) of extract for isolate BS-4.

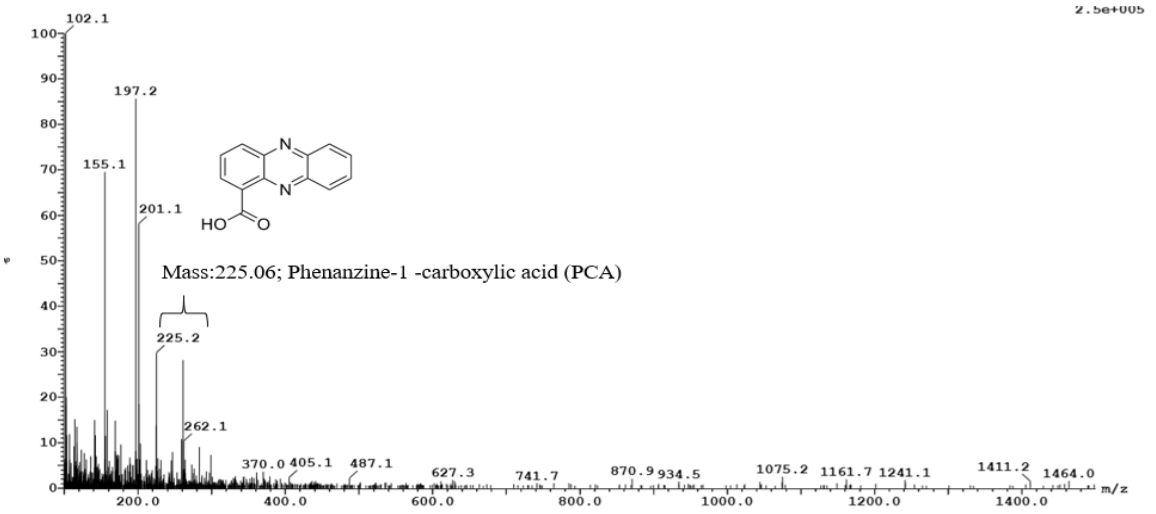


Fig 5.12 Mass spectra of LC-MS/MS based fragmentation analysis of phenazine 1- carboxylic acid (PCA) (m/z 225) of extract for isolate BS-4.

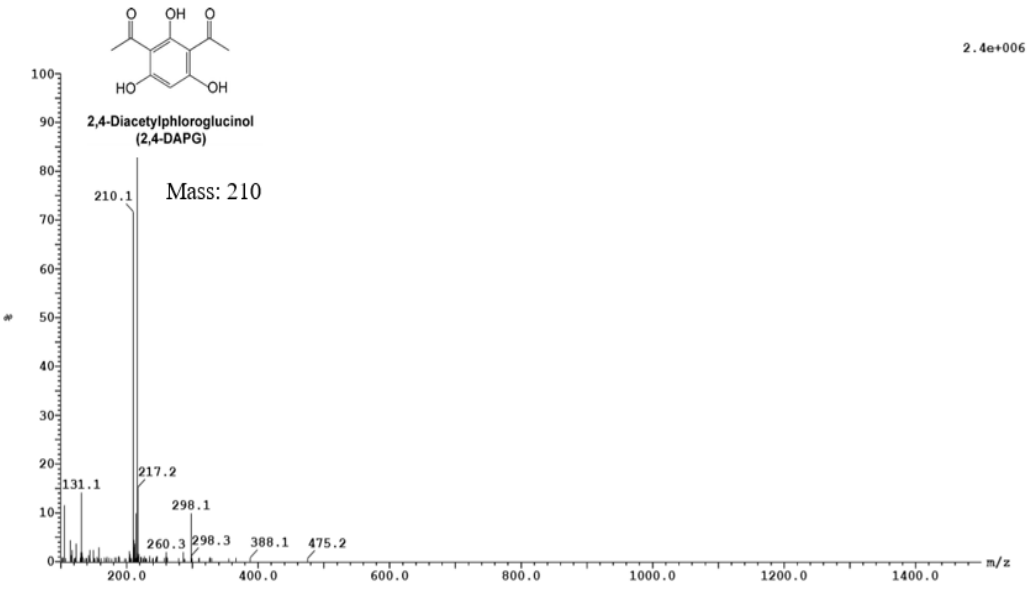


Fig 5.13 Mass spectra of LC-MS/MS based fragmentation analysis of 2,4-Diacetylphloroglucinol (DAPG) (m/z 210) of extract of isolate BS-4.

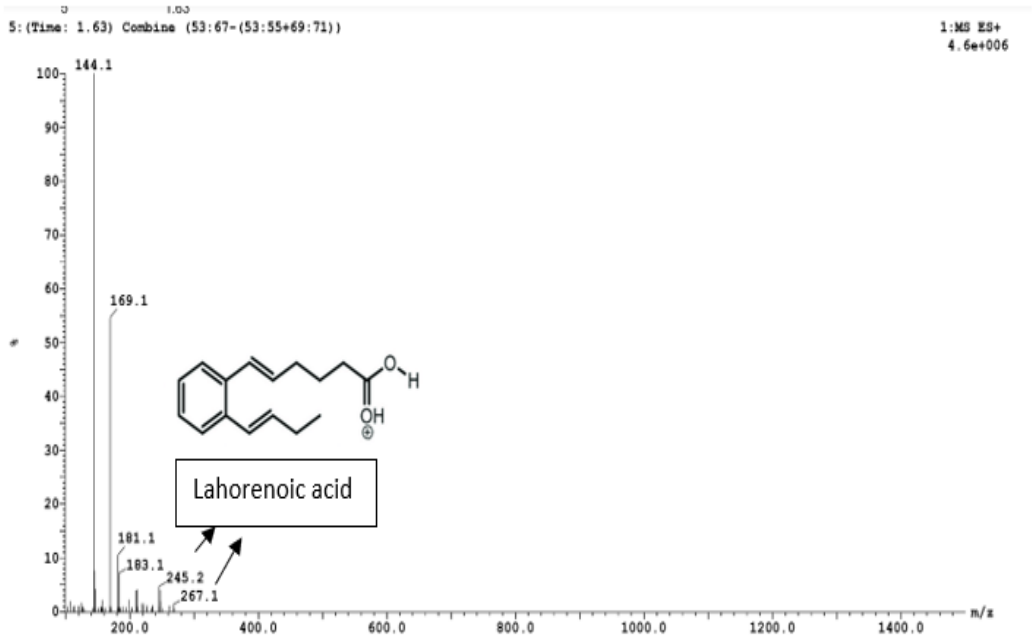


Fig 5.14 Mass spectra of LC-MS/MS based fragmentation analysis of Lahorenoic acid at m/z 245 $[M+H]^+$, 267 $[M+Na]^+$ of extract for isolate BS-4.

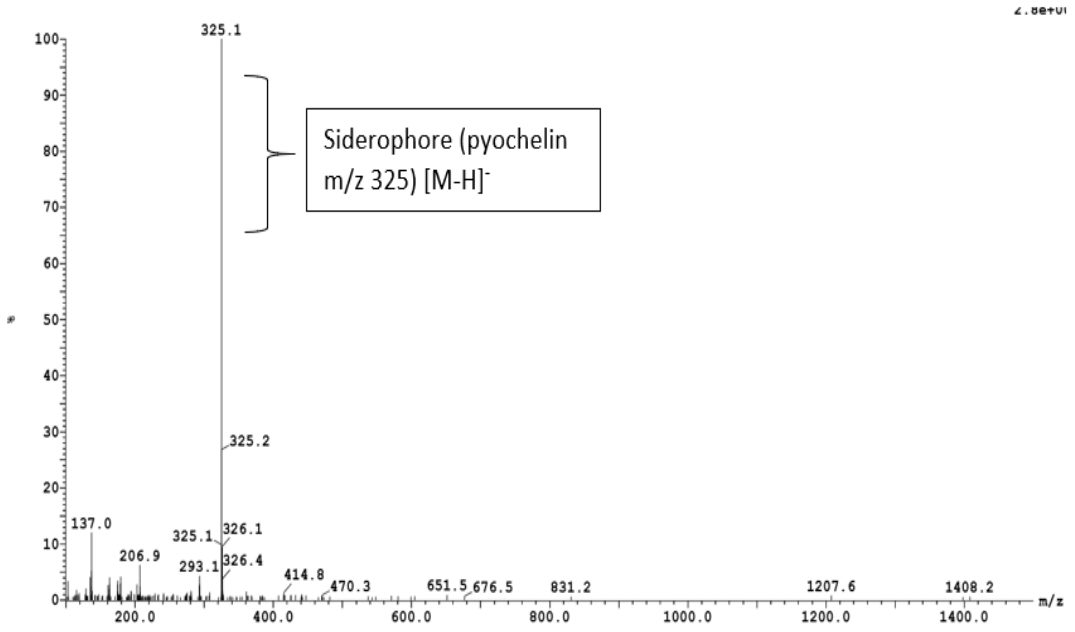


Fig 5.15 Mass spectra of LC-MS/MS based fragmentation analysis of Siderophore at m/z 325 of extract for isolate BS-4.

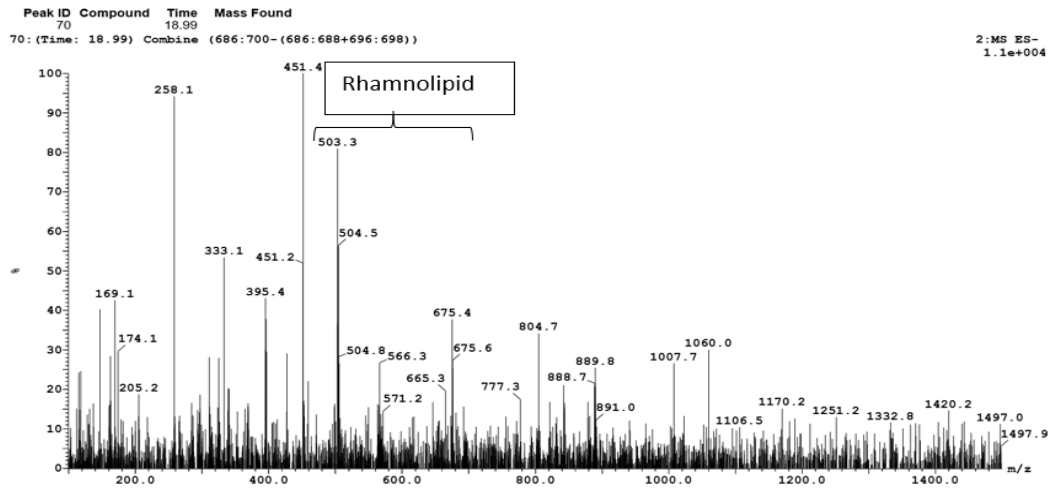


Fig 5.16 Mass spectra of LC-MS/MS based fragmentation analysis of Rhamnolipid (m/z 503) of extract for isolate BS-4.

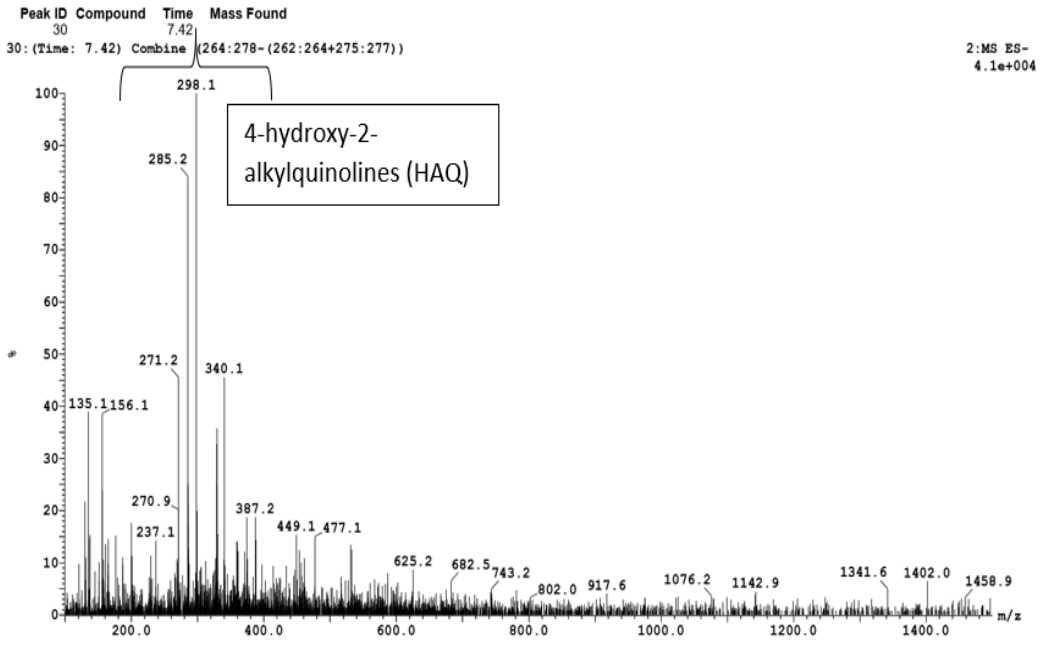


Fig 5.17 Mass spectra of LC-MS/MS based fragmentation analysis of 4- hydroxy-2-alkylquinolines (HAQ) in the range of m/z of 214 to 340 having a prominent peak at 271, 298 and 340) of extract for isolate BS-4.

ii) LC-MS/MS Analysis for Isolate R5:

Similarly, mass spectral analysis for crude extract of isolate R5 demonstrated the possibility of antifungal compound surfactin isomer C-13 having m/z at 1008.8 $[M+H]^+$ (Fig 5.18); C-14 surfactin having m/z at 1022 [M+H]+ (Fig 5.19); C-15 surfactin at m/z 1036.7 [M+H]+ (Fig 5.20) at positive mode. However, their confirmation peak was also observed at Negative ion mode at m/z 992, 1006 and 1020 $[M-H]^-$ (Fig 5.21). The peak for Bacillomycin (Fig 5.22) was also observed at m/z (1031.8) and iturin family might found to be detected in positive mode as sodiated peak of C-16 isomer of iturin was at m/z 1096 $[M+Na]^+$ (Fig 5.23) and protonated peak of C-14 isomer of iturin was also found at m/z 1043 $[M+H]^+$ (Fig 5.24). Besides these, lipopeptide kurstakin family might also be present which was observed at m/z 887.6 and 903 $[M+H]^+$ (Fig 5.25) and fengycin A was observed at peak 1463.6 $[M+H]^+$ (Fig 5.26).

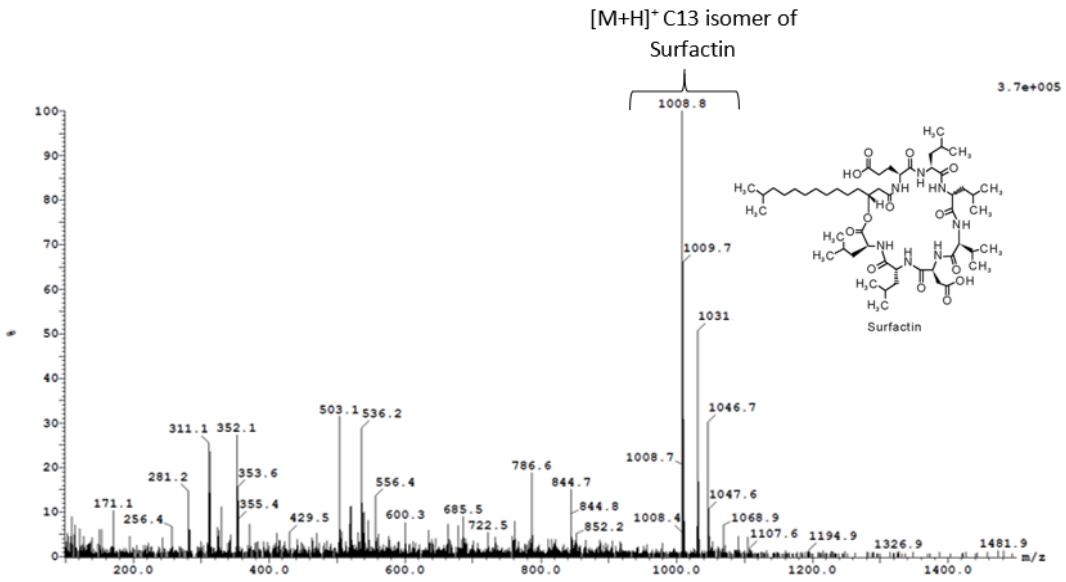


Fig 5.18 Mass spectra of LC-MS/MS based fragmentation analysis of C13 Surfactin (m/z 1008) of extract for isolate R5.

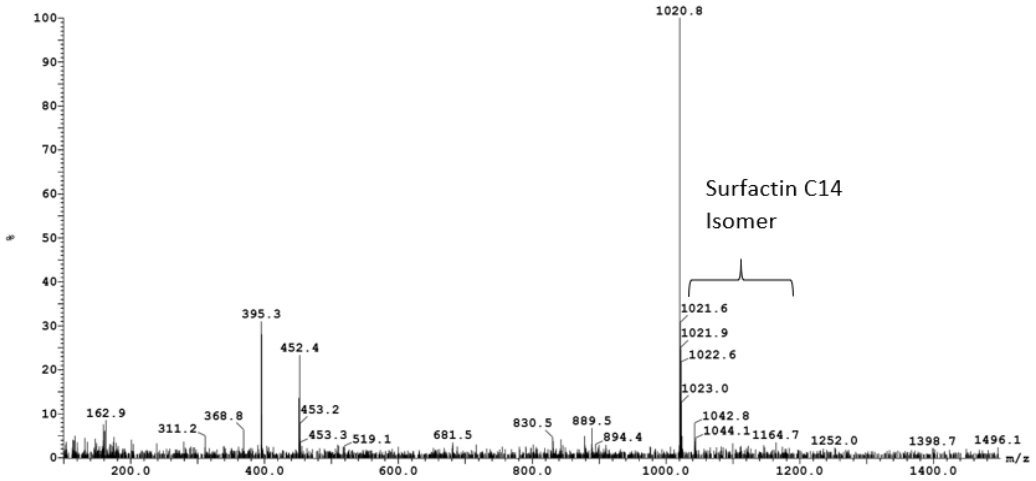


Fig 5.19 Mass spectra of LC-MS/MS based fragmentation analysis of C14 Surfactin (m/z 1022) of extract for isolate R5.

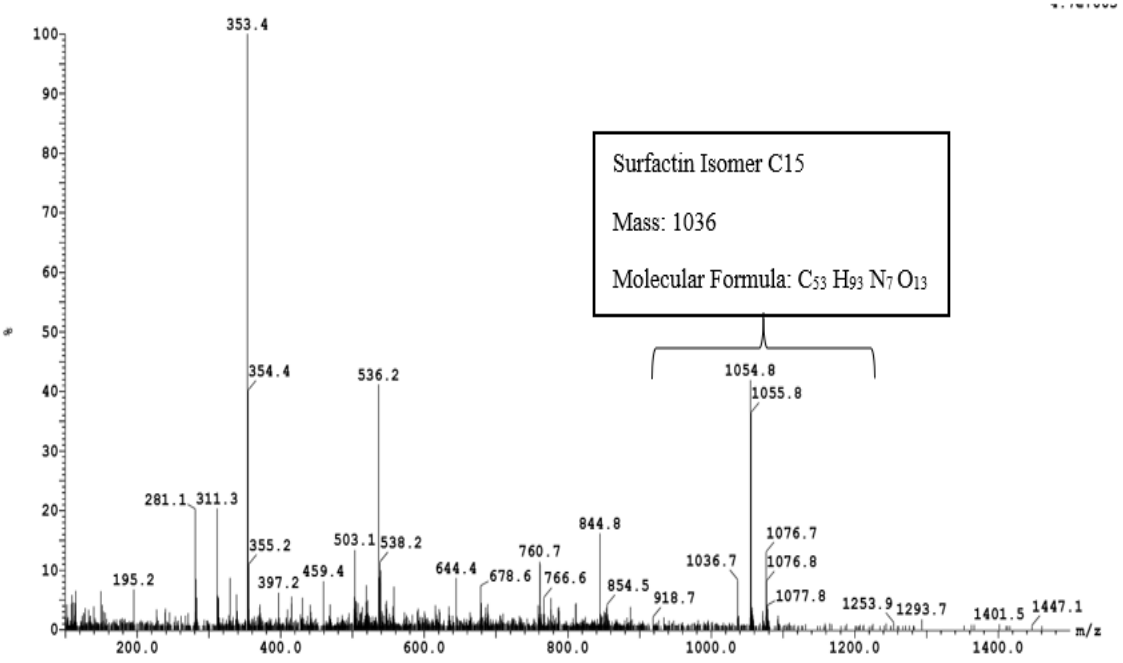


Fig 5.20 Mass spectra of LC-MS/MS based fragmentation analysis of C15 Surfactin (m/z 1036.7) of extract of isolate R5**.**

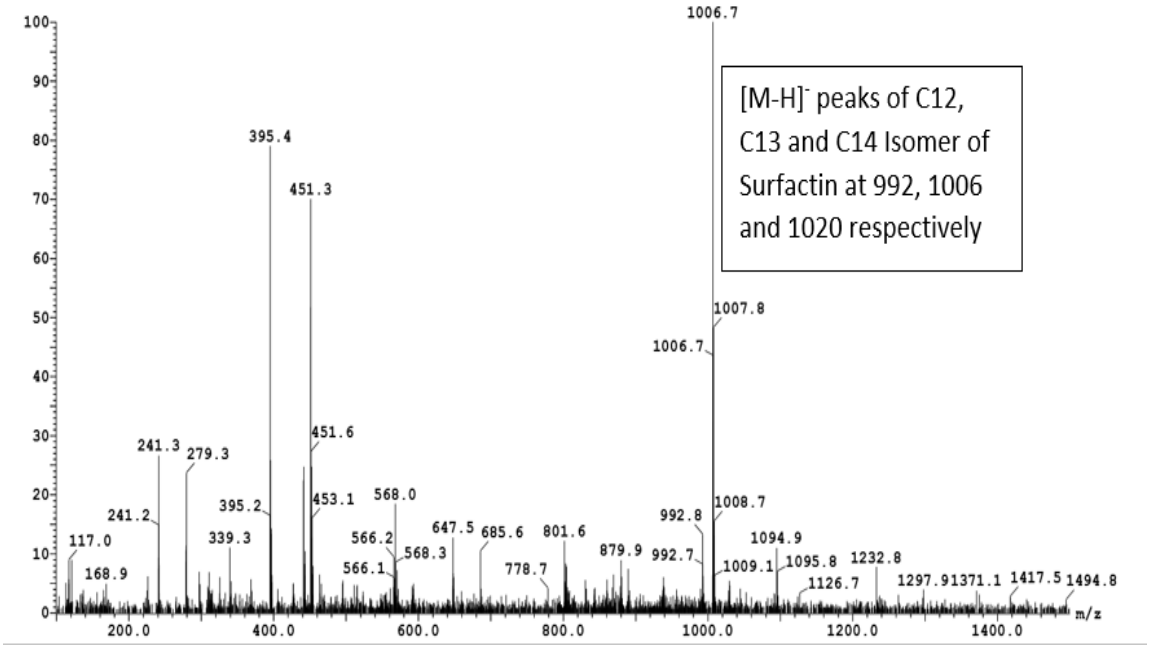


Fig 5.21 Mass spectra of LC-MS/MS based fragmentation analysis of C12, C13 and C14 isomer of Surfactin at 992, 1006 and 1020 respectively at the negative mode of extract for isolate R5.

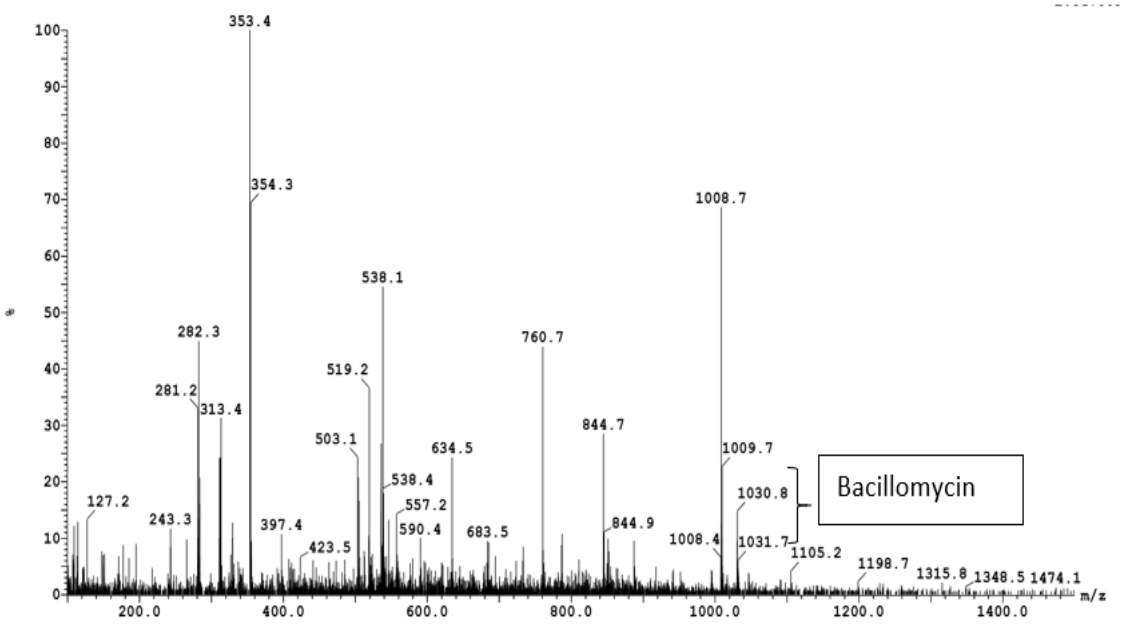


Fig 5.22 Mass spectra of LC-MS/MS based fragmentation analysis of Bacillomycin (m/z 1030.8) of extract for isolate R5.

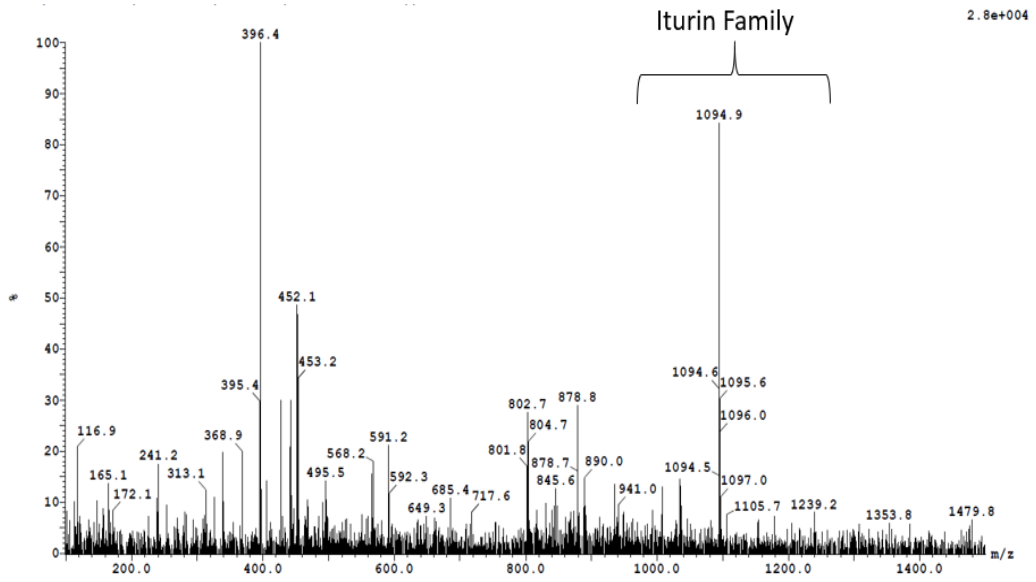


Fig 5.23 Mass spectra of LC-MS/MS based fragmentation analysis of Iturin family (m/z 1094-1105) with a sodiated peak of isomer C-16 Iturin at m/z 1096 of extract of isolate R5.

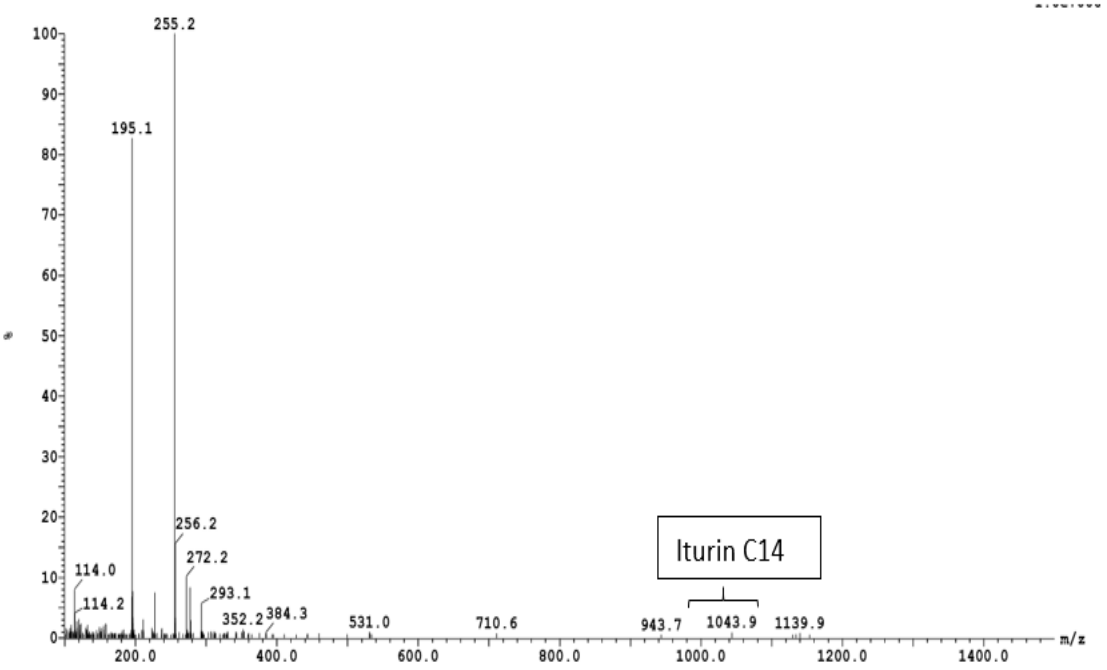


Fig 5.24 Mass spectra of LC-MS/MS based fragmentation analysis of protonated peak of C14 isomer of Iturin at m/z 1043.9 of extract for isolate R5.

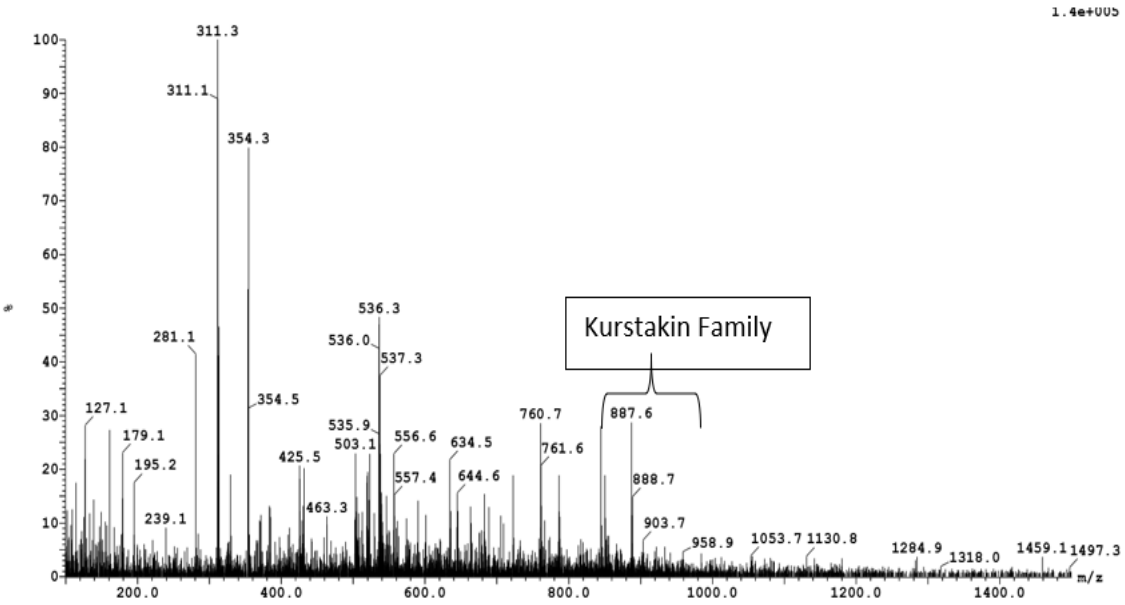


Fig 5.25 Mass spectra of LC-MS/MS based fragmentation analysis of Kurstakin m/z (887.6 and 903) of extract for isolate R5.

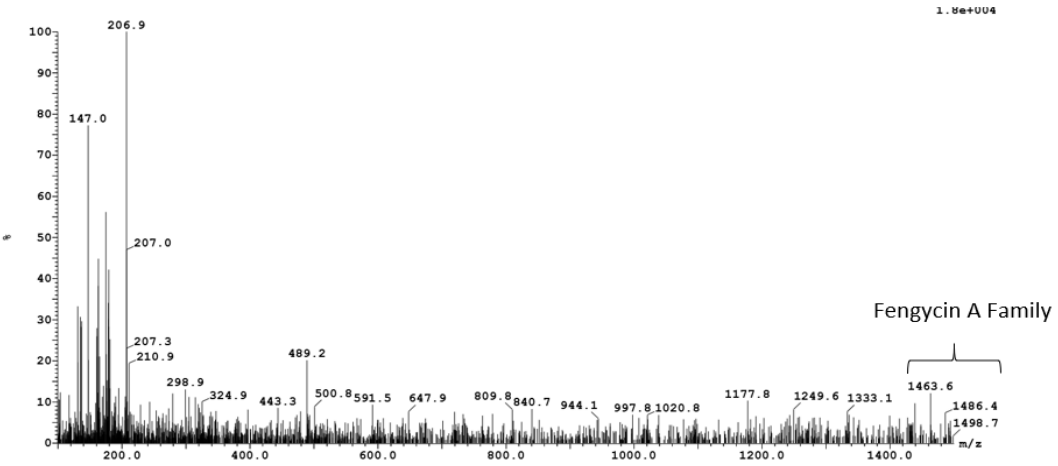


Fig 5.26 Mass spectra of LC-MS/MS based fragmentation analysis of Fengycin A m/z (1463.6) of extract for isolate R5**.**

iii) LC-MS/MS Analysis for Isolate S12:

In comparison with R5 and BS-4 isolates; the other two isolates S8 and S12 detected the probability of less lipopeptide antifungal compound. The crude extract of S12 revealed the presence of Kurstakin might be present in the range of m/z 850-950 $[M+H]^+$ (Fig 5.27) and Iturin at the peak m/z of 1057.1, 1072.6, 1084.4 $[M+H]^+$ (Fig

5.28). The possible peak for fengycin was also detected in the range of m/z of 1450-1550 (Fig 5.29).

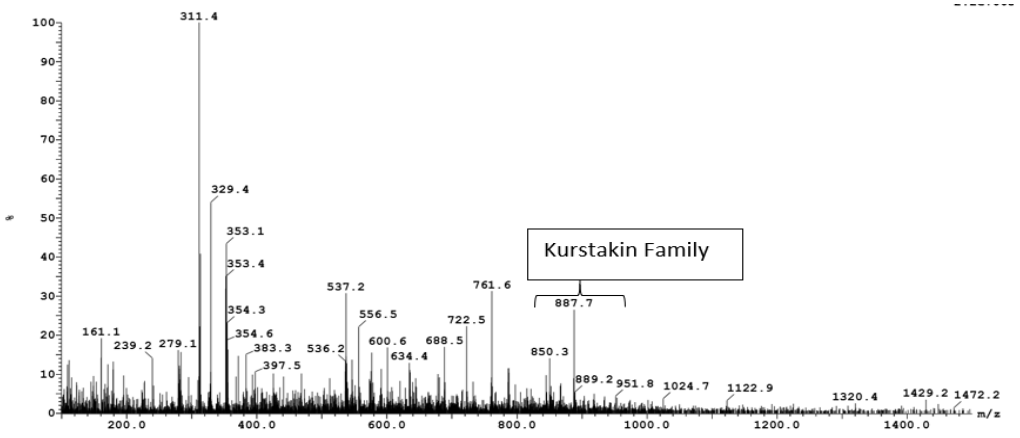


Fig 5.27 Mass spectra of LC-MS/MS based fragmentation analysis of Kurstakin of m/z 850-950 $[M+H]^+$ of extract for isolate S12.

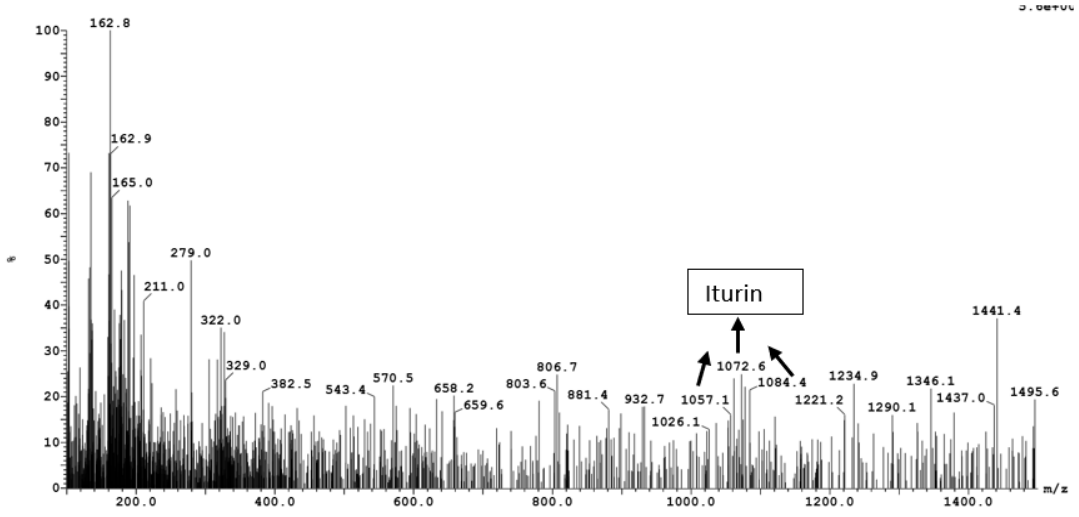


Fig 5.28 Mass spectra of LC-MS/MS based fragmentation analysis of Iturin at the peak m/z of 1057.1, 1072.6, 1084.4 $[M+H]^+$ of extract for isolate S12**.**

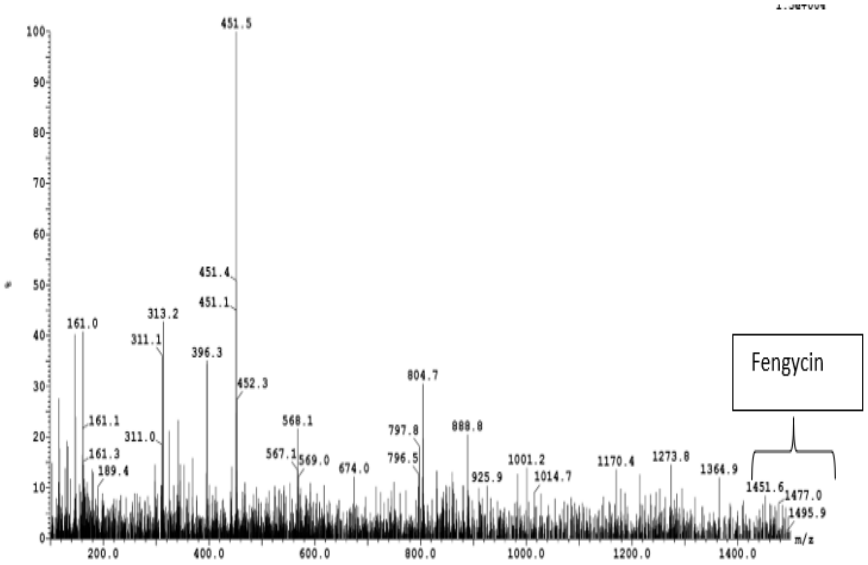


Fig 5.29 Mass spectra of LC-MS/MS based fragmentation analysis of Fengycin at the m/z 1450- 1550 of extract of isolate S12.

iv) LC-MS/MS Analysis of Isolate S8:

The S8 crude extract showed the presence of Kurstakin Na adduct of C10, C11 isomer might be present at 887 and 901 $[M+Na]^+$ (Fig 5.30) and Bacillomycin at m/z 1031.8 $[M+H]^+$ (Fig 5.31).

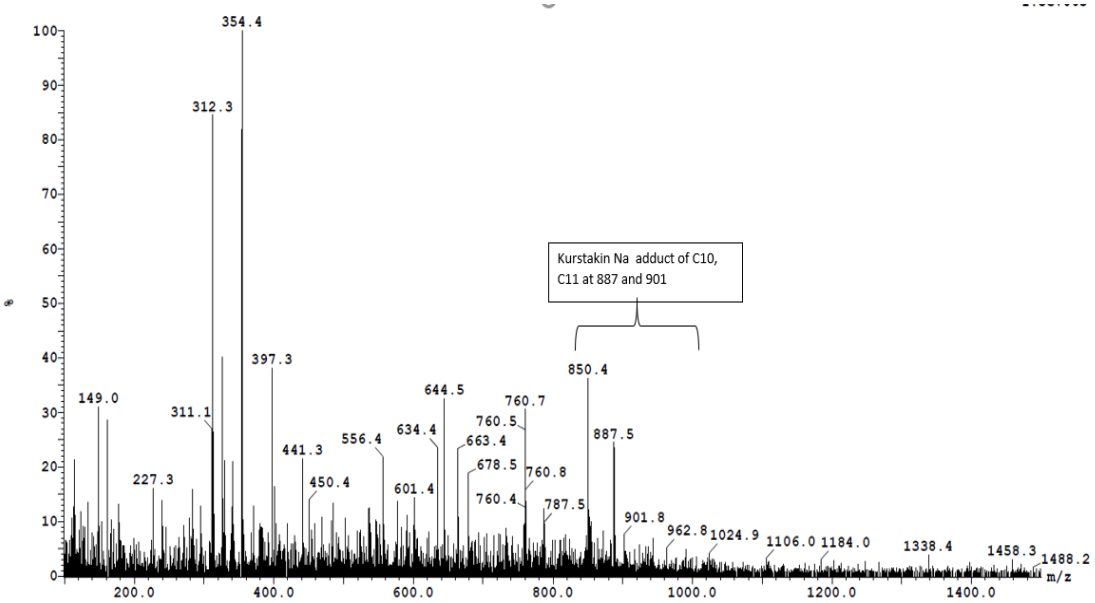


Fig 5.30 Mass spectra of LC-MS/MS based fragmentation analysis of Kurstakin Na adduct of C10, C11 isomer at 887 and 901 $[M+Na]^+$ of extract for isolate S8.

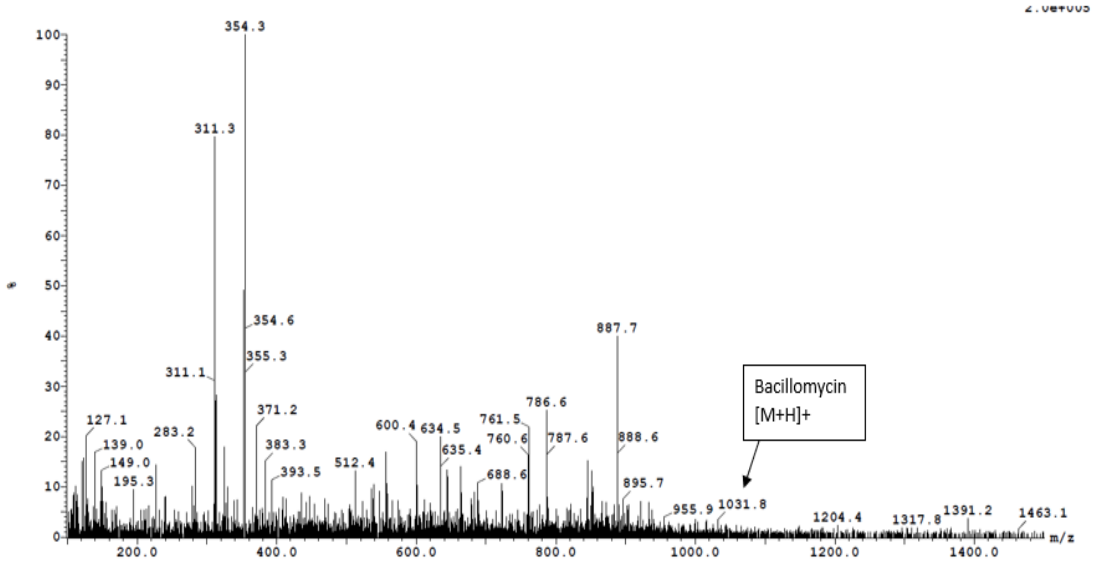


Fig 5.31 Mass spectra of LC-MS/MS based fragmentation analysis Bacillomycin at m/z 1031.8 $[M+H]^+$of extract for isolate S8.

5.3.7 GC-MS analysis for detection of volatile compounds of the isolates:

The volatile organic compounds (VOCs) present in the crude extract of isolates BS-4, R5, S12 and S8 were identified by GC-MS analyses. The GC-MS chromatogram of ethyl acetate extracts of isolates BS-4, R5, S12 and S8 revealed that the presence of various compounds with corresponding peaks at different retention times Figures 5.32- Fig 5.35. The retention time, area, peak area percent, and name of the compound were represented in the Tables 5.2- 5.5.

i) GC-MS analysis for isolate BS-4:

The GC- chromatogram for isolate BS-4 revealed the presence of various volatile compound (Fig 5.32). However, the major antifungal volatile compounds identified and confirmed for BS-4 were Benzoic Acid, 2,6-Bis(Trimethylsiloxy), Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), Octadecanoic Acid, Methyl Ester, Dibutyl phthalate,6-Octadecenoic acid, methyl ester, (Z) 1,4-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester, and gamma.-Tocopherol (Table 5.2).

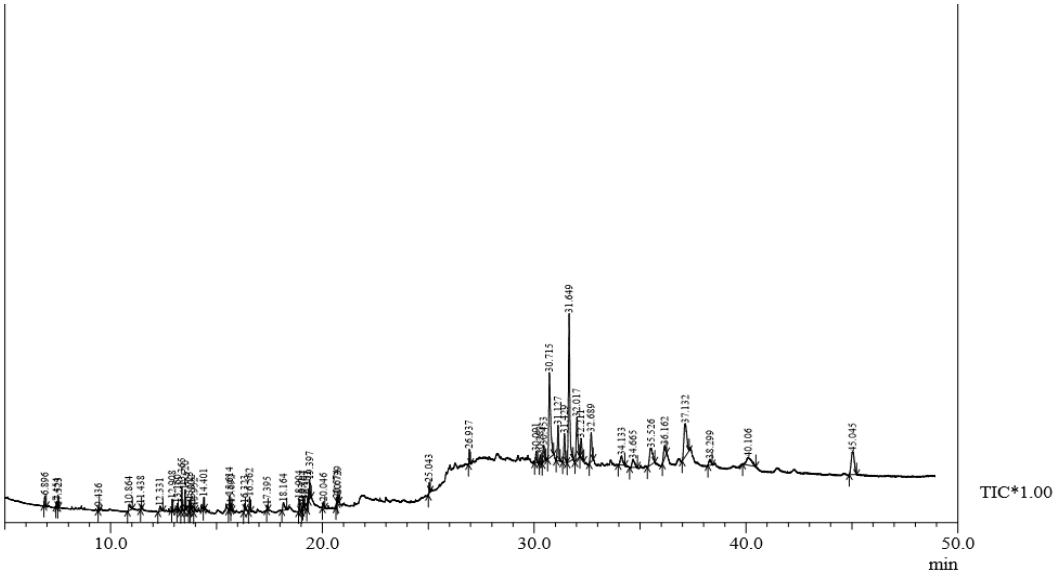


Fig 5.32 Chromatogram of GC-MS analysis of extract BS-4 isolate.

Table 5.2 List of VOCs detected by GC-MS analysis from the extract of isolate BS-4.

Peak#	Retention Time	Area	Area%	Name
1	6.896	209513	0.73	Undecane
2	7.454	65277	0.23	Cyclohexene, 1-Methyl-4-(1-Methylethenyl)
3	7.523	66449	0.23	Eucalyptol
4	9.436	42517	0.15	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-
5	10.864	216524	0.76	Linalyl acetate
6	11.438	56433	0.20	Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester
7	12.331	188202	0.66	3-Cyclohexene-1-Methanol, .Alpha.,.Alpha.,4-
8	12.908	132823	0.46	Cyclohexane, 1-Ethenyl-1-Methyl-2,4-Bis(1-M
9	13.160	89756	0.31	(-)-Alpha-Gurjunen
10	13.365	324769	1.13	Bicyclo[7.2.0]Undec-4-Ene, 4,11,11-Trimethyl-8-
11	13.520	252772	0.88	(-)-Sinularene
12	13.723	114063	0.40	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl)o
13	13.802	81057	0.28	Seychellene
14	13.952	57610	0.20	1h-3a,7-Methanoazulene, 2,3,6,7,8,8a-Hexahyd
15	14.401	146709	0.51	4,11,11-Trimethyl-8-

				Methylenebicyclo[7.2.0]U
16	15.614	244710	0.85	Isofuranodienone
17	15.692	70356	0.25	Benzoic Acid, 2,6-Bis(Trimethylsiloxy)-, Trime
18	16.323	111887	0.39	(7a-Isopropenyl-4,5-Dimethyl-Octahydro-Ind
19	16.562	258482	0.90	Patchouli alcohol
20	17.395	53109	0.19	Cyclohexasiloxane, dodecamethyl-
21	18.164	344985	1.20	Pyrrolo[1,2-A]Pyrazine-1,4-Dione, Hexahydro-3-(2-Methylpropyl)
22	18.904	164935	0.58	7,9-Di-Tert-Butyl-1-Oxaspiro(4,5)Deca-6,9-Diene-2,8-Dione
23	19.037	48643	0.17	Octadecanoic Acid, Methyl Ester
24	19.104	144042	0.50	Benzenepropanoic Acid, 3,5-Bis(1,1-Dimethylethyl)-4-Hydrox
25	19.303	106079	0.37	Pyrrolo[1,2-A]Pyrazine-1,4-Dione, Hexahydro-3-(2-Methylpro
26	19.397	404687	1.41	Dibutyl Phthalate
27	20.046	143521	0.50	Ethylene brassylate
28	20.674	88324	0.31	Linoelaidic acid
29	20.729	131999	0.46	6-Octadecenoic acid, methyl ester, (Z)-
30	25.043	159703	0.56	Bis(2-ethylhexyl) phthalate
31	26.937	266577	0.93	1,4-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester
32	30.091	303196	1.06	(3S,8S,9S,10R,13R,14S,17R)-17-((2R,5R)-5,6-Dimethylhep
33	30.283	208477	0.73	Stigmastanol
34	30.453	637026	2.22	(R)-6-Methoxy-2,8-dimethyl-2-((4R,8R)-4,8,12-trimethyltrid
35	30.715	3835358	13.38	.gamma.-Tocopherol
36	31.127	1082581	3.78	9,19-Cycloergost-24(28)-En-3-Ol, 4,14-Dimethyl-
37	31.429	879006	3.07	Stigmast-5-En-3-Ol, Oleate
38	31.649	5202124	18.14	(-)-1,2,3,4,4a,5,6,7-Octahydro-1-Methyl-7-Isopro
39	32.017	1644430	5.73	Vitamin E
40	32.211	675208	2.35	9,19-Cycloergost-24(28)-En-3-Ol, 4,14-Dimethyl-, Acetate, (3.
41	32.689	1091511	3.81	Glutaric Acid, Hex-4-Yn-3-Yl 4-(4-Methoxyphenyl)Cyclohexyl
42	34.133	533569	1.86	Ergost-5-En-3-Ol, (3.Beta.,24r)-
43	34.665	469751	1.64	Cholest-5-en-3-ol, 4,4-dimethyl-, (3.beta.)-

ii) GC-MS analysis for isolate R5:

Similarly, The GC-MS analysis also confirmed the presence of various volatile compound in the crude extract of R5 isolates which play a significant role in antibiosis and disease management. The chromatogram for extract of R5 is given below (Fig 5.33) and compounds are listed in table 5.3. Some compounds such as Phenol, 3,5-bis(1,1-dimethylethyl)-, Hexadecane, Santalol, cis,.alpha, 1-Tetradecanol, Pentadecane ,6-Oxabicyclo[3.1.0]hexan-3-one, 2,2,4,4-tetramethyl-, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, Dibutyl phthalate, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-, Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1), Tris(2,4-di-tert-butylphenyl) phosphate.

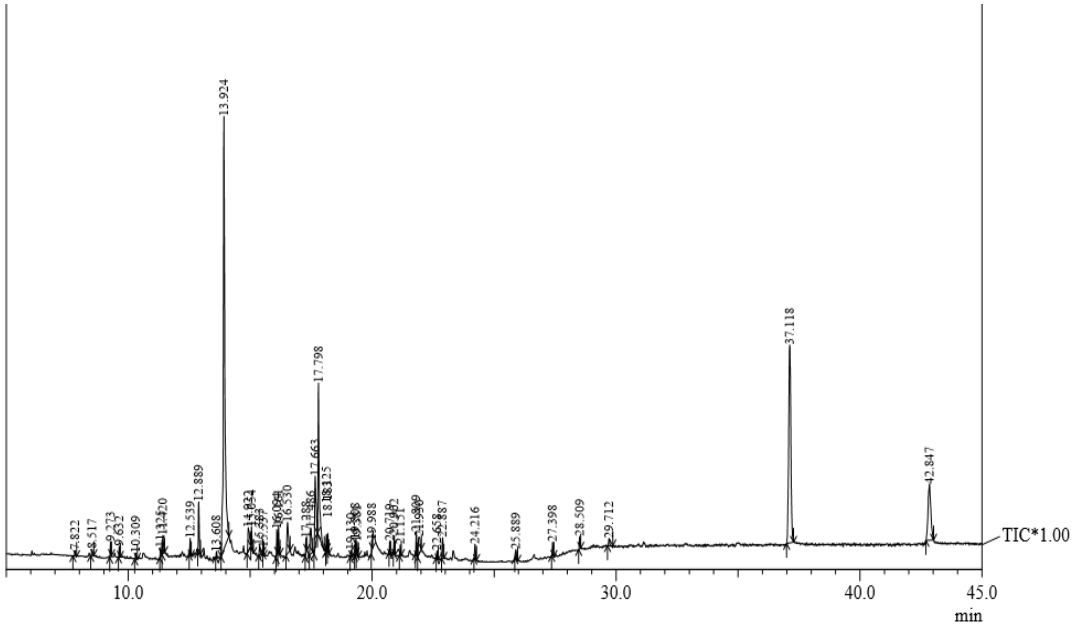


Fig 5.33 Chromatogram of GC-MS analysis of extract R5 isolate.

Table 5.3 List of VOCs detected by GC-MS analysis from the extract of isolate R5.

Peak#	R. Time	Area	Area %	Name
1	7.822	21009	0.21	Silane,Dimethoxymethyl(1-Methylpropyl)-
2	8.517	28712	0.28	Dodecane
3	9.273	48181	0.47	Benzene, 1,3-Bis(1,1-Deceneethyl)-
4	9.632	33623	0.33	Hexane, 3,3-Dimethyl-
5	10.309	21721	0.21	Hexane, 2,4-Dimethyl-
6	11.325	43082	0.42	1-Octyl Trifluoroacetate
7	11.420	92526	0.90	Tetradecane
8	12.539	56180	0.55	Sulfurous Acid, 2-Ethylhexyl Isohexyl Ester
9	12.889	221375	2.16	Phenol, 3,5-Bis(1,1-Dimethylethyl)-
10	13.608	2244	0.02	1,1,3,3-Tetramethoxypropane
11	13.924	3331162	32.51	Hexadecane
12	14.922	196464	1.92	Santalol, Cis,.Alpha.-
13	15.054	93004	0.91	Octadecane
14	15.383	40141	0.39	6-Methyl-1,4-Cyclooctadiene
15	15.537	16680	0.16	Octane, 2-methyl-
16	16.094	83565	0.82	1-Tetradecanol
17	16.161	92304	0.90	Pentadecane
18	16.530	244783	2.39	6-Oxabicyclo[3.1.0]hexan-3-one, 2,2,4,4-tetramethyl-
19	17.288	49008	0.48	Dodecane, 5-methyl-
21	17.663	492451	4.81	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
22	17.798	867807	8.47	Dibutyl phthalate
23	18.125	46774	0.46	2-Dodecanol, 1,1-Dichloro-
24	18.183	51271	0.50	Sulfurous acid, 2-ethylhexyl isohexyl ester
25	19.130	20991	0.20	Undecane, 2,8-dimethyl-
26	19.308	56753	0.55	Nonane, 4,5-Dimethyl-
27	19.381	46329	0.45	Eicosanoic Acid, Methyl Ester
28	19.988	32106	0.31	2-Dodecanol, 1,1-Dichloro-
29	20.719	34453	0.34	Palmitic Acid, TBDMS derivative
30	20.902	58103	0.57	Undecane, 5,7-dimethyl-
31	21.151	40100	0.39	Nonane, 4,5-Dimethyl-
32	21.809	77405	0.76	Octadecane
33	21.930	159636	1.56	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)
34	22.658	25881	0.25	Stearic acid, TBDMS derivative
35	22.887	91199	0.89	Octadecane
36	24.216	72848	0.71	Sulfurous acid, 2-ethylhexyl isohexyl ester
37	25.889	53130	0.52	Decane, 1-iodo-
38	27.398	59351	0.58	Sulfurous acid, 2-ethylhexyl isohexyl ester
39	28.509	51179	0.50	Decane, 2,3,5,8-tetramethyl-
40	29.712	28644	0.28	Octane, 3,4,5,6-tetramethyl-
41	37.118	2175706	21.23	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)
42	42.847	788411	7.69	Tris(2,4-di-tert-butylphenyl) phosphate

iii) GC-MS analysis for isolate S12:

Similarly, The GC-MS analysis also confirmed the presence of total 37 various volatile compound in the crude extract of S12 (Table 5.4). Out of them, following compounds are being known as potential antifungal such as Phenol, 3,5-bis(1,1-dimethylethyl)-, Hexadecane, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, 1,2-Benzenedicarboxylic Acid, Dibutyl Ester, Octadecane, Pyrrolo [1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-, Sulfurous acid, 2-ethylhexyl iso hexyl ester, Sulfurous acid, 2-ethylhexyl isohexyl ester, Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1) and Tris(2,4-di-tert-butylphenyl) phosphate. The chromatogram for S12 is given below (Fig 5.34)

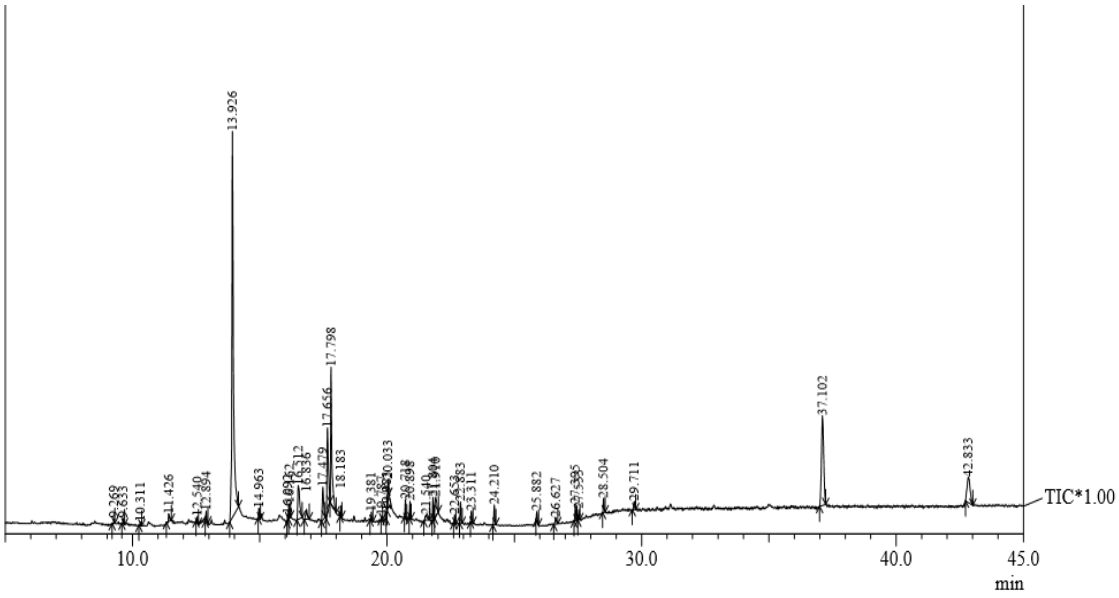


Fig 5.34 Chromatogram of GC-MS analysis for extract of isolate S12.

Table 5.4 List of VOCs compound detected by GC-MS analysis for the extract of isolate S12.

Peak#	R. Time	Area	Area %	Name
1	9.269	11361	0.16	Trans-2-[Bis(2,2,2-Trifluoroethoxy)Phosphoryl]-5-Tert-Butyl-1,3-Dith
2	9.633	16772	0.24	Nonane, 1-Iodo-
3	10.311	7801	0.11	1-Methyl-2,4-Diamino-Cyclohexane
4	11.426	33180	0.47	Undecane, 4,7-Dimethyl-
5	12.540	21107	0.30	Butane, 2,2-Dimethyl-
6	12.894	42593	0.60	Phenol, 3,5-Bis(1,1-Dimethylethyl)-
7	13.926	2714325	38.08	Hexadecane
8	14.963	37372	0.52	Cyclopentane, 1,3-Dimethyl-2-(1-Methylethenyl)-, (1.Alpha.,2.Alpha.,3.Beta.)-
9	16.092	19214	0.27	1-Heptadecanol
10	16.162	46725	0.66	Octadecane
11	16.512	300097	4.21	Pyrrolo[1,2-A]Pyrazine-1,4-Dione, Hexahydro-3-(2-Methylpropyl)-
12	16.836	70111	0.98	1,2-Benzenedicarboxylic Acid, Diundecyl Ester
14	17.656	545964	7.66	Pyrrolo[1,2-A]Pyrazine-1,4-Dione, Hexahydro-3-(2-Methylpropyl)-
15	17.798	728495	10.22	1,2-Benzenedicarboxylic Acid, Dibutyl Ester
16	18.183	30605	0.43	Pentadecane
17	19.381	27659	0.39	Hexanoic Acid, 2-Methyl-
18	19.787	10137	0.14	Dodecanal Dimethylacetal
21	20.718	53680	0.75	Palmitic Acid, TBDMS derivative
22	20.898	48901	0.69	Undecane, 5,7-dimethyl-
24	21.804	76585	1.07	Octadecane
25	21.910	150718	2.11	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-
26	22.653	30200	0.42	Stearic acid, TBDMS derivative
27	22.883	90693	1.27	Sulfurous acid, 2-ethylhexyl isohexyl ester
28	23.311	49272	0.69	1,2-Benzenedicarboxylic Acid
29	24.210	91396	1.28	Sulfurous acid, 2-ethylhexyl isohexyl ester
30	25.882	45006	0.63	Decane, 1-iodo-
32	27.395	70284	0.99	Sulfurous acid, 2-ethylhexyl isohexyl ester
33	27.533	36315	0.51	5,9,13-Pentadecatrien-2-One, 6,10,14-Trimethyl-, (E,E)-
34	28.504	55410	0.78	Nonadecane
35	29.711	39685	0.56	Hexadecane, 1-iodo-
36	37.102	907582	12.73	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)
37	42.833	360135	5.05	Tris(2,4-di-tert-butylphenyl) phosphate

iv) GC-MS analysis for isolate S8:

The GC-MS chromatogram for isolate S8 (Fig 5.35) revealed many peaks at different retention time and also detected the presence of various antifungal compounds (Table 5.5) such as Tetradecane, Phenol, 3,5-bis(1,1-dimethylethyl)-,Hexadecane, Patchouli alcohol, 1-Pentadecene, Pentadecane, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, 1,2-Benzenedicarboxylic Acid, Dibutyl Ester, Palmitic Acid, TBDMS derivative, Undecane, 5,7-dimethyl-, Sulfurous acid, 2-ethylhexyl isohexyl ester, Spiro[Cyclopentane-1,2'(1'H)-Quinoxaline], 3'-(4-Morpholinyl)-6',8'-Dini, and Tris(2,4-di-tert-butylphenyl) phosphate.

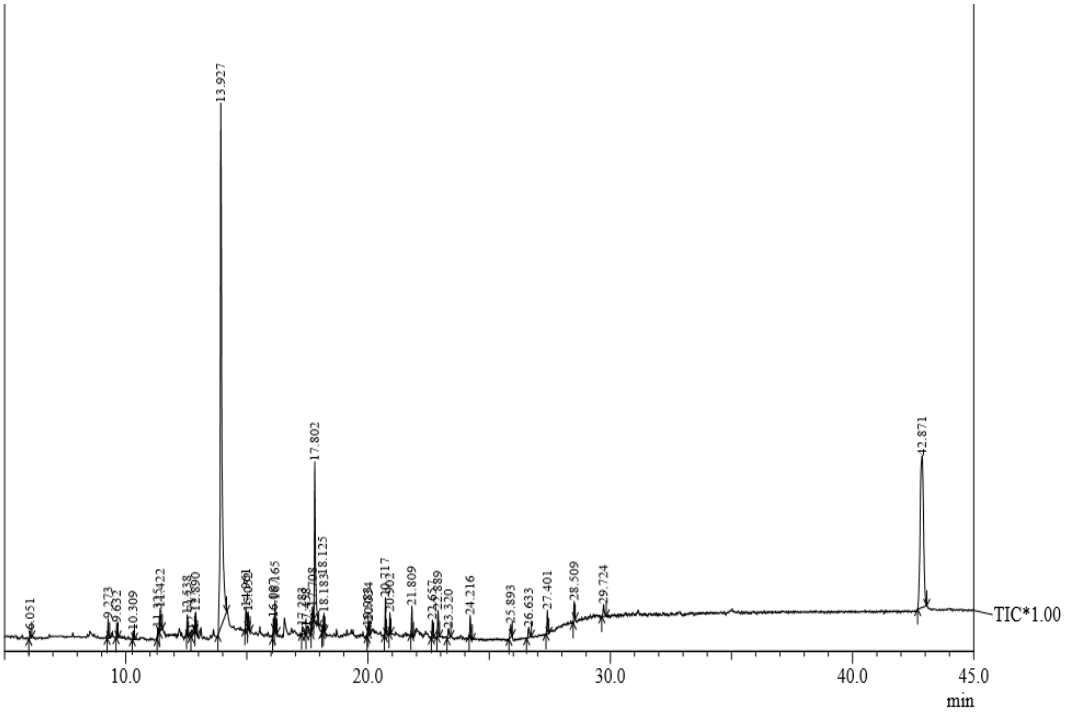


Fig 5.35 Chromatogram of GC-MS analysis for extract of isolate S8.

Table 5.5 List of VOCs detected by GC-MS analysis for the extract of isolate S8.

Peak#	R. Time	Area	Area %	Name
1	6.051	22369	0.32	Butane, 2,2-dimethyl-
2	9.273	48213	0.68	1,2-Di-Tert-Butylbenzene
3	9.632	34580	0.49	Hexane, 3,3-Dimethyl-
4	10.309	22477	0.32	Heptane, 3-Methyl-
5	11.325	32062	0.45	Cyclopentane, 1,1,3-Trimethyl-
6	11.422	87698	1.24	Tetradecane
7	12.538	44197	0.63	Octadecane
8	12.717	45284	0.64	1-Phenyl-2-hexanone
9	12.890	56004	0.79	Phenol, 3,5-bis(1,1-dimethylethyl)-
10	13.927	3022192	42.77	Hexadecane
11	14.961	72827	1.03	Patchouli alcohol
12	15.052	58064	0.82	Nonane, 4,5-Dimethyl-
13	16.097	40872	0.58	1-Pentadecene
14	16.165	82747	1.17	Pentadecane
15	17.283	25356	0.36	Hexane, 3,3-Dimethyl-
16	17.458	43847	0.62	Tridecanoic Acid, Methyl Ester
17	17.708	74387	1.05	Pyrrolo[1,2-A]Pyrazine-1,4-Dione, Hexahydro-3-(2-Methylpropyl)-
18	17.802	614544	8.70	1,2-Benzenedicarboxylic Acid, Dibutyl Ester
19	18.125	14540	0.21	1-Heptadecanol
20	18.183	36596	0.52	Sulfurous Acid, 2-Ethylhexyl Hexyl Ester
21	19.983	14613	0.21	2-(1-Chloro-2,2,2-Trifluoroethylidene)Cyclopentanone
22	20.034	21220	0.30	Undecane, 4,7-Dimethyl-
23	20.717	82083	1.16	Palmitic Acid, TBDMS Derivative
24	20.902	42301	0.60	Undecane, 3,7-Dimethyl-
25	21.809	76814	1.09	Undecane, 5,7-Dimethyl-
26	22.657	50533	0.72	Stearic Acid, TBDMS Derivative
27	22.889	77739	1.10	Sulfurous Acid, 2-Ethylhexyl Isohexyl Ester
28	23.320	40983	0.58	Ditridecyl Ester Of Phthalic Acid
29	24.216	72483	1.03	Spiro[Cyclopentane-1,2'(1'h)-Quinoxaline], 3'-(4-Morpholinyl)-6',8'-Dini
30	25.893	58145	0.82	Diethyl(Decyloxy)-Borane
31	26.633	49025	0.69	Octane, 2-chloro-
32	27.401	75312	1.07	Sulfurous acid, 2-ethylhexyl isohexyl ester
33	28.509	58277	0.82	Decane, 2,3,5,8-tetramethyl-
34	29.724	49788	0.70	Nonadecane
35	42.871	1818190	25.73	Tris(2,4-di-tert-butylphenyl) phosphate

5.4 Discussion:

Various modes of action have been reported to control pathogens, including substrate competition, the ability to colonize the ecological niche favoured by the pathogen, antagonism by antibiotics (Hoitink and Boehm 1999; Ben Slama et al. 2019) or cell-wall degrading enzymes (DasGupta et al. 2006; Aktuganov et al. 2008). β-1,3-glucanase and chitinase secreted by mycoparasites, termites and bacteria are involved in the biological control activity against fungal plant pathogens (Chatterton and Punja 2009; Hamilton et al. 2011; Alamri et al. 2012; Chen et al. 2015). In the recent year, the idea of shifting from chemical fungicides towards a searching of microorganisms possesing the hydrolytic enzyme satisfying the urge of using biological agent in combating the plant disease against fungal pathogen. As chitin and glucan are important constituent of cell wall of fungus and such enzyme are responsible for the lysis of pathogenic fungal cell wall; thus, helps in suppression of pathogen growth (Sa ´mi et al. 2001; Senol et al. 2014). Various reports have been documented for the production of chitinase and β-1,3-glucanase by the bacteria, fungi, insects, actinomycetes and as well by the plants (Asril et al. 2014; A Veliz et al. 2017). Numerous studies have been conducted on the production of hydrolytic enzyme by the microorganisms; however, very few findings have been done so far on the endophytic bacteria. Therefore, the present finding dealt with the presence of various active metabolites including hydrolytic enzyme present in the endophytic bacteria and their possible role in the inhibition of red rot pathogen directly. In the current study, almost selected endophytic bacterial isolates (R5, S12, S8, S17, and BS-4) revealed the significant production of Chitinase and β-1,3-glucanase activity when calculated in terms of enzyme activity (U/ml) in liquid broth medium. Thus, suggesting the potential role of isolates as biocontrol agents against fungal plant pathogen i.e.

Colletotrichum falcatum. The study was consolidated with the reports of Daulagala and Allan-Atkins (2015); Tan et al. (2015); Shabanamol et al. (2017) where, chitinase and β-1,3-glucanase enzymes present in the cell free extract of endophytic bacteria are considered to be involved in the antifungal mechanisms and thereby helps in suppressing the disease. Amongst the various species; *Bacillus* is the most often group known as biological control agent and various species *Bacillus cereus, B. licheniformis, B. thuringiensis, B. subtilis* have been cited already demonstrating the presence of hydrolytic enzyme (Asril et al. 2014; Senol et al. 2014; Lestari et al. 2017). However, present study is probably the first one highlighting the presence of chitinase and β-1,3-glucanase enzyme system in the *Bacillus paramycoides* strain R5 and *Bacillus aryabhattai* strain S12 isolated from sugarcane plant. Also, R5 isolates not only possessed the hydrolytic enzyme system but also demonstrated the highest activity amongst the isolates. The combination of two enzyme works together in a synergistic way in the lysis of fungal pathogen. As it has been observed that freshly synthesized hyphal tips of fungi contain chitin and β-1,3- glucan in a nascent stage and therefore are more susceptible to chitinase and β-1,3-glucanase enzyme (Mauch et al. 1988). Thus, it is evident from the study that hydrolytic enzyme are produced by the endophytic isolates as one of the main component and are responsible for inhibition of fungus cell wall. The isolate BS-4 also possessed strong *in vitro* antagonism against red rot fungus when checked. This might be due to the presence of chitinase enzyme which acts strongly on the fungus cell wall. The other endophytic isolates in the current findings, also revealed the enzymatic activity in considerable amount. As, isolates R5, S12, BS-4, S8 and S17 were producing significant amount of chitinase enzyme and isolates S17, R5 and S12 were producing the high amount of β-1,3 glucanase enzyme, therefore, later on they were considered for zymogram studies.

Zymogram analysis has been proved as one of the another important technique for the detection of active hydrolytic enzymes. In the present study, clear and specific band were observed in the zymographic electrophoretic run. The isolates exhibiting higher activity (R5, S12, S8, S17 and BS-4 for chitinase; and S17, S12 and R5 for β-1,3-glucanase) were analyzed for zymogram analysis and it confirmed the presence of respective hydrolytic enzymes with approximately molecular weight of 54 kDa in chitinase and ranging from 54 to 71 kDa in β-1,3-glucanase. The similar results were also found by Vázquez-Garcidueñas et al. (1998); Saadoun et al. (2009). Since, zymogram analysis has been proved as one of the best detection techniques for the detection of active enzyme by substrate gel and imprint techniques as employed in the present study (Trudel and Asselin 1990; Tronsmo and Harman 1993).

The molecular approach for screening of gene responsible for the synthesis of hydrolytic enzyme is also one of the trusted technique that have been employed in the present study. Through PCR amplification, expression of chitinase gene in the isolate R5, S12, S8 and BS-4; and for β-1,3-glucanase the expression was observed in the isolates R5, S12, and S17 with specific band of approximately 300 bp. The current study was found consolidated with the findings of Ramaiah et al. (2000); Venkatesan et al. (2015); Abdallah et al. (2017). The isolate thus proves to be a better and potential candidate for biocontrol of red rot disease against fungal pathogen *C. falcatum*. The newly isolated strain *B. paramycoides* R5 has better secretion capability for chitinase and β-1,3-glucanase which could be used as a potential antifungal agent against plant fungal pathogens. Also, other than R5; isolates especially BS-4, S8 and S12 also demonstrated good antagonism effect; therefore, further analysis for their metabolic approach was continued further.

The extracellular metabolomics is the study of low molecular weight metabolites that are produced extracellularly by the microorganisms into the surrounding environment probably into the culture medium (Pinu and Villas-Boas 2017). The supernatant obtained after the microbial growth generally contain all the secondary metabolites that are required for functioning of microorganism in adverse conditions. In today's scenario, metabolomics has emerged as a powerful tool in the quantitative identification of physiological and disease-induced biological status. The metabolome of endophytic microbiome plays a significant role in biotic and related abiotic stress. The various analytical techniques have been employed for the analysis of extracellular metabolites from microorganisms over the last two decades.

FTIR is one the earliest technique known for detecting the functional group of compound present in the metabolites. In the current study, FTIR analysis of crude extract detected the functional group of compounds of which some might belong to antifungal nature. As most of the stretching belonged to alcohol, phenol, aliphatic and amides region thus, representing the compounds might belonging to lipopeptide group or some another relevant compound belonging to some other group and therefore advanced study was necessary that could resolve the queries about the functional groups and metabolites.

The active bioactive metabolites including those of antibiotics and some other compounds are immensely produced by the bacteria that are useful for plant protection and help in the promotion of the plant growth. Such antibiotics and diffusible compound/metabolites were identified by the analytical technique LC-MS/MS. In the present study also, diverse secondary metabolites produced by the endophytic bacteria *B. paramycoides* strain R5, *P. aeruginosa* strain BS-4, *B. cereus* strain S8 and *B. aryabhattai* strain S12 were identified by the LC-MS/MS. However,

the versatile and diverse range of metabolites were detected in crude extract of isolates R5 and BS-4. Lipopeptide antibiotics (LPs) are the major antimicrobial compounds synthesized non-ribosomally by various endophytic bacteria belonging to *Bacillus* genera (Bacon and Hinton 2011; Abdallah et al. 2017). In the present study, active metabolites were detected in the extract of bacterial isolates R5 by LC-MS/MS which might includes the surfactin, iturins, fengycin, and bacillomycin. Also, kurstakin another form of cyclic lipopeptides was also detected in the crude extract of isolate R5. Lipopeptides play an important role in inducing defense mechanism and for their antibiotic property (Stein 2005; Raajmakers et al. 2010; Ek-Ramos et al. 2019). The various endophytic *Bacillus* group have been known for the production of lipopeptide that mainly target and binds to the organism's cell membrane directly and creates a pore in the membrane by which intracellular structure gets disturbed, and thus help in providing resistance mechanism against plant pathogen. Analysis of mass spectra revealed the peak in the range of 850-950 might corresponds with the kurstakin family, while iturin peaks were also observed for the extract R5. The peak in the range of 1450-1550 might correspond with the fengycin family and so peaks around 1030 corresponds with polyketide bacillomycin group. All the isomers of surfactin either belonging to C13, C14 or C15; were might to be present in the R5 extract. The findings also revealed that surfactin is also known for its antifungal nature due to its interaction with the cell membrane and thus, leads to membrane disruption (Maget-Dana and Ptak 1995; Grau et al. 1999; Carrillo et al. 2003; Eeman et al. 2006; Hazarika et al. 2019). Thus, endophytic R5 isolates produced an array of biologically active compounds including polyketides and lipopeptides antibiotic that might be the responsible for fighting against plant pathogens *C. falcatum*. Similar reports also detected the peak of these lipopeptide and suggested the biocontrol

behaviour of lipopeptides against many fungal pathogen (Jasim et al. 2016a,b; Dimkić et al. 2017). LC-MS/MS analysis detected the presence of lipopeptide compound in both the positive and negative modes. As lipopeptide contain peptide rings and aliphatic chain which was also confirmed by the presence of functional group analysis by FTIR. The various earlier reports found are in accordance with the present findings, where lipopeptide production from numerous species of *Bacillus* including *B. subtilis, B. amyloliquefaciens, B. pumilis, Bacillus* spp, was linked with the antifungal activity responsible for inhibition of various fungal pathogen (Ali et al. 2014; Jasim et al. 2016 a,b; Abdallah et al. 2017; Dimkić et al. 2017; Sarwar et al. 2018; Hazarika et al 2019). Thus, it is evident from the literature that till yet no studies have been done so far which elucidates the presence of bioactive molecule in the *Bacillus paramycoides* strain R5. Thus, it can be said that it is the first study highlighting the importance and presence of lipopeptide metabolite production in endophytic strain R5 which is helpful for antifungal mechanism(s). Thus, endophytic test isolate R5 can be considered as a potential candidate for biocontrol of red rot disease. Similarly, other two bacterial endophytic isolates of *Bacillus* in the study i.e *B.aryabhattai* strain S12 revealed the presence of iturin, fengycin and cyclic lipopeptide kurstakin might be present and *B. cereus* strain S8 elucidated the presence of only Kurstakin and bacillomycin peaks. Thus, the mass spectral analysis of these two endophytic isolates revealed the presence of peak for lipopeptide families. This was also in accordance with the study of Gond et al. 2015; Dimkić et al. 2017.

As lipopeptides metabolites are commonly found in Bacillus group; Similarly, many studies have been conducted which highlighted the importance of others metabolites/ compounds present in the Pseudomonad group that actively participate in the biocontrol mechanisms. The current study revealed that *P. aeruginosa* strain BS-4,

another important endophyte isolated in the current study from sugarcane crop also strongly inhibits the mycelial growth of fungal pathogen *C. falcatum* in *in vitro* studies. The LC-MS/MS analysis confirmed the presence of an array of antifungal metabolites such as 1-hydroxy phenazine (PHA), pyocyanin, phenazine-1-carboxylic acid (PCA), 2,4 Di-acetylphloroglucinol (2,4 -DAPG), Lahorenoic acid, siderophore (pyochelin), rhamnolipid and 4-hydroxy-2-alkylquinolines (HAQs).

The phenazine and pyocyanin has been known and studied for more than 60 years. Both the compounds have been effectively useful for biocontrol mechanism against fungal and bacterial pathogen (Chincholkar and Thomashow 2014). They are known for employing various mechanisms such as in cell signalling, biofilm formation, sequestering of iron and in the survival of bacteria. These feature makes the bacteria to survive and colonize in their native place and make them competitive to strongly compete with the pathogen (Pierson and Pierson 2010). The LC-MS/MS analysis of *P. aeruginosa* extract in the present study revealed the prominent peak of 1-hydroxy phenazine might be present at 197.1 $[M+H]^+$ and pyocyanin at m/z 211. Similarly, Siderophore production as evident from Chapter-4 were found to be produced by BS-4 isolates. Their production was again confirmed by the peak appearance at m/z 325 which indicated the pyochelin form of siderophore. Thus, the production of various metabolites by BS-4 isolates can be linked with their anti-fungistatic behaviour against red rot pathogen *C. falcatum.* Similar, results were also obtained by Yasmin et al. (2017), where Pyochelin produced by *P.aeruginosa* was considered responsible for showing antagonistic activity against *Xanthomonas oryzae*. The study of Audenaert et al. (2002) also demonstrated the inhibitory action of purified pyocyanin obtained from *P. aeruginosa* strain TO3 against *Macrophomina phaseolina* in tomato. The

prevalence of these metabolite helps the bacteria to survive and adapt in diverse conditions.

Phloroglucinol and its derivatives are phenolic compounds having broad spectrum antiviral, antibacterial, antifungal, antihelminthic and phytotoxic properties (Loper et al. 2012). 2, 4-diacetylphloroglucinol (2,4 DAPG) are phloroglucinol compound involved in the biocontrol of pathogen. The present findings revealed the presence of prominent peak of DAPG might be present at m/z 210 in the LC-MS/MS analysis of BS-4 extract. Also, several findings are found in accordance with the present study which confirmed the nature of DAPG as a major antimicrobial metabolite involved in biocontrol of phytopathogens (Sonnleitner and Haas 2011). The study of Reddy et al. (2007) also confirmed the presence of 2,4-DAPG at m/z 210 in LC-MS/MS and found to show antifungal activity against *Magnaporthe grisea, Dreschelaria oryzae, Rhizoctonia solani* and *Sarocladium oryzae* that are known to attack rice plants.

Rhamnolipids are another class of glycolipids that are widely reported from *Pseudomonas* sp. (Chong and Li 2017). In the present study, rhamnolipids peaks were confirmed in the range of m/z 500-600 that might be one of the metabolites responsible for inhibition of *C. falcatum* under *in vitro* study. The LC-MS analysis conducted by (Reddy et al. 2016) also confirmed that the rhamnolipids production by *P. aeruginosa* DR1 inhibited the growth of different plant pathogens like *F. oxysporum, Sclerotium rolfsii, Phytophthora nicotianae* and *M. phaseolina*. In this report, the crude extract analysis of BS-4 isolates also confirmed the presence of Phenazine-1-carboxylic acid (PCA) and lahorenoic acid A which might be also responsible for showing *in vitro* antagonism against red rot pathogen. The antifungal nature of Phenazine-1-carboxylic acid (PCA) and lahorenoic acid A have already been established by Mehnaz et al. (2013) and Shahid et al. (2018). Another metabolite

reported from the crude extract of BS-4 confirmed the presence of HAQ compounds which was supported by the findings of Yasmin et al. (2017) where HAQ was considered in biocontrol mechanisms. Thus, the isolate BS-4 is responsible for the suppression of pathogen growth under *in vitro* conditions due to the production of diverse exo-metabolites. Thus, biocontrol strain BS-4 would be providing a great opportunity for sustaining a higher population and also help in protection against a fungal pathogen.

Thus, variety of compounds have been detected by the powerful analytical technique LC-MS/MS. It revealed that BS-4 and R5 contain a diverse range of active metabolites that would be considered as potential biocontrol endophytic agents against *C. falcatum* pathogen and would help in the management of red rot disease under field conditions. Although S8 and S12 isolate also produced antifungal metabolites.

Endophytic bacteria are also known for production of a variety of volatile organic compounds (VOCs) that play an important role in the biocontrol mechanism. The volatile compounds are usually gaseous compounds produced by numerous endophytic microorganisms and can be detected by analyzing the crude extracellular extract of bacteria samples in the GC-MS. The GC-MS provides precise information on the metabolites and their metabolic pathway. The high effectiveness of volatile compounds at very low concentration makes them the best alternative for chemical fertilizers, pesticides, fungicides, and bactericides (Kanchiswamy et al. 2015). Interestingly, VOCs produced by plant growth-promoting bacteria act as effective biocontrol against numerous pathogens, by activating the plant defense responses and inducing systemic resistance (ISR) (Ryu et al. 2004; Farag et al. 2006; Raza et al. 2016). Also, various studies illustrated the importance of microbial VOCs serving as

the sustainable approach of agriculture by promoting as well as protecting the crop against various biotic and abiotic stresses (Morath et al. 2012, Selim et al. 2017). In the present chapter, the crude extract analysis of endophytic bacteria also revealed the presence of various volatile compounds. The detailed interpretation revealed that most of the compounds were found commonly present in the crude extracts of all the four endophytic isolate R5, S12, S8 and BS-4.

The compound Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) and Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) were found to be present in all the extract of all the isolates that might be responsible for their *in vitro* inhibition of fungal pathogen *C. falcatum* when checked for their volatile metabolite detection (Chapter 4). Similar findings have been reported by Sheoran et al. (2015); Kannabiran (2016); and Jasim et al. (2016 a,b) where antifungal nature of the same compound was observed and produced by endophytic bacterium. The long chain hydrocarbons i.e. hexadecane were found to be abundantly present in the extract of endophytic *Bacillus* isolate R5, S12 and S8 when analysed by GC-MS. This suggested their role in plant protection of sugarcane crop against red rot pathogen. The study of Bee Park et al. (2013) also confirmed the role of hexadecane in the protection of *Arabidopsis* from infection by a biotrophic pathogen, *P. syringae pv maculicola* and a necrotrophic pathogen, *Pectobacterium carotovorum subsp carotovorum*. Thus, current study revealed that the presence of hexadecane in higher amount can be directly linked to sugarcane plant resistance against *C. falcatum* pathogen. The volatiles compounds act strongly by possessing antagonistic mechanisms from a long distance and directly inhibiting the pathogen (Fialho et al. 2011). The Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro- 3-(2-methylpropyl); Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) compounds were also detected in the GC-MS

analyses and established their antifungal role against *Pyricularia oryzae* (Awla et al. 2016).

The volatile compound Phenol, 2,4-bis(1,1-dimethylethyl) also known as 2,4 -di-tert butyl phenol was also present in a higher amount in the crude sample of S12 and R5 isolates. Previous studies also reported the antifungal ability of Phenol, 2,4-bis(1,1-dimethylethyl) against *Colletotrichum acutatum, Phytophthora capsici and Fusarium oxysporum* (Sang et al. 2011; Sang and Kim 2012; Dharni et al. 2014). In the study of Padmavathi et al. (2014) phenol, 2,4-bis(1,1-dimethylethyl) was found to be linked with inhibiting the quorum sensing pathway mediating biofilm formation in the uropathogen *Serratia marcescens*. Thus, anti-biofilm is associated with inhibiting the signal between the pathogen and thereby helps in protection of plant from pathogens. Thus, from the present study it could be justified that both the isolates, S12 and R5 might be helpful in inhibiting the pathogen.

Dibutyl phthalate, another volatile compound was detected by GC-MS in the extract of R5 and BS-4 endophytic isolates which might be helpful in reducing the red rot disease concern of sugarcane. The present findings are consolidated with the study of Ahsan et al. (2017) who suggested that *Streptomyces* strain KX852460 had good general antifungal activity and might have potential biocontrol antagonist against *R. solani* AG-3 KX852461 to cure the target spot in tobacco leaf due to the presence of volatile compound Dibutyl phthalate.

The GC-MS/MS analysis of a crude extract of *Pseudomonas aeruginosa* strain BS-4 also confirmed the presence of gamma tocopherol in higher amount. Although till date their role has not been elucidated from *Pseudomonas aeruginosa* for antifungal

and antioxidant nature. This report is probably the first one to demonstrate the antifungal behaviour of tocopherol produced by the *P. aeruginosa* strain BS-4. The study of Reis et al. (2012) and Rhimi et al. (2018) suggested the antifungal behaviour of tocopherol. The bioactive properties of tocopherols include antioxidant capacity with the ability to prevent diseases correlated with increased formation of free radicals and oxidative stress (Ferreira et al. 2009; Carocho and Ferreira 2013). Antioxidant activity is related to inhibition of the oxidation of lipids, proteins, DNA or other molecules that occurs by blocking the propagation step in oxidative chain reactions (Carocho and Ferreira 2013; Stojković et al. 2015).

Also, various other metabolites produced by the endophytic isolates such as 1-Pentadecene, Pentadecane, 1,2-Benzenedicarboxylic Acid, 1,2-Benzenedicarboxylic Acid, Dibutyl Ester and others were also found to be involved in the antifungal mechanism. This was consolidated with the study of Sheoran et al. (2015); Ali et al. (2017); and Bhardwaj et al. (2017). Thus, GC-MS analysis revealed that isolates contain the volatile compound which play a significant role in antibiosis and disease management.

5.5 Conclusion:

The present study revealed the presence of a variety of metabolites produced extracellularly from endophytic isolates which were identified and confirmed by various analytical and molecular techniques. Although, all the selected isolates were able to produce one or the other antifungal metabolite; but isolates, *B. paramycoides* R5 and *P. aeruginosa* BS-4 possess diverse antifungal metabolite range that could be exploited for sustainable agriculture in biocontrol of red rot disease. This study confirmed that *in vitro* inhibition property of endophytes against *C. falcatum* could be

due to the presence of various diffusible, volatile compounds and lytic enzymes. Several studies demonstrated various metabolite production from the Bacillus group; but this is the first that highlighted the various important antifungal metabolites from *Bacillus paramycoides*. Also, it can be concluded that using such isolates containing maximum traits and metabolites would not only decrease the dependency on chemical fungicides against fungal pathogen but also help in enhancing the plant growth.

Chapter 6

To test the biocontrol efficacy of characterized endophytic isolates against Colletotrichum falcatum causing red rot in sugarcane in vivo condition.

Content of Chapter 6

To test the biocontrol efficacy of characterized endophytic isolates against *Colletotrichum falcatum* causing red rot in sugarcane *in vivo* condition.

6.1 Introduction:

Antagonism is the commonly reported phenomenon between the antagonistic microbe and the pathogen that results into suppressing or reducing the pathogen multiplication. An alternative mechanism generally referred to as induced resistance (IR) has been implemented for long in modern agriculture as an effective tool for plant disease management. Induced resistance is an intrinsic property of the host plant which is defined as a "state of increased defensive power of the host plant against a wide range of biotic and abiotic stresses elicited by chemical or biological stimulus". It is well reported that plants exhibit an array of defense machinery that activates in response to various biotic stresses including pathogen and parasites. The literature validates the fact that a variety of morphological, biochemical and physiological changes incurred in plant impart resistance against invasion by stress condition (Viswanathan and Samiyappan 2000).

A plant growth-promoting bacteria (PGPB) represents a special group of beneficial microbes which emerges out as potential biological stimulus instigating the induced resistance in the plant against wide spectra of plant pathogen; thereby, alleviating the disease and elevating the crop yield and productivity. Induced systemic resistance (ISR) and systemic acquired resistance (SAR) are the two well- known forms of induced resistance that are differentiated on the basis of the nature of inducer and the mechanism of induction pathways.

Induced systemic resistance (ISR) is defined as systemic enhancement of defense mechanism of host plant induced by beneficial microbes against necrotrophic pathogen (Kloepper et al. 1992). The mechanistic pathways for ISR are actively dependent on jasmonic acid (JA) and ethylene mediated pathways and not involved in the accumulation of pathogenesis-related proteins (PR) or salicylic acid (SA). However, Systemic acquired resistance (SAR) is another form of long-lasting induced resistance acquired by the plant either locally or systemically after being induced by pathogen. SAR provide broad-spectrum resistance against a wide range of pathogen and is dependent on salicylic acid-dependent pathways (Gao et al. 2015). Unlike ISR, SAR is associated with the accumulation of the production of pathogenesis-related (PR)-proteins. These PR proteins are soluble host-coded proteins including defense-related enzymes such as chitinase and β-1,3 glucanases; thus, having definite role in providing resistance to plant against pathogen attack. Induction of phenylalanine ammonia-lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO) and phenolic compound are the possible induced defense related enzyme/compound responsible for reducing the disease to some extent (Yasmin et al. 2016; Rais et al. 2017).

Both the form of induced resistance are interdependent and provide strong responsive defense action when plants are preconditioned with biological stimulus prior to infection or challenged inoculation by pathogen (Yi et al. 2013). Among the different potent candidate of PGPR for triggering an induced resistance; endophytic bacteria have attracted the attention of eco-warrior towards inferring the induced resistance in plants. They are considered as a prominent tool that can elicit unexpected physiological changes in the host plant rendering defense response against wide variety of pathogen as they are local resident of the host and can exhibit strong interaction with the plants.

Earlier studies have revealed that endophytes are able to elicit defense response against pathogen in a variety of crops, however, very less information is available for sugarcane crop.

Hence, the present chapter tries to enlighten the role of endophytic organisms in plant defense response. The chapter resolves the induction of various PR proteins and defense related enzymes in strengthening of plant cell walls by biocontrol agent treatments in response to infection by *C. falcatum* under *in vivo* condition.

6.2 Material and Methods:

6.2.1 Fungal pathogen and selection of endophytic biocontrol agent:

The procured test fungal pathogen *C. falcatum* was maintained on oatmeal agar medium at 4°C. The isolated endophytic antagonistic strain *Pseudomonas aeruginosa* (BS-4), *Bacillus paramycoides* (R5), and *Bacillus aryabhattai* (S12) (as discussed in Chapter-3, 4 and 5) based on the presence of multifarious metabolites were selected for evaluating their biocontrol efficacy against *C. falcatum* causing red rot in sugarcane plant under different pot trial experiment.

6.2.2 Plant material, experimental location and design:

Susceptible sugarcane cultivar Co 1148 was taken in this study. Three budded "sett" of selected variety were surface sterilized with 4.5% sodium hypochlorite (NaOCl), washed several times with sterile distilled water and blotted over sterilized filter paper under sterilized condition. After proper sterilization, sett was served as planting material and used for sowing in sterile pot (30 X 46 cm) containing 10 kg of potting mixture containing sterilized soil: sand: red earth: farmyard manure in the ratio of 4:2:2:2.

The experiment was conducted in the Lucknow-U.P. (India) for two consecutive years 2016-2017 and 2017- 2018. The experiment was laid out in a randomized block design with three replications in sterile pots each year, by sowing cane setts at 5 three budded setts in each pot, with the same treatment each year.

6.2.3 Preparation and Application of antagonistic endophytic bacterial strains:

The bacterial cells were freshly grown in LB and incubated in a shaking incubator for 24 h. Cells were harvested by centrifugation at the rate of 12,000 rpm for 3 min, then suspended in 100 ml of 0.85% saline to give a cell density of 10^9 cfu/ml. The cell number was estimated according to the optical density (0.5 absorbance at 595 nm gave approximately 10^{10} cells /ml, Da and Deng 2003). The prepared bacterial suspension was then applied by the method described by Viswanathan and Samiyappan (1999), Muñoz-Rojas and Mellado (2003), Hassan et al. (2010) and Hassan et al. (2011) with slight modification.

For the sett application, plantlets were soaked in the bacterial suspension for about one h and later incubated overnight (18 h) under sterile conditions before planting. For the control treatments, sterilized distilled water was used for soaking the setts.

For soil application, about 10 ml of the prepared bacterial suspension was drenched twice manually in the soil near the roots at different time interval as per the need of the experiment (as detailed below). While applying the bacterial suspension in the soil; the soil in the crop rhizosphere was removed and covered later.

For foliar application, 10 ml of bacterial suspension was sprayed over the standing cane.

6.2.4 Experimental setup based on pathogen inoculation and various designed treatment process:

The four different experiments or trials were designed and assayed at the same time to check the bio-efficacy of selected antagonistic endophytes against red rot disease under controlled pot culture. The pathogen inoculation and treatments in each trial are detailed below:

6.2.4.1 Trial-1 Soil Sick method (T-1):

The stalks of sugarcane infested with *C.falcatum* was chopped down into small bits and incorporated in the sterile soil 15 days prior to sowing the sett so as to get the pathogen infested soil as described by Viswanathan and Samiyappan (2008), Hassan et al. (2011) and Hassan et al. (2012). The pots were kept moistened till planting. After 15 days, washed and sterile setts (5 three budded) were planted in each pot. Each treatment has three replications and the bacterial inoculum was applied twice in the soil, i.e. at 4 months and 5 months after sowing the setts into the soil near the root at the rate of 10 ml per pot.

6.2.4.2 Trial-2 Stalk Inoculation method or Plug method (T-2):

In the second set of experiment, autoclaved sterile soil was used without any infestation of pathogen and three budded setts treated with overnight dipped bacterial formulations were planted (as described for sett application in section 6.2.3). After 4 and 5 months of sowing, 10 ml of bacterial inoculum were drenched manually in the soil near the root. The pathogen was multiplied on oat agar for 8 days and conidial suspension was prepared by mixing the spore with 10 ml of sterile distilled water. *C. falcatum* was challenge inoculated in cane stalks 6 months after planting or 1 month

after the last bacterial treatment. The 1 ml of conidial suspension (10^6 spore) was inoculated to the hole made by using the inoculator in the 3rd internode from the base by the standard plug method (Srinivisan and Bhatt 1961; Viswanathan 2010). After inoculating the spore suspension; hole was closed and sealed by using plastic clay.

6.2.4.3 Trial-3 Nodal swabbing method (T-3):

In this experiment also, sugarcane setts treated with overnight bacterial formulation were sowed in the sterile soil (section 6.2.3; sett application). After 4 and 5 months of sowing, bacterial treatment was applied in the soil (section 6.2.3; soil application). The pathogen was challenged inoculated by nodal swabbing method. This method was designed to assess induced resistance with limited injury to the cane tops. Here, 5th or 6th leaf from the top of the cane were removed and the conidial suspension (1 ml) was placed on the cotton pads and sealed with parafilm to retain moisture (Duttamajumdaar and Mishra 2012).

6.2.4.4 Trial-4 Modified soil sick method (T-4):

This experiment is the modification of soil sick method (T-1) and stalk inoculation method as described by Viswanathan and Samiyappan (2008), Hassan et al. (2011) and Hassan et al. (2012). In this experiment, sterile soil was infested with the pathogen by incorporating small chopped bits of infected stalk of sugarcane prior to sowing. The sugarcane sett dipped in bacterial formulation were sowed in the pot (same as sett treatment; section 6.2.3). After, 4 and 5 months of sowing, bacterial inoculum (10 ml) was applied in the soil as well as foliar spray (10 ml) respectively as per section 6.2.3. The methods differ from the above soil sick method (T-1) in the fact that this includes the usage of bacterial inoculum in the soil as well as above the plant.

Each trial has four different treatments with the usage of isolate viz. BS-4, R5, S12 and mixture of R5+S12 along with a control (without bacterial treated) as described below. The two bacteria (R5 and S12) only showed the compatible test; therefore, their mixture (R5+S12) were also evaluated for controlling the disease. The experiment was carried out with five treatments including control with three replications each in a randomized block design.

The different bacterial treatments in each trial were maintained in triplicates. The various conditions of the experimental set up for each trial are listed below in Table 6.1:

Table 6.1 The experimental set-up for each trial with different treatments.

Trial 1: **Soil- Sick Method (T-1)**	
Treatment 1 (BS-4)	10 kg sterile Soil + bits of stalk infested by pathogen + after 15 days, 5 three- budded setts were planted/ per pot (without any sett application) + Soil application of BS-4 bacterial inoculum (10 ml) at 4^{th} and 5^{th} months after planting.
Treatment 2 (R5**)**	10 kg sterile Soil + bits of stalk infested by pathogen + after 15 days, 5 three- budded setts were planted/per pot (without any sett application) + Soil application of R5 bacterial inoculum (10 ml) at 4^{th} and 5^{th} months after planting.
Treatment 3 (S12)	10 kg sterile Soil + bits of stalk infested by pathogen + after 15 days, 5 three- budded setts were planted/per pot (without any sett application) + Soil application of S12 bacterial inoculum (10 ml) at 4^{th} and 5^{th} months after planting.
Treatment 4 (R5+S12)	10 kg sterile Soil + bits of stalk infested by pathogen + after 15 days, 5 three- budded setts were planted/per pot (without any sett application) + Soil application of a mixture of R5+S12 bacterial inoculum (10 ml) at 4^{th} and 5^{th} months after planting.
Control	10 kg sterile Soil + bits of stalk infested by pathogen + after 15 days,

	5 three- budded setts were planted/per pot (without any sett application) + Soil application of sterile water (10 ml) at 4th and 5th months after planting.
Trial 2: Stalk Inoculation method or Plug method (T-2)	
Treatment 1 (BS-4)	10 kg sterile Soil + 5 three- budded setts treated with BS-4 bacterial suspension overnight were planted/per pot + Soil application (10 ml) of BS-4 inoculum at 4th and 5th months after planting + Spore suspension (1 ml) of *C. falcatum* inoculated in the 3rd internode of stalk from base + Sealed with clay.
Treatment 2 (R5)	10 kg sterile Soil + 5 three- budded setts treated with R5 bacterial suspension overnight were planted/per pot + Soil application (10 ml) of R5 inoculum at 4th and 5th months after planting + Spore suspension (1 ml) of *C. falcatum* inoculated in the 3rd internode of stalk from base + Sealed with clay.
Treatment 3 (S12)	10 kg sterile Soil + 5 three- budded setts treated with S12 bacterial suspension overnight were planted/per pot + Soil application (10 ml) of S12 inoculum at 4th and 5th months after planting + Spore suspension (1 ml) of *C. falcatum* inoculated in the 3rd internode of stalk from base + Sealed with clay.
Treatment 4 (R5+S12)	10 kg sterile Soil + 5 three- budded setts treated with mixture of R5+S12 bacterial suspension overnight were planted/per pot + Soil application (10 ml) of inoculum at 4th and 5th months after planting + Spore suspension (1 ml) of *C. falcatum* inoculated in the 3rd internode of stalk from base + Sealed with clay.
Control	10 kg sterile Soil + 5 three- budded setts treated with sterile water were planted /per pot + Soil application (10 ml) of sterile water at 4th and 5th months after planting + Spore suspension (1 ml) of *C. falcatum* inoculated in the 3rd internode of stalk from base + Sealed with clay.
Trial 3: Nodal swabbing method (T-3)	
Treatment 1 (BS-4)	10 kg **s**terile Soil + 5 three-budded setts treated with BS-4 bacterial suspension overnight were sown/ per pot + Soil application of BS-4 inoculum at 4th and 5th month after planting + Spore suspension (1

	ml) of *C. falcatum* inoculated in the 5th or 6th leaf sheath from top + Sealed with parafilm.
Treatment 2 (R5)	10 kg **s**terile Soil + 5 three-budded setts treated with R5 bacterial suspension overnight were sown/per pot + Soil application of R5 inoculum at 4th and 5th month after planting + Spore suspension (1 ml) of *C. falcatum* inoculated in the 5th or 6th leaf sheath from top + Sealed with parafilm.
Treatment 3 (S12)	10 kg **s**terile Soil + 5 three- budded setts treated with S12 bacterial suspension overnight were sown/per pot + Soil application of S12 inoculum at 4th and 5th month after planting + Spore suspension (1 ml) of *C. falcatum* inoculated in the 5th or 6th leaf sheath from top + Sealed with parafilm.
Treatment 4 (R5+S12)	10 kg **s**terile Soil + 5 three- budded setts treated with mixture of R5+S12 bacterial suspension overnight were sown/per pot + Soil application of mixture of R5+S12 inoculum at 4th and 5th month after planting + Spore suspension (1 ml) of *C. falcatum* inoculated in the 5th or 6th leaf sheath from top + Sealed with parafilm.
Control	10 kg **s**terile Soil + 5 three- budded setts treated with sterile water were sown/per pot + Soil application of sterile water at 4th and 5th month after planting + Spore suspension (1 ml) of *C. falcatum* inoculated in the 5th or 6th leaf sheath from top + Sealed with parafilm.
Trial 4: Modified soil sick method (T-4)	
Treatment 1 (BS-4)	10 kg sterile Soil + bits of stalk infested by pathogen + 5 three-budded setts treated with BS-4 bacterial suspension overnight were sown/per pot + Soil and Foliar application of BS-4 bacterial inoculum at 4th and 5th month after planting respectively.
Treatment 2 (R5)	10 kg sterile Soil + bits of stalk infested by pathogen + 5 three-budded setts treated with R5 bacterial suspension overnight were sown/per pot + Soil and Foliar application of R5 bacterial inoculum at 4th and 5th month after planting respectively.
Treatment 3	10 kg sterile Soil + bits of stalk infested by pathogen + 5 three-

(S12)	budded setts treated with S12 bacterial suspension overnight were sown/per pot + Soil and Foliar application of S12 bacterial inoculum at 4th and 5th month after planting respectively.
Treatment 4 (R5+S12)	10 kg sterile Soil + bits of stalk infested by pathogen + 5 three-budded setts treated with mixture of R5+S12 bacterial suspension overnight were sown/per pot + Soil and Foliar application of mixture of R5+S12 bacterial inoculum at 4th and 5th month after planting respectively.
Control	10 kg sterile Soil + bits of stalk infested by pathogen + 5 three-budded setts treated with sterile water were sown/ per pot+ Soil and Foliar application of sterile water at 4th and 5th month after planting respectively.

6.2.5 Inoculation of disease causing *C. falcatum*:

Fungal Inoculation was done by two methods:

6.2.5.1 Stalk inoculation: In "stalk inoculation" experiment, *C. falcatum* was inoculated in the stalk region (either by plug method or nodal method) of sugarcane plants so as to avoid the direct contact with the test endophyte bacterial inoculum and test the ability of the strains to suppress disease through the mechanism of induced systemic resistance as done in Trial-2 and Trial-3.

6.2.5.2 Soil inoculation: In "soil inoculation" experiments, the fungus was inoculated into the soil to check the efficiency of antagonistic strains in the pathogen-infested soil as done in Trial-1 and Trial-4.

6.2.6 Percentage Germination:

Percentage germination was recorded at 45th days after planting (DAP) and calculated with the following formula:

Percentage Germination = (No. of bud germinated/ No. of bud sown) X 100

6.2.7 Assessment of Disease in the various treatment:

Disease in plants inoculated with fungal spores by the plug method and nodal swabbing method (T-2 and T-3) was assessed after 60 days (8^{th} month after planting) of pathogen inoculation and symptoms like lesion width, top drying, transgression of lesions across the nodes and prominence of white spots in the stalks were observed. Disease intensity was scored on 0 to 9 scale (Table 6.2) as described by Srinivasan and Bhat (1961).

The disease in the plants grown in sick soil containing red rot debris (T-1 and T-4) was also assessed after 8 months of planting. The disease incidence and disease suppression were calculated by using the following formula:

$$\text{Disease incidence} = \frac{\text{infected tillers}}{\text{total tillers}} \times 100$$

$$\%\ \text{Disease suppression} = 1 - \frac{\text{Disease in treatment}}{\text{Disease in control}} \times 100$$

Table 6.2 Parameters used for determining red rot severity score (0-9 scale) in sugarcane caused by *Colletotrichum falcatum.*

Parameter	**Observation**	**Score**
Condition on top	Green	0
	Yellow/dry	1
Lesion extent	Very rare lesion	1
	Lesion spreading, but not covering entire cane area	2
	Lesion covering the entire inner cane area	3
White spot	Restricted	1
	Progressive	2
Nodal transgression	If 1 node crossed by fungal pathogen	1
	If 2 nodes crossed by fungal pathogen	2
	If 3 nodes crossed by fungal pathogen	3

6.2.8 Biochemical analysis of the plants for the various treatment:

Leaf samples were collected from individual treated canes of each Trial (T-1, T-2, T-3, T-4) to study the induction of PR proteins and defense-related enzymes in response to endophytic bacterial treatments in sugarcane plants upon challenged inoculation by *C. falcatum*. Leaf sample from treated as well as untreated plants were collected at 0, 7, 14, 21, 28 and 35 days after pathogen inoculation, and immediately stored in a deep freezer (-80°C) until biochemical analysis. Three replications were maintained for each treatment and each replicate consisted of 5 sample. One gm of the leaf sample was taken for extraction of individual enzyme by homogenization in different buffers at 4°C. The homogenates were centrifuged at 10,000 rpm for 15 min at 4°C and the supernatants were used for enzyme assays.

6.2.8.1 Assay of chitinase (E.C. 3.2.1.14):

One gram each of bacterial treated and control sugarcane leaf tissues were collected at 0, 7, 14, 21, 28 and 35 days after pathogen inoculation and the samples were immediately extracted with 10 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10,000 g at 4°C and the supernatant was used as enzyme source. The substrate used in the assay was 1% colloidal chitin prepared from crab shell chitin (Hi-Media) according to Berger and Reynolds (1958). For the colorimetric assay of chitinase, 1 ml each of enzyme extract substrate containing 1% colloidal chitin prepared in 1 M sodium acetate buffer (pH 4.0) was incubated for 60 min at room temperature. After incubation, the reaction was stopped by adding 2 ml of 1% 3,5 dinitrosalicyclic acid and boiled for 15 min in a water bath and cooled to room temp (Miller 1959). After, centrifugation at 10000 rpm for 3 min, absorbance was measured at 530 nm in UV spectrophotometer. The resulting

monomeric N-acetyl D-glucosamine (GlcNAc) was determined using standards of GlcNAc in the assay mixtures for calculations. Enzyme activity was expressed as µg GlcNAc equivalents min^{-1} g^{-1} fresh weight.

6.2.8.2 Assay of β-l,3-glucanase (E.C. 3. 2. 1. 39):

β-l,3-glucanase activity was assayed colorimetrically by the laminarin- dinitrosalicylic acid method (Miller 1959; Anand et al. 2007) with slight modification. Sugarcane leaf tissue (1 g) was extracted with 5 ml of 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4°C with a pestle and mortar. The extract was then centrifuged at 10,000 rpm for 15 min at 4°C and the supernatant was taken for enzyme assay. The reaction mixture consisted of 0.6 ml of 4% laminarin, 0.3 ml of 0.05 M sodium acetate buffer and 0.6 ml of enzyme extract. The reaction was incubated for 60 min at 40°C. After incubation, reaction was stopped by adding 3.75 ml of dinitrosalicylic reagent and heating the tubes for 5 min on a boiling water bath and vortexed. The resulting coloured solution was diluted with 4.5 ml of distilled water, vortexed, and its absorbance at 540 nm was measured. The blank was the crude enzyme preparation mixed with laminarin with zero-time incubation. The activity of β-1,3 glucanase was determined by measuring the release of reducing sugars by using laminarin (Sigma) as substrate and glucose as standard. Thus, the enzyme activity of β-1,3 glucanase was expressed as µg of glucose released per min per gm fresh weight.

6.2.8.3 Estimation of Phenylalanine ammonia lyase assay (PAL, E.C. 4.1.3.5):

Assay of phenylalanine ammonia-lyase (PAL) was performed using the procedure described by Dickerson et al. (1984) with slight modification of Sundar et al. (2009) and Xie et al. (2017). One gram of leaf tissue was homogenised in pre chilled mortar and pestle on ice containing 2 ml of 0.1 M sodium borate buffer, pH 8.8. The homogenate obtained was centrifuged at 12000 rpm for 20 min at 4°C and the

resulting supernatant was directly taken as enzyme source. The total reaction mixture of 3.5 ml for PAL consisted of 0.5 ml crude extract, 2.0 ml 0.1 M sodium borate buffer (pH 8.8), and 1ml of 12mm L-phenylalanine (Sigma) as a substrate which was incubated at 30°C for 30 min. The enzyme activity was measured against the control consisting of all the constituent as same in the reaction mixture except L-phenylalanine at 290 nm using UV/VIS spectrophotometer. The blank consisted of crude enzyme preparation mixed with L-phenylalanine with zero-time incubation. The extinction coefficient of cinnamic acid was 9630 M cm^{-1}. The activity of PAL was actually determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid. Hence, phenylalanine ammonia lyase enzyme activity was defined as the amount of enzyme forming 1µmole of trans-cinnamic acid from L-phenylalanine per min per gram fresh weight.

6.2.8.4 Estimation of Peroxidase assay (POX, E.C.1.11.1.7):

Peroxidase enzyme extract was prepared by grounding the 1 gm of leaf tissue in 2 ml 0.1 M sodium phosphate buffer, pH 7.0 at 4°C. The homogenate was centrifuged at 12000 rpm for 20 min at 4°C and the resultant supernatant was used directly as crude enzyme source. The POX activity was assayed with the method of Hammerschmidt et al. (1982) with a slight modification with Chance and Maehley (1955). The assay was performed with the total reaction mixture of 2.5 ml consisting of 1.5 ml 0.05 M pyrogallol, 0.5 ml crude enzyme extract and 0.5 ml of 1% H_2O_2 and incubated at room temperature (30°C). The change in absorbance was recorded against control after incubation at 30 s interval for 3 min at 420 nm. The glass tubes were protected by light by covering them by aluminium foil. Enzyme activity was calculated using the molar extinction coefficient of 2.47 mM^{-1} cm−1 (Chance and Maehle 1955). The activity of POX was actually determined as the rate of conversion of pyrogallol to Purpurogallin. The boiled enzyme preparation served as control and blank. The

enzyme activity was expressed as millimole (mmol) of purpurogallin produced per min per gram fresh weight of leaf.

6.2.8.5 Estimation of polyphenol oxidase (PPO, E.C.1.10.3.1):

The extract for PPO enzyme was prepared by homogenizing 1 g of leaf tissue in 2 ml 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 12000 rpm for 20 min at 4°C. The resulting supernatant was served as enzyme source. The enzyme activity of PPO was determined as per the methodology given by Mayer et al. (1966). For this, the reaction mixture consisted of 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added and the absorbance was read at 495 nm. The increase in absorbance was recorded at 30 s interval up to 3 min. The enzyme activity was expressed as changes in absorbance/O.D. per min per gram fresh weight of tissue.

6.2.8.6 Estimation of Total phenolics:

Phenolic content of sugarcane tissue was estimated by the procedure described by Zieslin and Ben-Zaken (1993) with slight modification. One g of leaf tissue was homogenized in 10 ml of 80% (v/v) methanol and agitated for 15 min at 70°C. One ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of Folin-Ciocalteau reagent (1N) and the solution was kept at 25°C for 3 min. After 5 minutes, one ml of a saturated solution of 8% Na_2CO_3 and 10 ml of distilled water were added and the reaction mixture was incubated for 1 h at 25°C. The absorption of the developed blue color was measured in a at 725 nm. The blank was performed using reagent blank with solvent. Gallic acid was used as standard and the content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reaction with a phenol solution (C_6H_60) and expressed as µg phenol equivalents per gram tissue.

6.2.9 Effect of inoculation with different endophytic bacterial treatments on growth parameters of sugarcane in the different experimental trials:

6.2.9.1 Cane Height: It is the total height from the first internode just above the ground to the first emergence of leaf of the top and was recorded at 300 days after planting (DAP).

6.2.9.2 Cane Girth: It is the diameter of mature internode (cm) and was measured with Vernier Calliper at 300 days after planting (DAP).

6.2.9.3 Cane Weight: It represents the average weight of cane (kg) and was measured at 300 DAP.

6.2.9.4 Total Number of Shoot: It was measured at the time of harvesting (300 DAP) by calculating the total number of shoots in each treatment.

6.2.9.5 Number of Internode: Number of Internode was also calculated in number starting from the first internode from the base to the top below the emergence of leaf.

6.2.10 Estimation of Chlorophyll a, chlorophyll b and total chlorophyll of the plants:

Physiological parameters, such as chlorophyll a, chlorophyll b, total chlorophyll content in leaves were also recorded using standard procedures (Arnon 1949).

One gram of leaf tissue was collected from each treatment of all trials and chlorophyll was extracted with 80% acetone in mortar and pestle followed by centrifugation of mixture at 5000 rpm for 5 min and the supernatant obtained was transferred into a 100 ml volumetric flask. The remaining residue was again grinded with 20 ml of 80 % acetone, centrifuged and supernatant was transferred into the volumetric flask. The process was repeated until the residue became colourless. The mortar and pestle was washed thoroughly with acetone and the clear washings were collected in the

volumetric flask. After, the volume was made upto 100 ml with 80% acetone and the absorbance of the solution was read at 645,663, and 652 nm against the solvent (80% acetone) blank., the calculation was done accordingly:

For Chlorophyll a:

mg chlorophyll a/g tissue = 12.7 (A_{663}) – 2.69 (A_{645}) * V/1000* W

For Chlorophyll b:

mg chlorophyll b/g tissue = 22.9 (A_{645}) – 4.68 (A_{663}) * V/1000 * W

And for Total Chlorophyll:

mg total chlorophyll/g tissue = 20.2(A_{645}) + 8.02 (A_{663}) * V/1000* W

Where, A= Absorbance at specific wavelengths

V= Final volume of Chlorophyll Extract in 80% acetone.

W=Fresh weight of the tissues extracted.

6.2.11 Statistical analysis:

The data were analyzed by ANOVA using Statistical Product and Service Solution (SPSS) version 20.0 software Developed by SPSS Inc., now IBM SPSS. All results were expressed at $P< 0.05$ to compare the means among the treatment means.

6.3 Results:

The three endophytic bacterial strain *B. paramycoides* (R5), *B. aryabhattai* (S12), *P. aeruginosa* (BS-4) and a mixture of *B. paramycoides* + *B. aryabhattai* (R5+S12) were demonstrated for checking the bioefficacy against *C. falcatum* under *in vivo* condition.

6.3.1 Efficacy of bacterial endophytes strains on percentage germination and disease development in different trial setup:

6.3.1.1 Trial-1 (Soil Sick Method):

In the first trial of 'soil sick' method (Fig 6.1), it was found that all the tested antagonistic endophytic bacteria showed suppression of disease (Fig 6.2). However, *P. aeruginosa* (BS-4) and *B. paramycoides* (R5) gave the statistically high suppression of disease by 35.71% in the Year-I (Table 6.3). The mixture of R5+S12 (*B. paramycoides* and *B. aryabhattai*) caused 26.53% suppression in the experiment while *B. aryabhattai* (S12) alone caused 14.29% suppression in the Year-I. All the bacterial endophytes treatments revealed statistically significant result as compared to control in the Year-I and Year-II (Table 6.3). In the year-II it was found that ability to suppress the disease was highest in the *P. aeruginosa* BS-4 with 41.67% disease suppression followed by *B. paramycoides* (R5) with 40.00 % respectively.

6.3.1.2 Trial-2 (Plug Inoculation Method):

In the second trial of plug method, the pathogen was inoculated at 3-4 internode above ground in the stalk region of the bacterial treated sett (Fig 6.2). During the Year-I of trial, it was found that the plant treated with the *B. paramycoides* (R5) showed significant higher suppression by 51.43% followed by *P. aeruginosa* (BS-4) by 42.86%. The mixture of R5+S12 gave higher suppression when compared with individual S12 strains by 37.14 and 34.29% respectively. However, much significant observation was made in the Year-II, where R5 was again demonstrating statistically highly significant result in the disease suppression by 52.94% (Table 6.4). The treatment with *P. aeruginosa* (BS-4) and a mixture of R5 +S12 significantly suppressed the disease by 44.12% and 32.35%., respectively. Ability of all the strains to suppress the red rot was statistically significant (Table 6.4)

6.3.1.3 Trial-3 (Nodal Swabbing Method):

In this method, pathogen was inoculated in the nodal region. It was found that only the control plants (without the bacterial treatment) showed disease development and

cane drying. While endophytic bacterial treated sett demonstrated reduction in the disease either by showing the limited lesion development or healthy cane (Fig 6.2). When the inoculated canes were split open, it was found that very limited lesion developed in the bacteria treated cane, while control had extensive lesion showing susceptible symptoms. When disease evaluation was done in the Year-I, it was observed that control plant had 8.00 disease score in the susceptible range (Table 6.5), while all the bacterial treated cane showed suppression in the disease. The least disease score of 3.75 was observed in treatment of *B. paramycoides* (R5) and suppression by 53.13% followed by 4.0 disease score in *P. aeruginosa* (BS-4) treatment and disease suppression by 50.00%. The mixture treatment of *B. paramycoides* and *B. aryabhattai* (R5+S12) showed higher suppression in disease by 46.88 % when compared with individual *B. aryabhattai* (S12) treatment (43.75 % ; Table 6.5). The similar results were obtained in the Year-II by the treatment of endophytic strain R5 with least disease score of 3.50 followed by BS-4 with 3.75. The suppression for red rot disease was recorded and it was observed that R5 leads in the suppression, followed by BS-4, mixture of R5+S12 and S12 strain. The effects of all the strains on red rot disease suppression was statistically significant in both the year (Table 6.5).

6.3.1.4 Trial-4 (Modified Soil Sick Method):

The fourth trial was modified Soil Sick in which setts pre-treated with bacterial strain were sown in pathogen-infested soil (Fig 6.1). Data indicated that in both Years (I and II), activity of *P. aeruginosa* (BS-4) treatment was significantly greatest with suppression of 60.00% and 60.71 % respectively (Table 6.6, Fig 6.2). The treatment with *B. paramycoides* (R5) suppresses the disease by 49.23% in the Year-I and by 51.11 % in the Year-II. The effect of other bacterial treatment including a mixture of *B. paramycoides* and *B. aryabhattai* (R5+S12) and *B. aryabhattai* (S12) on red rot

disease suppression was statistically significant when compared with the control in both the year (Table 6.6).

The biocontrol efficacy of the all bacterial treatments was statistically significant when compared with control in all the trial (T-1, T-2, T-3, and T-4). The efficiency of treated plant with *B. paramycoides* (R5) was found to be highest in the Trial-2 and Trial-3, followed by *P. aeruginosa* (BS-4) and other treatments. The activity of *P.aeruginosa* (BS-4) treatment was found to highest in both the soil sick method (T-1, and T-4) with enhanced activity in the T-4 trial.

6.3.1.5 Percentage Germination:

All the bacterial treated setts showed statistically significant increment in percentage germination when compared to control in all the trial except Trial-1 (Table 6.3, 6.4, 6.5, 6.6). In Trial-1, treatment with *P.aeruginosa* BS-4 resulted in highest germination of sett with 26.7% in the Year –I and in the Year-II *B. paramycoides* (R5) treatment resulted in highest germination with 28.8% (Table 6.3). In Trial-2, highest germination was recorded in R5 with 37.8%. The other strains also exhibited significantly high germination percentage when compared with control (Table 6.4). In Trial-3, R5 and BS-4 showed statistically equivalent germination with 42.2% followed by R5+S12 with 40% in the Year-I and data shown for both years depicted in Table 6.5. In the Trial-4 significantly higher germination was recorded for treatment with *P.aeruginosa* BS-4 followed by R5, R5+S12 and S12 in the Year-I and data for 1st as well as 2nd year depicted in Table 6.6.

Table 6.3 Effect of antagonistic endophytic bacteria on the incidence of red rot disease caused by *C. falcatum* on the sugarcane in "soil-sick method" (Trial-1).

Treatments	Year-I							Year-II						
	Total buds planted	Total buds germinated	Germination (%)	Total number of canes observed	Diseased canes	DI(%)	DS(%)	Total buds planted	Total buds germinated	Germination (%)	Total number of canes observed	Diseased Canes	DI(%)	DS(%)
BS-4	45	12	26.7a	12	6	50b	35.71	45	12	26.6a	12	5	41.67b	41.67
R5	45	11	24.4a	14	7	50b	35.71	45	13	28.8a	14	6	42.86b	40.00
S12	45	11	24.4a	12	8	66.67ab	14.29	45	12	26.6a	12	7	58.33ab	18.33
R5+S12	45	11	24.4a	14	8	57.14ab	26.53	45	12	26.6a	12	6	50ab	30.00
Control	45	10	22.2a	9	7	77.78a	0.00	45	8	17.7b	7	5	71.43a	0.00

Value are the mean of three replicates, and data in a column designated by the same letter(s) are not significantly different according to DMRT rule.
DI: Disease incidence; DS: Disease Suppression; Disease incidence was calculated on the basis of infected tiller to the total number of tillers.

Table 6.4 Effect of antagonistic endophytic bacteria on the incidence of red rot disease in "plug-inoculation method" (Trial-2).

Treatments	Year-I					Year-II				
	Total buds planted	Total buds germinated	Germination (%)	Disease Score	DS(%)	Total buds planted	Total buds germinated	Germination (%)	Disease Score	DS(%)
BS-4	45	16	35.6b	5b	42.86	45	15	33.3b	4.75bc	44.12
R5	45	17	37.8b	4.25b	51.43	45	17	37.7b	4.0c	52.94
S12	45	15	33.3b	5.75b	34.29	45	14	31.1b	6.0b	29.41
R5+S12	45	16	35.6b	5.5b	37.14	45	15	33.3b	5.75ab	32.35
Control	45	11	24.4a	8.75a	0	45	12	26.6a	8.5a	0

Value are the mean of three replicates, and data in a column designated by the same letter(s) are not significantly different according to DMRT rule.
DS: Disease Suppression; Disease score was calculated by observing different parameter such as condition of the top (green=0; dry=1), lesion width (very rare=1, spreading=2, covering the whole cane area=3), white spot (restricted=1, progressive=2) and nodal transgression of symptoms (across one node=1, across two nodes=2, across three nodes=3).

Table 6.5 Effect of antagonistic endophytic bacteria on the incidence of red rot disease in "nodal-swabbing method" (Trial-3).

Treatments	Year-I					Year-II				
	Total buds planted	Total buds germinated	Germination (%)	Disease Score	DS(%)	Total buds planted	Total buds germinated	Germination (%)	Disease Score	DS(%)
BS-4	45	19	42.2b	4.0b	50.00	45	18	40b	3.75b	53.13
R5	45	19	42.2b	3.75b	53.13	45	19	42.2b	3.50b	56.25
S12	45	16	35.6ab	4.50b	43.75	45	16	35.5ab	4.75b	40.63
R5+S12	45	18	40.0b	4.25b	46.88	45	17	37.7ab	4.50b	43.75
Control	45	13	28.9a	8.00a	0	45	13	28.8a	8a	0

Value are the mean of three replicates, and data in a column designated by the same letter(s) are not significantly different according to DMRT rule.
DS: Disease Suppression; Disease score was calculated by observing different parameter such as condition of the top (green=0; dry=1), lesion width (very rare=1, spreading=2, covering the whole cane area=3), white spot (restricted=1, progressive=2) and nodal transgression of symptoms (across one node=1, across two nodes=2, across three nodes=3).

Table 6.6 Effect of antagonistic endophytic bacteria on the incidence of red rot disease in "modified soil-sick method" (Trial-4).

Treatments	Year-I							Year -II						
	Total buds planted	Total buds germinated	Germination (%)	Total number of cane observed	Disease Cane	DI(%)	DS(%)	Total buds planted	Total buds germinated	Germination (%)	Total number of cane observed	Disease Cane	DI(%)	DS(%)
BS-4	45	18	40c	11	2	18.18b	60.00	45	17	37.7bc	14	3	21.43b	60.71
R5	45	17	37.8bc	13	3	23.08ab	49.23	45	16	35.5bc	15	4	26.67b	51.11
S12	45	14	31.1ab	12	5	41.67a	8.33	45	14	31.1ab	13	5	38.46ab	29.49
R5+S12	45	17	37.8bc	14	4	28.57ab	37.14	45	18	40c	14	5	35.71ab	34.52
Control	45	12	26.7a	11	5	45.45a	0.00	45	11	24.4a	11	6	54.55a	0.00

Value are the mean of three replicates, and data in a column designated by the same letter(s) are not significantly different according to DMRT rule.
DI: Disease incidence; DS: Disease Suppression; Disease incidence was calculated on the basis of infected tiller to the total number of tillers.

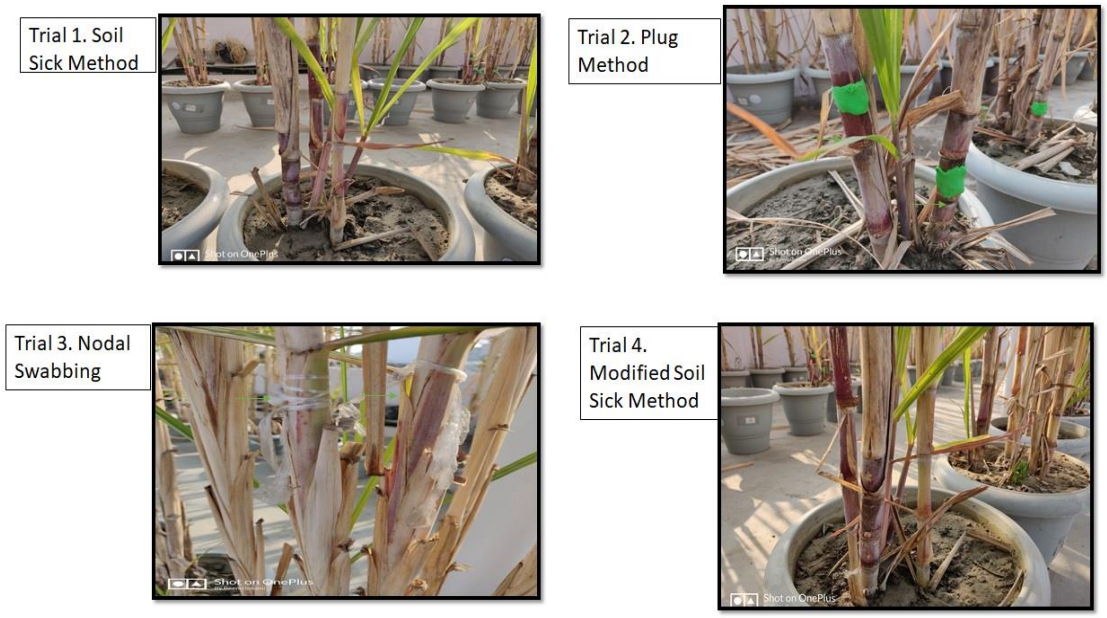


Fig 6.1 Pictorial representation of all the trials set-up of the experimental set-up.

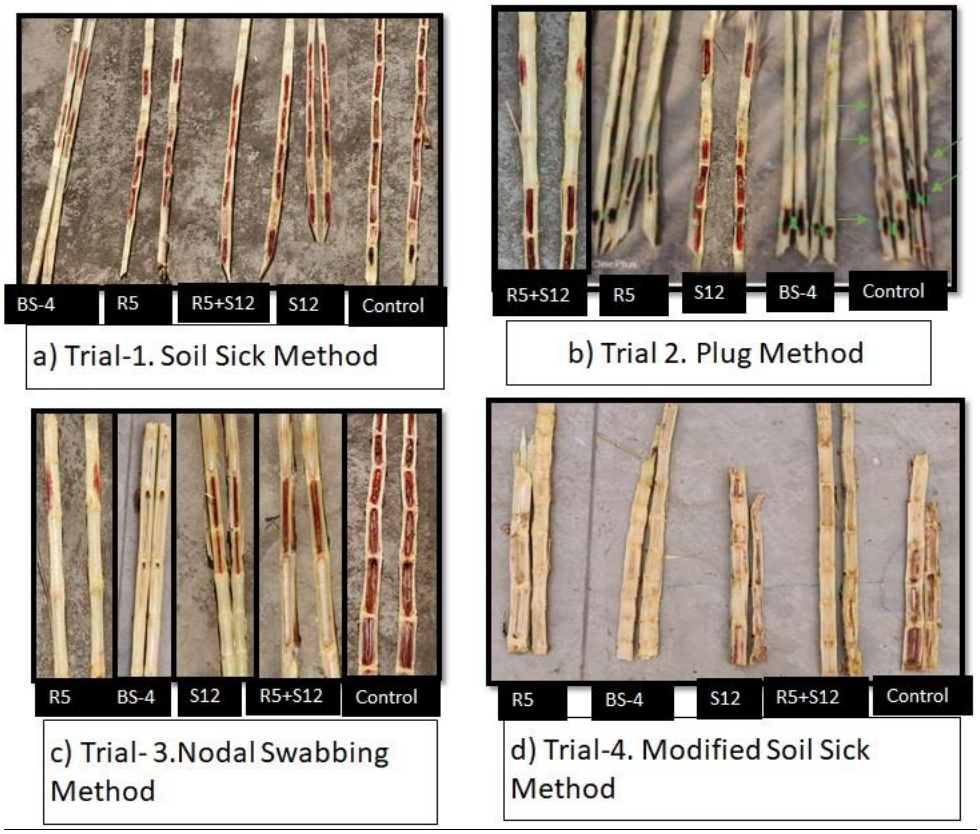


Fig 6.2 Disease assessment in the cane stalks at 8th month after planting sett treated with bacterial treatment (in all the trials)**.**

6.3.2 Induction of defense enzymatic activities:

In the present investigation, defense enzyme activity was observed mainly in the Trial-2 and Trial-3, where setts were sown after pre-treatment with endophytic bacteria followed by other soil application of bacteria and was challenge inoculated by red rot pathogen *C. falcatum* in order to observe the induced resistance in the plant. However, for the comparative analysis, defense enzyme mechanisms were also studied for the Trial-1 and Trial-2 where pathogen-infested soil was used, and therefore no challenged inoculation was done. It was observed that all the plants treated with bacterial inoculum demonstrated higher significant defense activity when compared with the control treatments (without bacterial treatments) in all the trial. In the Trial-2 and Trial-3 significant defense activity was observed as pathogen was challenge inoculated in pre-treated canes. However, when Trial-1 and Trial-4 were observed, results revealed that Trial-4 gave the enhanced defense activity when compared with the treatment in Trial-3.

6.3.2.1 Induction of PR protein

Induction of PR-proteins (chitinase and β-1,3 glucanase enzyme) was observed in the endophytic treatments of all the trials and also in the control plants.

6.3.2.1.1 Chitinase Enzyme:

The sugarcane leaves of treated plant with bacterial endophytes treatments were assayed for induction of chitinase enzymes. It was found that leaves of Trial-2 and Trial-3 marked higher expression of chitinase enzyme induction in all the bacterial treatments from the 1st day onward of pathogen inoculation. In the Trial-2 and Trial-3, higher activities were observed for R5 inoculation followed by BS-4 with maximum

increase up to 28 days and afterwards started declining gradually. The mixture of R5+S12 treated plant leaf also demonstrated highest activity on 28th day in Trial-3 and on 7th day in Trial-2. S12 treated plant leaf resulted into lower induction level of enzyme with highest on 7th day on both the Trials (2 and 3). However, in the Trial-1 and Trial-4, induction of enzyme occurred in all the bacterial treatments but with lower level of expression; as pathogen was not challenge inoculated. Thus, induction for enzyme started on the early days i.e. on the 3rd day and was maximum up to 14 days in the BS-4 inoculated plant's leaf in both the Trial-1 and Trial-4 followed by R5. The results also revealed that all the bacterial treated cane embarked with higher activity of enzyme as compared to control in all the trials (Fig 6.3).

6.3.2.1.2 β-1,3 glucanase enzyme:

Similarly, induction of β-1,3 glucanase enzyme was recorded for all the treatments of all the trials. A significant increase in the enzyme activity was noticed for all the endophytic bacterially treated plants over control one in all the trials. The study revealed that highest activity was found in treatment with R5 plant's leaf of Trial-2 (564.98 µg Glucose $min^{-1}g^{-1}$) and of Trial-3 (615.77 µg Glucose $min^{-1}g^{-1}$) on 14th day. Following R5; induction was also observed for BS-4 treated plant on the 14th day. Early induction of enzyme activity was seen from 1st day and maximum rise started from 3rd day up to 14th days of pathogen inoculation. Afterwards, it started declining gradually but maintained at higher level at all the time than the control. For the Trial-1 and Trial-4, prominent results were observed for BS-4 inoculated plants and maximum increase up to 14 and 28 days respectively (Fig 6.4).

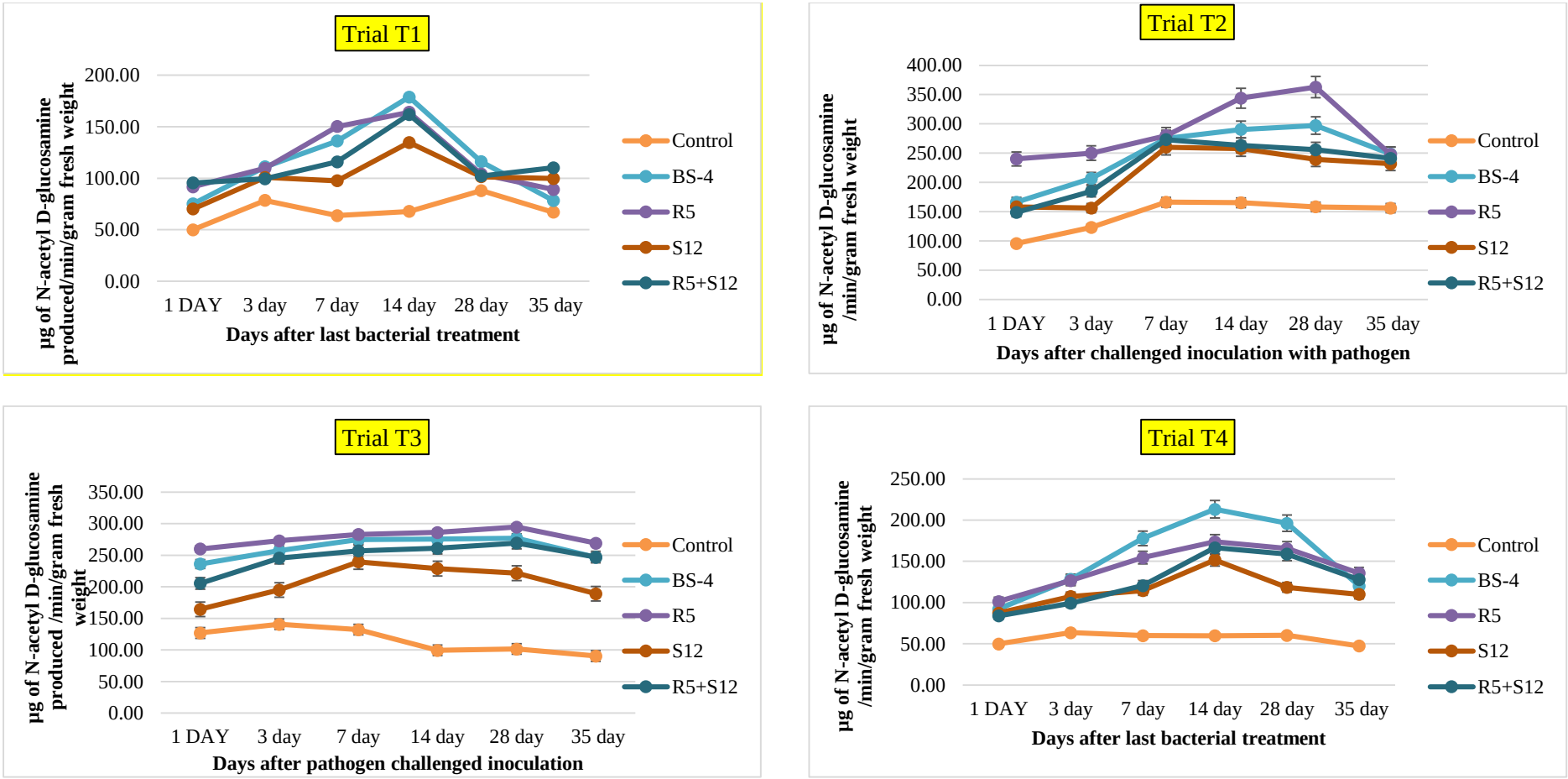


Fig 6.3 Induction of Chitinase enzyme activity in endophyte treated sugarcane plant challenged with *C. falcatum* (Trial-2 and Trial-3) and without challenged inoculation (Trial-1 and Trial-4). Control represent treatment without any endophytes inoculation in all the Trials. All values are the mean of three replicates. Vertical Bars represent the standard error of the means.

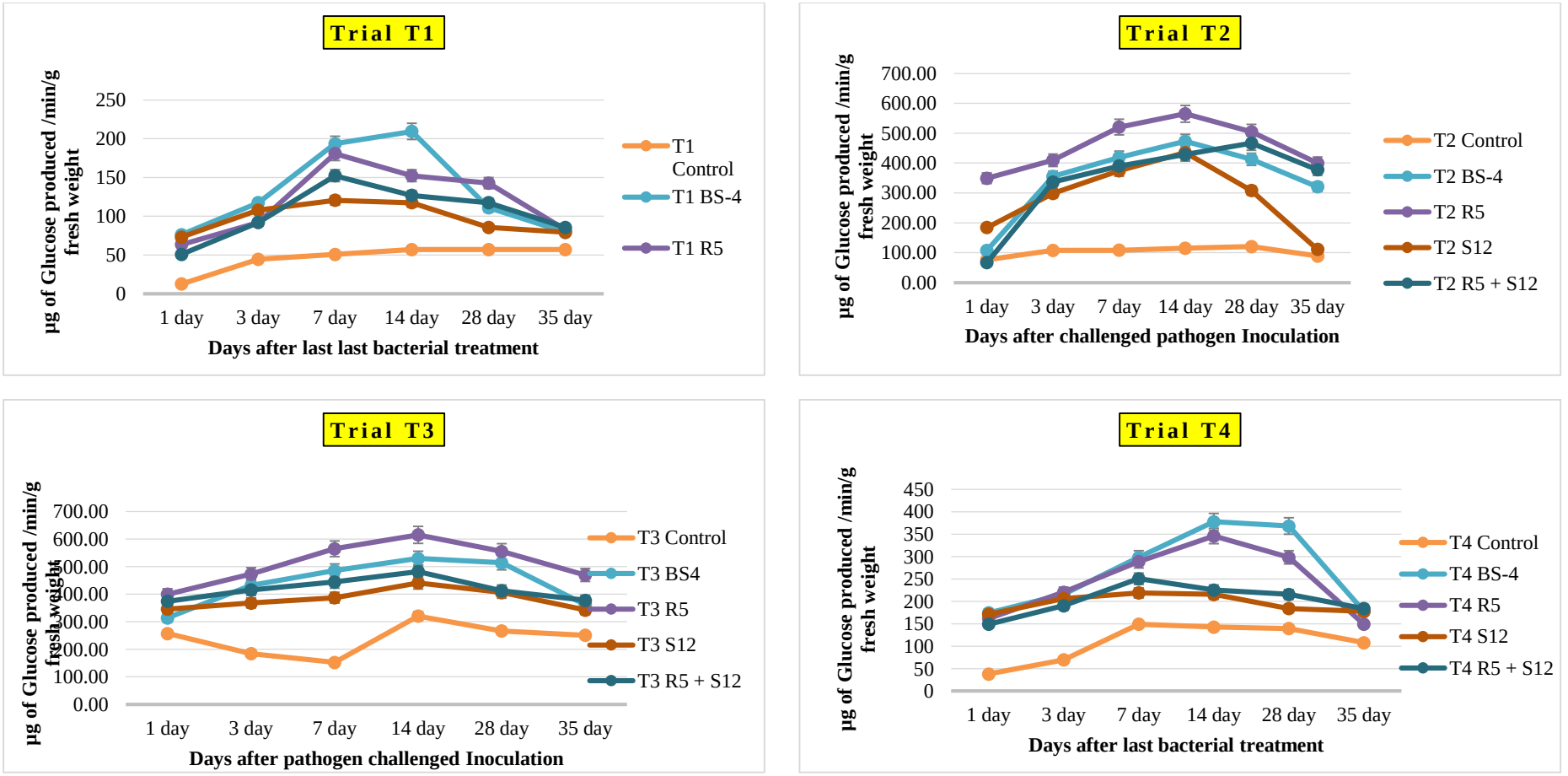


Fig 6.4 Induction of β-1,3 Glucanase enzyme activity in endophytic bacterially treated sugarcane plant challenged with *C. falcatum* (Trial-2 and Trial-3) and without challenged inoculation (Trial-1 and Trial-4). Control represent treatment without any endophytic bacterial inoculation in all the Trials. All values are the mean of three replicates. Vertical Bars represent the standard error of the means.

6.3.2.2 Induction of other defense related enzyme:

6.3.2.2.1 PAL activity:

A significant increase in PAL activity was noticed in all the antagonistic endophytic bacterially treated plants in all the trial when compared with the control of respective trial. In the Trial-1, the activity of PAL was enhanced in BS-4 treated cane which started increasing its activity from day 3rd, reached the higher level on day 7th and then highest up to day 14th and then declined slowly. Followed by BS-4, R5 also demonstrated a higher induction of PAL activity on the 14th day and declined afterwards. However, all the other treatments exhibited significantly higher PAL activity than the control plants at all the time intervals (Fig 6.5).

In the Trial-2, A time course study of PAL suggested that the plant treated with R5 revealed the highest activity from the day of pathogen inoculation and reaches the maximum at 14th day and then declined afterwards. In BS-4 treated plant increase in the activity was recorded from the first day onwards of pathogen inoculation and further increased till the maximum on the 14th day and beyond this time, it declined. However, treated plant with other treatments also showed an enhanced activity and maintained the higher level than those corresponding control during the experiment course (Fig 6.5).

Similarly, a significant increase in the activity of PAL has been recorded in Trial-3 for R5 treated cane from the day of pathogen inoculation and sharp increase on the 3rd day and maintained the higher level till the 28th day after pathogen inoculation. This activity further decreased and returned to lower level. A significant increment activity was also observed for BS-4 treated plants up to the 14th day. After reaching the

highest level, the enzyme activity started to decrease and thereafter returned to a relatively lower level.

Subsequently, when defense mechanisms were checked for Trial-4, it revealed that BS-4 treated cane presented marked increased in the activity of PAL followed by R5 till the 14th day after the last bacterial treatments.

Thus, after analysing all the treatments, the results demonstrated that in the Trial-1 and Trial-4; BS-4 demonstrated an enhanced activity followed by R5. In Trial-2 and Trial-3, there was a reshuffling and R5 treated plants induced more PAL activity followed by BS-4. However, it was also noticed that Trial-3 had the highest activity amongst all the treatments followed by Trial-2, Trial-4 and Trial-1.

6.3.2.2.2 Peroxidase (POX):

A significant increase in POX activity was detected in all the leaves upon treatment with endophytic biocontrol agent over control treatment in all the trials. However, treatment with BS-4 recorded significantly higher enzyme activity in Trial-1 and Trial-4 when compared with their respective control treatment. The activity of POX reached the highest level in all the treatment of Trial-1 and Trial-4 at 7th day after treatment and then slowly decreased (Fig 6.6).

However, in Trial-2 and Trial-3 marked increased in the enzyme activity was recorded for R5 followed by BS-4 treated setts upon challenged inoculation by pathogen. In the trial-2 and trial-3, highest activity was recorded at 14th day and 28th day post challenged inoculation respectively (Fig 6.6).

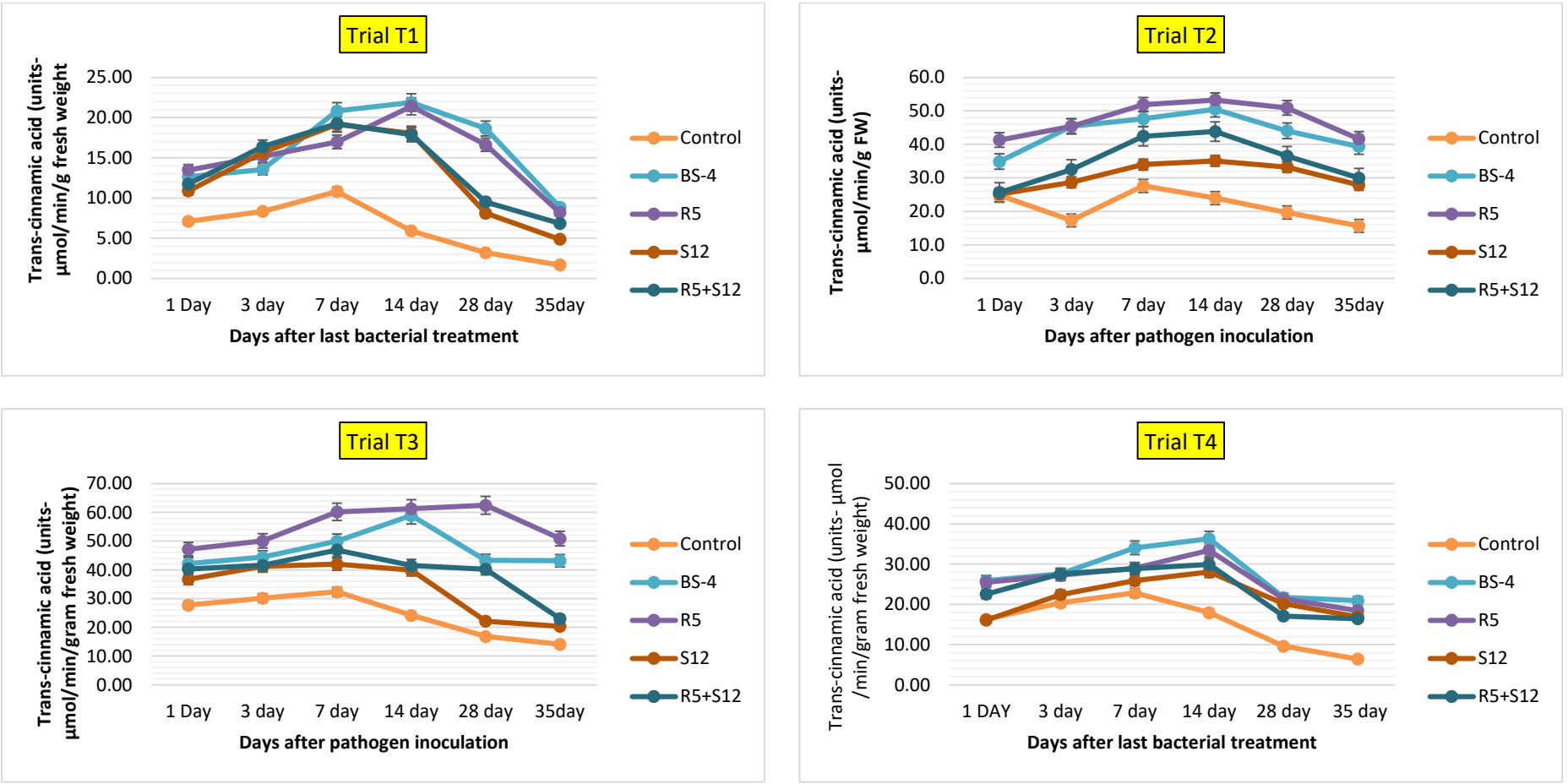


Fig 6.5 Induction of PAL enzyme activity in endophytic bacterially treated sugarcane plant challenged with *C. falcatum* (Trial-2 and Trial-3) and without challenged inoculation (Trial-1 and trial-4). Control represent treatment without any endophytic bacterial inoculation in all the Trials. All values are the mean of three replicates. Vertical Bars represent the standard error of the means.

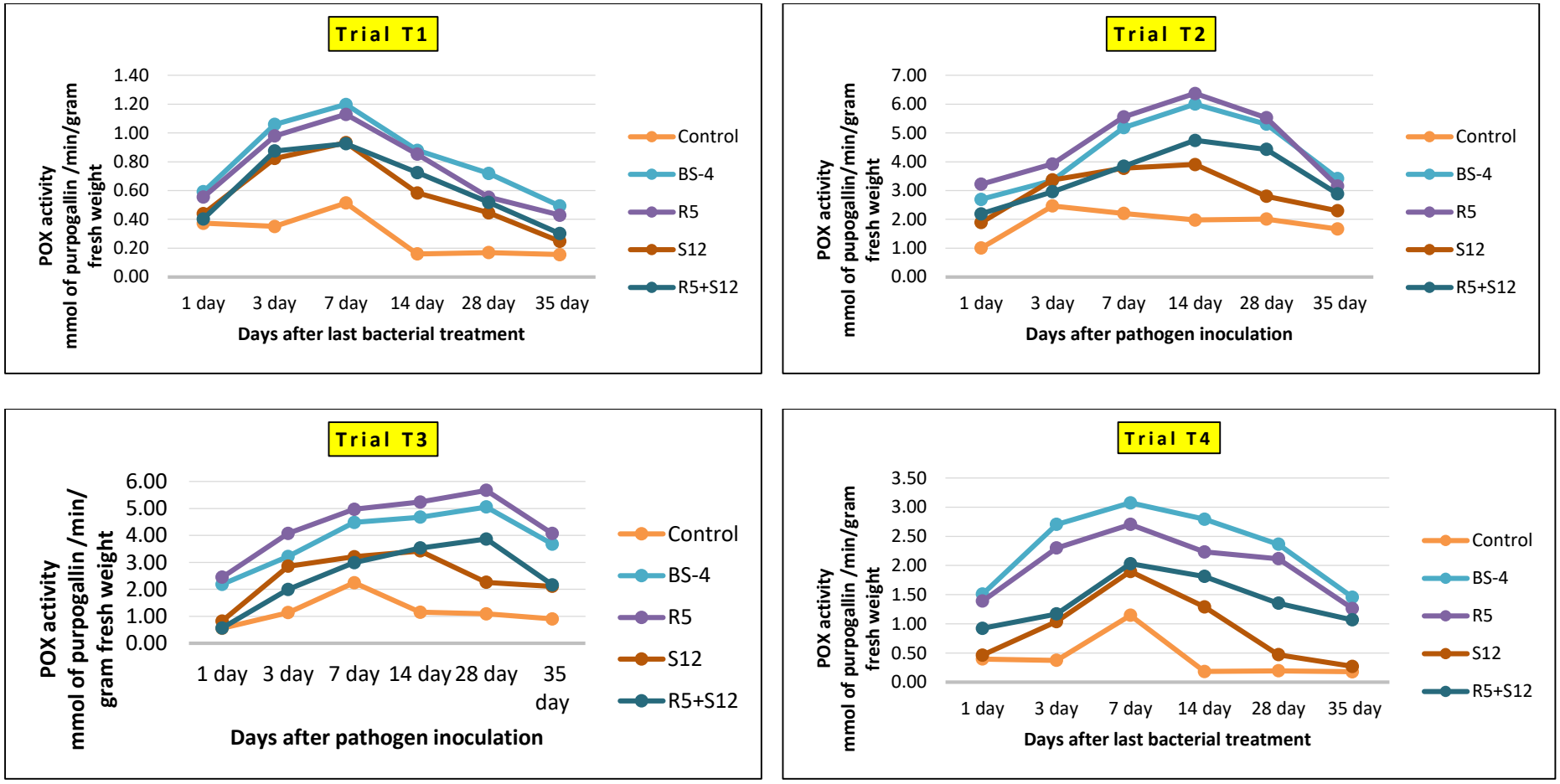


Fig 6.6 Induction of POX enzyme activity in endophytic bacterially treated sugarcane plant challenged with *C. falcatum* (Trial-2 and Trial-3) and without challenged inoculation (Trial-1 and Trial-4). Control represent treatment without any endophytic bacterial inoculation in all the Trials. All values are the mean of three replicates. Vertical Bars represent the standard error of the means.

6.3.2.2.3 PPO Activity:

A significant increase in PPO activity was detected in all the leaves upon treatment with endophytic biocontrol agents over control treatment in all the trials. However, in the Trial-1 and Trial-4, treatment with BS-4 recorded significantly higher enzyme activity on 7th day post pathogen inoculation when compared with their respective control treatment. In the Trial-1, PPO activity decreased after 7th day and increased further from 14th day till 28 day and started declining slowly. The activity of PPO reached the highest level in all the treatment of Trial-1 and Trial-4 at 7th day after treatment and then slowly decreased (Fig 6.7).

However, in Trial-2 and Trial-3, marked increase in the enzyme activity was recorded for R5 followed by BS-4 treated setts upon challenged inoculation by pathogen. In the Trial-2 and Trial-3, highest activity of R5 was recorded at 14th day and 28th day post challenged inoculation respectively (Fig. 6.7).

6.3.2.2.4 Total Phenol:

Treatment of sett with the endophytic bacterial treatments resulted in high phenol accumulation in the leaves of plants. A significant increase in phenolic content was observed 24 h after inoculation and phenolic content increased consistently up to 28 day of pathogen inoculation in the Trial -1 and Trial-4. Soil application of BS-4 resulted in the maximum accumulation of phenol when compared to the control. Treatment with R5 also recorded higher accumulation of phenol followed by R5+S12 and S12. The accumulation of phenol increased from the 1st day of treatment and increased upto 28th day (Fig 6.8).

However, in Trial-2 and Trial-3, R5 expressed higher accumulation of phenol and increased till the 28th day onward from the pathogen inoculation. BS-4 also induced the higher accumulation of phenol upto 28th day in Trial-2 and upto 7th day in Trial-3 (Fig 6.8).

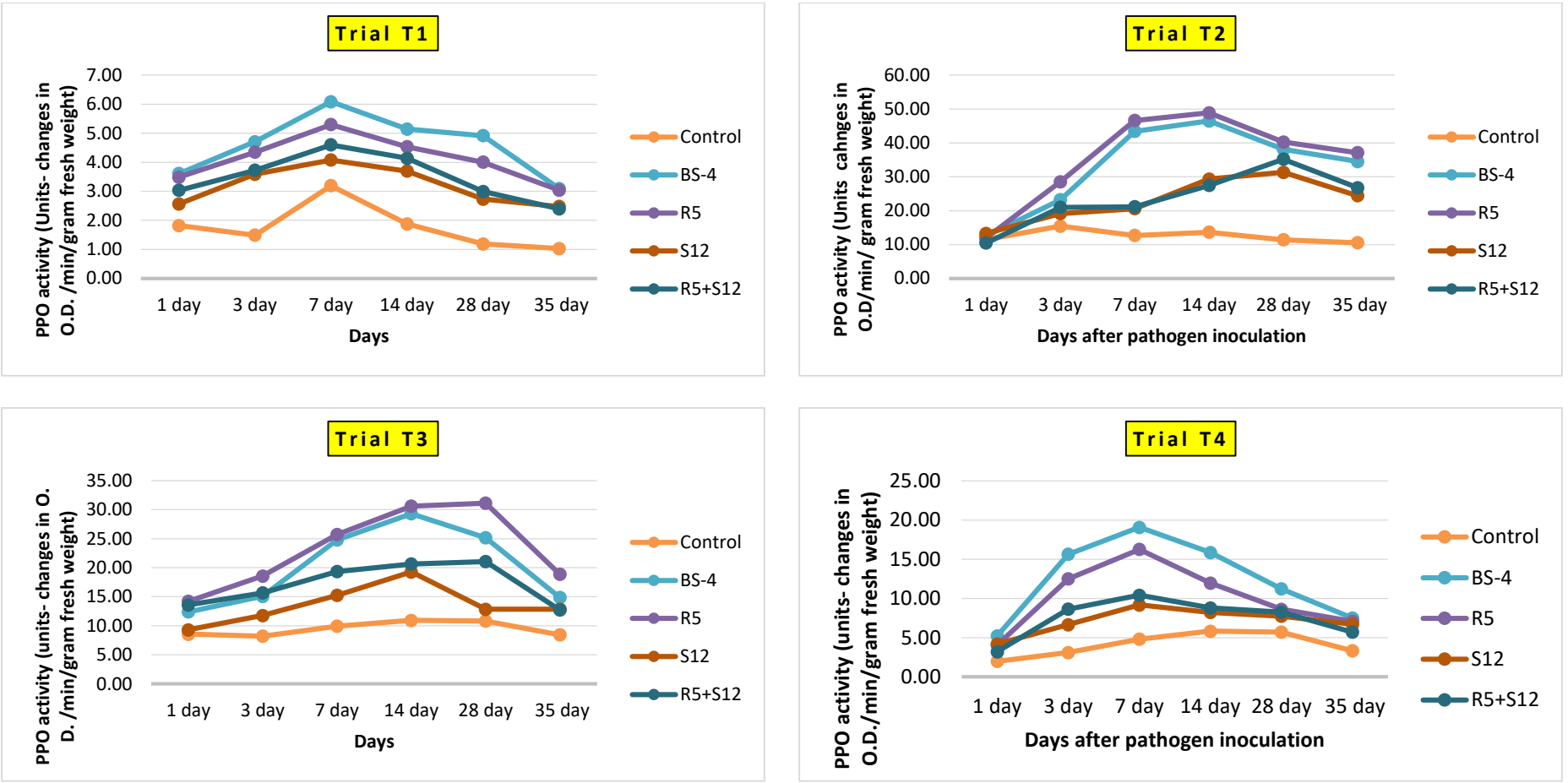


Fig 6.7 Induction of PPO enzyme activity in endophytic bacterially treated sugarcane challenged with *C. falcatum* (Trial-2 and Trial-3) and without challenged inoculation (Trial-1 and Trial-4). Control represent treatment without any endophytic bacterial inoculation in all the Trials. All values are the mean of three replicates. Vertical Bars represent the standard error of the means.

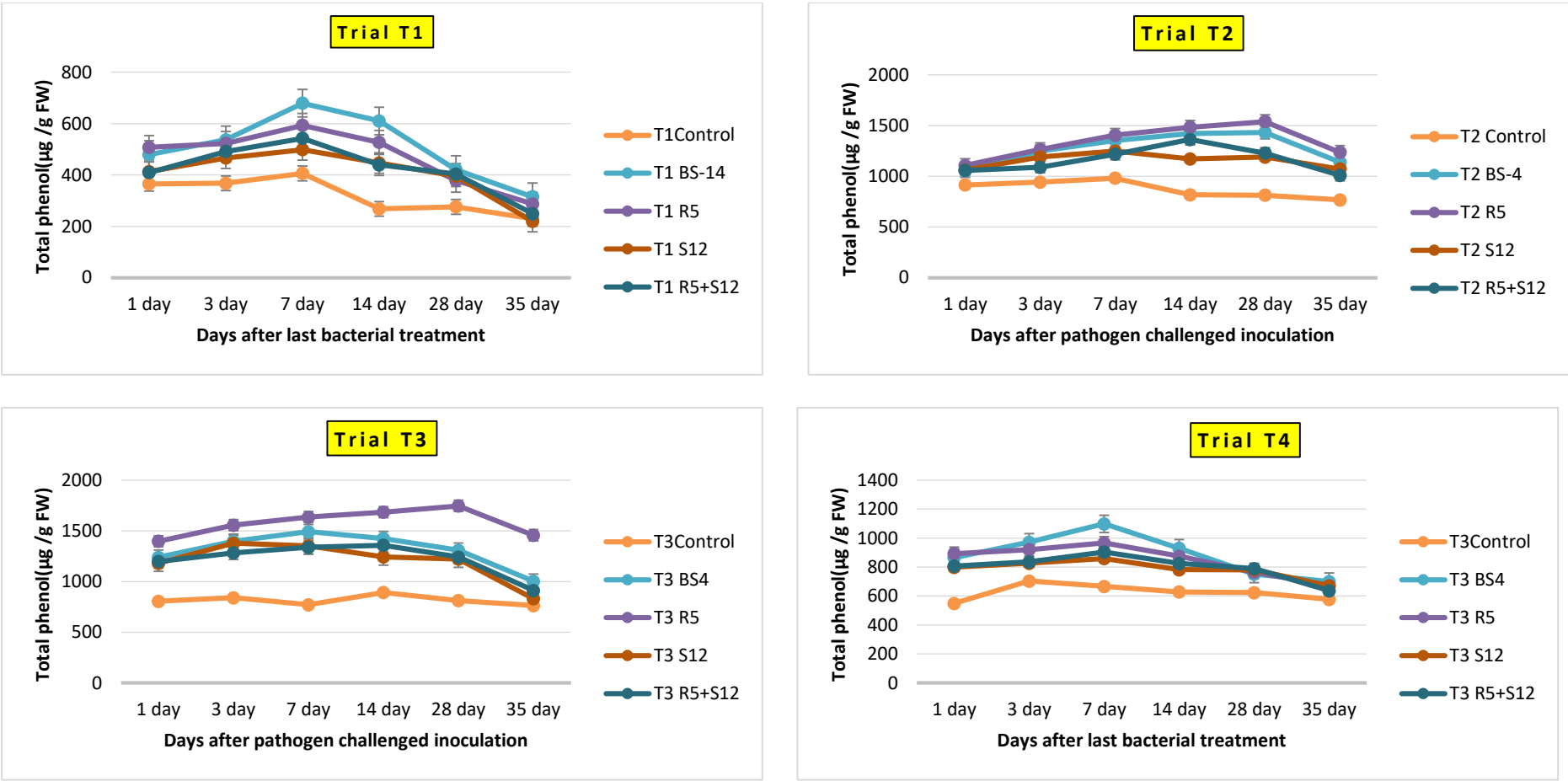


Fig 6.8 Total phenol accumulation in endophytic bacterially treated sugarcane plant challenged with *C. falcatum* (Trial-2 and Trial-3) and without challenged inoculation (Trial-1 and Trial-4). Control represent treatment without any endophytic bacterial inoculation in all the Trials. All values are the mean of three replicates. Vertical Bars represent the standard error of the means.

6.3.3 Effects of Endophytic bacterial treatments on various parameters of cane growth:

6.3.3.1 Percentage Germination: All the bacterial treated setts showed statistically significant increment in percentage germination when compared to control. The data of individual year (Year-I and Year-II) have already been illustrated in the Table 6.3-6.4 of all the trials. In this section, average of percentage germination for both the years of all the trials (T-1, T-2, T-3, T-4) have been demonstrated as pictorial representation. In the trial-1, *P.aeruginosa* BS-4 as well as *B. paramycoides* strain R5 resulted in highest germination of sett with 26.6% (Fig 6.9). In Trial-2, highest germination was recorded for R5. The other strains also exhibited significantly high germination percentage when compared with control (Fig 6.9). In Trial-3, R5 leads highest in germination followed by BS-4 (Fig. 6.9). In the Trial-4, higher germination was recorded for *P.aeruginosa* BS-4 followed by R5, R5+S12 and S12 (Fig 6.9) .

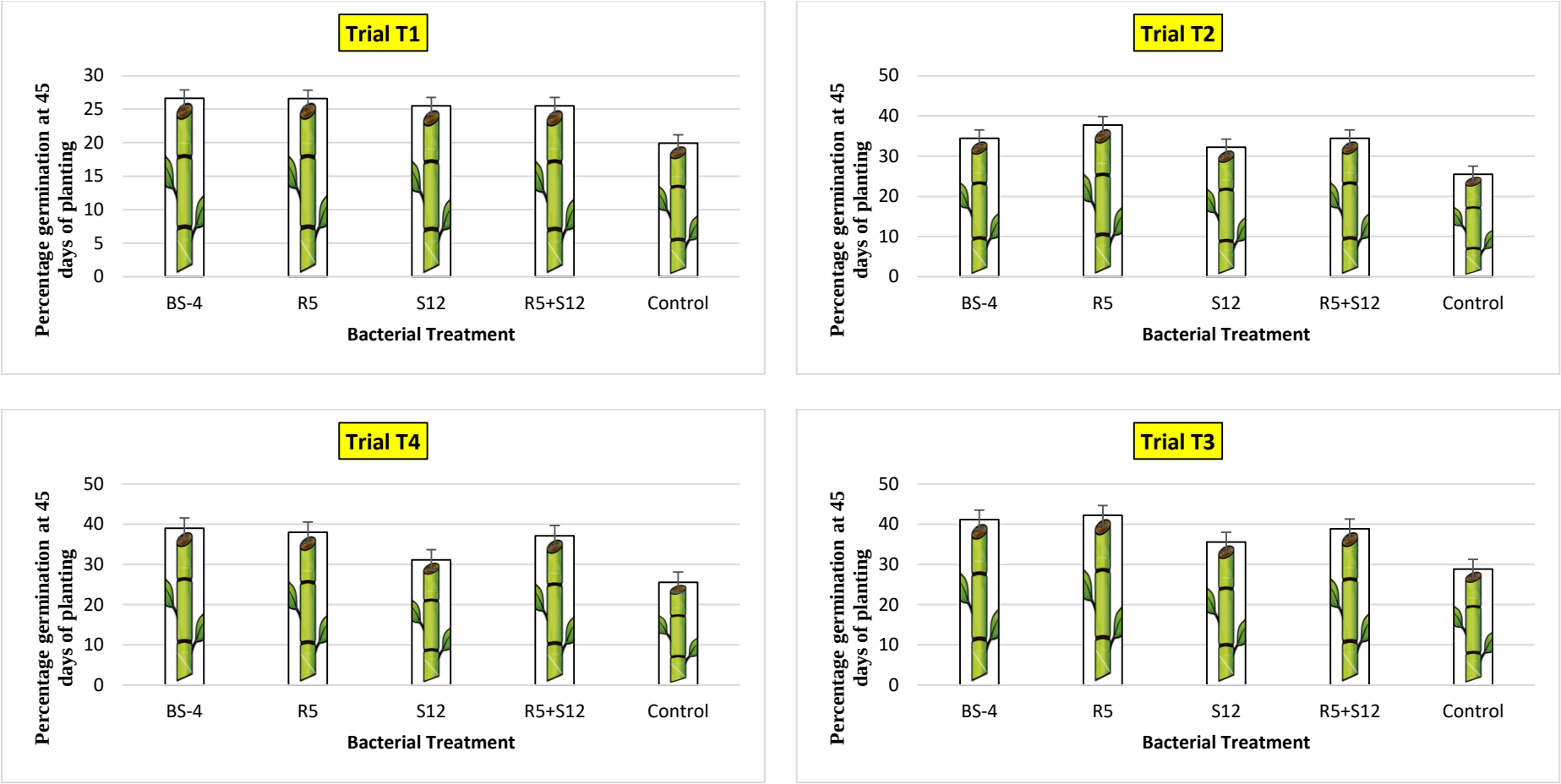


Fig 6.9 Germination (%) of sett treated with different endophytic bacterial treatments of all the Trials. Control represent treatment without any endophytic bacterial inoculation in all the Trials. All values are the mean of average data of both the years. Vertical Bars represent the standard error of the means.

6.3.3.2 Cane Height: Cane height was recorded at 300 days after planting (DAP) for all the trials and it was found that in the Trial-1, Trial-2 and Trial-3, R5 treatment had highest cane height with 206.7cm, 226.7 cm and 220 cm, respectively when compared with control of 155 cm, 155.7cm and 150 cm, respectively. However, in the Trial-3, a mixture of R5+S12 also revealed statistically significant cane height 217.3 cm when compared with other treatments (Table 6.7). Similarly, in the Trial-4, BS-4 exhibited highest cane height followed by R5 when compared with other treatments (Table 6.7). Overall, the greatest height was observed in the isolate R5, BS-4 and R5+S12. In Trial-1 and Trial-3, all the treatments showed significant difference while in Trial-2 only R5, BS-4 and R5+S12 represented higher significant value.

Table 6.7 Effect of bacterial treatment on the cane height after 300 DAP.

	Cane Height (cm)			
Treatments	Trial-1	Trial-2	Tial-3	Trial 4
Control	155a	155.7a	150a	164a
R5	206.7c	226.7b	220.3b	208b
S12	171.7ab	180a	177.3b	170a
R5+S12	175abc	218.3b	217.3b	177.7a
BS-4	192.3bc	223.3b	175.3b	217.3b

Data in a column designated by the same letter(s) are not significantly different according to DMRT rule.

6.3.3.3 Number of Shoots:

No. of shoots per treatment in each trial was counted at the time of harvesting i.e. 300 DAP. The total numbers of shoot were found to be higher in S12 treatment of all trial followed by a mixture treatment of R5+S12, R5 and BS-4 as clearly mentioned in the Table 6.8.

Table 6.8 Total number of shoots in different trial setup.

Total Number Of Shoots Per Bacterial Treatment				
TREATMENTS	Trial-1	Trial-2	Trial-3	Trial-4
CONTROL	7	11	10	11
R5	11	16	14	13
S12	14	19	18	16
R5+S12	12	17	16	14
BS-4	10	13	11	12

Data in a column designated by the same letter(s) are not significantly different according to DMRT rule.

6.3.3.4 Total number of internodes:

Total number of internodes were counted for each treatment in all the four trials at the time of harvesting. The data revealed that there was statistical difference in some of the treatment given in each trial (Table 6.9). In Trial-1, BS-4 had significantly large number of internodes i.e 25, while control treatments had 22 in numbers. In trial-2, both BS-4 and R5 had almost equal number of internodes i.e. 28. In a subsequent Trial-3, data revealed that cane treated with a mixture of R5+ S12 had 25 internodes. Similarly, when the internodes were counted in Trial-4, it was found that R5 and BS-4 had the highest number of internodes.

Table 6.9 Effect of bacterial inoculation on the internode in different trial setup.

Number of Internodes per treatment per trial				
Treatments	Trial-1	Trial-2	Trial-3	Trial-4
Control	22^{a}	23^{a}	22^{a}	22^{a}
R5	24^{ab}	28^{b}	24^{b}	27^{b}
S12	22^{a}	24^{a}	24^{ab}	24^{a}
R5+S12	24^{ab}	25^{a}	25^{b}	24^{a}
BS-4	25^{b}	28^{b}	24^{b}	27^{b}

Data in a column designated by the same letter(s) are not significantly different according to DMRT rule.

6.3.3.5 Cane girth (cm):

Cane girth was also measured for each cane treated with bacterial inoculation in different trial and found that girth of cane treated with bacteria was somewhat more in diameter as compared to control. However, when statistically differences were measured, it was found that in Trial -1 and Trial-2, significant difference were noticed in the cane girth; while such difference was not noticed in Trial-3 and Trial-4 (Table 6.10)

Table 6.10 Cane girth in different Trial set-up.

	Cane Girth (cm)			
Treatments	Trial-1	Trial-2	Trial-3	Trial-4
Control	2.1a	2.2a	2.0a	2.2a
R5	2.3ab	2.6b	2.3a	2.3a
S12	2.3ab	2.3ab	2.2a	2.2a
R5+S12	2.3ab	2.4ab	2.4a	2.2a
BS-4	2.4b	2.5b	2.4a	2.4a

Data in a column designated by the same letter(s) are not significantly different according to DMRT rule.

6.3.3.6 Cane weight: Another physiological parameter of sugarcane was measured that is cane weight and found that no statistical difference was measured in Trial-1; while in all the other three trials (Trial-2, Trial-3 and Trial-4), sett were sown after bacterial treatment and showed statistical difference in the weight of the cane (Table 6. 11)

Table 6.11 Cane weight of individual cane treated with bacterial endophytes.

	Cane weight (kg) of all the treatments in each trials			
Treatment	Trial-1	Trial-2	Trila-3	Trial-4
Control	0.7a	0.5c	0.6b	0.6b
R5	0.7a	0.9a	0.7ab	0.8a
S12	0.7a	0.7ab	0.6ab	0.7b
R5+S12	0.8a	0.8ab	0.8a	0.8a
BS-4	0.7a	0.7bc	0.6b	0.7b

Data in a column designated by the same letter(s) are not significantly different according to DMRT rule.

6.3.3.7 Chlorophyll content:

The effect of bacterial inoculation on the photosynthetic pigments was significantly higher in all the treatments of each trial when compared with the control (Table 6.12). The chlorophyll 'a' pigment was significantly higher in all the treatment of Trial-1 with equivalent value in R5 and R5+S12 treatment (0.71 mg/g) and equivalent value was also obtained for other treatment of S12 and BS-4 with 0.71 mg/g fresh weight. Similarly, Chlorophyll 'b' pigment was found to be highly significant in R5+S12 (0.59 mg/g fresh weight) followed by R5 (0.55 mg/g fresh weight) of Trial-1. The total chlorophyll pigment was found to be highest in R5+S12 (1.33 mg/g fresh weight) followed by R5 (1.29 mg/g fresh weight). (Table 6.12 and Fig 6.10)

Subsequently, in the Trial-2, Chlorophyll 'a' pigment were significantly higher in all treatment with highest in R5+S12 with 1.35 mg/g fresh weight. The other treatments demonstrated pigment in the range of 1.07 to 1.13 mg/g fresh weight. The chlorophyll 'b' pigment was highest in R5+S12 of Trial-2 with 0.85 mg/g fresh weight and R5 (0.82 mg/g fresh weight). The total chlorophyll was highest in R5+S12 of Trial-2 with 2.20 mg/g fresh weight (Fig 6.10).

The trial-3 revealed the highest Chl 'a' and total chlorophyll pigment in the plants treated with bacterial endophytes when compared with all the other trials treatments (Fig 6.10). A mixture of R5+S12 exhibited maximum chlorophyll 'a', chlorophyll 'b' and total chlorophyll with value having 1.68mg/g, 0.79mg/g and 2.47 mg/g, respectively. In the subsequent Trial -4, the maximum chlorophyll 'a' and total chlorophyll were found in plant inoculated with BS-4 with 1.65mg/g and 2.05 mg/g fresh weight. However, In Trial-4, the chlorophyll 'b' value obtained with plant inoculated with S12 was found highest followed by R5, BS-4 and R5+S12 (Fig 6.10)

Table 6.12 Effect of inoculation with bacterial endophytes on photosynthetic pigments of sugarcane.

Treatments	Trial-1			Trial-2			Trial-3			Trial-4		
	Chl a (mg/g)	Chl b (mg/g)	Total Chl (mg/g)	Chl a (mg/g)	Chl b (mg/g)	Total Chl (mg/g)	Chl a (mg/g)	Chl b (mg/g)	Total Chl (mg/g)	Chl a (mg/g)	Chl b (mg/g)	Total Chl (mg/g)
Control	0.59 a	0.26a	0.85a	0.87a	0.42a	1.28a	0.71a	0.58a	1.28a	0.77a	0.34a	1.11a
S12	0.71b	0.47 b	1.18b	1.07b	0.67c	1.74b	1.44b	0.67a	2.11b	1.03b	0.57e	1.60b
R5	0.74 b	0.55 c	1.29c	1.12b	0.82d	1.94c	1.65c	0.67a	2.32c	1.21d	0.50d	1.72c
BS-4	0.71b	0.49 b	1.20b	1.13 b	0.55b	1.68b	1.58c	0.60a	2.18b	1.12c	0.48c	1.59b
R5+S12	0.74b	0.59c	1.33c	1.35c	0.85d	2.20d	1.68c	0.79b	2.47d	1.65e	0.40b	2.05d

Data in a column designated by the same letter(s) are not significantly different according to DMRT rule.

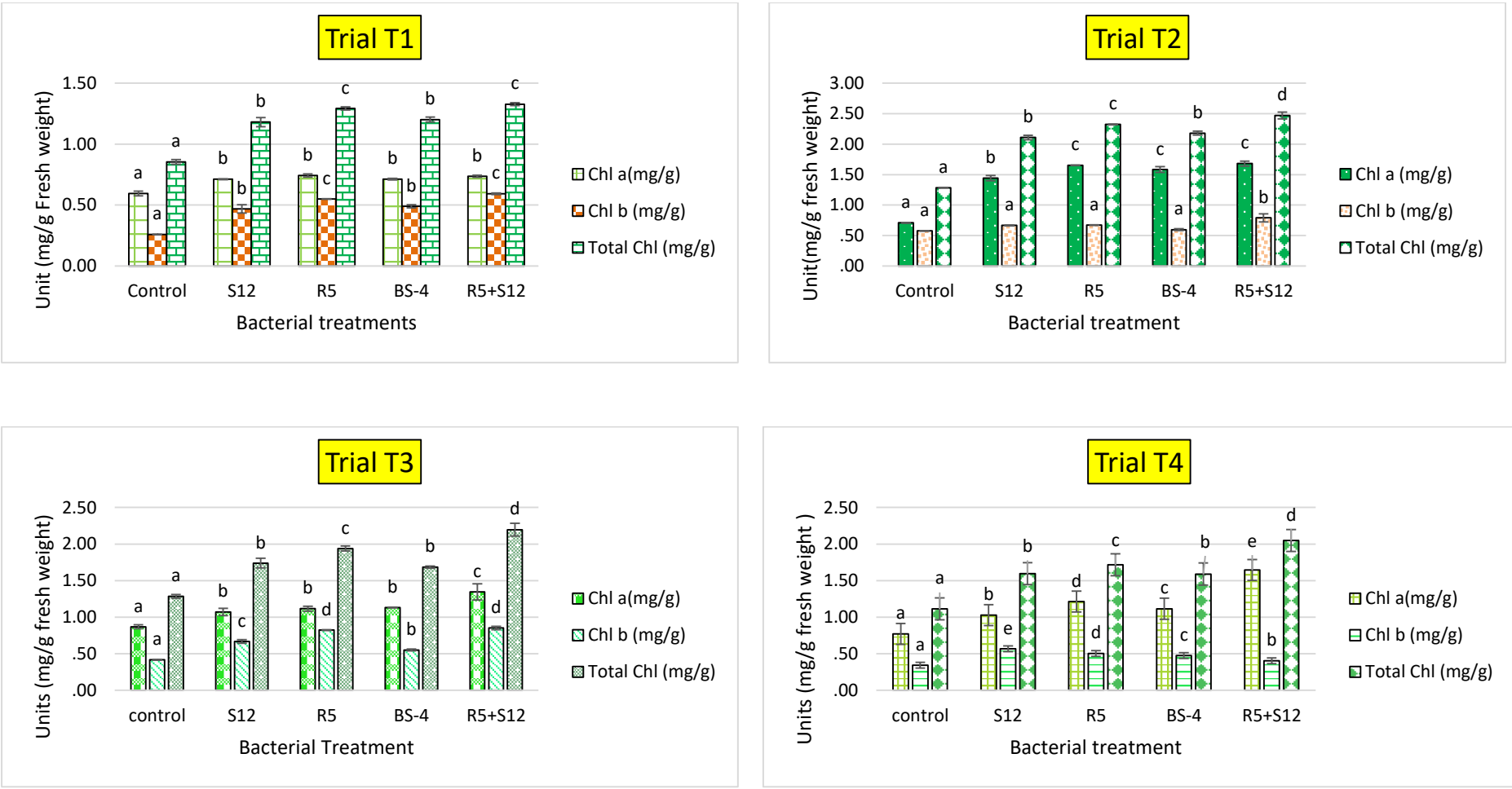


Fig 6.10 Chlorophyll content in leaves of sugarcane plant treated with different bacterial treatment in different Trials. Control represent treatment without any endophytic bacterial inoculation in all the trials. All values are the mean of three replicates. Vertical Bars represent the standard error of the means. Data in a column designated by the same letter(s) are not significantly different according to DMRT rule.

6.4 Discussion:

Biological control of red rot of sugarcane using endophytic bacteria is always an alternative management strategy to other control measures. The objective of this chapter was to evaluate the bio-efficacy of selected potent antagonistic endophytic bacteria found effective in *in vitro viz. Bacillus paramycoides* (R5), *Pseudomonas aeruginosa* (BS-4), *B. aryabhattai* (S12) and a mixture of *B. paramycoides* and *B. aryabhattai* (R5+S12) in suppressing the disease under *in vivo* condition. The endophytic biocontrol agents belonging to various species of *Bacillus* and *Pseudomonas* are well characterized and documented (Viswanathan and Samiyappan 2008; Hassan et al. 2012; Hassan et al. 2014; Patel et al. 2019) in sugarcane crop. The present study also revealed the potentiality of the various species of *Bacillus* and *Pseudomonas* including *P. aeruginosa* and *B. paramycoides* as an effective biocontrol agent for suppressing the red rot disease. The efficiency of *P. aeruginosa* has been already discussed as a biocontrol agent either with sugarcane or with other crops (Devi et al. 2017; Vinayarani and Prakash 2018). However, the present study is the first to report the potential of *B. paramycoides* as an endophytic agent and also for the suppression of red rot under *in situ* conditions.

In the present chapter, four different trials were conducted for the two years. The four trial were distinguished on the basis of difference in the inoculation of fungal pathogen *C. falcatum* inoculation; Trial-1 and Trial-4 had soil-based fungus inoculation before sowing the setts while in the Trial-2 and Trial-3, pathogen was inoculated in the stalk and nodal region of cane stalks far away from rhizosphere to induce the systemic resistance, respectively. In all the trials, the setts were sown after pre-treatment with the suspension of antagonistic endophytic bacterial followed by further application in the soil region except in the trial-1, where setts were planted

without any prior treatment with endophytic bacteria and only twice rhizosphere application was followed after the plantation in a specific period.

Although, the effect of all the endophytic bacterial treatment either as sett or soil or both application resulted in the reduction of disease in the stalk. However, the efficiency of individual strain *B. paramycoides* strain R5 and *P.aeruginosa* BS-4 through stalk or soil application effectively reduced the red rot incidence in all the trials and also promoted plant growth under field conditions. The above two individual treatments (R5 and BS-4) and a mixture effect of R5+S12 strain also imparted resistance against red rot pathogen. Though most of the study presented the dominant effect of consortia over a single strain (Molina-Romero et al. 2017; Bradáčová et al. 2019). Here, in the present study, R5 and BS-4 as an individual strain were most potent to protect the host against the disease than the mixture of R5+S12. The study conducted by Piakong and Zaida (2018) also revealed the efficacy of single strain over consortia. In the current study, the reason presumed for the low performance of R5+S12 *in vivo* condition over single strain R5 is either due to low efficiency of S12 strain in controlling the disease (less effective metabolites present as discussed in previous Chapter 5) or due to lesser colonization in the root system for effective action which are essential requirement of bioagent to get into action against the disease resistance.

Though pathogen infection was noticed in all the treatments but symptoms found were very less in all the bacterially treated canes of all the trials when compared to control where no bacterial treatments were given. Endophytic bacterial treatments from planting onwards showed comparatively higher resistance in all the treatments and this may be due to the early establishment of the bacterial population in the rhizosphere and showed better sett germination and subsequently stalk growth. In the

Trial-2 of Plug inoculation method, setts were once treated with the endophytic bacterial formulation; therefore, the spread of pathogen infection was limited to few internodes only while in the untreated cane, rapid progression was noticed from the point of pathogen inoculation. Similarly, in the Trial-3 of Nodal swabbing methods, pre-treatment of setts followed by subsequent application in the soil showed only limited lesion or remained healthy, while control treatment showed more progression in the disease development. Sett treatments followed by the soil applications were highly effective in inducing resistance against *C. falcatum*. Therefore, the treatment of Trial-2 and Trial-3 showed more suppression of red rot disease where pathogen was infested at an early stage. In between the Trial-1 and Trial-4, the later used the treated setts at the time of planting followed by subsequent soil application. Therefore, all treatments in the Trial-4 efficiently suppressed the red rot disease incidence in the plants more intensely than Trial-1. This study suggested that once the strain are introduced in the rhizosphere they can offer more resistance to the standing crop.

The study also revealed that by using the potent endophytic agent as a biocontrol in susceptible variety Co 1148 suppressed the disease more significantly. Thus, if the same inoculation would be used for resistant variety in future, the results would be more promising.

The results suggested that the endophyte biocontrol agent *B. paramycoides* strain R5 significantly suppressed disease in the "stalk inoculation" experiments i.e. in both Trial-2 and Trials-3, while the strain *P. aeruginosa* strain BS-4 exhibited best biocontrol activity in the "soil inoculation" experiments i.e. in the Trial-1 and Trial-4. The difference exhibited in the variation of the activity of two different strain i.e. R5 and BS-4 in the four different trial set-ups can be related to the variable metabolite production and their variable adoption mechanisms to suppress the fungal disease. It

can be seen that in Trial-2 and Trial-3 pathogen was inoculated in a way to avoid the direct contact with the antagonistic endophytic bacterial strains. However, in the "soil inoculation" experiments, i.e. the Trial-1 and Trial-4, both the pathogen and the antagonistic bacteria were inoculated in soil to facilitate direct physical contact between them. The mechanism generally known for rendering resistance to the plant against fungal pathogen upon stimulation by biological inducer without directly contacting with the pathogen is termed as Induced Systemic Resistance (ISR). Thus, the powerful phenomenon working for the R5 strain in the Trial-2 and Trial-3 is the ISR which suppresses the disease more efficiently in stalk- based experiment.

However, the BS-4 strain has some other mechanisms with the help of variety of metabolites produced by the strain (as discussed in Chapter-3 and Chapter-4) that became active in direct contact with the pathogen. Various studies have been carried out to elicit ISR mechanism against pathogen in a variety of crops after being stimulated by PGPR (Fallahzadeh et al. 2009; Kannojia et al. 2019). The efficacy of the strains used in the study for control of red rot was almost similar in both the years, perhaps the efficiency of some strain increased in the year-II while activity of some strains was lowered down from the Year-I. This could have been due to the prevailing of various environmental conditions that could make rapid progression of the pathogen over time. These findings suggest that multiple applications of antagonists after suitable time intervals may be necessary.

Involvement of ISR in the management of soil-borne and seed borne disease by endophytes in many crop have been well-documented (Rais et al. 2017). The mechanisms involved in the resistance to disease is complex process depending on multifactors. It is well known that plants have evolved efficacious active resistance machinery that endue them to safeguard themselves against invasion of pathogen.

Enhancement in the induction of resistance against pathogen attack is somewhat based on the innate defense property of plant which seems to be very optimistic approach to control disease (Suo and Leung 2002). The virulent pathogen when infects the plants, it either avoids engendering of defense response, or escapes from activated defense system of host. If defence mechanism of plants are stimulated by a stimulus prior to infection by a pathogen, the disease can be reduced to some extent (Conrath et al. 2001; Guo et al. 2015). However, prior priming of plants by biological stimulus reveal the enhancement in the induced resistance mechanism in the plants. Bacterized primed plants when challenged with a pathogen results in an induction and accumulation of variety of defense-related proteins such as peroxidases, phenylalanine ammonia lyase, peroxidases, polyphenol oxidase, chitinases and β-1, 3-glucanases (Anand et al. 2007; Fishal et al. 2010). These responsive enzymes get accumulated from local point to systemic one and results into either degradation of pathogen directly or production of some antimicrobial compound/s that restrict the entry of pathogens (Ku'c 1990). In the present study also, all the bacterial primed sett induced more resistance against red rot pathogen *C. falcatum* by induction of defense related enzyme. However, the extent of increase varied between treatments among all the trials. Here, the present study also demonstrated the plausible involvement of the important defense enzyme during ISR mediated by endophytic strain. Colonization of plants by biocontrol endophytes induces several cell-wall modifications, such as deposition of callose, pectin, cellulose and phenolic compounds which barricades the phytopathogen from entering the host (Benhamou et al. 1998; Benhamou et al. 2000). ISR can be elicited from the primed part to the distant part i.e leaves. Therefore, the experimental results of the present study demonstrated that endophytic mediated elicitation of systemic resistance in the sugarcane leaves against red rot disease leads

to the development of a complex response involving the earlier and higher expression of defense enzyme like chitinase, β-1,3 glucanase, PAL, POX, PPO and phenol. Activity of all the enzymes were found enhanced several times in the leaves of the bacterial treated sugarcane plants as compared to the control. Further, the increase in the expression of these enzyme was much higher after pathogen inoculation. Though there was an increase in the rapidity and the level of expression of all the enzymes; but sugarcane leaves of Trial-2 and Trial-3 responded more prominently and rapidly by the accumulation of high levels of PAL, POX, PPO and phenolics due to pre-treatments of setts with endophytic bacterial followed by challenge inoculation with *C. falcatum*. However, In the Trial-1 and Trial-4, where there was no challenged inoculation, response were seen in the activity of all the enzymes when compared with the control. As setts in the trial-4 were treated with bacterial endophytes, increase and expression were more pronounced in the Trial-4 as compared to Trial-1. The different temporal pattern of expression of these enzymes suggest their differential role in the protection against red rot. The present study was also consolidating with the study of Vivekananthan et al. (2004); Sundar et al. (2009).

Plants use various self defense mechanism to protect themselves from the pathogen infection. These mechanisms include the inducible modification in the morphology, histology, biochemical and physiology of the host plant directed by the synthesis of variety of toxic phytoalexins and the accumulation of Pathogenesis-related proteins (PR proteins). PR proteins are defined as plant coded protein that are induced by pathogen and other abiotic stress conditions (Van Loon et al. l998). ISR is also associated with the accumulation of PR protein which are not only present in the infected leaves but also in the non-infected leaves of the same plant (Uknes et al. 1992). Normally application of endophytic PGPR results into several changes in the

host plant which make them aware from the pathogen attack. Though large families of PR protein are known till date; however, two of them chitinases (PR-3) and β-l,3-glucanases (PR-2) appear to be a potential candidate since more than decades for their antifungal nature. Chitinase (E.C. 3.2.1.14) and β-l,3- glucanases (E.C 3.2.1.39) have the potential to manage the fungal diseases as they have potential to hydrolyze chitin and β-l,3-glucan respectively; the main component of fungal pathogen, leading to the lysis and direct inhibition of fungal growth (Kauffmann et al. l987; Legrand et al. l987). Chitinase and β-l,3- glucanase enzyme may directly inhibit the mycelial growth of fungus occupying the intercellular spaces and possible release of oligosaccharides from the fungal cell wall which further act as inducer of several defense responses in plant (Ren and West 1992). In addition, it is also reported that the synergistic action of the combination of two enzymes can strongly suppress the fungal growth by causing lysis in the hyphal tips of various fungus like those suppressed by individual enzyme alone (Srivastava and Lal 2019). It is well established that microbes also produce extracellular chitinase and β-l,3-glucanases to lyse the fungal pathogen. Similarly, such enzymes are constitutively present in the plants and get induced upon treatment with biotic as well as abiotic inducers (Viswanathan and Samiyappan 2001). In the present investigation also, chitinase and glucanase activities significantly increased in all the endophytic treated cane leaves over control cane. In cane leaves treated with R5 and BS4, biocontrol agents, increased and higher enzyme activity was recorded over the control Various previous studies have indicated a possible role of the PR proteins in conferring red rot resistance in sugarcane (Viswanathan and Samiyappan 2001; Viswanathan et al. 2005). It is also evident that chitinase and β-l,3- glucanases are synthesized more rapidly in resistant variety than susceptible variety. However,

susceptible variety also depicts higher induction of enzymatic system with endophytic bacterial treatment but is less as compared to resistant varieties as also observed in the present study. Karthikeyan et al. (2005) demonstrated that soil and foliar application of *P. fluovescens* enhanced the glucanase activities in onion plants. Synthesis and accumulation of PR-proteins have been reported to play an important role in plant defense mechanisms (Van Loon et al. l998). Colonization of bean roots by rhizobacteria was correlated with induction of PR proteins resulting in induced systemic resistance against *Botrytis cinerea* (Zdor and Anderson l992). The study of Chandrasekaran and Chun (2016) also consolidated with the present study where, effectiveness of a biocontrol agent *Bacillus subtilis* CBR05 was evaluated for control of soft rot disease (*Erwinia carotovora*) in tomato and mechanisms responsible for protection was possible induction of defense-related genes expression and antioxidant enzyme.

Phenylalanine ammonia-lyase (E.C. 4.1.3.5) is the leading enzyme of phenylpropanoid pathways that aid in the conversion of L- phenylalanine to trans-cinnamic acid, which then enters into different biosynthetic pathways leading to lignin synthesis; an important defense compound in phenylpropanoid metabolism. Thus, formation of lignin acts a shield for the plant and protect them from invasion of pathogen attack. Besides, lignin; variety of other secondary metabolites are synthesized by the PAL enzyme which are associated with the disease resistance (Campos et al. 2003). Activity of PAL could be induced in response to pathogen infection in plant-pathogen interactions and also in endophyte bacterial elicitor treatment. As in the current study also, it was well established that early induction of enzyme system occurred in the pre-treated endophyte plant. In the treated cane, increased activity of PAL was recorded in R5 (Trial2 and Trial-3) and BS-4

(Trial-1 and Trial-4) strain followed by a mixture of R5+S12 and S12 treated plants; while in the control plants, it remained low. The time duration for activation and expression of defense enzyme system is also a deciding factor for the suppression of the invading pathogen. Therefore, in the present study, earlier and higher level of enzyme expression is established for all the endophytes treated especially with R5 and BS-4 treatments. Subsequently following expression, accumulation of inhibitory substance is activated at the site of infection and thus, prevent the further colonization of pathogen. Several studies have demonstrated the induced effect of PAL activity treatment with bacterial strains such as *Pseudomonas sp., Bacillus sp., Burkholderia sp* (Chen et al. 2000; Sundaravadana 2002; Dalal et al. 2015; Rais et al. 2017)

Peroxidase (E.C.I.ll.I.7) is another defense related enzyme, which plays a key role in plant-pathogen interactions. Peroxidases are not known for their direct antifungal role as shown by other systemic inducible enzymes such as chitinase, β-1, 3-glucanase (Boller 1987). However, they may function indirectly by affecting the biochemical process which in turn have impact on the disease resistance. With its broad-spectrum enzymatic activity POX system becomes activated and responds well against pathogen infection by biosynthesizing variety of plants cell wall components contributing in the lignification, suberin formation and many other fungitoxic compound which restricts the spreading of pathogen (Grisebach 1981; Bruce and West l989). Reactive oxygen species (ROS) are known for its defensive response in plants against pathogen and formation of hydrogen peroxide (H_2O_2) also act as local signal for hypersensitive cell death and also as a diffusible signal for the induction of defensive genes in adjacent cells thereby increasing the general resistance to pathogen challenge (Alvarez et al. 1998). In the present study, a rapid increase in POX

activity was recorded in plants treated with an individually potent antagonistic endophytes i.e R5 in Trial-2 and Trial-3; and BS-4 in Trial-1 and Trial-4 which might have contributed to induced resistance against invasion by *C. falcatum*. Present study results also showed that the content of peroxidase (POX) increased in all the endophyte treated plants after challenged inoculated with *C. falcatum* and were maintained at higher level in comparison to control. Also, studies were focused on the effect of *Pseudomonas fluorescens* strain Pf10 treatment and *Fusarium oxysporum* f.sp. *cubense* (*Foc)* inoculation on the induction of banana plant enzymes and compounds known to be related with defense responses such as POX and other defense enzyme (Thangavelu et al. 2003).

The third enzyme in the list of defense related enzyme is the polyphenol oxidase (PPO). PPO (E.C.1.14.18.1), is an oxygen dependent copper- containing enzyme that catalyses the conversion of phenols to highly toxic quinones. Quinones are antimicrobial compound, highly toxic to pathogen and also contributes in the lignin synthesis. Thus, indirectly helps in suppressing the pathogen invasion and play a key role in providing resistance against disease progression. The enzyme has been implicated in cellular protection; as enzyme is localized in the plastids, whereas its phenolic substrates are present mainly in the vacuole (Mayer and Harel 1979; Vaughn and Duke 1984). Thus, PPO has participated in the defense mechanism against highly toxic plant pathogens (Thipyapong and Steffens 1997; Li and Steffens 2002). Primed plants are able to protect themselves against the pathogen attack by producing a broad-spectrum PPO enzyme. Thus, present study also validates the induction and early establishment of PPO enzyme in the endophytic bacteria treated plant which might be the contributing reason for suppression of red rot disease in the primed cane.

Phenolic compounds also fungitoxic in nature and increase the mechanical strength of the host cell wall by synthesizing lignin. Another study by M'Piga et al. (l997) also provided the supporting data for fungitoxic behaviour of phenol as it was found that the hyphae of the pathogen surrounded by phenolic substances exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content. The plants adopted more immunization against pathogen; if planted seed are bacterized with PGPR as the phenolic compound started accumulating at the invasion of pathogen (Wei et al. 1991). Previous study also exhibited correlation between the induction and enhancement of phenolic or other compounds with the protection of Pea plant against powdery mildew infection; when treated with foliar application of PGPR strains namely *P. fluorescens* (Pf4) and *P. aeruginosa* (Bahadur et al. 2007). In the present findings also, sett and soil application of endophytic bacterial (especially with R5 and BS-4 treatments) offered great resistance against red rot disease; the reason attributed for this might be an enhanced level of accumulation of phenols as observed in the leaves of treated cane. Benhamou et al. (2000) reported that an endophytic bacterium *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots and offered resistance to *P.ultimum* infection. The study conducted by Shabanamol et al. (2017) also concluded that endophytic diazotroph *L. sphaericus* act as a potent plant growth promoter with excellent biocontrol efficiency as it induces systemic resistance as evident from the significant accumulation of defense enzymes such as peroxides, polyphenol oxides and phenylalanine ammonia in addition to the increase of phenolic compounds. Since the production of phenolic compounds depend upon PAL activity (Graham and Graham, l99l), increased phenolic synthesis in treated sugarcane plants may be due to increased activity of PAL. Various studies have documented enlisting the

importance of PO, PPO and PAL in ISR studies against *R. solani* and other fungal pathogens in rice and other diverse crops (Jayaraj et al. 2004; Chitrasree et al. 2011; Rajendran and Samiyappan 2008).

According to study of Zhang et al. (2004), relationship between the elicitation of plant growth promotion and ISR was established. It was evident from the study that seed treated could alone elicit the growth promotion of plants but not provide resistance to disease. However, application of endophytes as seed followed by soil application could elicit both ISR and growth promotion of plant. Therefore, in the current study also, besides the reduction in the disease due to induction of systemic resistance, it was also noticed that sett treatment with endophytic strain has resulted in enhanced vegetative sett germination in the *in vivo* conditions. As the present study clearly indicated that the inoculated sugarcane plants with endophytic strains were significantly superior in terms of cane height, shoot counts, cane weight and various other parameters. *Bacillus* spp. and *Pseudomonas* spp. have been cited in various studies for growth promotion in grape wine, tomato, maize, rice and sugar beet through various mechanisms (Mirza et al. 2006; Mehnaz 2011). Alongwith the germination percentage, number of various parameters were recorded for improvement in the cane height, number of shoots, cane weight and number of internodes. The reasons for improving these physiological parameters might be due to use of the antagonistic endophytic strain with multifarious plant growth promoting traits (as discussed in chapter-2) which have been reported to enhance the growth by several ways. The PGPR strain have been demonstrated previously on their positive role in improving the germination, cane growth (Viswanathan and Samiyappan 1999a, 2002). The effect of endophytic bacterial inoculation on rice seed significantly and consistently increased shoots and root length and plant biomass (Walitang et al.

2017). Similar study was also conducted by Egamberdieva et al. (2017) where endophytic *B. subtilis strain* NUU4 proved significant reduction in the infection of root rot in chickpea caused by *F. solani* as well as improved the plant growth and promoted yield under field condition.

The height of cane showed a significant increase in the R5 and BS-4 treated sett, followed by R5+S12, however, in the trial-3, R5+S12 showed an increase height of cane. The response might be due to the combined effect of PGP trait of both strains (R5 and S12) enhancing their growth. It can be clearly understood from the study that R5+S12 mixture might not be contributing well in the biocontrol mechanisms, but contributing well in the growth parameter of sugarcane.

In the present study, the number of shoots was higher in endophytic bacterial inoculated treatments than in the control. However, the increased number of cane was observed in S12 treated cane at the time of harvesting. The reasons might be slow germination rate in the initial stage and after a certain time period followed by more doses of inoculum, germination got increased in the setts. Thus, the slow performance of S12 strain cane be effectively increased *in vivo* condition by increasing the doses of inoculum. Therefore, at the time of harvesting, number of shoots was more. Positive and significant effects of inoculation of endophytes other than *Pseudomonas* spp. and *Bacillus* spp. on sugarcane have been reported by Sevilla (1998).

The efficiency of endophytic treated cane is also related to the performance of photosynthetic apparatus of the plant. Chlorophyll a, chlorophyll b and total chlorophyll contents were significantly higher in all the inoculated treatments compared with the control at all the time intervals studied. Higher chlorophyll content

in sugarcane treated with bacteria was also reported by Muthukumarasamy et al. (1999), Sevilla (1998), and Chauhan et al. (2013).

6.5 Conclusion:

Thus, from the study, a coordinated induction of various enzymes and metabolites involved in host defence would have reduced the pathogen colonization and further spread inside the stalk tissues and this was reflected in bioassay studies using the tissue bits directly. In bioassay, the pathogen growth was restricted by metabolites from the primed tissues. The present results suggest that catalase, peroxidase, chitinase, PAL and PPO were good markers of resistance after infection with the pathogen. The application of PGPR as seed treatment could prove to be a beneficial component of integrated disease management. These bacteria, apart from their action against pathogens, are good growth promoters, which is an added advantage for any practical agricultural system. The pre-treatment with these inducers significantly arrested the pathogen growth and reduced the disease severity in the pathogen-inoculated canes.

Chapter 7
Summary

SUMMARY

- The sugarcane crop provides a treasure trove of valuable products right from the intact canes to the crushing of crop. The sugarcane has established itself as an important cash crop and opened up new opportunity for agro-processing industry. However, sugarcane production does not correspond with the cultivated area in India. Several factors are responsible for low sugarcane productivity; among them, the major constraint in the successful crop production is the occurrence of several deadly diseases which not only affects its yield but also quality. Red rot is the key menance of sugarcane in India. The fungal pathogen *Colletotrichum falcatum* is responsible for causing devastating red rot disease in the standing crop.

- Numerous chemical methods have been employed for combating the disease; however, such methods failed as synthetic fungicides are resistant to degradation and their residue gets accumulated in the soil affecting the health of other beneficial microorganisms and their environment. The use of organically and biologically safe practices has drawn the attention of various eco-warriors, researchers, and scientists for manoeuvring such methods that are safe and eco-friendly especially using the microorganism through endophytic bacteria.

- Thus present study "**Characterization of endophytic bacterial metabolites and their usage for biocontrol of *Colletotrichum falcatum* causing Red Rot in Sugarcane crop**" was aimed to study endophytic bacteria and explore their multifarious metabolites that would contribute to red rot disease protection in

sugarcane crop against fungal pathogen *C. falcatum*. Besides, protection to disease resistance; various other growth-promoting and other traits were also explored for endophytic bacterial isolates.

- The three different cultivars namely Co 1148, CoJ 64 and CoS 767 of sugarcane were collected from the Indian Institute of Sugarcane Research (IISR), Lucknow and washed properly to remove the soil and processed for the isolation of endophytic bacteria. The culture plate of red rot pathogen i.e. *C. falcatum* (CF01) was also generously procured from the Division of Crop Protection, IISR-Lucknow.

- A total of 118 endophytic bacteria were isolated from the root and stalk tissue of sugarcane varieties (Co 1148, CoS 767, and CoJ 64). The variety Co 1148 yielded the highest number of endophytic bacterial colonies (59) followed by CoJ 64 (36) and CoS 767 (23) respectively from roots and stalk tissue. Isolation was done on different nutrient-rich media (LB, NA, PDA, KB, and LGIP media) and selective media (3% agar medium). The results for the isolation of endophytic bacteria also revealed that root tissue from all the cane varieties produces a higher number of distinct endophyte bacteria.

- After isolation of 118 endophytic bacterial isolates, they were checked for their *in-vitro* antagonism against *C. falcatum* on PDA medium. As antagonistic endophytic bacteria was the prime need of the study.

- Out of 118; only 29 isolated endophytic bacteria demonstrated *in vitro* inhibition against red rot fungus. It was further noticed that out of 29 isolates; a majority of strain (20) isolated from sugarcane variety Co 1148 showed inhibition against pathogen followed by variety CoJ 64 (7) and CoS 767 (2), respectively. Overall,

only two strain B2 and B7 showed low *in vitro* inhibition with 47.57 ± 5.52 % and 48.57± 5.34%; while the remaining 27 endophytes showed more than 50% mycelial inhibition of fungal pathogen. Isolates S8, S11, S12 (from Co 1148), BS-4 (from CoS 767) and BS14, BS29 (from CoJ 64) showed maximum inhibition with 82.97 ± 2.15%,80.54 ± 2.15%, 85.00 ± 1.46%, 80.54 ± 5.68%, 81.62 ± 5.15% and 80.54 ± 2.15%, respectively.

- The selected 29 antagonistic endophytic isolates were also checked for another strong attribute i.e. sucrose tolerance (3-19%). The results revealed that all the twenty-nine endophytic bacterial isolates tolerated sucrose up to 11%. However, when further sucrose level was increased, only five isolates S8, S17, S12, R5, and BS-4 showed the highest tolerance level i.e. up to 19%.

- The morphological and biochemical characterization of all the twenty-nine endophytic isolates with antifungal activities were characterized. All the selected endophytic test isolates were distinct in their cultural characteristics. Morphological results revealed that nearly 79.31% of selected endophytes belonged to gram positive rod-shaped and rest belonged to gram-negative rod-shaped bacteria. All the 29 isolates were found to be motile. Various biochemical tests such as catalase, urease, IMViC, oxidase, and oxidation fermentation (O/F) were also conducted. Biochemically, sugars are very important for most of the microbe; therefore, carbohydrates utilization pattern were also recorded for all the twenty-nine isolates.

- After, morphological and biochemical characterization; the most efficient endophytic bacterial isolates were completely identified by using 16S rRNA sequencing analysis. Six endophytic bacteria (S8, S12, S17, S26, R5, and BS-4)

based on *in vitro* antagonism, sucrose tolerant and other PGP characteristics (discussed further) were selected for molecular identification. On the basis of 16 S rRNA sequencing; S8 was identified as *Bacillus cereus* with accession number (MG966498*)*, S12 was identified as *B. aryabhattai* (MH298519), S17 as *B. subtilis* (MK411294), S26 as *B. licheniformis* (MK411295), R5 as *B. paramycoides* (MN318075) and BS-4 as *Pseudomonas aeruginosa* (MG966460).

- The 29 isolates along with antifungal property against *C. falcatum;* were further screened for the presence of various plant-growth-promoting and other antifungal metabolites or lytic enzymes in order to select the best isolates that possess multiple beneficial traits in the single isolate.

- The selected antagonistic endophytic bacteria when checked for their PGP traits, it was found that most of the bacteria had one or more PGP characteristics. IAA producing bacteria causes changes in the structure of root by stimulating root hair formation thus, ultimately increasing root surface area for mineral uptake and root exudation. In the current study; almost 51.72% (15 out of 29) of isolated endophytes showed IAA production. In present study, all the isolates produced different amount of IAA ranging from minimum 29.99 ± 2.59 µg/mL in the isolate BS-4 to maximum 112.92 ± 4.16 µg/mL in the isolate BS14.

- Endophytes are known to improve plant growth by phosphate solubilization after releasing organic acid, polysaccharides and the presence of phosphatase enzyme (Schachtman et al. 1998). In the present study, nearly 93.10% of isolated endophytes were found to have the potential to solubilize phosphorous. SI (Solubilization Index) of the isolates ranged from 1.20±0.03 in isolate S22 to 2.67±0.29 in isolate S26. Only two isolates i.e. B8 and B9 were not showing any

halo zone. Rest all of the endophytes were showing positive confirmation for P-solubilization. The quantitative estimation of available phosphate revealed that the highest amount of phosphate was present in BS-4 (272.85 µgPO^{3-}_4) and lowest in B12 isolate (11.28 µgPO^{3}_4). Followed by BS-4; the higher amount of phosphate was also detected in the isolate R5, S-26, S17, and S8.

- Various species of *Bacillus* are known for IAA production and phosphate solubilization; but in the present study, entirely different species of *Bacillus*, which has never been reported in sugarcane and could be the novel strains i.e. *Bacillus aryabhattai* (S12) and *B. paramycoides* (R5) strain were found positive for these IAA and phosphate solubilization traits.

- All the 29 endophytic bacteria were showing positive results for ammonia production (another attribute that is linked with indirect plant growth promotion and used for biocontrol mechanisms). However, isolate S8, S17, R5, and BS-4 showed strong ammonia production after addition of Nessler's reagent that resulted in dark yellow to brownish colour.

- ACC deaminase; an another PGP trait that helps to alleviate the stress condition and **s**creening result noticed that out of 29 endophytic bacteria only three (S8, S26, and R5) were found positive for the production of ACC deaminase as indicated by their growth on the DF medium.

- Besides, the above characteristics; HCN is an important PGP attribute and found that ten isolates *viz.* B2, B5, B8, B12, S11, S17, R5, BS-4, BS8, and BS15 endophytic bacteria showed positive test for HCN production as observed by change in the colour of filter paper from yellow to brown.

- The 29 endophytic bacterial isolates were also screened for siderophore production using the chrome azurol S (CAS) agar and only six bacteria namely S8, S12, S17, S26, R5 and BS-4 were found positive for siderophore production by change in colour of dye from blue to yellow- orange zones around the bacterial isolates in CAS-agar plates Further confirmation of these isolates for their siderophore presence in liquid assay was expressed in terms of percentage siderophore unit and it ranged from 10.58±4.24% PSU in S26 to maximum 93.16±1.44% PSU in BS-4. Therefore, the present study favoured the ability of siderophore production in appreciable amount by endophytic bacteria BS-4, S17 and R5, which are universally recognized biocontrol and plant growth promoting agents.

- Petri dish-based qualitative assays revealed that endophytic bacterial isolate produced variety of lytic enzymes such as chitinase, β-1,3 glucanase, protease, amylase, pectinase, and cellulase and that play an important role in lysis of fungal pathogen.

- Chitinase production was observed by a halo zone around the bacterial colony. The isolates S8, S12, S17, S26, R5 and BS-4 demonstrated the positive chitinase enzyme production. Similarly, S12, S17, R5 and BS-4 revealed the promising result for β-1, 3 glucanase enzyme production by formation of halo zone around the colony.

- Similarly, pectinase was observed in five isolates namely, R5, BS-4, S8, S12 and S17; caseinase enzyme was recorded in 23 isolates only; Cellulase, an important lytic enzyme responsible for degradation of cellulose was observed in the 16 isolates out of 29; while the amylase production was also found to be positive for

the 27 endophytic isolates. The isolates B1 and B7 did not show any amylolytic activity due to the absence of yellow zone around colonies after flooding the plate with iodine solution.

- As justified from the above findings that various attributes are present in endophytic bacteria that are responsible for revealing strong antagonism against red rot fungus. Besides, several other volatile and diffusible metabolites are also responsible for suppression of fungal pathogen. Therefore, *in vitro* analysis of volatile and diffusible compound were also conducted. It was revealed from the experiment that all the 29 antagonistic endophytic bacteria produced anti-fungal volatile compound(s) (VOCs) as evident from the growth inhibition of *C. falcatum*. In addition, aerial mycelial growth was also reduced due to the effect of volatile metabolites. Raza et al. (2016) demonstrated the role of VOCs produced by *Pseudomonas fluorescens* WR-1 in biocontrol activities. Kandel et al. (2017) and Lee et al. (2017) have also recently reported that VOCs mediate antifungal activities. Similarly, all the isolates were able to inhibit the mycelial growth due to the action of its diffusible metabolites.

- In order to evaluate the involvement of another putative antifungal compound in their suppressive effect, the antifungal activity of cell-free supernatant from the culture of all the 29 isolates were analysed. Cell-free supernatant of the isolates BS13, BS11, S21, S37 and B9 were able to inhibit the radial mycelial growth of *C. falcatum* with less than 50% inhibition. While rest all the isolates demonstrated strong inhibition against *C. falcatum* with highest in S12 isolates with 85.95%. These findings provide evidence that extracellular metabolites in the cell-free culture filtrate of isolates inhibited the growth of pathogenic fungi which prompted the further investigation of the effects caused by their

metabolites on the growth of the hyphal structure. There is a greater probability that the increased antifungal activity against the pathogen fungi tested by the culture filtrate of the isolates is a consequence of the production of extracellular secondary antifungal metabolites.

- Study of fungal-antagonist interaction from dual plate antagonism under simple microscopy revealed the distortion of hyphal structure in the growth of the test pathogen by all the endophytic strains. The isolates R5, BS-4, S8, S12, S26 and S17 were selected due to the possession of maximum PGP and antifungal lytic enzyme traits. Therefore, the antagonistic mechanisms of isolates S12, S8, S26, S17, R5 and BS-4 against hyphae of *C. falcatum* were also observed by scanning electron microscopy (SEM). These findings prompted the investigation that distortive effects (hyphal swelling, and distortion of mycelium) in pathogenic fungi, *C. falcatum* were due to the extracellular metabolites in the culture filtrates. Further, hydrolytic enzymes and secondary antifungal compounds may also be responsible for the distortion in the mycelium of the fungal pathogen.

- The present study filtered and selected the isolates R5, BS-4, S8, S12, S26 and S17 for future identification and application based on multiple beneficial traits.

- **Determination of Chitinolytic activity:** The enzyme activity of crude enzyme of six strain (R5, S12, S8, S17, S26 and BS-4) were investigated for the production of extracellular chitinase spectrophotometrically. The results revealed that all the six strain demonstrated chitinase production; however, the strain R5 exhibited maximum chitinase with 116 U mL^{-1} followed by BS-4 and S17 isolate with 84 U mL^{-1} and 79.4 UmL^{-1}, respectively at pH 7.0 and temperature 30° C. The isolate S26 detected low activity.

- **Determination of β-1,3 glucanase enzyme:** The qualitative test revealed the presence of β-1,3 glucanase enzyme in four isolates i.e. S12, S17, R5 and BS-4. Similarly, the activity of crude enzyme of the test isolates (S12, S17, R5, and BS-4) was evaluated for β-1,3 glucanase enzyme detection and the results revealed that R5 possessed the maximum enzyme activity with 415.8 U ml^{-1} followed by S17 and S12 with 385.3 U ml^{-1} and 225.1 U ml^{-1} respectively at pH 7.0 and temperature 30° C. While BS-4 detected low activity.

- **SDS and Zymogram of Chitinase activity:** Since the chitinase activity was found higher in five isolates viz. S8, S17, S12, BS-4 and R5 out of six. Therefore, the molecular weight of protein present in crude enzyme was assessed for five isolates S8, S17, S12, BS-4 and R5 only under SDS and it revealed various protein band profile. Chitinolytic zymography assay used in this study revealed a high resolution of single zymographic band for all the five isolates which had molecular weight of approximately ~54 kDa where lysis occured. The zymogram study of crude chitinase revealed that activity was present in all the 5 isolates and band was approximately ~54 kDa.

- **SDS and Zymogram of β-1,3 glucanase:** On the basis of qualitative and quantitative assay, strain R5, S12 and S17 were selected for characterizing the enzyme activity by electrophoresis and zymogram studies. The results for SDS-PAGE analysis revealed that several protein band were present in the crude supernatant detected after staining with Coomassie blue staining. However, single band of protein was observed as clear zone on the agarose gel by zymography. The band of S12 corresponds with size ~ 54 kDa, molecular weight of protein

band for isolate S17 corresponds to ~60 kDa and that of R5 with ~ 59 kDa laminarin as substrate.

- **Detection of chitinase and β-1,3 glucanase gene in the isolates:**

  Chitinase gene was screened for five isolates *viz.* S8, S17, S12, BS-4 and R5 and result revealed that only four isolates *viz.* R5, S8, S12 and BS-4 possesed the gene with appoximately 300 bp amplicon. While β-1,3 glucanase gene with 300 bp was found in R5, S12 and S17 isolates.

- Further, the isolates R5, S12, S8 and BS-4 were selected on the basis of collective information on enzymatic activity, presence of gene and also due to the presence of various other metabolite. Therefore, the FTIR spectra of crude supernatant produced by endophytic isolates (R5, S12, S8 and BS-4) were analyzed and it revealed the presence of different bands corresponding to the following functional groups present in the molecular structure. Observed results demonstrated that FTIR spectrum of crude antifungal extracellular filtrate of isolates (BS-4, R5, S8 and S12) had characteristic peaks between 3746 - 698 cm^{-1}.

- **Identification of Antifungal compound by LC-MS/MS analysis:** Filter sterilized cell free extract of isolate BS-4, R5, S12 and S8 was also studied for bioactive antifungal compound by LC-MS/MS analysis. After 96 hr of bacterial growth, cell-free supernatants were extracted and analysis of crude extract of isolates BS-4 and R5 demonstrated the predominance of variety of metabolites as compared to S8 and S12 isolate.

- The antifungal metabolites from BS-4 extract in LC-MS/MS includes 1-hydroxy phenanzine, Pyocyanin, phenanzine 1- carboxylic acid (PCA), 2,4-

Diacetylphloroglucinol (DAPG), and Lahorenoic acid at positive mode. However, Siderophore (pyochelin), rhamnolipids, and presence of 4 hydroxy-2-alkylquinolines (HAQ) were present in negative mode.

- Similarly, crude extract of isolate R5 demonstrated the presence of multiple antifungal compound C-13, C-14, C-15 isomer of Surfactin, Bacillomycin, C-16 and C-14 isomer of Iturin, Fengycin A and cyclic lipopeptide Kurstakin.

- However, in comparison to R5 and BS-4 isolates; other two isolates S8 and S12 detected the presence of less lipopeptide antifungal compound. The crude extract of S12 revealed the presence of Kurstakin, Fengycin and Iturin only. The S8 crude extract showed the presence of C10, C11 Na adduct isomer of Kurstakin and Bacillomycin.

- **GC-MS analysis for detection of volatile compounds of selected isolates:**

  The volatile organic compounds (VOCs) present in the crude extract of isolates BS-4, R5, S12 and S8 were identified by GC-MS analyses. The GC-MS chromatogram of ethyl acetate extracts of isolates BS-4, R5, S12 and S8 revealed the presence of various compounds with corresponding peaks at different retention times. Some of the common volatile antifungal compound in all the four crude extract of isolates found were Hexadecane, Pyrazine derivative, Dodecane, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), Dibutyl phthalate, Phenol, 2,4-bis(1,1-dimethylethyl)-, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl), 1,2-Benzenedicarboxylic Acid, Eicosane etc.

- **Efficacy to check bio-antagonistic endophytic bacteria under *in situ* condition:**

  After analyzing all the necessary techniques to reveal the potential metabolites of the selected isolates, three endophytic bacterial strain *Bacillus paramycoides* (R5), *B. aryabhattai* (S12), *P.aeruginosa* (BS-4) and a mixture of *B. paramycoides* + *B. aryabhattai* (R5+S12) (as found compatible) were used for checking the bioefficacy against *C. falcatum* under *in vivo* conditions. The two isolates selected viz. *B. aryabhattai* (S12) and *B. paramycoides* (R5) were the novel endophytes in the sense that they have been reported from sugarcane and compatibility was found between these two isolates only, while, *P. aeruginosa* (BS-4) was selected due to the presence of multifarious metabolic compound.

- In the current study, four different methods/trials were adopted for checking the efficacy of bacterial endophytic strains for disease development in different trial setup: The four different trial were performed for the two consecutive years 2016-2017 and 2017-2018. The four trial (T-1, T-2, T-3 and T-4) were distinguished on the basis of differences in the inoculation of fungal pathogen *C. falcatum*; Trial-1 and Trial-4 had soil-based fungus inoculation in the sterile soil 15 days before sowing the setts while in the Trial-2 and Trial-3 pathogen was inoculated in the stalk and node region of cane far away from rhizosphere to induce the systemic resistance, respectively. In the trials (T-2, T-3 and T-4), the setts were sown after pre-treatment with the antagonistic endophytic bacteria followed by further application in the soil region and foliar spray in Trial-4. In the Trial-1, setts were planted without any prior treatments with endophytic bacteria and only two times rhizosphere application was followed after the plantation in a certain period.

- Although the effect of all the endophytic bacterial treatment either as sett or soil or foliar application resulted in the reduction of disease occurrence in the stalk of sugarcane. However, the efficiency of individual strain *B. paramycoides* strain R5 and *P.aeruginosa* BS-4 through sett or soil application effectively reduced the red rot incidence in all the trials and also promoted plant growth under field conditions better than the mixture /consortium of R5+S12. This finding is not in conformity with the work reported by other researchers (Molina-Romero et al. 2017; Bradáčová et al. 2019) while the finding is in conformity with the findings of Piakong and Zaida, (2018), who have reported efficacy of single strain over consortia.

- In the current study, the reason presumed for the low performance of mixture of R5+S12 in the *in vivo* condition over single strain R5 is either due to low efficiency of S12 strain in controlling the disease (less effective metabolites present) or due to lesser colonization in the root system for effective action which are essential requirement of bioagent to get into action against the disease resistance.

- The results suggested that the endophytic biocontrol agent *B. paramycoides* strain R5 significantly suppressed disease in the "stalk inoculation" experiments (where pathogen was inoculated above plant parts) i.e. in both Trial-2 and Trials-3, while the strain *P. aeruginosa* strain BS-4 exhibited best biocontrol activity in the "soil inoculation" experiments (where pathogen was inoculated in the soil) i.e. in the Trial-1 and Trial-4. The difference exhibited in the variation of the activity of two different strains i.e. *B. paramycoides* (R5) and *P. aeruginosa* (BS-4) in the four

different trial set-ups can be related to the variable metabolite production and their variable adoption mechanisms to suppress the fungal disease.

- Bacterized primed plants when challenged with a pathogen results in an induction and accumulation of a variety of defense-related proteins such as peroxidases (POX), phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), total phenol, chitinases and β-1, 3-glucanases (Anand et al. 2007, Fishal et al. 2010). In the present study also, all the bacterial primed sett induced more resistance against red rot pathogen *C. falcatum* by induction of defense related enzyme. ISR can be elicited from the primed part to the distant part i.e leaves. Therefore, the experimental results of the present study demonstrated that endophytic mediated elicitation of systemic resistance in the sugarcane leaves against red rot disease leads to the development of a complex response involving the earlier and higher expression of defense enzyme like chitinase, β-1,3 glucanase, PAL, POX, PPO and phenol. Activity of the all the enzymes were found increased several times in the leaves of the bacterial inoculum treated sugarcane plants as compared to the control. Further, increase in the expression of these enzymes was much higher after inoculation of pathogen.

- Though there was an increase in the rapidity and the level of expression of all the enzymes; but Sugarcane leaves of Trial-2 and Trial-3 responded more prominently and rapidly by the accumulation of high levels of PAL, POX, PPO and phenolics due to pre-treatments of setts with endophytic bacterial followed by challenge inoculation with *C. falcatum*. However, in Trial-1 and Trial-4 where there was no challenged inoculation, active response were seen in the activity of all enzyme as compared to control. As setts in trial-4 were pre-treated with bacterial endophytes; rise and expression were more pronounced in Trial-4 as

compared to Trial-1. The present study was also consolidating with the study of Vivekananthan et al. 2004; Sundar et al. 2009.

- In the present investigation also, chitinase and glucanase activities significantly increased in all the endophytic treated cane leaves over control cane (Sugarcane). In the present study also, significant increased and highest activity was recorded in cane leaves treated with biocontrol agents R5 and BS-4. Thus, present study demonstrated the higher induction of the enzymatic system in susceptible variety treated with endophytic bacterial treatment.

- **Effects of Endophytic bacterial treatments on various parameters of cane growth:**

  Percentage Germination: All the bacterial treated setts showed statistically significant increment in percentage germination when compared to control. In Trial-1, *P.aeruginosa* BS-4 resulted in highest germination of sett with 26.7% in the Year –I and in the Year-II *B. paramycoides* (R5) treatment resulted in highest germination with 28.8%. In Trial-2, highest germination was recorded in R5 with 37.8%. The other strains also exhibited significantly high germination percentage when compared with control. In Trial-3, R5 and BS-4 showed statistically equivalent germination with 42.2% followed by R5+S12. In the Trial-4 significantly higher germination was recorded for treatment with *P.aeruginosa* BS-4 followed by R5, R5+S12 and S12.

- In the present study, various other parameters such as cane height, shoot counts, cane weight, cane girth, number of internodes and chlorophyll content were recorded. From the present study; it was found that plant inoculated with bacterial endophytic bacteria demonstrated improved cane height, number of shoots, cane

weight, cane girth and number of internodes. The reasons for improving these physiological parameters might be due to the antagonistic endophytic strain with multifarious plant growth promotion which have been reported to enhance the growth in several ways. The PGPR strain have been demonstrated previously on their positive role in improving germination and cane growth (Viswanathan and Samiyappan 1999a, 2002).

- The efficiency of endophytic treated cane is also related to the performance of photosynthetic apparatus of the plant. Chlorophyll a, chlorophyll b and total chlorophyll contents were significantly higher in all the inoculated treatments compared with the control. Higher chlorophyll content in sugarcane treated with bacteria was also reported by Sevilla et al. (1998), Muthukumarasamy et al. (1999), and Chauhan et al. 2014.

- Therefore, the present study revealed the application of endophytic plant growth promotory bacterial strains as sett treatment followed by soil application could prove to be a beneficial component of integrated disease management as such combination resulted into higher accumulation of PR-protein and other defense enzyme response. These bacteria, apart from their action against pathogens are good growth promoters, which is an added advantage for any practical agricultural system.

Chapter 8

Conclusions and Future Prospective

CONCLUSIONS AND FUTURE PROSPECTIVE

Sugarcane is one an important cash crop of country and the Indian economy is highly dependable on it. Red rot disease of sugarcane is one of the serious concerns in today's scenario as it affects the production and productivity of the crop by 10-50 %. Although chemical known methods have been implemented for so long; but it either resulted in excessive chemical accumulation in the environment or developing resistant pathovar. Biological control practices provide an extensive approach for the cultivation of organically important cash crops such as sugarcane and many other crops. Therefore, such practices are in greater demand as alternatives to synthetic pesticides. Because of great interest in the ecologically safe agricultural product such as biological fertilizers/pesticides their availability is limited in the agricultural market. Subsequently, this led to the present study to explore the number of effective/potent biocontrol agents. Over a decade, endophytic bacteria have stolen the show in the field of agriculture, due to their intense colonization with the host plants and providing resistance to various biotic and abiotic stresses. Thus, in the present study, endophytic bacteria isolated from the sugarcane plant were assayed for their antagonistic activity against phytopathogen *C. falcatum*. The objective of the present study was bifurcated into four different chapters and conclusion drawn from individual objectives are described below:

From the first objective; it can be concluded that though endophytes have been reported from a wide variety of plants but the functional role is known only with the limited number of isolates. Sugarcane crop is associated with a diverse community of

microorganisms but their full potential is yet to be explored for other functional attributes. Besides this, less literature is available for the role of endophytes as a biocontrol agent, rather diazotrophs have been studied extensively. Therefore, in the present study different species of *Bacillus* and *Pseudomonas* have been isolated from the root/ stalks of different sugarcane varieties which are ubiquitous in nature and easy to isolate than diazotrophs. This study proved that these endophytes showed potent *in vitro* antagonism against red rot fungus *C. falcatum*. Along with inhibitory action against a pathogen, further evaluation for other PGP traits would need to be explored so that these endophytes have potential applicability in the biocontrol of red rot disease of sugarcane. Also being endophytic in nature, they reside in the same niche as with the host plant, thus communicate and protect the plant in an effective manner. Considering all these factors, the study indicated that isolates with antifungal activity must be expected to be a potential source for bioactive metabolites which would require further evaluation to develop a strong biofertilizer/bio-fungicides for sugarcane crop.

The second objective dealt with the exploration of plant growth-promoting and some other antifungal metabolites as well as lytic enzymes. Thus, the current study finds the selection of potent endophytic bacterial isolate exhibiting extraordinary features of plant growth promotion (biofertilzer) and biocontrol of pests (biopesticides/biocontrol agent). The present finding is one of the few reports which unravel biocontrol traits of endophytic bacterial strains against red rot pathogen *C. falcatum* alongwith plant growth promotion. In conclusion, selected endophytic bacteria showed important requirements for valuable biological agents, which turn them into promising candidates to be included in biocontrol management against red rot of sugarcane caused by *C. falcatum*.

The third objective used the various techniques to uncover some more metabolites that would help in controlling the red rot disease of sugarcane. Diverse types of metabolites produced extracellularly from endophytic isolates were identified and confirmed by various analytical (FTIR, LC-MS/MS, GC-MS) and molecular techniques (gene detection of lytic enzyme). Although, all the selected isolates were able to produce one or the other antifungal metabolite; however, strain *B. paramycoides* (R5) and *P. aeruginosa* (BS-4) possessed diverse antifungal metabolite range that could be exploited for sustainable agriculture in biocontrol of red rot diseases. This study confirmed *in vitro* inhibition property of endophytes against *C. falcatum* that could be due to the presence of various diffusible, volatile and lytic enzymes. Several studies demonstrated the various metabolite production from the Bacillus group, but this is the first finding that highlighted the various important antifungal metabolites from strain R5 *Bacillus paramycoides*. Also, it can be concluded that using such strain containing maximum traits and metabolites would not only decrease the dependency on chemical fungicides against fungal pathogens but also help in plant growth promotion.

Finally, from the last objective, the conclusion drawn is, that under *in vivo* conditions the individual strain *B. paramycoides* (R5) and *P.aeruginosa* (BS-4) demonstrated astonishing results in red rot disease suppression due to the coordinated induction of various enzymes and metabolites involved in host defense by reducing pathogen colonization and further aggression into the stalk tissues better than mixture (R5+S12). The bacterially primed setts restricted the pathogen growth more efficiently. These endophytic bacteria (R5 and BS-4), apart from their action against pathogens, are good growth promoters, which is an added advantage for any practical agricultural system. The mechanisms involved in suppression included the ISR

phenomenon that was triggered by the biological stimulus i.e. endophytic bacteria which induced the defense mechanism of the plant.

Collectively, the present study investigated the various metabolites that helped in the suppression of fungal pathogen. Also, various research-based findings are still prompted in the future to explore the range of metabolites produced by endophytes. As endophytes are an untapped source of the bioactive compound; therefore, the potential to exploit their unique characteristics have raised the hopes in finding biotechnological solutions that would be more applicable for sustaining agricultural solution. These aspects have been dealt in detail as evidenced through current scientific understanding coupled to the future perspectives.

Various studies confirmed the beneficial effects of endophytic bacteria and their adaptability to survive in stress conditions. Therefore, understanding of composition and functioning of plant-associated microbial communities as well as control of the structure of endophytic bacterial populations through the development of environmentally benign agricultural practices has a large potential for improved plant performance and application of the integrated plant disease management systems required for sustainable agricultural production.

An additional challenge in future research is the detection and characterization of metabolites formed in niches on and in plants or under specific circumstances under natural conditions only, as indicated by the partial realization of the metabolic potential of bacteria grown under *in vitro* conditions.

Chapter 9

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Scientific Publications and Achievements

ACHIEVEMENTS:

- Achieved **Best Research Scholar Award- 2018** in the field of Environmental Microbiology on the occasion of 2nd International Conference "Advances in Agricultural, Biological and Applied Sciences for Sustainable Future (ABAS-2018).

- Achieved **Best Poster award** on the paper entitled "Exploration of biocontrol and growth promoting activity of bacterial strains isolated from sugarcane crop." In the 1st International Conference on Biotechnology & Biological Sciences-BIOSPECTRUM 2017 organised by University of Engineering and Management, Kolkata in association with Springer-Nature Publisher.

SCIENTIFIC PUBLICATION:

RESEARCH PAPER:

- **Beenu Shastri** and Rajesh Kumar **(2019)**. Comparative efficacy of potentially active antagonistic endophytic and rhizospheric bacteria against red rot of Sugarcane. International Journal of Emerging Technologies and Innovative Research, ISSN:2349-5162, 6(6):916-921.

- **Beenu Shastri,** Rajesh Kumar and Ram Ji Lal **(2019)**. Evaluation of plant growth promoting traits of rhizospheric and endophytic bacteria isolated from sugarcane crop. International Journal of Research and Analytical Review, ISSN:2349-5138, 6(2):712-717.

- **Beenu Shastri,** Rajesh Kumar and Ram Ji Lal **(2019)**. Isolation of endophytic bacteria associated with sugarcane and their *in vitro* antagonistic activity against

red rot pathogen *Colletotrichum falcatum.* Indian Journal of Sugarcane Technology. 34 (02) (In Press).

- **Beenu Shastri,** Rajesh Kumar and Ram Ji Lal **(2019)**. A research article entitled "Isolation and Identification of Antifungal metabolite producing endophytic *Bacillus subtilis* S17 and its role in controlling *in vitro* red rot causing *Colletotrichum falcatum* (Under communication) in Vegetos Journal of Springer.
- **Beenu Shastri,** Rajesh Kumar and Ram Ji Lal **(2019)**. A research paper entitled "Isolation, characterization and identification of indigenous endophytic bacteria exhibiting PGP and antifungal traits from the internal tissue of sugarcane crop" (Under Revision) in Sugar Tech Journal of Springer.

PROCEEDING RESEARCH PAPER:

- **B**. **Shastri,** A. Kumar, and R. Kumar **(2019)**. Exploration of Biocontrol and Growth-Promoting Activity of Bacterial Strains Isolated from the Sugarcane Crop. In *Advances in Plant & Microbial Biotechnology* (pp. 129-134). Springer, Singapore.
- **B Shastri,** R.Kumar **(2016).** Screening of antagonistic endophytic bacteria against *Colletotrichum falcatum* causing red rot in Sugarcane crop. In Proceeding; Recent Trends and Experimental Approaches in Science, Technology and Nature" published by OURA Prakashan in association with Society for Science and Nature (ISBN: 978-81-932601-6-6).

BOOK CHAPTER:

- **B Shastri,** R Kumar **(2018).** Microbial secondary metabolite and plant-microbe communications in rhizosphere. In: Microbial Resources in Soil and Plant Productivity Management. (Eds. JS Singh) Elsevier (In Press).

- R Kumar, **B Shastri (2017).** Role of Phosphate-solubilising microorganisms in sustainable agriculture development. In: Agro-environmental Sustainability – Managing Crop Health (Vol.-I), (Eds. JS Singh & Sneiviratne G.) Springer.pp-271-303.

- A J Das, **B Shastri,** S Lal, R Kumar **(2016).** Bioremediation of Petroleum Hydrocarbons and Heavy Metal Contaminated Sites by Biosurfactants: An Eco-friendly and Sustainable Technology. In: Bioremediation of Industrial Pollutants, (Eds. RN Bhargava and G Saxena) Write and Publication, India.

NATIONAL TRAINING ATTENDED:

- Attended Eight days (21st Feb 2017- 28th Feb 2017) National training on "Biological Control of Plant Pathogens: Classical to Modern Approaches Conducted by NBAIM-Mau.

WORKSHOP ATTENDED:

- Attended Two-day workshop on "High Performance Liquid Chromatography (HPLC) and Its Application" organized by Divison of Biotechnology, Cytogene Research & Development, Lucknow (2018).

PAPER / POSTER PRESENTED:

- Presented **Oral paper** on "Isolation of novel endophytic bacteria and investigation of its bio-fungicidal activity against red rot disease" in 4th National Conference of ASIAN PGPR (2018) on "PGPR for Sustainability of Agriculture and Environment" jointly organized by Department of Biotechnology, Mizoram University, Aizawl, Mizoram.

- Presented **Oral paper** on the paper entitled **"**Exploration of biocontrol and growth promoting activity of bacterial strains isolated from the sugarcane crop" in 1st International Conference on Biotechnology & Biological Sciences 'Biospectrum-2017' organized by University of Engineering & Management, Kolkata held on 25th -26th August, 2017 **(Best Oral Award)**.

- Presented **Poster** entitled on "Intellectual Property Rights (IPR): Role and Its Importance in Sugarcane Development Research" in the National Symposium on "IPRs in Agricultural Research" held on August 30th -31st 2017 jointly organized by Babasaheb Bhimrao Ambedkar University, Lucknow & U.P. Council of Agricultural Research, Lucknow.

- Presented **Poster** entitled on "Screening of antagonistic endophytic bacteria against *Colletotrichum falcatum* causing red rot in Sugarcane crop" in International seminar on Recent Trends and Experimental Approaches in Science, Technology and Nature held at IISR, Lucknow jointly organised by Society for Science and Nature & Oura Prakashan (2016).

- Presented **Poster** entitled "Isolation of plant growth promoting and antagonistic endophytic bacteria against C*olletotrichum falcatum* fungus causing Red Rot in Sugarcane crop" in **57th Annual Conference of AMI** & International Symposium On Microbes and Biosphere: What's New and What's Next organised by Guwahati University at Guwahati (Assam) - 2016.

- Presented **Poster** in **56th AMI- 2015** & International Symposium on "Emerging Discoveries in Microbiology" organised by JNU, New Delhi.

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Comparative efficacy of potentially active antagonistic endophytic and rhizospheric bacteria against red rot of sugarcane

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Abstract: Sugarcane crop has attained the most significant position in the agrarian based economic country of India. The crop is source for the production of various valuable products which have become a part and parcel of daily needs. However, occurrence of disease especially; red rot disease is affecting the health status of crop. The disease is adversely affecting the economic status of crop. It not only affecting the growth and yield; but also, degrading the quality of juice. Thus, various biological strategies have been adopted for their effective management of red rot. As such, the present study dealt with comparing the *in vitro* efficacy of endophytic and rhizospheric bacteria isolated from sugarcane crop for suppressing the red rot pathogen. In the current study, total 51 bacteria were isolated from the sugarcane plant; 16 bacteria from the internal root tissues and 35 bacteria from the rhizospheric soil. Further, when the isolates were checked for *in vitro* antagonism against *C. falcatum*; it was found that a major proportion of endophytic bacteria isolated showed *in vitro* inhibition with above 50% inhibition when compare with rhizospheric bacteria. Therefore, it can be concluded that the *in vitro* biocontrol efficacy was higher in endophytic bacteria over rhizospheric ones.

Keyword: Rhizopsphere, Endophytes, Sugarcane, Red rot.

I. Introduction:

Sugarcane is an important agro-industrial cash crop cultivated in tropical and sub- tropical regions of the world. Globally, sugarcane is an important source for production of sugar and a raw material for sugar industry.Apart from, various other produce of sugarcane such as bagasse, molasses, filter wax, jaggery, khandsari and ethanol are being commercialized at large scale. Sugarcane is one of the most crucial crops of India where majority of population are dependent on it for their daily need. Various factors affecting the well-being of the crop are biotic and abiotic. Red rot is one of the threatening diseases widely affecting the sugarcane crop in the country. The fungal pathogen mainly responsible for such adverse condition is *Colletotrichum falcatum* which affects the well-being of standing crop. It causes severe loss in yield and quality of the susceptible cultivars in the Indian subcontinent (Satyavir, 2003; Duttamajumder, 2008). *C. falcatum* trigger severe loss of yield in many parts of sugarcane growing states in India. It can reduce cane weight by up to 29% and loss in sugar recovery by 31% (Hussnain and Afghan, 2006). The red rot pathogen hydrolysed the stored sucrose by producing the enzyme invertase which breaks the sucrose molecule into its components namely glucose and fructose. As a result, the quantity of molasses increases (Sehtiya, 1993).

Red rot disease of sugarcane is known to India in ancient times and measures to combat such disease are being taken since then. Therefore, continuous efforts are required for the effective improvement of this economical crop. A variety of biological controls are available for use, but further development and effective adoption will require a greater understanding of the complex interactions among plants, people, and the environment. Rhizopheric bacteria are known for production of various stable secondary metabolites responsible for increasing the yield of crop as well as reduction of disease. Presently, another term "endophytic bacteria' is being used frequently as biocontrol agent isolated from inner side of crop plant. The endophytic bacteria are found to be closely associated with the plant and largely regarded as a untapped resource for the discovery of isolates with novel antifungal and plant growth-promoting traits (Lodewyckx et al., 2002; Rosenblueth et. al., 2006; Sturz et.al., 2008). For several crops, rhizospheric and endophytic bacteria have shown antagonistic effect against pathogen by providing beneficial effect on the health status of plant and inhibiting the pathogen growth. However, the main modes of action described are nitrogen fixation, production of phytohormones and antifungal compounds, and induced systemic resistance (Compant, 2005; Iniguez, 2004; Kuklinsky-Sobral, 2004). Biocontrol agents provide an excellent alternative to chemical pesticides and fertilizers. Thus, plant growth promoting rhizobacteria and endophytes are important, potentially active antagonistic agent capable of suppressing multiple pathogens and enhancing crop growth as well as yield. Consequently, sustaining and enhancing the growth and yield of sugarcane have become a major focus of research. Thus, present study aims to screen the potent antagonistic bacteria isolated from the rhizospheric and inner side tissue of root responsible for the *in vitro* suppression of plant diseases and to compare their efficacy in controlling the red rot fungus.

II. Methodology:

Sample collection:
Red rot susceptible healthy sugarcane plant and the rhizospheric soil surrounding the sugarcane crop were collected in a sterile plastic cover and processed immediately for the isolation of bacteria.

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Evaluation of plant growth promoting traits of rhizospheric and endophytic bacteria isolated from sugarcane crop

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ABSTRACT

Sugarcane (*Saccharum spp.* hybrids) is one of the significant economical crop of India. Its productivity and production mainly depends on the potential of a cultivar. Plant growth promoting rhizobacteria (PGPR) not only help in the upgradation of health status of crop plants but also aid in enhancing their resistance against various plant pathogens. In recent years, they are being extensively explored for improving plant nutrient acquisition and disease resistance in several crops. In the present study, a total of 51 rhizospheric and endophytic bacterial isolated from the rhizospheric soil and as well as internal root tissues of sugarcane were evaluated for various plant growth promoting (PGP) attributes, which either directly or indirectly exerts beneficial effects on host plant. Data indicated that most of the isolates of rhizospheric and endophytic bacteria exhibited Indole acetic acid (IAA), phosphate solubilization (PS), hydrogen cyanide (HCN) and ammonia production ability. Thus, the effective PGPR obtained from this study could be used as an effective tool for plant growth promotion as well asa bio-control agent for the management of some of the sugarcane diseases.

Keywords: PGPR, Endophytes, IAA, Phosphate Solubilization, Ammonia, HCN production

INTRODUCTION

In the current scenario of urbanization, rapid demand of crop production has led to the indiscriminate use of chemical pesticides/chemical fertilizers that adversely affect our eco-system as well as environment. It has been estimated that global population will reach to approx 9 billion by 2050, which would require about 60% additional foods from the present (Singh *et al.* 2019). Therefore, alternative to chemical fertilizers for enhancing the crop productivity for ever growing population, use of microorganisms are the only eco-friendly approach to maintain soil as well as plant health.

Microorganisms residing in the close vicinity of root zone which help in the growth promotion of plants are called plant growth-promoting rhizobacteria (PGPR); Kloepper *et al.*(1989). Bacteria of diverse genera have been identified as PGPR, of them *Bacillus* and *Pseudomonas* spp. are predominant ones (Podile and Kishore 2006). Endophytic bacteria isolated from the interior part of plants also offer several ecological benefits to plant like that of PGPR. The only difference in endophytic bacteria to that of rhizobacteria is that they reside in close

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Isolation of endophytic bacteria associated with sugarcane and their *in vitro* antagonistic activity against red rot pathogen *Colletotrichum falcatum*

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ABSTRACT

Red rot is one of the most threatening diseases of sugarcane crop that adversely affect the yield and productivity. Therefore, in the present study *in vitro* antagonistic activity of endophytic bacteria isolated from the stalk region of the sugarcane variety Co767 was assessed against red rot pathogen(*Colletotrichumfalcatum*Went). The findings of the present study revealed that the 21 isolates were obtained by proper sterilization technique. The isolates when checked for dual culture *in vitro* antagonism against pathogen; only 18 isolates were able to inhibit the mycelial growth of the pathogen. However, of them 11 isolates exhibited strong antagonisms with more than 70% inhibition. The highly effectiveness in the inhibition of fungus mycelium might be due to the production of various metabolites and other related compounds that needs to be explored. Therefore, these effective endophytic bacterial isolates could be further exploited as an bioagent for the management of red rot disease of sugarcane.

Key words: Red rot, *Colletotrichum falcatum*, Endophytic bacteria, Antagonism

INTRODUCTION

Sugarcane (*Saccharum* spp. hybid) commands an important position among the various commercial crops of India.It is the second most important agro-industrial crop, next only to cotton (Jayashree et.al. 2010). The crop is mainly cultivated in most of the states of India. Globally, sugarcane is an important source of commercial sugar accounting for 75% of world sugar production and almost 100% of sugar production in India (Srivastava and Lal 2019). More recently, it has been exploited for the production of renewable bio-fuel ethanol for blending with petrol. Consequently, sustaining and enhancing the growth and yield of sugarcane is an important aspect of research. The growth and performance of the crop in the field is adversely affected by a number of abiotic and biotic factors. About one hundred diseases of sugarcane have been reported from different parts of the world (Rott *et al.* 2000; Sharma and Tamta 2015). In India, the estimated loss in crop production due to fungal diseases is about 18-31% (Jayashree *et al.* 2010). Generally, fungi, bacteria, virus and phytoplasmas are mainly responsible for causing serious threat to sugarcane cultivation (Viswanathan and Rao 2011, lal, 2016). It has been also estimated that sugarcane diseases alone reduce the crop yield by 20% every year (Lal 2019).

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Red rot, wilt, smut and sett rot are the main fungal diseases causing significant loss to sugarcane production.

Red rot is an important disease of sugarcane caused by the fungus *Colletotrichum falcatum*Went. It causes severe losses in yield and quality of the sugarcane in the Indian sub-continent (Satyavir 2003; Duttamajumder 2008). It can reduce cane weight to 29% and causes loss in sugar recovery by 31% (Hussnain and Afghan 2006). Although several commercial varieties have been developed with higher cane and sugar yield; but they cannot be cultivated continuously due to frequent development of new pathotypes of the pathogen resulting in knockdown of varieties (Viswanathan 2017). Current control strategies involve the use of resistant varieties and fungicidal applications. However, the efficacy of both control measures are limited, and there is an urgent need for novel and environmentally sound strategies to manage the disease. In recent years, the uses of biological agents are the only cost-effective and promising method for the management of this devastating disease. Currently, endophytic microorganisims (fungi and bacteria) are attaining a most prestigious approach for the management of plant diseases. The endophytes comprise of two Greek word "endon" meaning within, and "phyton" meaning plant. Thus, endophytic microorganisms cane be defined as such fungi and bacteria that reside the plant endosphere during all or part of their life cycle without causing any harm to the host

Sugar Tech

Isolation, characterization and identification of indigenous endophytic bacteria exhibiting PGP and antifungal traits from the internal tissue of sugarcane crop

--Manuscript Draft--

Manuscript Number:	SUTE-D-19-00330R1
Full Title:	Isolation, characterization and identification of indigenous endophytic bacteria exhibiting PGP and antifungal traits from the internal tissue of sugarcane crop
Article Type:	Original paper
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Funding Information:	
Abstract:	Using different isolation approach, 118 endophytic bacteria (beyond N-fixer diazotroph) were isolated collectively from the roots and shoots of three sugarcane varieties Co 1148, CoS 767 and CoJ 64 in this study. Further, these endophytes were tested for their in vitro inhibition against the red rot pathogen (Colletotrichum falcatum). Out of 118, only 29 bacteria were able to show in vitro inhibitory action against the pathogen. Beside inhibiting their mycelial growth, some of these endophytes were able to synthesize various PGP traits such as IAA, phosphate solubilization, siderophore, HCN, ACC deaminase, and ammonia production. These endophytes were found helpful in promoting the growth of the plant in a direct and indirect way. Six bacterial isolates with more than 75% inhibitory activity against the pathogen and PGP attributes were further characterized through 16S rRNA gene sequencing. On the basis of the phylogenetic analysis, the six endophytic antagonists were identified as Bacillus cereus (S8), B. aryabhattai (S12), B. subtilis (S17), B. licheniformis (S26), B. paramycoides (R5), and Pseudomonas aeruginosa (BS-4). This study demonstrated that endophytic bacteria are an untapped source of natural products and can be exploited for yield attributes and red rot control in sugarcane. Keywords: Endophytes, Bacillus spp., Pseudomonas, PGP and antifungal traits, Colletotrichum falcatum
Response to Reviewers:	Response to Reviewers Manuscript No.: SUTE-D-19-00330 Sugar Tech Journal Title: Isolation, characterization and identification of indigenous endophytic bacteria exhibiting PGP and antifungal traits from the internal tissue of sugarcane crop. Dear Sir, We are very thankful to Reviewer 1 for his/her suggestions to improve the quality of the manuscript. According to their comments/suggestions, we have incorporated all possible suggestions and redrafted the manuscript. Also space in the variety is also made. Morphological and Biochemical table has been attached in supplementary file.

Vegetos

Isolation and Identification of Antifungal metabolite producing endophytic Bacillus subtilis S17 and its role in controlling in vitro red rot causing Colletotrichum falcatum

--Manuscript Draft--

Manuscript Number:	VTOS-D-19-00176
Full Title:	Isolation and Identification of Antifungal metabolite producing endophytic Bacillus subtilis S17 and its role in controlling in vitro red rot causing Colletotrichum falcatum
Article Type:	Research Articles
Funding Information:	
Abstract:	Red rot, a threatening disease of sugarcane caused by fungal pathogen Colletotrichum falcatum Went adversely affecting the crop. Therefore, it is of prime importance for eco-warriors to encompass such methods of protection which are organically safe especially with the use of bioagents. Currently, usage of endophytic bacteria as biocontrol agent have become a focussed approach in the field of biological control and widen up the eyes of researchers in enhancing crop productivity. Hence, this study was centred on endophytic bacteria S17 Bacillus subtilis (identified by 16S rRNA analysis) isolated from the sugarcane stalk tissues. An in vitro study showed that isolate S17 inhibited the mycelial growth of C. falcatum by 76.22±2.34% on dual-culture antagonism assay. Scanning Electron Microscopy (SEM) revealed the distortion and destruction in the hyphae of the fungal pathogen by isolate S17. Further investigation proved that the hyphal disorientation was by the secretion of various enzymes (chitinase, β-1,3 glucanase, protease, pectinase, and amylase) as well as other inhibitory substances including siderophore, ammonia, hydrogen cyanide (HCN) and other volatile compounds. The volatile and diffusible metabolites exhibited strong inhibition (74.57±1.45% and 81.86±2.34%), against C. falcatum respectively. Similarly, cell-free culture supernatant that contains extracellular metabolites exhibited mycelial inhibition (70.52±0.95%) against C. falcatum . Thus, the present finding increased our understanding of how the different metabolites as well as various exo-enzymes produced by Bacillus subtilis S17 exert inhibition of red rot pathogen and besides the mechanism of the antagonism. Keywords : Sugarcane, Red rot, Colletotrichum falcatum Went, Endophyte, Bacillus subtilis , Lytic enzyme
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Author Comments:	To, The Editor-In-Chief Vegetos Subject: Submission of the manuscript. Dear Editor, I am enclosing herewith a manuscript entitled “Isolation and Identification of Antifungal

Exploration of Biocontrol and Growth-Promoting Activity of Bacterial Strains Isolated from the Sugarcane Crop

17

Beenu Shastri, Anil Kumar, and Rajesh Kumar

Abstract

Naturally occurring bacteria were isolated from the internal tissues of stalks as well as from roots of sugarcane crop and from the rhizospheric soil. The highest numbers of bacterial populations were isolated from the rhizospheric zone. Isolated bacterial strains were subjected to antagonistic activity in vitro against *Colletotrichum falcatum* fungus causing red rot disease in sugarcane crop. Most of the isolated bacteria showed antagonistic activity against *C. falcatum* in vitro. Isolated antifungal isolates were identified morphologically and biochemically. Further, the potential strains were examined for various plant growth promoting traits and hydrolytic enzymes production. Bacteria isolated from rhizospheric as well as from endophytic zone of sugarcane crop showed the inhibition of red rot pathogen as well showed the in vitro plant growth promotory traits. Thus, isolates help in biocontrol of red rot as well as can be used for increment of sugarcane yield.

Keywords

Sugarcane · Endophytes · Rhizospheric bacteria · *Colletotrichum falcatum*

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Chapter 13
Role of Phosphate-Solubilising Microorganisms in Sustainable Agricultural Development

Rajesh Kumar and Beenu Shastri

Abstract Phosphorous (P) is an essential macronutrient required for plant growth and development and comes next to Nitrogen (N). The quantity of phosphorous present in soil is huge but is unavailable to the plants due to its fixation with the other elements in soil necessitating the application of chemical phosphatic fertilisers to the soil for plant growth and development. Injudicious use of phosphatic fertiliser though has resulted in enhancement of crop yield but had left an adverse effect on the ecosystem. In the present scenario, to manage the nutritional security and the environment, sustainable agriculture holds the key which uses phosphate solubilising microorganisms (PSM's) as an important alternative, which can solubilise soil phosphate and supply it to the plants in a more eco-friendly and sustainable manner. PSM's are diversified in nature and are abundant in normal to stressed environments. They include bacteria, fungi, algae, actinomycetes and mycorrhizae which solubilises soil phosphate by different mechanisms including production of organic acids and enzymes, thus making phosphorous available to the plants for their growth and development. Molecular biotechnology brings out a better technique that could help researchers to understand the mechanisms responsible for solubilisation and also improve the performance of PSM's by manipulating the genes responsible for phosphorous solubilisation for the betterment of crops and also in managing a sustainable environment system.

Keywords Sustainable agriculture • Phosphate-solubilising microorganisms • Bioinoculant • Ecosystem • Genetic engineering

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Chapter 3

Bioremediation of Petroleum Hydrocarbons and Heavy Metal Contaminated Sites by Biosurfactants: An Eco-friendly and Sustainable Technology

AMAR JYOTI DAS, BEENU SHASTRI, SHATTROHAN LAL AND RAJESH KUMAR[1]

SUMMARY

With the rapid industrialization and increasing demand for petroleum hydrocarbons as a source of energy, has resulted in its increased extraction, refinement and use. But, this increase in extraction and refinement has led to the contamination of soil and groundwater and needs utmost attention as it finds an easy entry through the food chain. Besides this, heavy metal contamination is another problem, which has aggravated due to industrialization. Various remediation technologies have been proposed for management of petroleum hydrocarbons and heavy metal contaminated soil but most of them are expensive and lead to incomplete decomposition of contaminants. So, large attention has been paid on the development and implementation of new

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Appendices

Appendices I

Chemicals, Reagents, Solvents, and Microbiological Media's

All the required chemicals, reagents, and solvents were used of analytical grade (highest purity ≥ 99%) and purchased from **Sigma-Aldrich (St. Louis, MO, USA)** whereas microbiological media was purchased from **Hi Media Laboratories (Mumbai, MH, IN).**

Appendices II

Glasswares and Plasticware's

All the laboratory glassware's were purchased from **Borosil Glass Works Ltd.,** Mumbai, Maharashtra; **J-SIL Scientific Industries,** Agra, Uttar Pradesh; **Loba Chemie Pvt. Ltd.,** Mumbai, Maharashtra (India); and **ASGI (India) Industries,** Agra, Uttar Pradesh whereas the laboratory plastic wares were purchased from **Lab Line Traders**, Lucknow, Uttar Pradesh, India.

Appendices III

Laboratory Tools and Equipments

Autoclave (SM-102, S M Scientific Instruments Pvt. Ltd., UP, IN)

Temperature controlled incubator shaker (New Brunswick Innova 4230, NJ, USA)

Refrigerated centrifuge (REMI Instruments Pvt. Ltd., Mumbai, MH, IN)

Veriti™ 96-Well Thermal Cycler (Applied Biosystems™ Inc., CA, USA).

Spectrophotometer (Thermo Scientific™ Evolution 201, Australia)

Nicolet FT-IR Spectrometer (Model Nicolet 6700, Thermo Fisher Scientific, MA, USA).

GC-MS (Thermo Fisher Scientific, FL, USA).

Biographical Sketch

Ms. Beenu Shastri was born in the state of Uttar Pradesh, India and completed her schooling from Lucknow Public School (10th 2006) and Lucknow Public School (12th 2008), Lucknow. She received B.Sc. degree (2012) in Zoology, Botany and Chemistry from C.M.S. Girls Degree College, affiliated to Lucknow University, Lucknow (U.P.), India and then moved to Babasaheb Bhimrao Ambedkar (Central) University, Lucknow (UP) India where completed M.Sc. degree (2014) in Environmental Microbiology. Because of her keen interest in environmental protection, she joined Babasaheb Bhimrao Ambedkar (Central) University, Lucknow (UP) India to further pursue doctoral research work in environmental microbiology at the Rhizopshere Biology Laboratory, Department of Environmental Microbiology (DEM). Her research interest includes Environmental Microbiology, Agricultural Microbiology, Plant-microbe interaction, Endophytic bacteria and Biotic stress management. For this she attended various training and workshop to learn the technique. She is a dual gold medalist in M. Sc Environmental Microbiology and has received RGNF for pursuing Doctorate degree. She has qualified (2016) National Eligibility Test (NET) and GATE (2015). She has published research, chapter and proceeding articles in reputed national and international journal and has presented papers/poster at conferences. She has won Best Poster award at International Conference on Biotechnology & Biological Sciences- BIOSPECTRUM 2017 organised by University of Engineering and Management, Kolkata in association with Springer-Publication, India. She has also achieved Best Research Scholar Award-2018 in the field of Environmental Microbiology on the occasion of 2nd International Conference "Advances in Agricultural, Biological and Applied Sciences for Sustainable Future (ABAS-2018). She also holds the life memberships of the Association of Microbiologists of India (AMI), The Indian Science Congress Association (ISC) and Honorary Life Membership of ASIAN PGPR Society of Sustainable Agriculture USA.