Endophytic Bacteria and their Bioactive Metabolite for Enhanced Crop Productivity and Induction of Systemic Resistance in Rice Plant against Sheath Blight Disease

by

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Gopika V. Krishnan

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List of Abbreviations

µg	: micrograms
µl	: microlitres
µm	: micrometres
16S rRNA	: 16S ribosomal ribonucleic acid
6PP	: 6-pentyl-α-pyrone
ACC	: 1-aminocyclopropane-1-carboxylate
AIA	: Actinomycetes isolation agar
ANOVA	: One-way analyses of variance
Avr	: avirulence
BF	: Bioformulation
BLAST	: Basic Local Alignment Search Tool
BLB	: Bacterial Leaf Blight
BOD	: Bio-Oxygen Demand
℃	: degree Celsius
CAS	: chrome azurol S
CFU	: Colony forming units
CFU	: Colony forming unit
cm	: centimetres
CMC	: Carboxymethylcellulose
COMs	: Crude organic metabolites
CRD	: Completely randomised design
CWG	: Cell Wall Glucan
DAMPs	: Damage-associated molecular patterns
DCA	: 3,5-dichloroanthranilic acid
DDD	: Dichlorodiphenyldichloroethane
DDE	: dichlorodiphenyldichloroethylene
DDT	: Dichlorodiphenyltrichloroethane
DMRT	: Duncan's Multiple Range Test
DNA	: Deoxyribonucleic acid
dNTPs	: deoxynucleotide triphosphate
dS	: decisiemens

EDTA : Ethylenediaminetetraacetic acid

EI : electronic ionisation

ESI : Electrospray ionisation probe

ET : Ethylene

ETI : Effector-triggered immunity

FAO : Food and Agriculture Organisation

Fe^{3+} : Ferric ion

$FeCl .6H_2O$: Iron (III) chloride hexahydrate

FTIR : Fourier-transform infrared spectroscopy

g : g-force

g : grams

GAs : Gibberellic acids

GB : Glycinebetaine

GC-MS/MS : Gas Chromatography Tandem Mass Spectrometry

GI : Growth inhibition

GIAHS : Globally Important Agricultural Heritage System

GR : Granules

h : hours

H^1NMR : Hydrogen-1 nuclear magnetic resonance

$H_2PO_4^-$: Dihydrogen phosphate

ha : hectares

HCl : Hydrochloric acid

HCN : Hydrogen cyanide

HDTMA : Hexadecyltrimethylammonium bromide

HPLC : High Performance Liquid Chromatography

HPO_4^{2-} : Monohydrogen phosphate

HR : Hypersentitive reaction

HRGPs : Hydroxyproline-rich glycoproteins

IAA : Indoel-3-acetic acid

IARI : Indian Agricultural Research Institute

IBM : International Business Machines

ICAR : Indian Council of Agricultural Research

IRRI : International Rice Research Institute

ISR : Induced systemic resistance

ITCC	: Indian Type Culture Collection
JA	: Jasmonic acid
K_2HPO_4	: Dipotassium phosphate anhydrous
KBSFS	: Kuttanad Below Sea-Level Farming System
KH_2PO_4	: Potassium dihydrogen phosphate
KV	: Kilovolts
LB	: Luria bertani
LC-MS/MS	: Liquid Chromatography-Tandem Mass Spectrometry
LOX	: Lipoxygenase
LPS	: Lipopolysaccharide
LS	: Longitudinal section
M	: Molar
m	: metres
MAMPs	: Microbe-associated molecular patterns
MAPKs	: Mitogen-activated protein kinases
MEGA	: Molecular Evolutionary Genetics Analysis
mg	: milligram
$MgSO_4.7H_2O$	: Magnesium sulphate heptahydrate
MHA	: Mueller Hinton agar
min	: minutes
mL	: millilitres
mM	: millimolar
mm	: millimetres
MRM	: Multiple reaction-monitoring mode
MS	: Mass Selective Detector
N	: Normal
NA	: Nutrient agar
NaOH	: Sodium hydroxide
NCBI	: National Center for Biotechnology Information
NGS	: Next generation sequencing
NH_3	: Ammonia
NIIST	: National Institute for Interdisciplinary Science and Technology
NIST	: National Institute of Standards and Technology

NLRs : Nucleotide-binding and leucine-rich repeat receptors

nm : nanometres

NO_3^- : Nitrate

NPR1 genes : Nonexpressor of Pathogenesis-Related Genes 1

OD : Oil dispersion

OF : Oil miscible flowable concentrate

ORP : Operational Research Project on Integrated Rice Pest Control

P : Phosphorus

pA : picoamperes

PAL : Phenylalanine ammonia lyase

PAMP : Pathogen-associated molecular pattern

PBS : Phosphate buffer saline

PCR : Polymerase chain reaction

PDA : Potato dextrose agar

PGP : Plant growth-promoting

PGPR : Plant growth promoting rhizobacteria

pH : Potential of hydrogen

PIA : Pseudomonas isolation agar

PIPES : 1,4-Piperazinediethanesulfonic acid

pKa : Acid dissociation constant

PNS : Plant nutrient solution

POX : Peroxidase

PPO : Polyphenol oxidase

PR : Pathogenesis-related

PRR : Pattern recognition receptors

PSMs : Phosphate solubilizing microorganisms

PTI : PAMP-triggered immunity

RLCKs : Receptor-like cytoplasmic kinases

RNAs : Ribonucleic acids

ROS : Reactive oxygen species

rpm : revolutions per minute

s : seconds

SA : Salicylic acid

SAP : Shrimp alkaline phosphatase

SAR	: Systemic acquired resistance
SCs	: Suspension concentrates
SEM	: Scanning electron microscopy
ShB	: Sheath Blight
SI	: Solubilization Index
SPSS	: Statistical Package for the Social Sciences
SU	: Suspension
TDS	: Total dissolved salts
TFs	: Transcription factors
TLC	: Thin-layer chromatography
TLRs	: toll-like receptors
TSA	: Tryptone soya agar
ULV	: Ultralow volume
V	: Volts
VOCs	: Volatile organic compounds
WAKs	: Wall-associated kinases
WG/WDG	: Wettable/water dispersible granules W
WPs	: Wettable powders
YMB	: Yeast mannitol agar

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Chapter 1

Introduction and Review of Literature

1.1.Introduction

Rice (*Oryza sativa* L.) is an important cereal crop and staple food for more than 50% of the world's population (Kumar et al., 2020; Raman, 2021). Asia accounts for 90% of the total rice production, and India is the second largest producer, recording 105 million tonnes from 44 million ha in 2021 (Surendran et al., 2021b). Rice production has a vital role in the economic landscape of many countries, especially in Asia. This agricultural sector serves as the principal means of income and employment opportunities for over 200 million households residing in developing countries (Muthayya et al., 2014). The world faced a record rise in rice production during the Green Revolution, which introduced novel rice varieties and sophisticated farming techniques. In India's case, the Green Revolution's initiation in the 1960s was motivated by the urgent need to alleviate the profound poverty that had plagued the nation between 1947 and 1960. Notably, the introduction of high-yielding monohybrid crops capable of withstanding the application of chemical fertilisers played a pivotal role in this transformative process. Consequently, the adoption of chemical fertilisers witnessed a significant upsurge after the advent of the Green Revolution, particularly evident in the period spanning from 1981 to 1982. Although these advancements yielded superior results, they also left us with more significant issues of grave concern. The excessive use of chemical fertilisers resulted in the physical and chemical deterioration of the soil, manifesting in the alteration of its natural flora, escalation of alkalinity and salinity levels, soil acidification, and reduction of soil organic matter (Adeleke et al., 2022; Nelson Eliazer et al., 2019).

1.2.Rice production in Kerala

Rice cultivation spans almost half of the country's states, Kerala being one of them. Traditionally, rice cultivation has held a prominent position within the agrarian economy of Kerala, serving as the primary food source for the people. The visually stunning paddy fields, lush with vibrant greenery, have long been a captivating feature of Kerala's distinctive landscape. However, in recent times, there has been a decline in paddy cultivation in Kerala, resulting in a significant decrease in agricultural productivity. This unfortunate trend has exacerbated issues such as food insecurity, rural unemployment, environmental degradation, and ecological damage. Currently, rice production in Kerala has been surpassed by the cultivation of coconut and rubber, ranking it as the third most important crop. This decline in rice cultivation must be reversed to ensure a sustainable future, as Kerala's rice production only

accounts for 15 per cent of the total demand. Furthermore, preserving paddy fields is crucial for maintaining the delicate balance of the region's environment (Thomas, 2011).

In Kerala, Palakkad and Alappuzha are the two prominent rice producing districts. More precisely, the region of Kuttanad is endowed with extensive backwaters and also bestowed upon it the esteemed title of the 'Rice Bowl of Kerala" (Lakshmi, 2018). The Kuttanad wetland is distributed across three districts, namely Alappuzha, Kottayam, and Pathanamthitta. The geographic area spans from 9°17' to 9°40' N latitude and 76°19' to 76°33' E longitude (Fig 1.1). Kuttanad is a remarkably fertile area worldwide where rice is cultivated below sea level, approximately at depths ranging from 4 to 10 feet. As a result, the Kuttanad Below Sea-Level Farming System (KBSFS) has emerged as a distinctive practice characterised by expansive flat paddy fields covering an area of 50,000 hectares, predominantly composed of reclaimed delta swamps (FAO, 2012). The farmers in this region have gained significant renowned for their biosaline farming techniques. Recognising its global significance, the Food and Agriculture Organisation (FAO) has designated Kuttanad farming as a Globally Important Agricultural Heritage System (GIAHS) (Nair, 2013). However, despite being hailed as a "biodiversity paradise," the Kuttanad wetland ecosystem, which serves as the rice granary, faces serious challenges, including rice diseases. One such disease that affects rice plant is sheath blight disease, which causes up to 50% loss of yield, causing it a yield-limiting disease in paddy.

1.3.Diseases of rice plants

Plant disease can be defined as "anything that prevents the plant from performing to its maximum potential". These factors can be classified into abiotic and biotic categories. Abiotic factors encompass nutritional deficiency, soil composition, ice, salt injury, and sun scorch. Unlike biotic factors, abiotic factors are non-infectious and cannot spread from one plant to another. On the contrary, biotic factors that causes disease are infectious, and are attributed to the activities of living organisms such as fungi, bacteria, viruses, protozoa, insects, and parasitic plants, commonly referred to as pathogens. Pathogens have the capability to infect any part of the plant, including the root, stem, and leaf (Cropwatch, 2023).

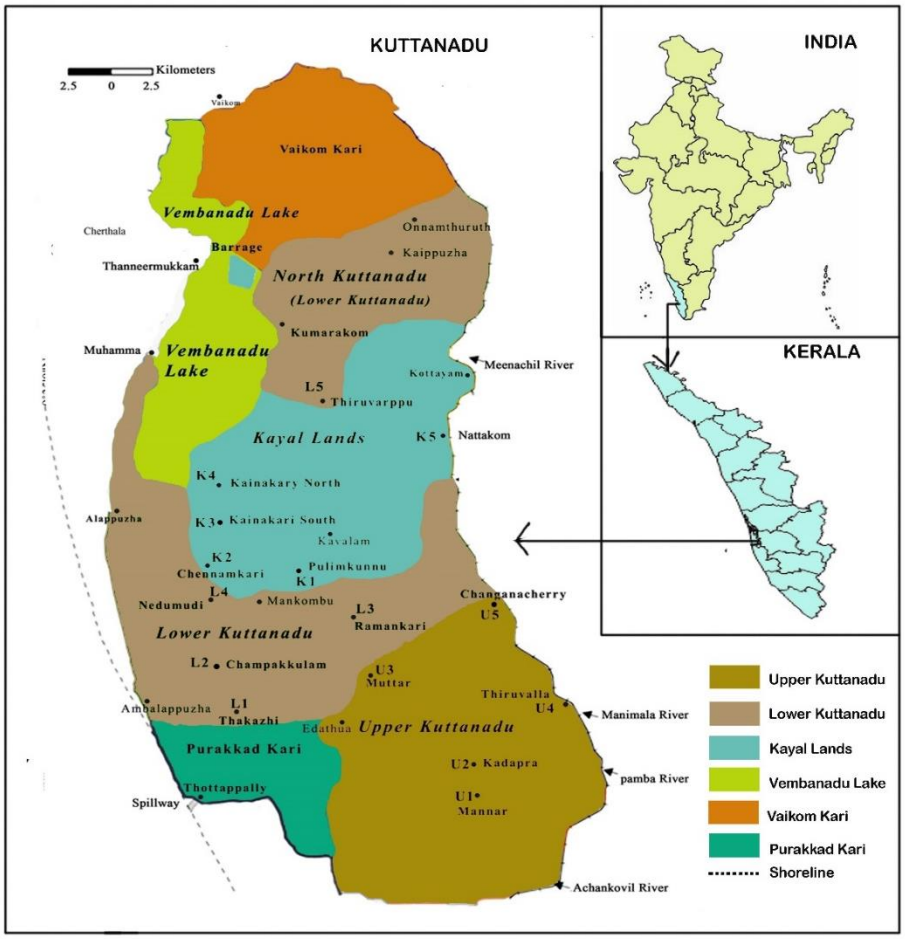

Fig 1.1 Geographical area of Kuttanad, Kerala, India (modified from Vijayan and Ray, 2015)

With the advancement of agriculture, the prevalence of infectious diseases has emerged as a significant determinant impacting crop yields and economic efficiency (Nazarov et al., 2020). Moreover, the productivity of rice is susceptible to the impact of different diseases. It has been reported that a considerable proportion of rice production in Asia, precisely 10-15%, is adversely affected by severe rice diseases (Shrivastava et al., 2019).

A variety of diseases have been observed to impact rice plants. Rice blast disease incited by *Magnaporthe oryzae*, Bacterial Leaf Blight (BLB) disease caused by *Xanthomonas oryzae*, and Sheath Blight (ShB) disease infected by *Rhizoctonia solani* are the most devastating diseases affecting rice plants. It is worth noting that sheath blight has emerged as the most economically destructive disease in several major rice-growing countries worldwide (Shrivastava et al., 2019). Sheath blight disease was initially documented in Japan in 1910. Subsequently, it disseminated across various rice growing regions. As the disease spread to

other Asian countries, it acquired multiple names such as 'Oriental leaf and sheath blight', 'Pellicularia sheath blight', 'Sheath blight', 'Sclerotial blight', and 'Banded blight of rice' (Singh et al., 2019). ShB disease was observed in India by Butler in 1918 (Raman, 2021). The severity of the disease is contingent upon the growth stage of the rice plant during the time of infection, cultivation techniques, the susceptibility of the rice variety, and the utilisation of nitrogen fertilisers. Additionally, the disease is exacerbated by a combination of high humidity (approximately) and warm temperatures (28-32°C) (Singh et al., 2019). Heavy yield loss is a significant constraint of ShB disease (Raman, 2021). According to the findings provided by the International Rice Research Institute (IRRI), none of the rice varieties tested (30,000 rice lines) against the ShB disease exhibited a consistent and dependable resistance level. The difficulty in breeding rice for resistance stems from a dearth of germplasm with complete resistance (International Rice Research Institution, 2023). Therefore, a variety with less susceptible and moderate resistance can opt or alternative methods, such as applying chemical fungicides, which have been employed to prevent ShB disease (Raman, 2021).

Rice diseases have had a devastating impact on rice production and the livelihoods of farmers. To address the problem of pests in the Kuttanad *padasekharams,* a project known as the *Operational Research Project on Integrated Rice Pest Control in Kuttanad* (ORP) was implemented in this region from 1975 to 1992. This endeavour was a collaborative effort between the Kerala Agricultural University and the State Agricultural Department, with funding provided by the Indian Council of Agricultural Research (ICAR). The project employed a comprehensive approach involving the application of genetic, cultural, and biological methods, in addition to the use of chemical pesticides. Starting from the 1970s, the local market became inundated with chemical pesticides and insecticides, leading farmers to use them indiscriminately. Until recently, farmers would apply these pesticides up to four times during a single crop. According to the Indo-Dutch Mission, in the year 1987-88, a total of 293,660 litres of liquid pesticide and 190.97 tonnes of powder were used in the Kuttanad region. In addition to the permitted pesticides, the banned substances Dichloro Diphenyl Trichloroethane (DDT), as well as its derivatives Dichloro Diphenyl Dichloro ethylene (DDE) and Dichlorodiphenyldichloroethane (DDD), were frequently used in paddy fields even after the flowering stage. The farmers did not understand the harmful effects of these highly toxic pesticides well (Thomas, 2002). The excessive use of toxic pesticides has not only disrupted the ecological balance but has also harmed natural biocontrol agents, such as beneficial microbes. This leads to additional complications as the soil lacks inherent suppressiveness,

such as antagonistic microorganisms that regulate pathogenic microbes. This negative effect can be reversed by implementing environmentally friendly practices. Substituting chemical fertilisers with organic fertilisers containing beneficial microorganisms, the degraded soil can be restored to a more advanced functional state (Nature with us, 2021).

1.4.Sheath blight disease and *R. solani*

In most instances, *R. solani* infection arises as a consequence of the presence of sclerotia from the previous cropping season. Initially, the fungus manages to survive as sclerotia under conditions that are unfavourable and retains its viability for 2-3 years. The sclerotia, which are initially white, transformed to a dark brown following maturation due to the synthesis of melanin in the cell wall. Melanin, being a phenolic compound that has undergone oxidation and possesses hydrophobic properties, imparts reduced permeability to the cell wall, thereby safeguarding the cells against biological degradation. Furthermore, the sclerotia are replete with a nutrient reserve consisting of glycogen, lipids, proteins, and polyphosphates within the cytoplasm. This nutrient reserve serves as an energy source during extremely unfavourable conditions and facilitates reinfection. When conditions become favourable, the sclerotia infiltrate the rice plant either through the cuticle or stomata, thereby instigating the production of infection cushions or lobate appressoria. Subsequently, the hyphae spread horizontally across the plants, thus propagating the disease at a rapid pace. Once inside the plant, *R. solani* generates RS toxin, a combination of N-acetyl glucosamine, N-acetyl galactosamine, glucose, and mannose. The pathogen also releases pathogen effectors such as glycosyltransferase, peptidase inhibitor I9, and cytochrome C oxidase CtaG/cox11, all of which are associated with the virulence of the pathogen. The formation of appressoria instigates enzymatic degradation, resulting in necrosis of the host plant and facilitating the colonisation of the fungus. The pathogen colonises various parts, starting from the leaf sheath and extending to the leaf blades, tillers, and panicle. This colonisation is characterised by the presence of green ellipsoid lesions (0.5 and 3 cm), which undergo bleaching in the centre while maintaining a purple brown border. Ultimately, these lesions merge, affecting the entire stem and leaves. This extensive colonisation results in stem lodging, which in turn disrupts the photosynthetic activity of the plant. Consequently, the process of grain filling is significantly hindered, ultimately leading to the death of the plant. Following the harvest, the sclerotia, which are present in the soil, persist for a period of three years as a reservoir of infection for subsequent crop seasons (Singh et al., 2019; Tezpur University, 2023) (Fig 1.2).

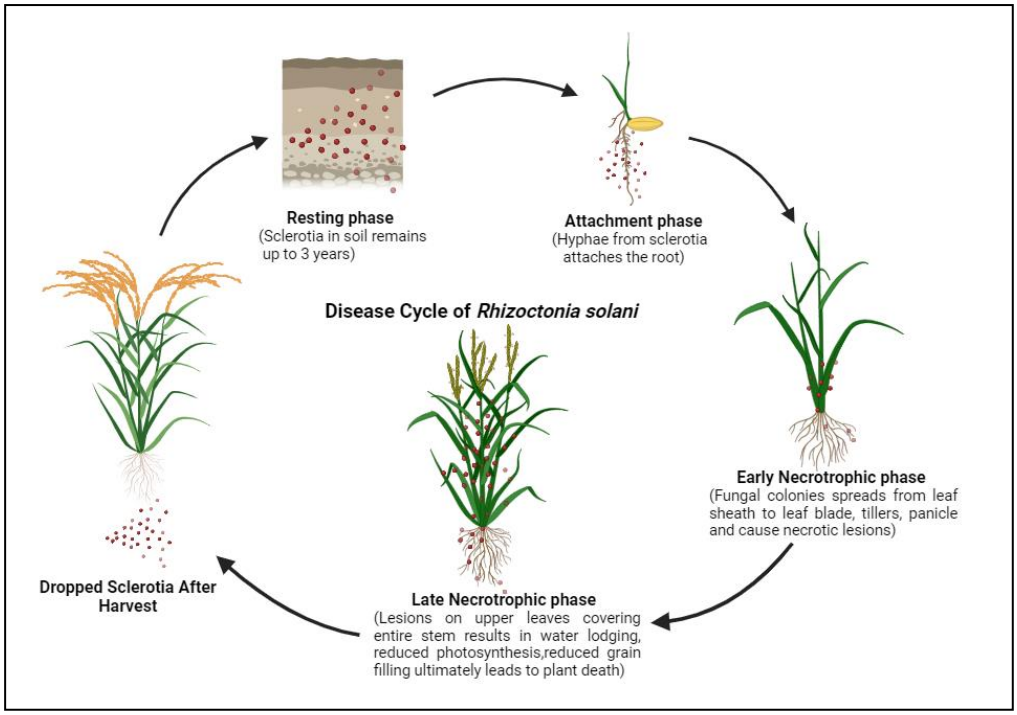

Fig 1.2 Disease cycle of *R. solani* in rice plant comprises resting sclerotia, attaching and germination of sclerotia in rice plants, early necrotrophic phase, late necrotrophic phase and shedding the sclerotia after harvest (image drawn using BioRender software)

Eco-friendly methods such as employing bioinoculants derived from plant-based microbes have proven to be suitable for enhancing both soil quality and agricultural productivity without agrochemicals. Moreover, scientific evidence supports the use of endophytic microbes as bioinoculants to promote soil health and enhance crop productivity (Adeleke et al., 2022). In the scenario of rice plant, which established a wide range of symbiotic relationships with various bacterial species, spanning from the root system to the apex of the plant, thereby facilitating the assimilation and utilisation of essential nutrients from the surrounding soil. Some notable examples of these bacteria include *Bacillus, Rhizobium, Burkholderia, Methylotropicus, Pantoea, and Pseudomonas* (Pal et al., 2019).

1.5.Endophytes

A wide array of microorganisms can be found worldwide, each carrying out distinct functions. However, their minuscule size makes them invisible to the naked eye and can only be observed using a microscope. Hence, these entities are referred to as microorganisms or microbes. These microorganisms can be found in the atmosphere, soil, or within living organisms such as plants

and animals. Several microbes inhabit the vicinity of the plants, specifically close to the roots, the surface of the plant, and within the plants themselves. The microorganisms residing near the root area, or rhizosphere, are referred to as rhizobacteria, while those coexisting with the plant make up the plant microbiome. This microbiome encompasses a vast array of diverse microorganisms from various plant components, including the roots, stems, leaves, flowers, fruits, and seeds. The microbial populace residing externally on the plants is designated epiphytes, whereas the microorganisms occupying the tissues or organs internally are termed endophytes (Kumar et al., 2020). Microbial endophytes have been discovered in nearly all plant species. Their distinctive capacity to invade, coexist, and interact with the host plant renders them unique in the world. These microorganisms assume critical functions in fostering the growth of plants and enabling their successful acclimatisation to diverse ecological conditions (Verma et al., 2021).

The term endophyte was initially coined by De Bary in 1866 and described in terms of their types and relationships (Khare et al., 2018). Endophytes encompass a diverse range of microorganisms, including bacteria, fungi, and actinomycetes. To elaborate, endophytes refer to microbes obtained from healthy plant tissue, undergo superficial disinfection, and do not inflict harm upon the host (Bhattarai et al., 2015). Instead, they serve as a reservoir for numerous biologically active compounds such as terpenoids, saponins, steroids, quinones, alkaloids, and phenolic acids, which possess antimicrobial, anticancerous, and insecticidal properties (Gouda et al., 2016). Endophytes are highly host-specific. The occurrence and efficacy of these endophytes are subject to the influence of host genotypes, geographical circumstances, and soil characteristics. Presumably, endophytes possess distinct roles, adaptations, specialisation, and competence, as the plant provides a unique niche. Specialised endophytes possess the ability to colonise and endure within the reproductive organs of the host plant. Metagenomic analysis of bacterial endophytes present in roots predicted the metabolic properties and other important characteristics associated with the endophytic lifestyle of these bacteria (Walitang et al., 2017).

1.6.Types of endophytes

Endophytes are ubiquitous in plants, with diverse groups exhibiting unique characteristics and functions. The most extensively studied are fungal endophytes, known for their symbiotic relationships with various plant species. Bacterial endophytes are another significant group contributing to plant growth promotion, nutrient acquisition, and defence against pathogens.

While fungal and bacterial endophytes are the most extensively studied, other groups of endophytes also play important roles in plant-microbe interactions. Actinobacteria, for instance, are known for their ability to produce bioactive secondary metabolites with diverse biological activities. Algal endophytes contribute to nutrient cycling and carbon fixation within plant tissues. Endophytic viruses, protozoa, and nematodes are less commonly reported but can still influence plant health and physiology (Aleynova & Kiselev, 2023). Based on their life strategies, the endophytic communities have been categorised as either obligate or facultative endophytes, with both types maintaining associations with their respective host plants. Obligate endophytes depend on the host's metabolism for their growth, survival, and transmission to other plants, which can occur vertically or through vectors. On the other hand, facultative endophytes spend a certain period of their life cycle outside the host but remain connected to the surrounding soil environment and atmosphere (Gouda et al., 2016). As a result, the life cycle of a facultative endophyte is considered biphasic, as it alternates between the plant and its environment. From a more extreme perspective, bacterial phytopathogens can function as either obligate or facultative endophytes, with their avirulent forms persisting in certain plants (Hardoim et al., 2008). A notable example is the avirulent form of *Ralstonia solanacearum* biovar 2, which can be found in water systems but can also exist in tomato plants without causing harm for an extended period (Overbeek et al., 2004).

1.7.Functions of endophytes

The prime feature of endophytes is that they inhabit the host without causing any outward symptoms or expressions of their presence (Rana et al., 2021; White et al., 2021). They elicit plant growth promotion by various mechanisms such as nitrogen fixation, production of indole-3-acetic acid (IAA), phytohormones or enzymes, solubilisation of phosphate, iron chelation through siderophore production and suppressing the disease by preventing the pathogen through the production of antifungal or antibacterial compounds (Krishnamoorthy et al., 2020). These traits were classified as direct and indirect mechanisms (Ali et al., 2017). Direct mechanisms include the traits that directly promote plant growth, such as the production of IAA, gibberellic acid, 1-aminocyclopane-1 carboxylic acid (ACC), zinc and so on, whereas, in indirect mechanism, endophytes act as a biocontrol agent to improve the plant growth (Ali et al., 2021). These endophytes also produced specific secondary metabolites to control the attack of pathogens on plants. The native bacterial endophytes perform better as biocontrol agents as they have wider adaptability with the host plant in the same climatic conditions (Kumar et al., 2020). Other benefits include promoting host plant growth in nutrient-limited conditions

(Kandel et al., 2017). It has been found that endophytes isolated from nutrient-deprived regions and extreme climates such as the Indian Himalayas also exhibit plant growth-promoting characteristics (Rana et al., 2021; Walitang et al., 2017).

1.8.Bacterial endophytes

Research on the presence of endophytic microorganisms within plant structures was initiated during the 1870s by Pasteur and his research team. Despite the prevailing inclination to perceive these microbes as pathogenic, Perotti, in 1926, made the first documentation of non-pathogenic bacterial strains residing within the root tissue of plants (Pal et al., 2019). This was identified as endophytes. Most of the endophytes that have been later studied are fungi derived from economically important crops and grasses. Bacterial endophytes have been studied to a much lesser extent when compared to their fungal counterparts. This may be attributed to the fact that the former group is more abundant in plants. Nevertheless, bacterial endophytes have been successfully isolated from various host plants (Ali, 2014). Table 1.1 provides a list of different bacterial species, including *Acinetobacter, Bacillus, Bravibacillus, Burkholderia, Corynebacterium, Curtobacterium, Enterobacter, Erwinia, Microbacterium, Pantoea, Paenibacillus, Pseudomonas* among others, that have been isolated from different plants.

Table 1.1 Diversity of bacterial endophytes from various plants

Sl. No.	Plant	Endophyte isolated	Plant parts	Reference
1.	*Gynura procumbens*	*Psuedomonas resinovorans,* *Paenibacillus polymaxa,* *Acinetobacter calcoaceticus*	Leaves	(Bhore et al., 2010)
2.	Sugarcane (*Saccharum*)	*Bravibacillus* sp. *Curtobacterium* sp. *Enterobacter* sp. *Erwinia* sp. *Klebsiella* sp. *Pantoea* sp. *Pseudomonas* sp. *Staphylococcus* sp.	Leaves and stem	(Magnani et al., 2010)
3.	*Pairs polyphylla* var. *yunnanensis*	*Staphylococcus epidermids* *Achromobacter xylosoxidans* *Lelliottia amnigena* *Achromobacter spanius* *Achromobacter marplatensis*	Rhizome	(Tao et al., 2022)

		Serratia plymuthica *Alcaligenes sp* *Klebsiella aerogenes* *Pantoea agglomerans* *Bacillus megaterium*		
4.	*Artemisia annua* L.	*Staphylococcus equorum* *Kocuria rosea* *Glutamicibacter creatinolyticus* *Pseudomonas stutzeri* *Planomicrobium glaciei* *Microbacterium saperdae* *Corynebacterium humireducens* *Pseudomonas brassicacearum* *Corynebacterium glutamicum* *Bacillus subtilis* *Sphingobacterium daejeonense* *Kocuria atrinae*	Whole plant	(Husseiny et al., 2021)
5.	Soybean (*Glycine max* (L.) Merril)	*Stenotrophomonas maltophilia* *Alcaligenes aquatili*	Stem and root	(Jaiswal et al., 2023)
6.	*Teucrium polium* L.	*Bacillus cereus* *Bacillus subtilis*	Leaves	(Hassan, 2017)
7.	Onion	*Burkholderia phytofirmans* PsJN	Root	(Weilharter et al., 2011)
8.	Holy basil (*Ocimum tenuiflorum* L.)	*Bacillus altitudinis* *Bacillus tequilensis* *Bacillus safensis* *Bacillus haynesii* *Bacillus paralicheniformis* *Bacillus pacificus* *Bacillus siamensis*	Root, leaf, and shoot	(Sahu et al., 2020)
9.	*Paullinia cupana* var. *sorbilis* (Mart.) Ducke	*Serratia marcescens*-R381 *Bacillus cereus*-R8 *Bacillus aerophilus*-R114 *Bacillus nodosa-R*195 *Rhodopseudomonas boonkerdii*-R102 *Pantoea ananatis-R*309 *Nocardioides aromaticivorans*-R21 *Pseudomonas plecoglossicida*-R382 *Streptomyces thermoviolaceus* -R183 *Pantoea coffeiphila*-R34	Root, leaf, and seed	(Liotti et al., 2018)

10.	Cucumber (*Cucumis sativus)*	*Herbaspirillum lusitanum* *Acinetobacter johnsonii* *Stenotrophomonas rhizophila* *Agrobacterium tumefaciens* *Rhizobium radiobacter* *Micrococcus yunnanensis* *Paenibacillus graminis* *Bacillus pumilus* *Bacillus cereus* *Bacillus muralis* *Terribacillus goriensis*	Root, shoot and leaf	(Gamalero et al., 2020)
11.	Sorghum (*Sorghum bicolor)*	*Bacillus cereus* *Pseudomonas migulae* *Pseudomonas* sp. *Pseudomonas brassicacearum* *Paenibacillus lautus* *Brevibacterium frigoritolerans* *Bacillus anthracis* *Paenibacillus illinoisensis* *Bacillus muralis* Bacillaceae bacterium *Micrococcus luteus*	Root	(Gamalero et al., 2020)
12.	Tomato (*Solanum lycopersicum)*	*Bacillus safensis* Bacillaceae bacterium *Acinetobacter lwoffii* *Bacillus cereus* *Bacillus thuringiensis* *Bacillus muralis* *Bacillus megaterium* *Bacillus tequilensis* *Bacillus aerophilus* *Acinetobacter johnsonii* *Microbacterium schleiferi* *Bacillus subtilis* *Paenibacillus* sp. *Bacillus niacin* *Kochuria palustris*	Root, shoot and leaf	(Gamalero et al., 2020)

Bacterial endophytes are isolated from the above and below ground parts of plants, with a higher concentration in the roots. They exhibit a high level of diversity and are characterised by their polyphyletic nature (Burragoni & Jeon, 2021). In a study conducted by Hardoim et al., the diversity of endophytes was investigated using a meticulously curated database

encompassing all available 16S rRNA gene sequences (limited to those longer than 300bp) of cultured isolates and uncultured organisms. The findings revealed a total of 21 bacterial phyla, with only four phyla accounting for 96% of the observed diversity. These prominent phyla include *Proteobacteria* (54%), *Actinobacteria* (20%), *Firmicutes* (16%), and *Bacteroidetes* (6%) (Hardoim et al., 2015). Most of the cultured isolates were found to be members of the *Proteobacteria* phylum, while *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* were less frequently observed. The genera *Bacillus* and *Pseudomonas* were the most prevalent in agricultural crops. The occurrence of various endophytic bacteria is primarily influenced by the genetic makeup of the plant and bacteria, as well as abiotic and biotic environmental factors (Miliute et al., 2015).

1.9.Plant-Endophyte relationships

Plants possess the remarkable capability to engage with diverse microorganisms, including bacteria, fungi, archaea, and more. The pivotal occurrence of early land plants establishing symbiotic relationships with terrestrial fungi stands as a crucial milestone in the development of terrestrial ecosystems (Papik et al., 2020). These relationships may initiate the endophytic nature of microorganisms. The endophytes frequently originate from the soil and initially infect the plants by colonising through the cracks formed in the junction of lateral roots, subsequently spreading rapidly in the intercellular spaces of the root. Additional entry points may occur via regions of wounds caused by microbial or nematode pathogens or through stomatal openings. As colonisation through root cracks is of great importance, endophytes that infect plants from the soil must be able to colonise roots effectively (Hardoim et al., 2008). When an endophyte infiltrates the plant, it is recognised by the plant via intercommunication of signal molecules. The endophytes chemotactically react to the root exudates of the host plant, which contain an abundance of biomolecules that attract beneficial microorganisms. Nutrient and water-rich exudates attract all sorts of microorganisms, while exudates abundant in flavonoids specifically facilitate the interaction between endophytes and root hairs. Numerous other root exudates, such as amino acids, sugars, organic acids, phenolic compounds, and other secondary metabolites, selectively entice mutualistic microorganisms, particularly endophytes (Khare et al., 2018).

In *planta*, endophytic microorganisms can inhabit both intercellular and intracellular spaces. Nevertheless, the organisation and composition of these endophytic communities are influenced by numerous biotic factors, including the developmental stage of the host plant, the

plant species or cultivar, and the interactions with other microbes associated with the host plant. Furthermore, abiotic factors such as seasonal variations, levels of radiation, fluctuations in temperature, and the chemical composition of the soil also exert an influence on the structure of these endophytic communities. Endophytes fulfil various functions within plants, encompassing the acquisition of nutrients via nitrogen fixation, the solubilisation of phosphate, the production of siderophores and phytohormones, as well as protection against biotic stresses, such as phytopathogens and abiotic stresses, including drought, water, heat, cold, salinity, and heavy metal pollution (Papik et al., 2020) (Fig 1.3).

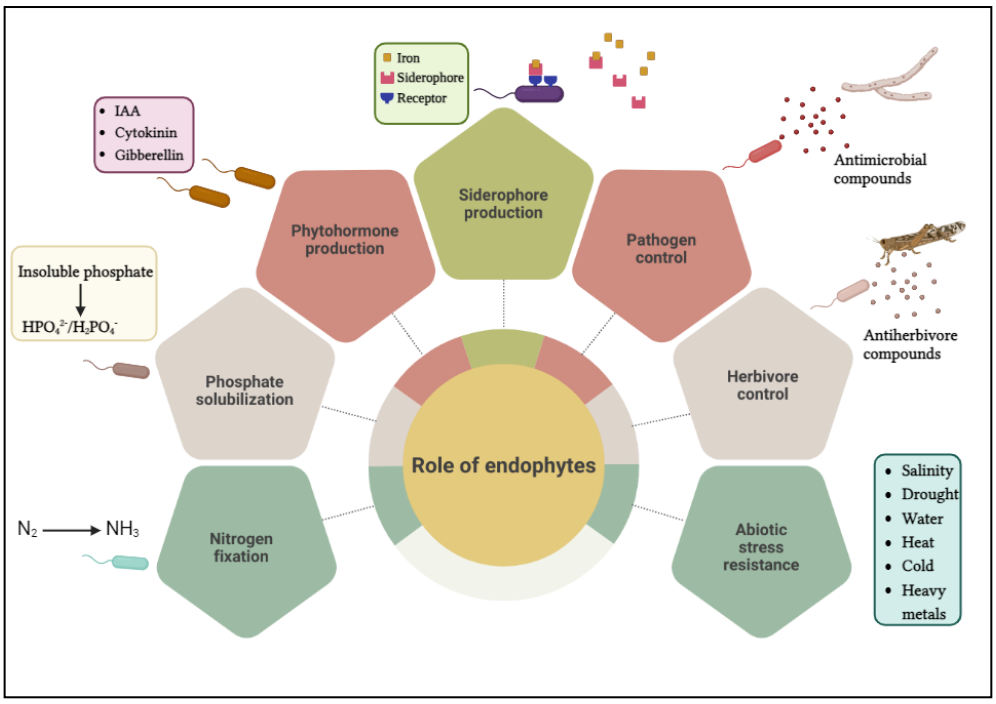

Fig 1.3 Role of endophytes in plants includes nitrogen fixation, phosphate solubilisation, production of phytohormone, siderophore, control of pathogenic microbes, herbivores, and abiotic stresses (image created using BioRender software)

1.10. Endophytes in rice plant

Rice plants also host various endophytes, which include fungi and bacteria. The endophytic bacteria that have been isolated from various sections of the rice plant are enumerated in Table 1.2.

Table 1.2 Bacterial endophytes isolated from various parts of rice plant

Sl.No.	Endophytic bacteria	Plant part	Reference
1.	*Micrococcus yunnanensis* RWL-2 *Micrococcus luteus* RWL-3 *Enterobacter soli* RWL-4 *Leclercia adecarboxylata* RWL-5 *Pantoea dispersa* RWL-6 *Staphylococcus epidermidis* RWL-7	Seed (*Oryza sativa* L. var. Jin so mi)	(Shahzad et al., 2017)
2.	*Enterobacter aShBuriae* *Pantoea dispersa* *Pseudomonas putida*	Seed (*O. sativa* L.)	(Verma et al., 2017)
3.	*Curtobacterium citrum* (B8, B9, B14, B18, B26)	Stem (*O. sativa* Bu-24, *O. eichingeri* Wl521, *O. longiglumis* Wl228, *O. rujipogon* Wl989, *O. punctata* Wl564)	(Elbeltagy et al., 2000)
	Microbacterium sp.	Stem *(O. officinalis* WOOl2)	
	Aureobacterium testaceum	Stem *(O. rujipogon* Wl989)	
	Corynebacterium aquaticum	Stem *(O. punctate* Wl564)	
	Azospirillum amazonense	Stem (*O. alta* WOOl8)	
	Sphingomonas adheasiva	Stem (*O. rufipogon* W1964)	
	Rhodopseudomonas palustris	Stem (*O. ridleyi* WOOOl)	
	Herbaspirillum seropedicae	Seed (*O. meridionalis* WI627)	
	Pan toea ananas	Seed (*O. alta* WOO 17)	
	Cytophagales str.	Stem (*O. sativa* Bu-24)	
	Flavobacterium gleum	Stem (*O. alta* WOO17)	
	Klebsiella oxytoca	Seed (*O. sativa* Bu-24)	
	Methylobacterium sp (BI, B13, B15, B20, B21, B22, B25, B28, B32, B33, B34, B38)	Stem (*O. sativa* SC-41) Stem (*O. longiglumis* WI228) Stem (*O. brachyantha* W0656) Stem (*O. sativa* 1-10 I) Stem (*O. sativa* Bu-24) Leaf sheath (*O. sativa* SC-41) Stem (*O. latifolia* WOO19) Stem (*O. longiglumis* WI220) Stem (*O. rufipogon* WI989) Stem (*O. minuta* W1318) Stem (*O. rufipogon* WI964) Seed (*O. meridionalis* WI627)	
4.	*Micrococcus* *Chryseobacterium* *Flavobacterium*	Root (*O. sativa*)	(Raweekul et al., 2016)

	Myroides *Pedobacter* *Bacillus* *Fictibacillus* *Halobacillus* *Paenibacillus* *Staphylococcus* *Acinetobacter* *Citrobacter* *Cronobacter* *Dickeya* *Enhydrobacter* *Enterobacter* *Escherichia* *Novosphingobium* *Pseudomonas* *Sphingomon*		
	Curtobacterium *Microbacterium* *Chryseobacterium* *Mucilaginibacter* *Bacillus* *Fictibacillus* *Lactococcus* *Lysinibacillus* *Staphylococcus* *Acinetobacter* *Aeromonas* *Burkholderia* *Enterobacter* *Klebsiella* *Novosphingobium* *Ochrobactrum* *Pantoea* *Pseudacidovorax* *Pseudomonas* *Sphingomonas*	Stem (*O. sativa*)	
5.	*Burkholderia* sp. KJ006	Root (*Oryza sativa* L.)	(Kwak et al., 2012)

1.11.Management of sheath blight disease through endophytes

Earlier, sheath blight disease in rice was managed with chemical pesticides, which had harmful environmental effects. Therefore, a new approach involving alternative biocontrol agents, especially the endophytes, has been adopted. Endophytic bacteria interact closely with host plants, potentially serving as efficacious biocontrol agents in sustainable crop production. *Bacillus*, a frequently encountered bacterium, exhibits the propensity to colonise plants as endophytes, a capability that could assume a pivotal function in the biocontrol against phytopathogens (Nagendran et al., 2014). Various reports suggest the use of *Bacillus* to control sheath blight disease in rice. For instance, a report by Zheng et al. demonstrated the effectiveness of *Bacillus velezensis* isolated from *Fraxinus hupehensis* in controlling sheath blight disease in rice plant (Zheng et al., 2021). Another study explained the efficacy of antifungal metabolites produced by the endophyte *Lysinibacillus sphaericus* KJ872548 against the sheath blight pathogen *R. solani* (Shabanamol et al., 2021). *B. subtilis* (EBPBS4) also showed an inhibiting effect against *R. solani* and reduced sheath blight disease with enhancement in the growth and yield of rice plants (Durgadevi et al., 2015). Apart from *Bacillus*, *Pseudomonas aeruginosa* has biocidal and plant growth-promoting characteristics. A recent study by Rath and Danger revealed that *Pseudomonas aeruginosa* can restrict the growth of the ShB pathogen while promoting the growth of rice plants (Rath & Danger, 2018). One more study disclosed four endophytic bioactive isolates, including *Streptomyces* sp. (AcRz21), *Alkalihalobacillus* sp. (PtL11), *Bacillus* sp. (TgIb5), and *Priestia* sp. (TgIb12) derived from medicinal plants possess the capacity to augment the root and shoot biomass in both pathogen challenged and pathogen free field conditions. Furthermore, these isolates effectively mitigate the sheath blight disease (Khunjamayum et al., 2022). Research has also shown that endophytic fungi, namely *Trichoderma virens* and *Aspergillus fumigatus*, effectively reduce the sheath blight disease and enhance the phenotypic characteristics of rice plant. These characteristics encompass plant height and fresh and dry weight compared to infected plants (Safari Motlagh et al., 2022).

1.12.Mechanism of disease resistance

Plants are prone to pathogen attack by various pathogenic microorganisms, herbivores, and insects. In response to such attacks, plants activate their defence mechanisms. The disease resistance is underpinned by numerous functional processes and systems, ultimately impacting the infiltrated pathogen. The plant immune system depends on its capacity to identify

pathogens, execute signal transduction, and initiate defensive responses *via* various pathways encompassing numerous genes and their corresponding products. The initial line of defence constitutes mechanical barriers, such as the cuticle, cell wall and wax layer, which impede the entry of fungal pathogens. Pathogens produce cutinases to degrade the plant surface cutin. These cutin derivatives stimulate the plant's defence response to the pathogen's elicitors. Once the cuticle is breached, the pathogen exerts mechanical and hydrolytic pressure on the plant cell wall. Fungal pathogens produce multiple enzymes that degrade the cell wall, including polygalacturonases, cellulases, xylanases, and proteinases. The resulting cell wall fragments have the ability to further stimulate the defence response in plants (Glazebrook, 2005; Huckelhoven, 2007). Oligogalacturonic acid derived from the activity of polygalacturonase induces defence responses such as the synthesis of antibacterial proteins and reactive oxygen species (ROS) (D'Ovidio et al., 2004). Moreover, plants are also capable of identifying fungal polygalacturonase. An exemplary instance of this is the activation of plant defence independent of enzymatic activity by endopolygalacturonase 1 from *Botrytis cinera* (Poinssot et al., 2003). The polygalacturonase inhibitor protein in the host organism reacts with the fungal enzyme in the plant cell wall. This interaction relies on the leucine-rich repeats of the inhibitor protein, which bind to the hydrolases and alter the molecular structure of pectin fragments, producing more elicitor-active fragments. Consequently, the enzyme that inhibits polygalacturonase combines cell wall protection with communication to the symplast[1] by compelling the fungal enzyme to release signal molecules.

When a pathogen undergoes apoplastic[2] growth, it liberates nonspecific exogenous and endogenous elicitors, encompassing monomeric and oligomeric fragments of the cuticle, cell wall and conserved structures of pathogen like fungal chitin, flagellin, cryptogein, and other substances. The plant recognises this nonself activity in the apoplast and triggers an innate defence response. The conserved structures of the pathogen, the pathogen-associated molecular pattern (PAMP), were detected by toll-like receptors (TLRs) and pattern recognition receptors (PRR) leading to PAMP-triggered immunity (PTI) or first layer of immune defence (Li et al., 2020). Wall-associated kinases (WAKs) located on the cellular membrane are responsible for

[1] Symplast is the continuous network of cytoplasm that connect all living cells in a plant. It is composed of cytoplasm and the plasmodesmata

[2] Apoplast is non-living place outside the cell membrane. It composed of cell wall and intercellular spaces

the detection of damage-associated molecular patterns (DAMPs), which arise as a consequence of cellular damage occurring during an infection (Andersen et al., 2018).

The secondary layer of defence commences with the recognition of pathogen avirulence (Avr) effectors by the nucleotide-binding and leucine-rich repeat receptors (NLRs), as well as other forms of cytoplasmic proteins. This process often culminates in establishing a robust, race-specific effector-triggered immunity (ETI). The PRR, NLRs, and WAKs were initiated signalling cascades to prevent further infection. Both the PTI and ETI pathways involve a multitude of defense-signalling genes. Among them are genes responsible for encoding enzymes involved in epigenetic regulation and protein degradation, receptor-like cytoplasmic kinases (RLCKs), mitogen-activated protein kinases (MAPKs), transcription factors (TFs), and various other signalling molecules. Additionally, the pathogen stimulates the expression of several pathogenesis-related (PR) genes and leads to the production of antimicrobial proteins, such as proteases, defensins, protease inhibitors, or enzymes that participate in the generation of ROS. Accumulation of ROS, antimicrobial proteins, and phytoalexins leads to hypersensitive reaction (HR) that manifests as rapid cell death occurring at or in close proximity to the site of infection to limit the pathogen spread. This localised cellular self-destruction serves to further impede the growth of the biotrophic[3] pathogen. Conversely, necrotrophic[4] pathogens exploit these deceased cells as a means to acquire nutrients that facilitate their invasive growth. Furthermore, this stimulation also facilitates the accumulation of secondary metabolites that contribute to cell wall cross-linking, as well as the deposition of callose and lignin (Andersen et al., 2018; Huckelhoven, 2007; Li et al., 2020; Mahlein et al., 2019). The intricate and precise immune system in plants has evolved as a result of the competition between the host and pathogens. This development enables beneficial microorganisms to stimulate immunity by selectively influencing the crucial components involved in the processes of PTI and ETI through the regulation of small RNAs (Yu et al., 2022).

1.13.Disease resistance through Induced Systemic Resistance

Plant disease resistance is of two kinds: localized and systemic. The localized resistance responds directly to an infection at the site of attack, whereas systemic resistance provides protection throughout the entire plant against future pathogen invasion. Systemic resistance

[3] Biotrophs are pathogen derive nutrient from the living cells
[4] Necrotrophs are pathogen derive nutrient from dead or dying cells

can be classified into two categories: induced systemic resistance (ISR) and systemic acquired resistance (SAR). ISR is induced by non-pathogenic microbes, while SAR is induced by pathogenic microbes. When a beneficial microbe colonises a plant, it triggers a physiological state known as "priming". Once priming is activated, the plant exhibits a more robust and quicker defence response against pathogens. This enhanced defence response is a characteristic feature of systemic resistance induced by beneficial microbes (Fig 1.4). The term SAR was introduced by Ross during the 1960s and represents a phenomenon wherein unaffected systemic parts of plants enhance their resistance in reaction to a localised infection occurring elsewhere within the plant (Pieterse et al., 2014). It is initiated by the production of a signalling molecule called salicylic acid (SA) at the site of infection. SA then spreads throughout the entire plant and activates PR genes. However, in 1991, three separate research groups independently observed that beneficial microbes induce defence responses in plants through ISR (Yu et al., 2022). In 1996, Pieterse et al. reported that ISR does not rely on SA or PR proteins but instead depends on the jasmonic acid (JA) and ethylene (ET) pathways (Pieterse et al., 1996). This distinction became the primary difference between SAR and ISR. However, subsequent reports have demonstrated the involvement of both SA and JA/ET pathways in ISR triggered by beneficial microbes, leading to a more complex understanding of the signalling pathways involved in ISR.

ISR is initiated by the action of various plant hormones in response to elicitors. These elicitors encompass microbe-associated molecular patterns (MAMPs[5]), volatile organic compounds (VOCs[6]), siderophores[7], and other substances that induce a response. PPRs in plants recognise MAMPs, while other receptors recognise other types of elicitors. Once perceived, these elicitors activate plant hormones, resulting in the initiation of a defence response. In certain instances, microbes themselves produce hormones such as auxin and cytokinin, which are recognised by plant hormone receptors and subsequently lead to changes in root physiology and morphology. The beneficial microbes that induce ISR do not directly activate the defence response but rather prime the plant to mount an intense and rapid defence response upon invasion by pathogens (Romera et al., 2019; Villena et al., 2018). Two factors must be considered to express ISR in plants: the time required for ISR development and the number of bacterial cells needed to initiate ISR. It is crucial that the population of beneficial

[5] MAMPs are microbe-associated molecular patterns produced by non-pathogenic microbes.
[6] VOCs are low molecular weight compounds produced by various biosynthetic pathways with high vapour pressure and can evaporate and disperse easily
[7] Siderophores are Fe chelating agents produced by bacteria to sequestrate Fe from the medium

bacteria reaches a minimum of 10^5 to 10^7 colony forming units (CFU) per gram of plant root for a sustained period (Bakker et al., 2013; Jankiewicz & Kołtonowicz, 2012). It is also worth noting that the abundance of microbial density in the rhizosphere is typically around 10^8 to 10^9 bacteria per gram, but their diversity is generally lower compared to the bulk soil due to the plant's secretion of exudates that selectively enhance or suppress certain members of the microbial community, thereby shaping the root microbiome (Romera et al., 2019).

The activation of signalling pathways leads to the upregulation of defence-associated genes, resulting in the biosynthesis of antimicrobial compounds, pathogenesis-related proteins, and various other defence mechanisms, thereby resisting the pathogen. In a recent investigation conducted by Lavanya et al., the eliciting properties of 3,5-dichloroanthranilic acid (DCA), lipopolysaccharide (LPS), cell wall glucan (CWG), and glycinebetaine (GB) were examined in relation to downy mildew of pearl millet caused by *Sclerospora graminicola*. The study observed the modulation of gene expression pertaining to defense enzymes such as β-1,3-glucanase, lipoxygenase (LOX), phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), peroxidase (POX), and defence protein hydroxyproline-rich glycoproteins (HRGPs) (Lavanya et al., 2022). Similarly, the utilisation of SA and IAA augmented the concentrations of flavonoids and phenolics, along with the inductions of PAL, POX, and PPO, in tomato plants that were infected with *Orobanche ramose*, as compared to the healthy plants (Al-wakeel et al., 2013). Several other reports provide evidence of beneficial microorganisms enhancing the resistance of infected plants compared to healthy ones. The majority of the defence responses observed were associated with an increased expression of defence enzymes such as PAL, POX, PPO, LOX, NPR1 genes, and chitinase when plants were treated with plant growth-promoting rhizobacteria (PGPR) or endophytic microbes. The application of endophytic *Bacillus subtilis* var. *amyloliquefaciens* (FZB24) isolated from rice plants demonstrated disease resistance against sheath blight disease, with enhanced expression of defence-related enzymes PAL, POX and PPO, alongside increased production of phenolic compounds (Nagendran et al., 2014). Another *Bacillus* sp. improves the growth of chilli by suppressing the anthracnose disease caused by *Colletotrichum capsica* with an increased production of PAL, POX, PPO and chitinase (Jayapala et al., 2019). Moreover, *Pseudomonas* isolates were found to enhance the expression of PAL and NPR1 genes, consequently reducing the incidence of sheath blight disease in rice plants (Elsharkawy et al., 2022).

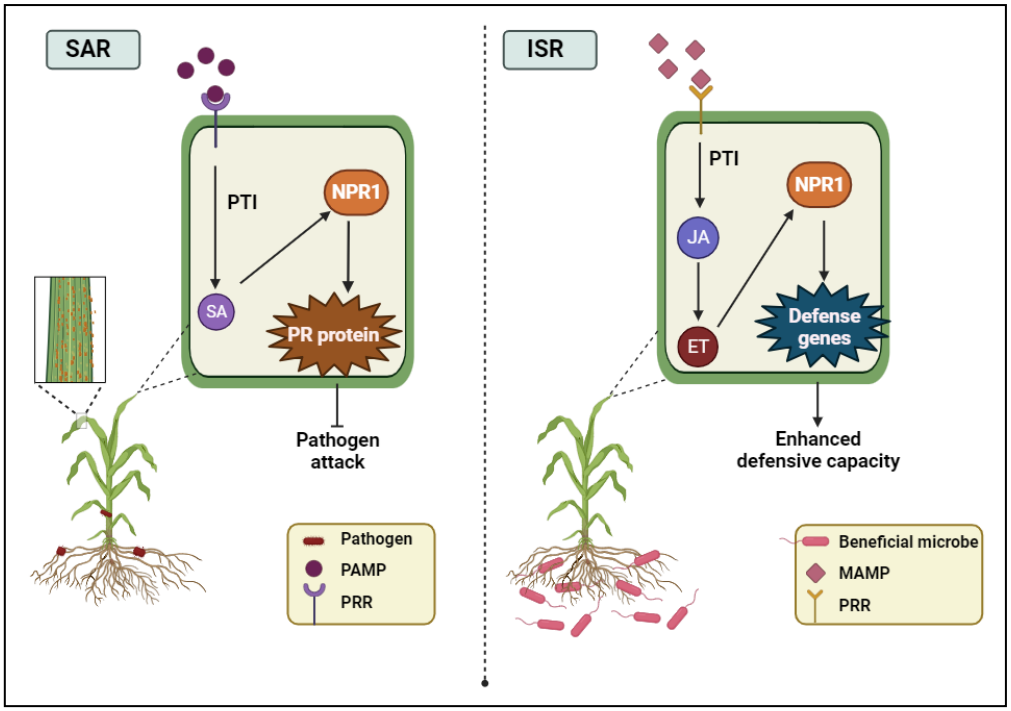

Fig 1.4 Two kinds of plant resistance: Systemic acquired resistance (SAR) involves pathogen-associated molecular pattern (PAMP) recognised by pattern recognition receptors (PRR) and activates pattern-triggered immunity (PTI) through salicylic acid (SA) signalling pathway resulting in the action of pathogenesis-related (PR) proteins. Induced systemic resistance (ISR) primes the plant by microbe associated molecular patterns (MAMP) produced by beneficial microbe and triggers the jasmonic acid (JA)/ ethylene (ET) pathway, resulting in defense gene response (image created using BioRender software)

1.14.Plant growth-promoting characteristics of endophytes

In addition to the induction of systemic resistance, endophytes contribute to enhancing plant growth through multiple other mechanisms. To establish a stable symbiotic relationship with plants, they produce various compounds that facilitate the growth and development of the plant. Endophytes and PGPR have the ability to alleviate multiple stresses such as heat, salinity, water scarcity, drought, and oxidative stress. Endophytes such as *Enterobacter*, *Achromobacter*, *Bacillus*, and *Stenotrophomonas*, which were isolated from the roots of *O. sativa* in saline environments, were reported to possess a wide range of plant growth promoting traits, including nitrogen fixation, production of IAA, and phosphate solubilisation under saline conditions. These traits greatly contribute to the growth and productivity of the plant (Jhuma et al., 2021). Therefore, the plant growth-promoting (PGP) traits, including phosphate solubilisation, nitrogen fixation, production of growth hormones such as IAA, cytokinin, and

gibberellic acid, as well as siderophore production, significantly enhance the growth and development of the plant. The major plant growth-promoting traits are discussed below.

1.14.1. Biological nitrogen fixation

Nitrogen is one of the essential nutrients that is vital for the growth of plants. The original form of atmospheric nitrogen cannot be absorbed by the plant. Therefore, it undergoes a biological process known as nitrogen fixation, carried out by specific bacteria called nitrogen-fixing bacteria, to be converted to either ammonia (NH_3) or nitrate (NO_3^-) (Franche et al., 2009). The global nitrogen cycle has the potential to contaminate groundwater and increase the likelihood of chemical spills. The reduced availability of nitrogen, resulting from significant loss through emission or leaching, is a limiting factor in agricultural ecosystems. Consequently, bacteria that possess the ability to make atmospheric nitrogen accessible to plants play a crucial role in reducing the reliance on chemical fertilisers and mitigating their negative impacts on the environment (Martinez-Viveros et al., 2010; Tahir & Sarwar, 2013). Certain endophytes possess the capability to perform nitrogen fixation and establish a symbiotic relationship with specific plant species. One well-known example is nitrogen-fixing endophytes, namely *Rhizobium* and *Bradyrhizobium,* which establish a mutualistic association with leguminous plants, including *Pisum sativum, Glycine max, Vicia faba,* and *Lens culinaris* (Boivin et al., 2021; Prusinski et al., 2020). In the case of non-leguminous plants, the endophytic interaction with diazotrophic organisms plays a crucial role in enhancing nitrogen availability. However, it is important to emphasise that non-leguminous plants are incapable of forming nodules for nitrogen fixation purposes (Nag et al., 2020). Several studies have identified certain endophytic bacteria with the ability to fix nitrogen, such as *Azoarcus* spp. (Krause et al., 2006), *Herbaspirillum* (Elbeltagy et al., 2001) and *Gluconacetobacter diazotrophicus* (Dent & Cocking, 2017).

1.14.2. Solubilisation of phosphate

Phosphorus is the second crucial nutrient requirement in plants, following nitrogen. Interestingly, soils often possess a significant reservoir of total phosphorus (P), although the portion available to plants is typically quite small in comparison. The limited availability of phosphorus to plants is attributed to the fact that most soil phosphorus is present in insoluble forms. Nevertheless, plants can assimilate it in two soluble forms: monobasic ($H_2PO_4^-$) and diabasic (HPO_4^{2-}) ions. This is where the significance of phosphate-solubilising bacteria comes into play. Numerous phosphate solubilising microorganisms (PSMs) have been observed to

convert insoluble phosphorus into a soluble form through processes such as acidification, organic acid or proton secretion, chelation, and exchange reactions (Bhattacharyya & Jha, 2012). Phosphate-solubilising bacteria such as *Pantoea vagans* IALR611, *Bacillus safensis* IALR1035, *Bacillus subtilis* IALR1033, *Pantoea agglomerans* IALR1325, and *Pseudomonas psychrotolerans* IALR632 markedly facilitated the growth of potato and tomato plants through this process (Mei et al., 2021).

1.14.3. Production of phytohormones

Evidence suggests that PGPR modulates the growth and development of plants by producing phytohormones such as auxins, cytokinins, and gibberellins. However, the genetic foundation and the signal transduction components that mediate the positive impacts of PGPRs on plants remain poorly understood. Auxin, specifically IAA, is a crucial phytohormone linked to the stimulation of root proliferation, resulting in enhanced nutrient uptake by associated plants. The effect of IAA on plant growth is dependent on its concentration, as low levels can promote root growth while high concentrations can hinder it. Moreover, IAA elicits both rapid responses, such as increasing cell elongation, and long-term responses, such as cell division and differentiation (Goswami et al., 2016). The production of IAA by *Enterobacter cloaca*, an endophyte isolated from *Ocimum sanctum*, was confirmed using thin-layer chromatography (TLC), Fourier-transform infrared spectroscopy (FTIR), and hydrogen-1 nuclear magnetic resonance (H^1NMR) analysis. Furthermore, it revealed that the presence of the endophyte led to a significant enhancement in the growth of four different crops, including *Oryza sativa*, *Arachis hypogaea*, *Vigna mungo*, and *Brassica rapa* var. Toria in terms of improved germination, increased shoot and root biomass, as well as enhanced seed vigour when compared to control plants (Panigrahi et al., 2020).

Another proposed mechanism through which PGPR enhances plant growth is by the action of cytokinins. Cytokinins are N6-substituted aminopurines that influence various physiological and developmental processes in plants. Like IAA, the exogenous application of cytokinins leads to increased cell division, enhanced root development, improved root hair formation, inhibition of root elongation, shoot initiation, and other specific physiological responses. Furthermore, cytokinins play a role in developmental processes such as embryo vasculature formation, nutritional signalling, seed germination promotion, root growth, chlorophyll production, leaf expansion, branching, and delay of senescence (Goswami et al., 2016). Inoculating zeatin-producing and non-producing strains of *Azospirillum brasilense* on

the growth of *Triticum aestivum* L. results stimulated plant growth. Activated antioxidant and physiological systems in wheat are evidence of this action (Zaheer et al., 2022).

Gibberellins, or gibberellic acid (GAs), represent a diverse group of 136 structurally distinct structures. These compounds stimulate rapid shoot and root growth, enhance seed germination rates, and induce mitotic division. The effect of gibberellins is dose-dependent, with low amounts yielding significant effects and excessive amounts leading to the opposite outcome. Gibberellins A1, A3, and iso-A3 were identified from aseptic cultures of the *Azospirillum lipoferum* strain op 33 using capillary gas chromatography-mass spectrometry (GCMs) and GC-MS selected ion monitoring. These particular gibberellins form the basis for the growth-promoting effects of *Azospirillum* sp. on plant growth and yield (Bottini et al., 2004). Furthermore, various endophytes capable of producing gibberellin were isolated from the roots of different plants, such as rice, sugarcane, corn, maize, sunflower, and chilli. These endophytes were then tested in maize seedlings to determine their potential for promoting plant growth, and a significant increase in germination, shoot length, shoot fresh weight, and dry weight was found in the treated seedlings compared to the control group. The qualitative analysis of the gibberellin produced by these bacteria was conducted using TLC and FTIR spectroscopy (Ei et al., 2024).

1.14.4. Production of siderophore

Iron, a vital element for the proliferation of living organisms globally, elicits intense competition due to the scarcity of bioavailable iron in soil and on plant surfaces. The conditions of iron limitation inflict significant harm upon plants. However, PGPR counteracts this adversity by producing low molecular weight compounds called siderophores, which engage in the competitive acquisition of ferric ions (Backer et al., 2018). These siderophore-producing bacteria, which establish residence within plants, serve as valuable assets as they procure iron through siderophore and subsequently transfer it to the plants. As a result, siderophores play a vital role in the growth process and contribute to the plant's resistance against pathogens by possessing iron chelators that scavenge the soluble form of iron (Fe^{3+}). Consequently, pathogens are deprived of iron, leading to their demise, while plants benefit from acquiring iron, which facilitates their growth. The classification of siderophores into three groups is predicated upon the moiety responsible for donating the oxygen ligand for Fe^{3+}, namely (i) catecholates or phenolates, (ii) hydroxamates or carboxylates, and (iii) the mixed types. Examples of siderophores in the catecholate group include enterobactin, pyochelin, and

vibriobactin. Hydroxamate encompasses alcaligin, staphyloferrin, and the mixed type comprises mycobactin and petrobactin (Jacob et al., 2020). Siderophore-producing endophytes were isolated from nodules and roots of *Cicer arietinum* and *Pisum sativum* plants. The majority of the isolated siderophores belonged to the hydroxamate and carboxylate categories and exhibited characteristics that promote the growth of plants. (Maheshwari et al., 2019).

1.15.Bioformulation with endophytes

Bioformulations are products that contain biologically active substances derived from microbes or products that contain microbes and their metabolites. They serve as a greener alternative to chemical pesticides and fertilisers, as they can enhance soil fertility, promote plant growth, and suppress disease. The most commonly utilised bacterial genera for developing bioformulations include *Rhizobium, Mesorhizobium, Bradyrhizobium, Azotobacter, Pseudomonas,* and *Bacillus*. Additionally, the fungal genus *Trichoderma* is also frequently employed. These microbes secrete a variety of metabolites that possess antibiotic and antifungal activities (Aamir et al., 2020).

In essence, there are two main types of bioformulations available: solid and liquid bioformulations. However, other types of bioformulations are used worldwide. One such type is encapsulation. The most commonly encountered solid formulations are granules (GR), wettable/water dispersible granules (WG/WDG), wettable powders (WPs), and dusts. Granular bioformulations contain dry particles containing the active ingredient, carrier, and binder. Based on particle size, they are classified as coarse particles (100-1000 µm) and microgranules (100-600 µm). WG/WDG, also known as dry flowable, possess a high concentration of dispersing agents and exhibits excellent shelf life (Mishra & Arora, 2016). On the other hand, WPs are the oldest type of bioformulation and have a prolonged shelf life. They typically contain 50–80% technical powder, 1–10% dispersant, 15–45% filler, and 3–5% surfactant by weight (Brar et al., 2006). Dusts are also one of the original formulations and consist of a finely ground mixture of active ingredients, usually comprising 10% of the formulation, with particle sizes ranging from 50 to 100 µm (Aamir et al., 2020; Mishra & Arora, 2016).

Liquid bioformulations, on the other hand, are flowable or aqueous suspensions that are made in water, oil, or a combination of both. A typical liquid formulation is composed of 10–40% microorganisms, 35–65% carrier liquid (oil or water), 1–3% suspender ingredient, 3–8% surfactant, and 1–5% dispersant (Brar et al., 2006). These liquid formulations are available in the form of suspension concentrates (SCs), ultralow volume (ULV) suspension (SU), oil

miscible flowable concentrate (OF), and oil dispersion (OD). SCs are formulated by combining the active ingredient with the most negligible water solubility and satisfactory stability to hydrolysis. ULV SU is a suspension that is ready to use through ULV equipment in the form of an extremely fine spray. In contrast, OF is a stable suspension of active ingredient(s) in a fluid intended for dilution in an organic liquid before use, whereas OD is a stable suspension of active ingredient(s) in a water-immiscible solvent or oil (Aamir et al., 2020; Mishra & Arora, 2016). Studies showed the efficacy of a commercial liquid formulation containing *Bacillus subtilis* strain MBI 600 against *R. solani* both in *in vitro* experiments (Krishna Kumar et al., 2013) and in *planta*, which also exhibits the ability to promote plant growth (Kumar et al., 2012). Several other research state that liquid bioformulation is better than solid bioformulation (Khan et al., 2023).

Encapsulation entails the application of a polymeric material to coat and encapsulate microbial cells, resulting in the formation of permeable beads that allow for the passage of gas, nutrients, and metabolites, thereby preserving the viability of the cells contained within the beads. Encapsulations offer adequate protection to active ingredients against adverse environmental conditions. There are two distinct types of encapsulations based on the size of the polymeric beads: macro-encapsulation, which encompasses beads ranging in size from a few millimetres to centimetres, and microencapsulation, which encompasses beads ranging in size from 1 to 1000 μm, typically less than 200 μm in size (Mishra & Arora, 2016).

Most of the bioformulations currently available consist of a single strain. However, it has been observed that a consortium or combination of multiple strains of PGPR can lead to more favourable outcomes in terms of overall plant growth and development. Several studies have provided evidence for the synergistic effects of all microorganisms in the consortium. Endophytic and rhizospheric microbes, when used as a microbial consortium, result in improved growth and physiological parameters in foxtail millet. These outcomes were superior to those observed in untreated control groups and groups treated solely with fertilisers (Kaur et al., 2023). Another microbial endophytic consortium comprised of *Halomonas aquamarina* EU-B2RNL2, *Erwinia persicina* EU-A3SK3, and *Pseudomonas extremorientalis* EU-B1RTR1 led to an augmentation in the shoot/root biomass and length, as well as the number of branches, leaves, and fruits per plant. Furthermore, it resulted in notable changes in various physiological parameters, such as the chlorophyll content, total soluble sugar, flavonoids, and phenolics of the treated plants, surpassing those of both the individual strain-inoculated plants and the untreated control (Devi et al., 2022). The efficacy of bioformulations can be significantly

improved by incorporating adjuvants or carriers. For instance, the bioformulation consisting of *Azotobacter chroococcum* 76A, *Trichoderma afroharzianum* T22, and a *Trichoderma* secondary metabolite known as 6-pentyl-α-pyrone (6PP), combined with a carboxymethyl cellulose-based biopolymer, exhibited an increase in the fresh weight, photosynthetic efficiency, and plant metabolome of sweet basil (*Ocimum basilicum* L.) (Comite et al., 2021). Hence, it is apparent that utilising a microbial consortium with an adjuvant yields the desirable outcomes of prolonging the shelf life and enhancing the effectiveness of plant growth and development while concurrently fortifying the plants against diseases by stimulating the activation of induced systemic resistance. These benefits surpass those achieved through the utilisation of single bioformulations.

1.16.Relevance of the present study

Rice cultivation in Kerala, particularly in Kuttanad, encounters significant challenges arising from diverse diseases. Among these, sheath blight disease stands out as a prominent one, presenting a considerable threat to paddy yields by potentially causing a loss of up to 50%. Consequently, it ranks among the diseases that restrict the maximum achievable yield in paddy. Moreover, this situation is exacerbated by the occurrence of sudden and intense floods in the years 2018, 2019, and 2020, resulting in substantial destruction of cultivated land and affecting both the environmental microorganisms and the spread of pathogenic microbes (Divakaran et al., 2019; Surendran et al., 2021a). Consequently, disease control measures are imperative for the sustainable production of rice. In the past, various chemical methods were employed for crop growth, yield enhancement, and disease management. However, these approaches have progressively degraded the ecological niche. To overcome the limitations of conventional chemical treatments, utilising natural alternatives, such as endophytic bacteria, shows immense potential. Nonetheless, the effectiveness of endophytic bacteria-based microbial consortia and their capacity to control sheath blight disease in rice plants through induced systemic resistance (ISR) holds great promise.

1.17.Gap areas identified

Despite the efforts to manage the sheath blight disease, several gap areas remain that hinder its effective control and management, impacting rice yields and farmer livelihoods. One of the major gaps is the inadequate implementation of integrated pest management (IPM) strategies. IPM involves combining various control methods, including biological, chemical, and cultural practices, to manage pest populations effectively. In Kuttanad, there is often a lack of awareness

and training among farmers regarding these integrated strategies. This results in over-reliance on chemical fungicides, which can lead to resistance in pathogens, making the disease harder to control over time.

Another gap is the limited access to effective fungicides and resistant rice varieties. While chemical control can be effective, the availability of fungicides that are both affordable and suitable for local conditions is often constrained. Additionally, there has been insufficient progress in developing and distributing disease-resistant rice varieties that could offer long-term protection against sheath blight disease.

Lastly, environmental management practices are often overlooked at important places like the Kuttanad. The unique water management system and high humidity levels at this region contribute to the proliferation of sheath blight disease. Along with better water management practices, there is a need for more robust agronomic interventions that can help to reduce disease pressure. To achieve this target, there is a need for research to understand the specific environmental conditions and pathogen strains in Kuttanad, which would help develop tailored and more effective control measures. Furthermore, it provides extension services that educate farmers on the latest management practices and technologies. By focusing on these deficiencies, Kuttanad can improve its disease management strategies, leading to more sustainable rice production and enhanced food security.

1.18.Objectives of the present study

The primary goals of this study are to screen promising endophytes capable of resisting sheath blight disease, while promoting rice plant growth and to develop a bioformulation with the potential endophytes isolated to enhance both rice plant growth and disease resistance.

The main objectives are to -

- Explore the potential endophytic bacteria in rice plants for controlling sheath blight disease.
- Assess the effectiveness of selected endophytic bacteria to stimulate rice plant growth and strengthen defences against the sheath blight disease.
- Examine the role of secondary metabolites produced by endophytic bacteria in the growth of rice plants, and enhancing resilience against sheath blight disease.
- Create a bio-formulation with isolated endophytic bacteria, aimed at strengthening plant resistance against sheath blight and stimulating plant growth.

1.19.References

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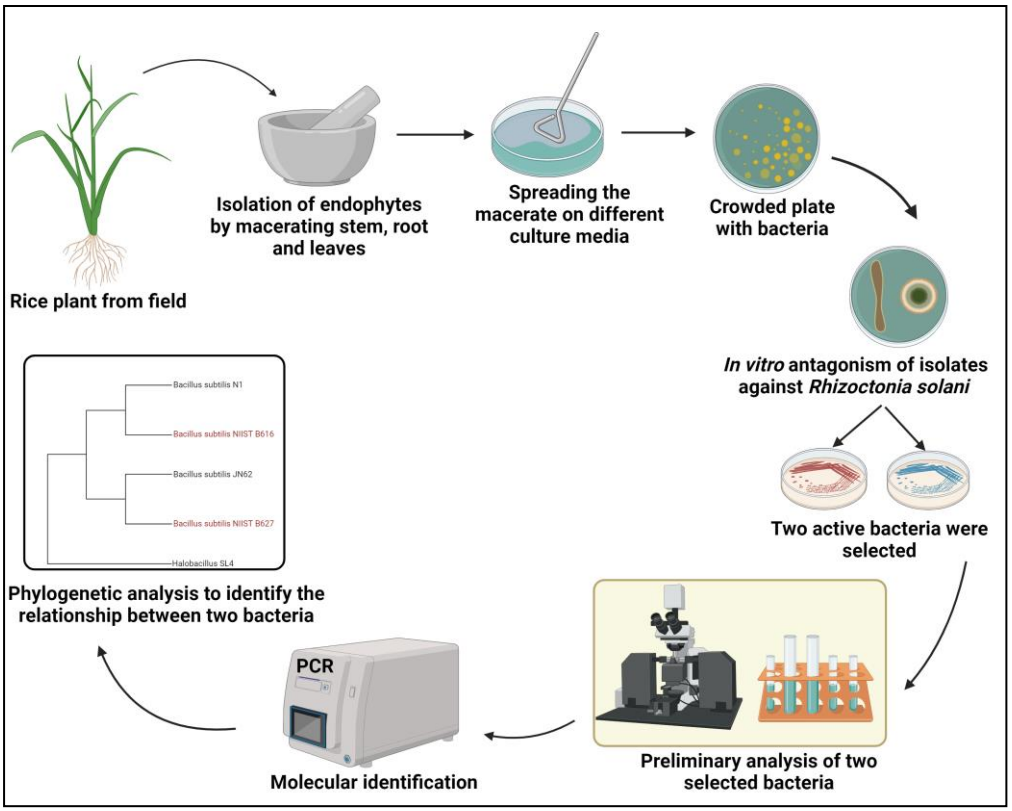
Chapter 2

Isolation, Screening, and Identification of Endophytes from Rice plants

Graphical Abstract



2.1.Introduction

Endophytes are defined as microbial communities that reside within the plants. In other words, the microbial community that can be isolated from surface-sterilized plant tissue or extracted from within the plants is called endophytes (Kuznair et al., 2019). Many endophytes benefit the host by improving plant growth, eliciting defence against pathogen attack and tolerance against stress, and insect resistance (Costa et al., 2012; Duan et al., 2013). Therefore, endophytes have gained significant attention in the field of microbiology due to their prominence in active secondary metabolites to promote plant growth and their contribution to defence mechanisms. Numerous studies showed that endophytes regulate plant growth by the action of nitrogen fixation, phosphate solubilisation, production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, siderophore, and indole acetic acid (IAA) (Yan et al., 2018). Endophytes secrete several phytohormones and other plant growth promoting substances that directly or indirectly help the host plant in its growth. This association between plants and endophytes has existed since the evolution of higher plants, but there remains a lack of detailed study on these groups. In particular, the process by which they recruit endophytes remains mostly unidentified (Kandel et al., 2017). Endophytes enter the plant tissue through wounds or openings or by the action of hydrolytic enzymes. These groups then acclimatised to the extreme condition and might have undergone genetic variations that led to the production of certain phytochemicals (Chatterjee & Abraham, 2020).

To further investigate endophytes, scholars have isolated endophytes from various plant species and conducted comprehensive research on them. The process of isolating endophytes is a detailed procedure due to the potential risk of contamination from epiphytic organisms. Nevertheless, despite these challenges, endophytes have successfully been isolated from numerous plant species, such as rice (*Oryza sativa* L.) (Bertani et al., 2016; Laskar et al., 2012), wheat (*Triticum aestivum* L.) (Rana et al., 2020), tea (*Camellia sinensis*) (Yan et al., 2018), *Mimosa pudica* (Sánchez-Cruz et al., 2019), *Prosopis cineraria* (Gupta et al., 2015), sugarcane (*Saccharum officinarum*) (de Melo et al., 2021; Muangthong et al., 2015), potato (*Solanum tuberosum*) (Cui et al., 2019; Gorai et al., 2021) and medicinal plants such as *Fagonia mollis* Delile and *Achillea fragrantissima* (Forssk) Sch. Bip. (ALKahtani et al., 2020), among others. These isolated endophytes may fall into different categories, like bacteria, fungi, or actinomycetes. Studies revealed that endophytes were isolated from the mangroves environment, containing bacterial species such as *Bacillus* sp., *Pseudomonas* sp., *Stenotrophomonas* sp., *Sporosarcina* sp., *Serratia* sp., *Staphylococcus* sp., and fungi such as

Aspergillus sp., *Penicillium* sp., *Cladosporium* sp., *Fusarium* sp., and *Trichoderma* sp. Additionally, there are actinomycetes like *Nocardia* sp., *Microbispora* sp., *Micromonospora* sp., and *Streptomyces* sp. in mangroves (Chatterjee & Abraham, 2020). Studies reported that bacterial endophytes in rice plants improve their growth, yield and resistance against fungal pathogens. Various investigations were conducted on isolating bacterial endophytes from wild and traditional rice varieties from different locations. It is also reported that endophytes were isolated from the ovule and seed endosphere to determine the plant growth-promoting activities (Kumar et al., 2020).

Recognizing the potential of native endophytic bacteria to combat pathogens, this study explored the bacterial endophytes present in rice plants from the Kuttanad region. Native endophytes are thought to be more adaptable and effective than non-native ones. Therefore, this study focused on identifying highly potent native endophytic bacteria that can promote plant growth and enhance disease resistance. This chapter details the isolation and identification of endophytic bacterial strains that exhibit antagonistic activity against *Rhizoctonia solani*, the fungal pathogen responsible for sheath blight disease.

2.2.Materials and Methods

2.2.1.The site and sample collection

Rice samples were collected from different parts of Kuttanad (9° 25′ 30″ N, 76° 27′ 50″ E), Kerala, India. 650 samples were collected from different areas, viz. Ambalappuzha, Illimuri, Kainakkary, Karuvatta, Kavumpuram, Kochukayal, Manappallichira, Pallipad, Pazhaveedu, Pazhayakari, Ponga, Punnappra, Purakkad Kari, Puthenkari, Thekke 900, Thoothukkulam, Vaisyambhagam, Vandanam of Kuttanad. The collected samples, consisting of rice plants with adjoining soil particles from the root region, were stored in ice boxes and kept in a cold room at 4°C until the isolation of the endophytic organisms was complete.

2.2.2.Isolation of endophytes

The rice plant samples were rinsed with flowing tap water in order to eliminate any attached soil particles. Various parts of the plant, such as the leaves, pseudostem, and roots, were cut and submerged in 70% ethanol for 3 min. Subsequently, they were rinsed with a fresh sodium hypochlorite solution (containing 2.5% available chlorine) (HiMedia, India) for 5 min, followed by five washes with sterile distilled water. The final rinse was scrutinised to ensure the absence of any bacteria or fungi attached to the surface, achieved by streaking the sample

on Luria Bertani (LB, HiMedia, India) agar and potato dextrose agar (PDA, HiMedia, India) plates. The samples (pseudostem, root, leaf) were then crushed using a sterile mortar and pestle and plated on various media, namely actinomycetes isolation agar (AIA, HiMedia, India), nutrient agar (NA, HiMedia, India), pseudomonas isolation agar (PIA, HiMedia, India), and yeast mannitol agar (YMB, HiMedia, India), LB agar, PDA (Elbeltagy et al., 2000). All the plates were kept in a BOD incubator (Rotek, India) at 28 ± 2℃ for 2 to 5 days. Following the incubation, the plates were examined for the presence of bacterial growth, allowing for the estimation of endophytic microorganisms.

2.2.3.*In vitro* antibiosis

2.2.3.1.Test organism

The antagonistic potential of endophytes was examined against the fungal pathogen *Rhizoctonia solani* ITCC 6882. The fungal culture was procured from the Indian Type Culture Collection (ITCC), Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi, India, and maintained on PDA slants at 4℃.

2.2.3.2.In vitro antagonism

In vitro antagonism of the isolates against *R. solani* was examined by modified dual culture technique in PDA plates (Dileep Kumar et al., 2001). For this, a loopful of bacterial inoculum (18 h old) of the respective test strain was streaked on one side of the PDA plates approximately 2.0 cm inside from the periphery, and an actively growing circular mycelial disc (6.0 mm diameter) of *R. solani* was placed opposite (approximately 5.0 cm) from the bacterial streak. The plates with no bacteria served as a control. All the plates were kept in a biological oxygen demand (BOD) incubator (Rotek, India) at 28 ± 2℃, and the inhibition zone (in mm) was recorded as the distance between the mycelial and bacterial growth after seven days. The readings were taken from three replicates each.

2.2.3.3.Scanning electron microscopic analysis for hyphal deformities

Hyphal deformities of *R. solani* developed due to inhibition caused by the bacterial isolates in *in vitro* plates were observed under scanning electron microscopy (SEM). To accomplish this, fungal mycelia from the point of inhibition and mycelia from control plates were carefully extracted and positioned on double adhesive tape on the stub. The sample was then dried, and a thin layer of gold was coated using a sputter coater. The gold-coated metal stub was viewed through the scanning electron microscope (Zeiss Evo 40 EP) at an accelerating voltage of 20

KV and a probe diameter of 102 pA to obtain secondary electron images (Fischer et al., 2012). The field was scanned to check the variation in hyphal morphology. Suitable fields in the preparation were photographed.

2.2.4. Morphological characterization of selected isolates

The selected endophytic isolates were then morphologically analysed. The cell morphology of bacteria was observed under light microscopy after Gram staining. The size of the cell was observed under scanning electron microscopy (SEM). Spore staining was conducted to view the endospores produced by the endophytes.

2.2.4.1.Light microscopic analysis

The selected isolates were grown for 24 h in LB agar plates. Subsequently, the isolates were subjected to Gram-staining and observed under a light microscope. Using a sterilised inoculation loop, a thin film of the sample was prepared on a glass slide. For that, a single colony from the bacteria grown plate was picked and resuspended in a drop of sterile water previously applied to the slide. The prepared bacterial smear was then air-dried and heat-fixed by passing over the flame. Following this, the smear was stained with crystal violet and allowed to sit for 30 s. The stain was then rinsed away under a steady stream of water and substituted with Gram's iodine for one min. The stain was once again washed off with water, and the smear was decolourized using a decolourising solution until the purple hue faded. After one more wash, the smear was counterstained with safranin. The stain was removed by rinsing it with water, and the smear was allowed to dry. The dried smear was examined under a light microscope equipped with oil immersion (Olympus X41, Japan) (Coico, 2005).

2.2.4.2.Scanning electron microscopic analysis

The selected isolates were subjected to analysis using a SEM. Bacterial specimens in the mid-exponential growth phase grown in Luria Bertani (LB) broth were washed, and the pellets were resuspended in phosphate buffer saline (PBS). After that, the cells were fixed in 2% glutaraldehyde for 12 h at 4℃. Following this fixation step, the samples were washed with PBS and then postfixed in 1% osmium tetroxide for 1 h at 4℃. Afterwards, the samples were rewashed with PBS and underwent dehydration by a series of ethanol concentrations (30%, 50%, 70%, 80%, 95%, 100%). The dehydrated cells were diluted with 100% ethanol, deposited onto a stub, and allowed to air-dry. The stub, along with the sample, was later coated with a layer of gold. The gold-coated metal stub was viewed through the scanning electron

microscope (Zeiss Evo 40 EP) at an accelerating voltage of 20 KV and a probe diameter of 102 pA to obtain secondary electron images. Appropriate regions of interest were then captured.

2.2.4.3.Spore staining

A thin smear of bacteria was prepared onto a glass slide and subsequently flooded with malachite green. The slides were positioned on top of a beaker filled with water atop a heated plate, thus allowing it to undergo steaming for 2 to 3 min. Following this step, the slides were rinsed in cold water and subjected to a counterstain of safranin for 30 s. A further rinse with water was carried out, followed by gentle removal of excess moisture using absorbent bibulous paper. Finally, the prepared slides were examined under a light microscope.

2.2.5. Cultural characterisation of selected isolates

The cultural attributes of the chosen isolates were assessed using Tryptone soya agar (TSA, HiMedia, India), LB agar, PDA, and NA medium. All the isolates were evenly spread across each agar plate, and after 2-5 days of incubation, various features of the colonies, including colour, form, elevation, and margin, were documented.

2.2.6. Biochemical characteristics of selected isolates

Biochemical assays hold a significant position among the pertinent examinations employed to discern bacterial identification. Tests such as Gram staining, catalase, and hydrolysis of cellulose, protein, and starch were done using standard protocols. The Gram staining was carried out according to Cappuccino and Sherman, and the method was described above (Cappuccino & Sherman, 2005). The catalase test was determined by adding a 3% hydrogen peroxide drop to the glass slide containing the isolates (Cappuccino & Sherman, 2005). NA incorporated with 1% carboxymethylcellulose (CMC, HiMedia, India) was used for cellulose hydrolysis activity. These plates were inoculated with isolates and incubated at 28±2°C for 48 h. To visualise the hydrolysis zone, the plates were flooded with an aqueous solution of 0.1% Congo red (HiMedia, India) for 15 min and washed with 1 M sodium chloride (HiMedia, India) solution (Amore et al., 2012). Protein hydrolysis tests were conducted in NA supplemented with 5% skim milk (HiMedia, India). The agar plates were inoculated with the microbial isolates and subsequently incubated at 28±2°C for 72 h. Following incubation, the plates were carefully examined for clear zones indicating hydrolysis (Raveschot et al., 2020). Hydrolysis of starch was assessed by inoculating the strains in NA medium supplemented with 1% starch (HiMedia, India) and incubated at 28±2°C. After incubation, the plates were flooded with 1%

iodine solution (HiMedia, India) and observed for a clear hydrolysis zone around the bacterial growth (Aneja, 2003).

To evaluate the susceptibility of the chosen isolates to antibiotics, they were grown in LB broth for 24 h. The bacterial culture was then adjusted to a concentration of 10^8 colony forming units per milliliter (CFU/mL) and applied onto Mueller Hinton agar (MHA, HiMedia, India) plates. Following this, an antibiotic disc containing a known concentration was positioned at the centre of the medium and incubated for 24 h at 28 ± 2℃. The growth of the bacterial strains on plates containing antibiotic discs was compared to the growth on plates without antibiotic discs. The various zones of inhibition were assessed to the nearest millimetre and classified as sensitive, intermediate, or resistant according to the interpretative chart provided by the disc manufacturer (HiMedia, 2012).

2.2.7. Molecular identification of the selected isolates

16S rRNA gene sequencing is a prevailing molecular technique employed for microbial identification and categorisation, grounded on examining the 16S ribosomal RNA gene sequences. This method was used to ascertain the identity of the designated endophytic isolates.

2.2.7.1.Isolation of Genomic DNA

Genomic DNA of endophytic bacterial isolates was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions. The culture was collected in a microcentrifuge tube, followed by adding 180 µL of T1 buffer and 25 µL of proteinase K. This mixture was incubated at 56 ℃ in a water bath until complete lysis occurred. After lysis, 5 µL of RNase A (100 mg/mL) was added and incubated at room temperature for 5 min. To facilitate further processing, 200 µL of B3 buffer was added and incubated at 70℃ for 10 min. To this, 210 µL of 100% ethanol was added and mixed thoroughly by vortexing. The resultant mixture was pipetted into a NucleoSpin® Tissue column, placed in a 2 mL collection tube, and centrifuged at 11000 g for 1 min. The NucleoSpin® Tissue column was transferred to a new 2 mL tube and washed with 500 µL of BW buffer. This washing step was repeated using 600 µL of B5 buffer. Upon completion, the NucleoSpin® Tissue column was placed in a clean 1.5 mL tube, and DNA was eluted out using 50 µL of BE buffer.

2.2.7.2.Agarose gel electrophoresis

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1 µL of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH 8.0) was added to

5 µL of DNA. Then, samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/mL ethidium bromide. Electrophoresis was performed with 0.5X TBE as an electrophoresis buffer at 75 V until the bromophenol dye front migrated to the bottom of the gel. The gels were visualised in a UV transilluminator (Genei), and the image was captured under UV light using a Gel documentation system (Bio-Rad).

2.2.7.3.Polymerase Chain Reaction and Gel electrophoresis

The Polymerase chain reaction (PCR) amplification was performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). For that, 2X Phire Master Mix (5 µL), D/W (4 µL), Forward Primer (0.25 µL), Reverse Primer (0.25 µL), and DNA (1 µL) were used. The primers used were 16S-RS-F 5' CAGGCCTAACACATGCAAGTC 3' and 16S-RS-R 5' GGGCGGWGTGTACAAGGC 3'.

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/mL ethidium bromide. 1 µL of 6X loading dye was mixed with 4 µL of PCR products and was loaded, and electrophoresis was performed at 75 V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 h until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualised in a UV transilluminator (Genei), and the image was captured under UV light using a Gel documentation system (Bio-Rad).

2.2.7.4.DNA Sequencing using and sequence analysis

The PCR product (5 µL) is mixed with 0.5 µL of ExoSAP-IT and incubated at 37℃ for 15 min, followed by enzyme inactivation at 85℃ for 5 min. The sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following the manufacturer protocol. The Sequencing PCR mix consisted of D/W (6.6 µL),5X Sequencing Buffer (1.9 µL), Forward Primer (0.3 µL), Reverse Primer (0.3 µL), Sequencing Mix (0.2 µL), Exosap treated PCR product (1 µL).

The cleanup mixture consisted of D/W (5 µL), 125 mM EDTA (1 µL), 3 M sodium acetate pH 4.6 (0.1 µL), and ethanol (44 µL) were prepared and were properly mixed. 50 µL of the mix was added to each well in the sequencing plate containing the sequencing PCR product. The mixture was subjected to vortexing using the mixmate vortex apparatus and allowed to incubate at ambient temperature for 30 min. It was then spun at 3700 rpm for 30

min. The supernatant was decanted, and 50 µL of 70% ethanol was added to each well. Again, it was spun at 3700 rpm for 20 min. After that, the supernatant was carefully poured out, followed by a subsequent rinse with a 70% ethanol solution. The liquid portion was removed, allowing the solid residue to air dry. The cleaned up air-dried product was sequenced in ABI 3500 Genetic Analyzer (Applied Biosystems) using the Sanger DNA sequencing method.

The sequence quality was checked using sequence scanner software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond, 2010; Kearse et al., 2012).

2.2.8. Phylogenetic tree construction

The DNA sequences from 16S rRNA gene sequencing were compared with those in the National Center for Biotechnology Information (NCBI) database and EzBioCloud 16S database. A phylogenetic tree was constructed from the sequences obtained from the NCBI Basic Local Alignment Search Tool (BLAST) results. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA) 7 software based on the maximum likelihood method (Hall, 2013; Newman et al., 2016), and sequences were submitted to NCBI.

2.3. Results

2.3.1. Isolation of endophytes

A total of 225 endophytes were isolated from the rice plant samples collected from the Kuttanad region in Kerala (Fig 2.1). The number of endophytic microorganisms according to the agro-ecological zones of Kuttanad, most of them were from Lower Kuttanad (58%), Kayal Land area (35%) and Purakkadkari (7%) (Fig 2.2). The isolated endophytes demonstrated a nearly equivalent distribution within the root, stem, and leaf of the plant samples, with approximately 35% found in the root, 33% in the stem, and 32% in the leaf (Fig 2.3).

Fig 2.1 The Kuttanad region of Kerala

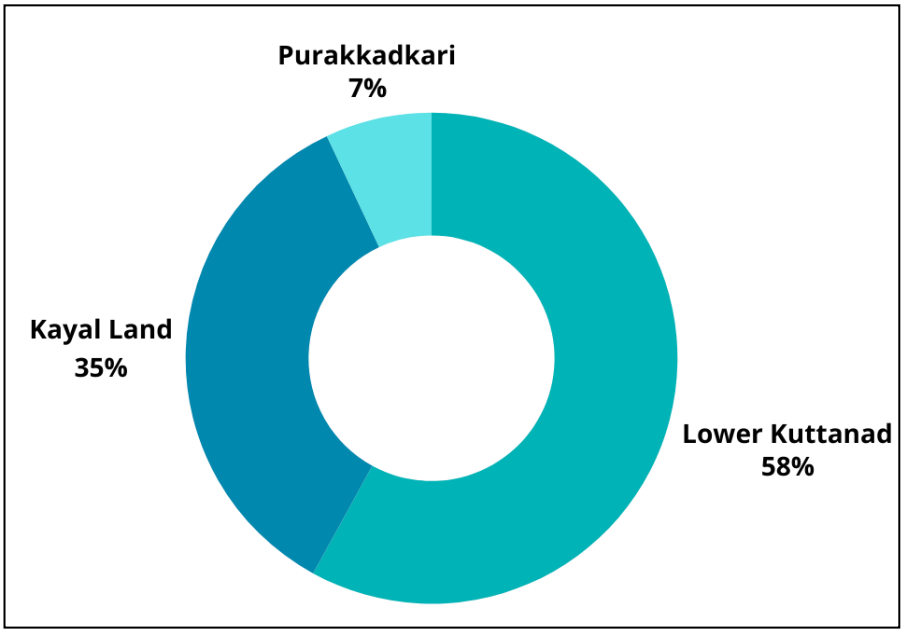


Fig 2.2 Percentage of endophytes isolated from agro-ecological zones of Kuttanad

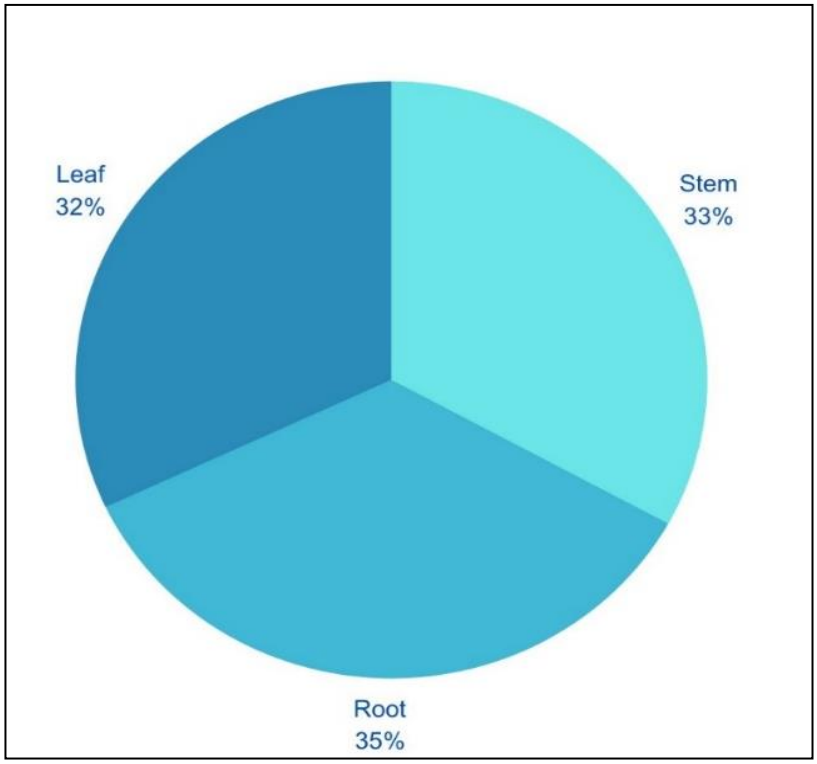


Fig 2.3 Percentage of total endophytes isolated from different parts of rice plant

2.3.2. *In vitro* antagonism of the isolates

The isolates were screened for their efficacy in inhibiting rice pathogen *R. solani*, which causes sheath blight disease in paddy. Among them, two endophytic strains isolated from roots, NIIST B616 and NIIST B627, were selected for further studies based on their *in vitro* antagonism. Both strains exhibited potent inhibition against *R. solani*, as demonstrated in Fig 2.4A. NIIST B616 exhibited an inhibition distance of 18±1.00 mm, while NIIST B627 demonstrated an inhibition of 12±1.00 mm. The SEM analysis of mycelial hyphae treated with both bacteria individually revealed cellular extrusion, abnormal bulbous-like formation, and shrinkage of the hyphae (Fig 2.4B).

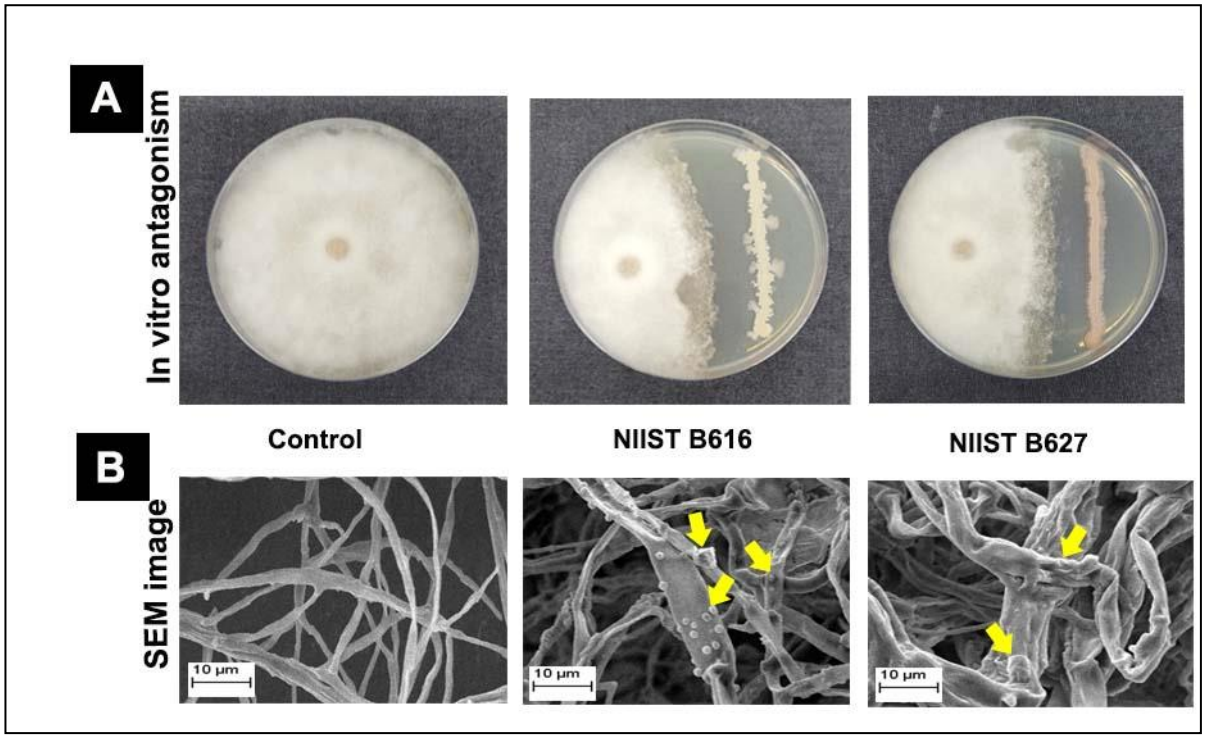


Fig 2.4 *In vitro* antagonism of (A) endophytic isolates against *R. solani* in such a way that *R. solani* was inoculated on the left (mycelial plug) and the *Bacillus* as a vertical streak on the right and (B) SEM images of the fungal hyphae from the point of inhibition. Arrow marks indicate the morphological changes of the mycelial hyphae

2.3.3. Morphological characterisation of selected isolates

On light microscopy, both bacteria were observed as cylindrical rod-shaped, Gram-positive bacteria. It was further confirmed in SEM and found that the bacteria NIIST B616 has a 1.84 µm length rod, and NIIST B27 has a 1.97 µm length rod (Fig 2.5). Spore staining of both bacteria revealed the presence of an elliptical centred endospore.

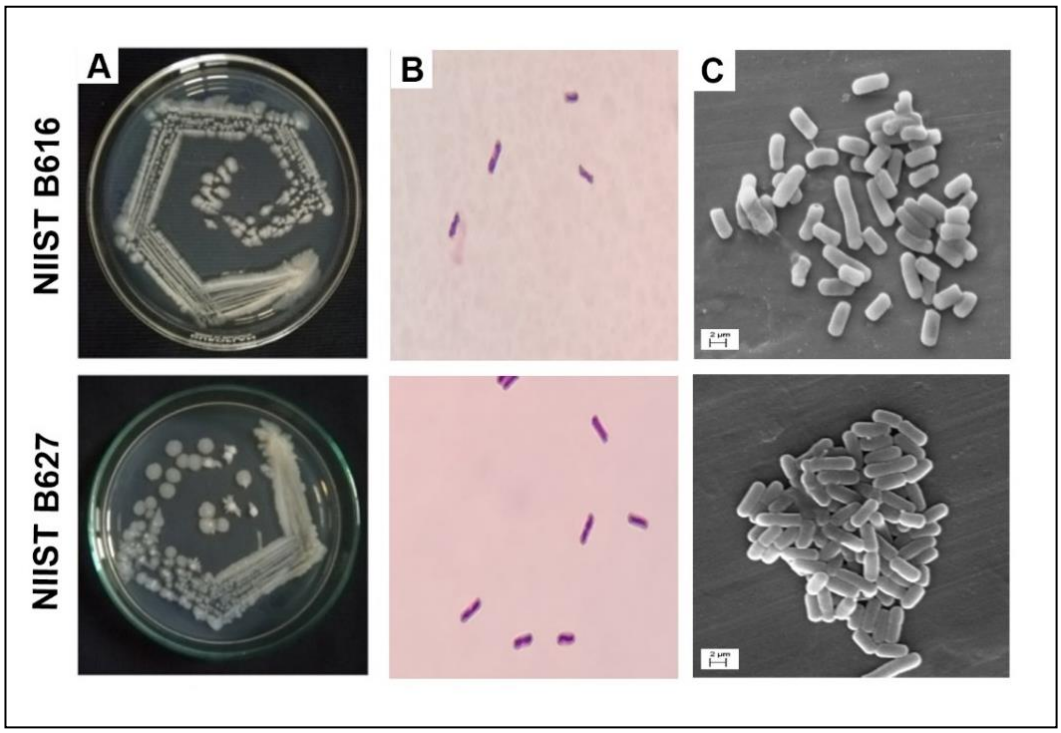


Fig 2.5 Endophytic bacterial isolates (NIIST B616 and NIIST B627) growth; **A**-on a agar plate, **B**-light microscopic image, and **C**-SEM image of respective isolates

2.3.4. Growth of isolated bacteria on different agar media

All bacterial isolates exhibit robust growth on all media types including TSA, LBA, PDA and NA. The morphological characteristics observed on each medium are summarised in Table 2.1.

2.3.5. Biochemical Characterisation of the selected isolates

The bacteria NIIST B616 and NIIST B627 were positive for the Gram staining and catalase test. Both bacteria give hydrolysis of cellulose and protein tests, but the hydrolysis of starch is only provided by NIIST B 627 (Fig 2.6). The antibiotic susceptibility tests revealed that both selected endophytes were sensitive to antibiotics such as ampicillin, chloramphenicol, ciprofloxacin, cinoxacin, imipenem, kanamycin, nalidixic acid, and rifampicin (Table 2.2).

Table 2.1 Morphological characters of NIIST B616 and NIIST B627 on different cultural media

Sl. No.	Morphological characters	NIIST B 616				NIIST B627			
		TSA	LBA	PDA	NA	TSA	LBA	PDA	NA
1	Form	Circular	Circular	Circular	Circular	Irregular	Irregular	Irregular	Irregular
2	Elevation	Raised	Raised	Raised	Raised	Flat	Flat	Flat	Flat
3	Margin	Curled	Entire	Curled	Entire	Curled	Curled	Curled	Curled
4	Colour	Creamy white	Creamy white	Creamy white	Creamy white	Cream	Cream	Cream	Cream
5	Optical characteristics	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
6	Colony size	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
7	Consistency	Buttery	Buttery	Buttery	Buttery	Dry	Dry	Dry	Dry

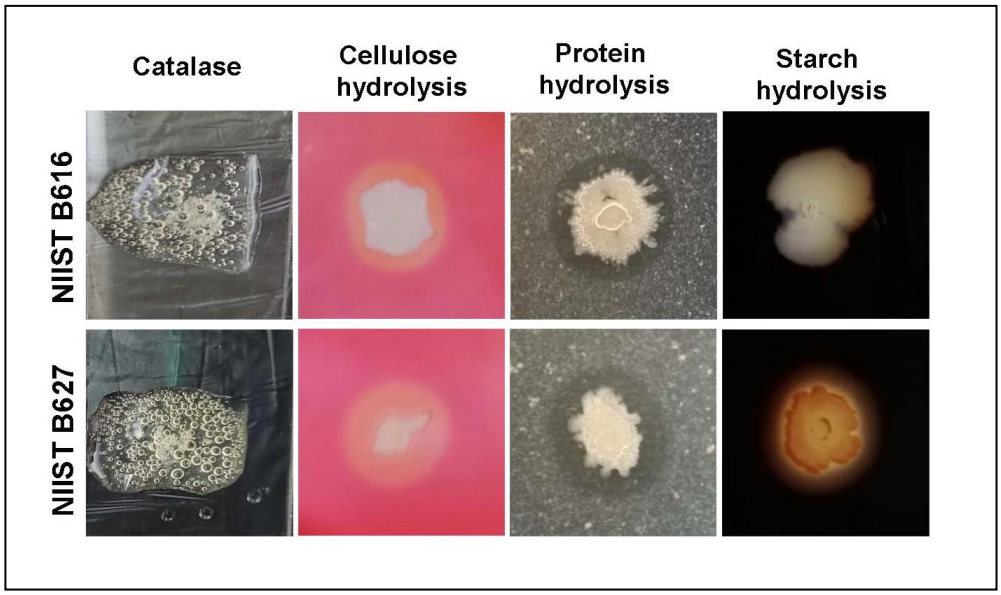


Fig 2.6 Biochemical test - catalase, cellulose hydrolysis, protein hydrolysis and starch hydrolysis tests of NIIST B616 and NIIST B627

Table 2.2 Antibiotic susceptibility tests of selected endophytic strains

Antibiotic disc (concentration in µg)	NIIST B616	NIIST B627
Ampicillin (10)	S	S
Chloramphenicol (30)	S	S
Cinoxacin (100)	S	S
Ciprofloxacin (5)	S	S
Imipenem (10)	S	S
Kanamycin (30)	S	S
Nalidixic acid (30)	S	S
Rifampicin (5)	S	S

S – Sensitive; I – Intermediate; R – Resistant

2.3.6. Molecular identification of the isolates

The analysis of the sequences obtained from the 16S rRNA sequencing method confirmed that both bacteria belonged to the *Bacillus subtilis* strain. The obtained sequences are given below.

NIIST B616

GAAGGGGGGGAAACTTTGGCTTCCTTGAATGTTTAGCGGCGGACGGGTGAGTAACACGTGGGTAA
CCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGC
ATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGT
TGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAA
AGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGG
AAGAACAAGTGCCGTTCAAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGG
GCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAA
ACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAG
ATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGAGTGCTAAGTGTTAG
GGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGGAGTACGGTCGCA
AGACTGAAACTCAAAGGGAATTTGACGGGGGGCCCCGCACAAAGCGGTGGGAGCAATGTGATTAT
CGGAAGCAACGCGAGAAACCTTAACCAAGGTCCTTTGTGA

NIIST B627

ATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGG
AAACCGGGGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCT
ACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAACG
ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTG
ATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGG
TACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGT
GAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAAAGGAG
AGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCG
ACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCT
AA

2.3.7. Phylogenetic tree construction

The phylogenetic tree of the two *Bacillus subtilis* strains, NIIST B616 and NIIST B627, were constructed from molecular data (DNA sequences) obtained from 16S rRNA gene sequencing and NCBI BLAST analysis. From the phylogenetic tree constructed in MEGA 7 software, it was noticed that the two bacteria, *Bacillus subtilis*, belonged to different strains (Fig 2.7). The sequences were then submitted to GenBank, and accession numbers ON054037 for NIIST B616 and KU577428 for NIIST B627 were obtained.

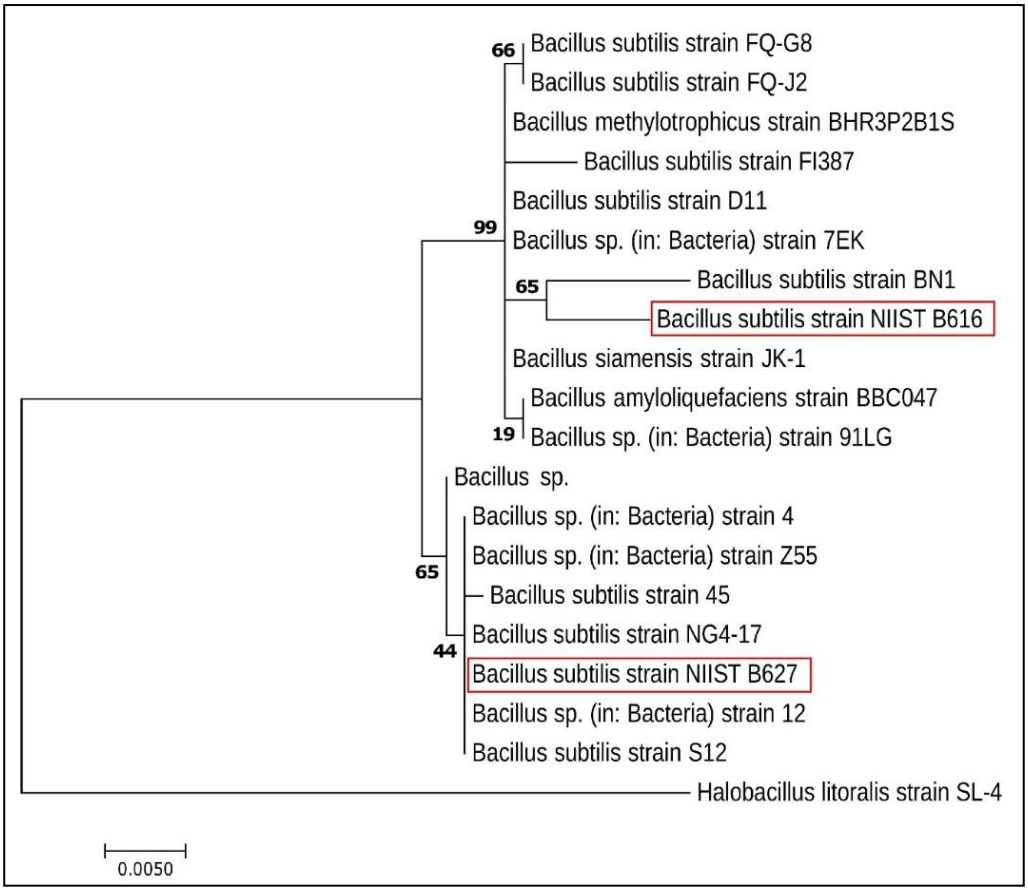


Fig 2.7 Phylogenetic tree constructed from 16SrRNA sequences of two endophytic isolates using the maximum likelihood method in MEGA software version 7.0

2.4. Discussion

Kerala has a flourishing culture of rice cultivation, which is considered a symbol of traditional lineage and prosperity (Krishnankutty et al., 2021). Rice production accounts for approximately 95% of the food grain output in the state (Abraham, 2019). Notably, Kuttanad stands as the sole area in India where rice is grown below sea level (FAO, 2012). This study endeavour entailed an examination of the bacterial endophytes found in rice plants cultivated within the Kuttanad locality, given their ability to enhance plant growth, productivity, and resistance against pathogenic agents causing diseases, specifically the sheath blight of paddy. Samples of rice plants from the Kuttanad area were systematically collected, and bacterial endophytes were subsequently isolated from different plant components. The endophytes exhibited a relatively equal distribution among the roots (35%), pseudostems (33%), and leaves (32%). Reports have shown that various types of endophytic bacteria have been isolated from rice plants, including *Azospirillum*, *Burkholderia*, *Bacillus*, *Pantoea*, *Pseudomonas*,

Methylobacterium, *Herbaspirillum* and *Rhizobium* (Mano & Morisaki, 2008). Research indicates that these endophytes in rice plants play a crucial role in enhancing rice growth and increasing yield (Kumar et al., 2015; Liu et al., 2020).

The isolated endophytes were screened against *R. solani*, the causative organism of sheath blight disease. Among the isolates, bacteria that exhibited good antagonistic behaviour against *R. solani* were selected for further studies. Two endophytes, NIIST B616 and NIIST B627, isolated from roots showed an activity of 18±1.00 mm and 12±1.00 mm of the zone of inhibition against the pathogen. The zone of inhibition between *R. solani* and the bacterial isolates indicates the presence of bioactive metabolites that diffuse into the medium and resist the pathogen. Thus, the *in vitro* antagonistic investigation disclosed the antifungal properties of the endophytes on the plate. Previous reports documented that two strains of the *Bacillus* genus, namely *Bacillus subtilis* and *Bacillus cereus*, demonstrate antagonistic behaviour towards *R. solani* on PDA. (Elkahoui et al., 2012). Studies showed that *Bacillus* spp. has emerged as a potent biocontrol against fungal pathogens (Solanki et al., 2012). *Bacillus amyloliquefaciens* MB101, and *Bacillus subtilis* MB14 are associated with the management of *R. solani* and showed *in vitro* antagonism against the same pathogen (Solanki et al., 2012, 2013). SEM analysis of fungal hyphae unfolded morphological changes at the point of inhibition. Similar results were obtained from malfunctioning fungal hyphae of *R. solani* on antibiosis with *Bacillus subtilis* (Krishna Kumar et al., 2013).

The two selected bacteria underwent a thorough assessment of their morphological traits by cultivation in various media, including TSA, LBA, PDA, and NA. Notable distinguishing features emerged for both strains. NIIST B616 presented a circular, raised, entire, and creamy white appearance in LBA and NA, while in TSA and PDA, they exhibited similar characteristics, except for a slightly curled margin. Conversely, NIIST B627 appeared irregular, flat, curled and cream-colored across all media examined. Gram staining further confirmed the Gram-positive nature of both strains with a distinct purple hue, observed under a light microscope (Coico, 2005). Subsequently, SEM analysis revealed that both bacteria were intact and rod-shaped. Further characterisation involved biochemical tests encompassing catalase, cellulose hydrolysis, protein, and starch assays. Both strains exhibited catalase activity and demonstrated hydrolytic activity towards cellulose and protein. However, only NIIST B627 displayed starch hydrolysis activity. These biochemical assays provided valuable insights into the enzymatic activities and substrate utilisation patterns of the endophytes, which are crucial for their identification and categorisation. Evaluation of antibiotic resistance profiles revealed minimal resistance in both strains, which is essential considering their potential

application as bioformulations in field conditions. Given the environmental implications of antibiotic resistance, it is imperative to thoroughly examine the resistance profiles of potential bioformulations. In the environment, particularly in soil, the transfer of antibiotic resistance genes from harmless bacterial species to other bacteria through horizontal gene transfer may be considered potentially pathogenic. Due to the widespread use of antibiotics, soil serves as a reservoir for resistant human pathogens. Additionally, the process of irrigation facilitates the transmission of pathogenic bacteria and antibiotic-resistant genes from organic fertilisers and manure to vegetables and fruits. Notably, neither strain exhibited an association with opportunistic human pathogens, and both showed low levels of resistance to the antibiotics tested in this study. This finding is significant, considering the potential for horizontal gene transfer and the transmission of antibiotic-resistant genes through agricultural practices and the food chain. As plants can absorb and transport antibiotics, the careful consideration of bacterial strains for agricultural use is essential to mitigate environmental and public health risks (Gamalero et al., 2020).

Identifying bacteria using these phenotypic characters is an arduous and tedious process. An alternative to this is 16S rRNA gene sequencing. The 16S rRNA gene is roughly 1500bp long and has nine variable regions (V1-V9) interspaced with conserved regions. As the 16S rRNA gene is present in all bacteria and its function over time has not changed, it has been widely used to identify and classify bacteria (Janda & Abbott, 2007; Winand et al., 2020). So, molecular analysis using 16S rRNA gene sequencing was executed to identify bacteria further. Subsequent sequence analysis via NCBI BLAST and EzBiocloud search identified both strains as *Bacillus subtilis*. A phylogenetic tree was constructed using MEGA 7 software to confirm their distinction, revealing that the two *Bacillus subtilis* strains belong to distinct lineages. Phylogenetic trees visually represent the evolutionary relationships among sequences or taxa and their common ancestors. While traditionally used for taxonomic classification, phylogenetic analysis now extends to understanding gene functions and elucidating mechanisms underlying microbial outbreaks (Hall, 2013). Consequently, it can be concluded that the two isolated bacteria are indeed disparate strains of *Bacillus subtilis*.

2.5. Conclusion

Endophytic bacteria were isolated from rice plants in the Kuttanad region of Kerala, yielding 225 bacteria from various plant parts, including roots, pseudostems, and leaves. The distribution of endophytes across different plant parts was found to be uniform. Screening against the sheath blight pathogen *R. solani* led to the identification of two endophytic bacteria, NIIST B616 and NIIST B627, isolated from the root that exhibited antagonistic activity, prompting their selection for further analysis. Initially, the morphological characteristics of these selected bacteria were examined through cultivation in diverse media, followed by observation under both light microscopy and scanning electron microscopy (SEM). Both bacteria were confirmed to be Gram-positive and rod-shaped. Moreover, biochemical characterisation involving Gram staining, catalase testing, and cellulose and starch hydrolysis assays were carried out. Subsequent identification was conducted via 16S rRNA sequencing, with sequence analysis through NCBI BLAST and EzBiocloud revealing both bacteria as *Bacillus subtilis*. Phylogenetic analysis using the maximum likelihood method in MEGA 7 software indicated that the two *Bacillus subtilis* strains belonged to distinct strains or lineages, highlighting their genetic diversity.

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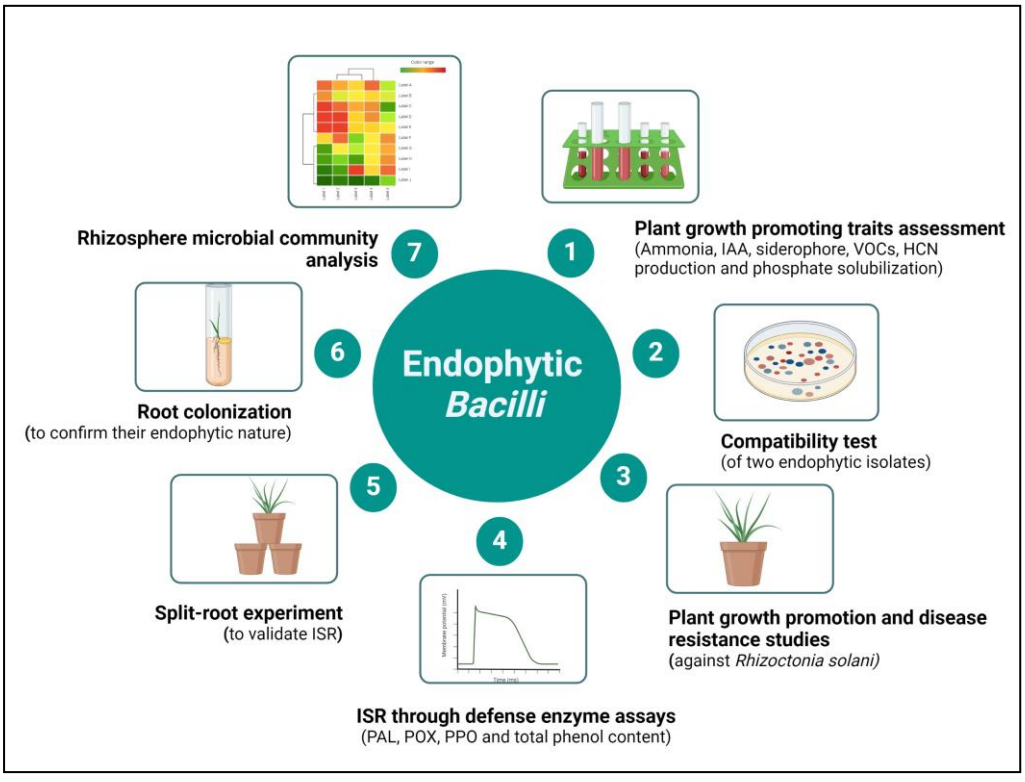
Chapter 3

Endophytic *Bacilli* for Enhanced Plant Growth and Induction of Systemic Resistance Against Sheath Blight in Paddy

Graphical Abstract



3.1.Introduction

Endophytes fulfil various functions within plants. One crucial function of endophytes is to enhance plant growth and yield by implementing diverse mechanisms. Additionally, the endophytic relationship presents a distinct opportunity to control harmful phytopathogens indirectly by synthesising specific metabolites and the induction of systemic resistance. The interaction between endophytes and the host plant can range from mutualism to exhibiting pathogenic characteristics at a later stage. In mutualism, the endophytic microorganism invades the host plant, providing a suitable environment, and reciprocally produces numerous active metabolites that augment the plant's absorption of nutrients, thereby advancing plant growth and development (Rana et al., 2020). Compared to their counterparts in the rhizosphere, endophytes establish a more effective and targeted interaction with the host. (Ali et al., 2017). In essence, endophytes exhibit a high level of host specificity. Despite that, a diverse array of endophytes was obtained from various plant species, encompassing *Bacillus, Pseudomonas, Burkholderia, Pantoea, Stenotrophomonas, Microbacterium,* and *Micrococcus* genera. Notably, *Bacillus* and *Pseudomonas* are the predominant ones (Hwang et al., 2021). Nevertheless, it is worth noting that research on endophytes remains relatively neglected despite being a crucial aspect of microbiological investigations (Etminani & Harighi, 2018).

Endophytes exert a positive influence on plant growth through a variety of mechanisms, including nitrogen fixation, the synthesis of indole-3-acetic acid (IAA), phytohormones or enzymes, phosphate solubilisation, and iron chelation via the production of siderophores (Krishnamoorthy et al., 2020). Furthermore, endophytes contribute to improving plant productivity by mitigating the incidence of diseases through the regulation of phytopathogens. Plant diseases disrupt normal physiological processes, leading to reduced growth, yield, and product quality, as well as postharvest deterioration and depletion of plantations, especially in perennial crops, while also increasing vulnerability to biotic and abiotic stressors (Tampakaki & Panopoulos, 2009). For instance, sheath blight disease, caused by the fungal pathogen *Rhizoctonia solani*, is a significant threat in the Kuttanad region of Kerala, often resulting in yield losses of up to 50%, depending on environmental conditions and crop growth stages (Surendran et al., 2021). However, endophytes present a promising solution to combat such diseases by producing antifungal or antibacterial compounds and inducing systemic resistance in plants against pathogens.

Most of the research has been focused on promoting plant growth by rhizobacteria, while there has been limited study on the effect of endophytes on plant growth. Endophytes' colonisation, maintenance, and impact on plant growth have yet to be thoroughly examined. However, a few studies have indicated the potential use of endophytes in phytoremediation projects. All available evidence suggests that endophytes employ mechanisms similar to those of rhizobacteria in promoting plant growth. Furthermore, it has been revealed that endophytic bacteria that promote plant growth are more effective than their non-endophytic counterparts. (Ali et al., 2017). Various bacteria such as *Burkholderia pyrrocinia* BRM 32113 (Arriel-Elias et al., 2019), *Azospirillum brasilense* (Thomas et al., 2019), *Paenibacillus polymyxa* Sx3 (Abdallah et al., 2019), *Bacillus pumilus* LZP02 (Liu et al., 2020) were showed increased growth, colonisation in roots, reduced disease progression in rice plants.

This chapter examines the plant growth-promoting traits and biocontrol potential of two newly isolated endophytic bacteria, NIIST B616 and NIIST B627. The primary objective is to assess their effects on rice plant growth, yield and disease resistance both individually and in combination, under conditions with and without the rice plant pathogen *R. solani*. Furthermore, the study investigates the role of these endophytes in inducing systemic resistance in rice plants, their colonization in roots, and their impact on the rhizospheric microbial community.

3.2.Materials and Methods

3.2.1. Microbial strains and culture conditions

The endophytic *Bacillus subtilis* (strain NIIST B616 and NIIST B627) used in this study were isolated from the roots of rice plants of Kuttanad (9º 25' 30" N, 76º 27' 50" E), Kerala. The details on their isolation as well as preliminary studies on their ability to resist sheath blight pathogen *in vitro* condition were already presented in the previous chapter.

The phytopathogen *Rhizoctonia solani* ITCC 6882 was procured from the Indian Type Culture Collection (ITCC), Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi, India. It was maintained in PDA (HiMedia, India) at 28±2°C and kept at 4°C for future investigations.

3.2.2. Experimental site

The study was conducted at the Agro-Processing and Technology Division of CSIR-NIIST, Thiruvananthapuram, Kerala, India, during 2018-19 period. This region has a hot tropical climate with abundant rainfall during monsoon seasons. The average minimum and maximum

temperature during the study is between 25.60 and 33.81℃, with an average rainfall of 5.2 mm and relative humidity of 84.15%.

3.2.3. *In vitro* screening of the endophytes for plant growth promotion traits

3.2.3.1. Test for ammonia production

A freshly grown culture was inoculated into peptone water and incubated for 48 h at 28±2°C to examine ammonia production by the endophytic isolates. The presence of ammonia in cell-free supernatant was estimated through the Nesslerization reaction, where 1 mL of Nessler's reagent was combined with 1 mL of the supernatant. A colour change from brown to yellow indicated a positive outcome for ammonia production (Cappucino & Sherman, 1992).

3.2.3.2. Test for HCN production

Qualitative estimation of HCN production was performed by a modified Picrate assay described by Castric (2010). Log phase cultures of the bacteria (25 µL) were inoculated into 5 mL of King's B agar supplemented with 4.4 g/L of glycine in sterile glass vials. To facilitate the measurement, filter paper strips soaked in a solution of picric acid (0.5% picric acid in 2% sodium carbonate) were inserted halfway into the vials and securely fastened with a screw cap. The vials were then sealed with parafilm and incubated for 72 h. The production of HCN was indicated by a colour change in the filter strips from yellow to brown to red. The intensity of the colour change was visually recorded (Dinesh et al., 2015).

3.2.3.3. Test for indole acetic acid (IAA) production

Screening for Indole acetic acid

The production of IAA by the endophytes is determined by Salkowski's reagent when the precursor L-tryptophan is provided (Sev et al., 2016). The bacterial cultures were cultivated in Luria Bertani (LB, HiMedia, India) broth supplemented with L-tryptophan at a concentration of 0.5 mg/mL and incubated at 37℃ for 7 days. Following the incubation period, the cultures were centrifugated at 10,000 rpm for 20 min. 2 mL of Salkowski's reagent was added to 1 mL of the supernatant, along with one drop of orthophosphoric acid. The mixture was then incubated at room temperature for 25-30 min. The development of pink colour indicated the presence of IAA.

Quantitative analysis of IAA production

For quantitative analysis, bacterial cultures were grown in LB broth provided with L-tryptophan and incubated at 37°C for 1-10 days. After incubation, it was centrifuged at 10,000 rpm for 20 min. Subsequently, 1 mL of the resulting supernatant was collected and mixed with 2 mL of Salkowski's reagent, to which a single drop of orthophosphoric acid was added. The mixture was then incubated for a period of 25 to 30 min. During this incubation, a distinctive pink colouration developed and its absorbance was quantified at a wavelength of 530 nm. The concentration of IAA was determined by utilising an IAA standard curve.

3.2.3.4.Test for volatile compound production

The antifungal volatile compounds were assessed through a double petri dish assay (Calvo et al., 2020) with some modifications. To perform this assay, a bacterial suspension (100 µL, 10^8 CFU/mL) was spread onto a petri dish containing LB agar, while a 6 mm agar plug of actively growing *R. solani* was placed at the centre of another petri dish containing PDA. These two half plates were positioned facing each other, ensuring no physical contact between them, and then sealed with parafilm to prevent the loss of any volatiles produced. The petri plates were then incubated at 28±2°C for 5 days, during which the growth of the pathogen was observed and compared to a control.

The fungal growth inhibition (GI) was calculated using the formula

$$\text{Percent growth inhibition,} \quad GI = \frac{C-T}{C} \times 100$$

(C = Growth in control; T = Growth in treatment)

3.2.3.5.Test for phosphate solubilisation

The selected endophytic strains were examined for their ability to solubilise phosphate under *in vitro* conditions by streaking bacterial isolates onto Pikovskaya agar medium. After 96 h of incubation at 28±2°C, the plates were observed for clear zones around the colonies (Pikovskaya, 1948). The Solubilization Index (SI) was measured using the following formula (Taguett et al., 2015).

SI = (Colony diameter + Halo zone diameter) /Colony diameter

3.2.3.6. Test for siderophore production

The universal chrome azurol S (CAS) assay for detecting siderophore production was modified based on the method described by Milagres et al., (1999). The protocol outlined by Schwyn & Neilands, (1987) was followed to prepare the CAS-blue agar. This involved dissolving 60.5 mg of CAS in 50 mL of distilled, deionised water and mixing it with 10 mL of an iron (III) solution containing 1 mM FeCl .6H_2O and 10 mM HCl. While stirring, this solution was slowly added to 72.9 mg of HDTMA[8] dissolved in 40 mL of water. The resulting dark blue liquid was autoclaved at 121℃ for 15 min. Another mixture of 750 mL of water, 15 g of agar, 30.24 g of PIPES[9], and 12 g of a 50% (w/w) NaOH solution was also autoclaved. This was done to raise the pH to the pKa[10] of PIPES (6.8). Finally, the dye solution was poured along the glass wall and agitated gently to prevent foaming. Petri dishes (90 mm in diameter) were prepared with 30 mL of appropriate medium for culturing each strain. The solidified CAS agar plates were punctured with 2.5 to 5 mm diameter holes using a gel puncher. Each hole was filled with 25 µL of the bacterial culture supernatant and incubated in darkness at 28℃ for 7 days (Jenifer & Sharmili, 2015).

3.2.4. Compatibility between selected endophytic isolates

The compatibility of the endophytic strains with each other was assessed *in vitro* using modified cross-streak and crowded plate methods. In the cross-streak method, both strains were streaked on LB agar plates in a manner that they radiated from the centre of the plate. These plates were then incubated at 28±2℃ for 24 h, and the resulting zone of inhibition was recorded (Prasad & Babu, 2017). In the crowded plate method, two bacterial cultures were separately inoculated in LB broth. After 24 h, these cultures were serially diluted, and the dilutions of the two endophytes together were spread onto LBA plates. The plates were then incubated at 28±2℃ for 24 h and observed for the zone of inhibition (Szermer-Olearnik et al., 2014).

[8] HDTMA; Hexadecyltrimethylammonium bromide

[9] PIPES; 1,4-Piperazinediethanesulfonic acid

[10] pKa; Acid dissociation constant

3.2.5. *In vivo* screening of the endophytes for plant growth promotion

3.2.5.1. *Physicochemical characteristics of soil*

The soil utilised in this investigation was subjected to air-drying at room temperature. Following this, it was meticulously ground using a mortar and pestle and passed through a stainless-steel sieve with a mesh size of 2 mm. The resulting finely ground soil sample was appropriately stored in polythene bags for analytical purposes, specifically to identify designated soil physicochemical characteristics. The pH of the soil was determined using a pH meter (Systronics, Inida). Electrical conductivity was measured using a conductivity meter (Systronics, Inida), 0.1 N of potassium chloride was used as the standard. The determination of available organic carbon in the soil was performed using the Walkley-Black titration and colorimetric method (Walkley & Black, 1934), while the analysis of phosphorus availability was carried out using the Bray No 1 extraction method (Bray & Kurtz, 1945). The availability of potassium was assessed using a flame photometer (Systronics, India), potassium chloride was used as the standard. Additionally, the analysis of the soil texture was also done (Bouyoucos, 1936).

3.2.5.2.Surface sterilisation of seed

The investigation was conducted on Jyothi rice seeds, known for their high susceptibility to sheath blight disease. These seeds were procured from the Regional Agricultural Research Station, Pattambi, Kerala. Surface sterilisation of rice seeds was carried out with a modified version of the method outlined by Dileep Kumar & Dube (1992). For that, seeds were soaked in 70% (v/v) ethanol for 2 min, followed by treatment with 2.5% (v/v) sodium hypochlorite. Subsequently, the seeds were rinsed five times in sterile, distilled water and dried under sterile conditions.

3.2.5.3.Growth promotion of rice plant by endophytic isolates

The experiments were executed in plastic pots measuring 18×22 cm, filled with sandy loam soil mixed with cow dung (3:1). A total of 200 pots were setup for the whole study, which were divided into four sections with 50 pots allocated for each treatment. The treatments include no bacteria (Control), individual bacteria alone (NIIST B616 or NIIST B627), and a combination of both bacteria (NIIST B616+627). The endophytic bacteria were cultured individually in LB broth, each inoculated with 1% (v/v) of a 24 h old inoculum of the respective strain. The cultures were then incubated in a shaking incubator at 130 rpm at 28±2°C for 72 h. A

suspension of NIIST B616 or NIIST B627 (50 mL, 1×10^8 CFU/mL each) and a combination of both strains (50 mL, 1×10^8 CFU/mL, mixed in an equal amount of 1×10^4 CFU/mL of both strains) were added to respective pots. The top layer was mixed up to an approximate depth of 2 cm using a sterile glass rod, and 20 surface sterilised seeds were introduced into the pots at a depth of 1 cm and grown under nursery conditions (Dileep Kumar & Dube, 1992). The control group consisted of pots with only surface sterilised seeds. The experiments were conducted in a completely randomised design (CRD), and data were collected from ten replications. Plant growth parameters such as shoot height, root length, dry weight, and total chlorophyll content (Hiscox & Israelstam, 1979) were recorded at intervals of 7 days for up to 28 days. After 30 days, a booster dose of foliar spray (50 mL) containing the abovementioned isolates was applied to the appropriate pots. Yield was determined by measuring the number of grains per pot, weight of 100 grains, number of tillers per plant, number of panicles per plant, dry shoot matter, and harvest index after 120 days of growth.

The harvest index was calculated using the following formula (Unkovich et al., 2010).

$$Harvest\ index = \frac{Grain\ yield}{Total\ shoot\ dry\ matter} \times 100$$

3.2.6. Growth promotion of rice plant by endophytic isolates in the presence of *R. solani*

The experiments were divided into five sections: no bacteria + no *R. solani* (Control), *R. solani* alone (Pathogen alone), NIIST B616 + *R. solani* (NIIST B616 + P), NIIST B627 + *R. solani* (NIIST B627 + P), combination + *R. solani* (NIIST B616 + 627 + P). A total of 250 pots were utilized in the experiment with 50 pots allocated for each treatment. The investigation was conducted as previously described, utilizing pots infested with respective bacteria and *R. solani*. The bacterial inoculum was prepared as mentioned above. The pathogen *R. solani* was cultured in potato dextrose broth (PDB, HiMedia, India) by inoculating it with a 96 h old fungal mycelial plug from an actively growing PDA plate. After seven days of incubation in a shaker maintained at 28±2°C and 130 rpm, the fungal mycelium was harvested and homogenized to create a uniform suspension. Subsequently, all pots, except for the control group, were infested with 50 mL of the homogenised *R. solani* suspension (10^8 CFU/mL) The booster dose was administered after 30 days of growth, and all growth and yield parameters were documented as previously described.

3.2.6.1.Disease control by endophytic isolates

The determination of disease syndrome occurrence and the lesion height after 90 days of treatment was estimated through the Rice Standard Evaluation System scale, developed by the International Rice Research Institute in the Philippines (IRRI, 2013). The following formula determined the percentage of disease incidence and relative lesion height:

$$Percentage\ of\ disease\ incidence = \frac{Number\ of\ diseased\ plants}{Total\ number\ of\ plants} \times 100$$

$$Relative\ lesion\ height = \frac{Lesion\ length}{Plant\ height} \times 100$$

Relative lesion heights were assessed using a scale that ranged from 0 (no infection) to 9 (more than 65%), with a score of 1 signifying a lesion limited to less than 20%, 3 representing 20-30%, 5 signifying 31-45%, 7 indicating 46-65%, and 9 signifying lesion more than 65% of the plant height. The values were obtained from three replicates.

3.2.7. Induction of systemic resistance against *R. solani* by endophytic isolates

Induced systemic resistance (ISR) was analysed through changes in the level of defence enzymes and total phenol content. In this study, five treatments were selected, which include no bacteria + no *R. solani* (Control), *R. solani* alone (Pathogen alone), NIIST B616 + *R. solani* (NIIST B616 + P), NIIST B627 + *R. solani* (NIIST B627 + P), combination + *R. solani* (NIIST B616+627+P). The experiment was conducted as mentioned above. The study focused on investigating three defence enzymes: L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.24), peroxidase (POX, EC 1.11.1.7), and polyphenol oxidase (PPO, EC 1.10.3.1*)* and total phenol content. The samples were collected at intervals of 10 days up to 80 days. Fresh leaf samples weighing 3 g (PAL, POX, and PPO) and 0.5 g (total phenol) were used for the assay. The samples for PAL were homogenised with 9 mL sodium borate buffer and 2-mercaptoethanol (0.8 mL/L) buffer in a pre-cooled mortar and pestle. The extract was then centrifuged at 12,000 *g* for 20 min at 5°C, and the supernatant was used as an enzyme sample for the assay. PAL activity was estimated following the method described by Sadasivam & Manickam (1991). The absorbance was measured in a UV-Vis spectrophotometer (Shimadzu, Japan) at 290 nm, and the reaction rate was noted as micromole trans-cinnamic acid formed (Units/g fresh weight).

POX activity was estimated according to Thimmaiah (1999). Leaf samples were extracted in 9 mL of 0.1 M phosphate buffer of pH 7.0 in a mortar and pestle and then

centrifuged at 18,000 *g* for 15 min at 5°C. The supernatant was used as an enzyme sample for the assay. Absorbance was recorded at 430 nm. The total activity of the peroxidase enzyme was calculated as units/g fresh weight of the sample, considering one unit of the enzyme as an increase in optical density (OD) by 1.0 under standard conditions.

For PPO, samples were homogenised in 0.1 M sodium phosphate buffer (6 mL) of pH 7.1, and the extract was centrifuged at 1,500 *g* at 5°C for 40 min. The supernatant was used as an enzyme sample. PPO activity was determined, as stated by Sadasivam & Manickam (1991). PPO activity was recorded as the change in absorbance per millilitre of enzyme extract per minute and was calculated in units/mg of fresh weight.

Total phenol content was assayed by extracting samples in 10 mL of 80% ethanol and centrifuging at 12,000 *g* for 20 min, and estimation was according to Mahadevan & Sridhar (1986). Total phenol was calculated with a standard graph made from catechol and expressed as mg/g of material (tissue weight). All readings were taken from three replicates.

3.2.8. Validation of ISR through split-root experiment

The split-root experiments were carried out to validate the elicitation of ISR by endophytic isolates against *R. solani* in rice plants. It was done according to Dutta et al., (2008) with a slight modification with three-cup systems (two lower cups and one upper cup). The experiment was conducted in eight sections with two bottom cups, each containing no bacteria/no pathogen (Control), *R. solani* / *R. solani* (P/P), NIIST B616/NIIST B616 (616/616), NIIST B627/NIIST B627 (627/627), combination/combination (616 + 627/616 + 627), NIIST B616/ *R. solani* (616/P), NIIST B627/ *R. solani* (627/P), combination/ *R. solani* (616 + 627/P). For this, seven day old seedlings in sterile soil were carefully uprooted and washed three times in sterile distilled water without disrupting the root system. The plant with similar root lengths was inserted through the upper cup so that half of the root went to each cup below, which contained different treatments as indicated above. Each individual bacterial treatment contains 10^8 CFU/mL, and the combination contains 10^4 CFU/mL of each bacterial strain, and the *R. solani* treatment contains 10^8 CFU/mL. It was ensured that the two cups below were not in direct contact. The development of the disease syndrome by *R. solani* was recorded up to 28 days at an interval of 7 days.

3.2.9. Root colonization

Root colonization was carried out to ensure the endophytic nature of two *Bacillus* species. For this, surface sterilised seeds were bacterised with two bacteria separately and grown in test tubes (2.5×15 cm) using plant nutrient solution (PNS) and 0.6% agar (Verma et al., 2018). After seven days, the plants were uprooted, and the longitudinal section (LS) of the root was examined through SEM for the presence of the introduced bacteria.

3.2.10. Rhizospheric microbial community analysis

The microbial community of the rhizosphere is analysed through the metagenomic next-generation sequencing (NGS) approach. Soil samples were taken 30 days after the application of bacterial cultures to respective rice plants. Rhizospheric soil was taken from four different treatments viz, control, NIIST B616, NIIST B627 and NIIST B616 + 627 pots. NGS analysis was done with the Illumina HiSeq 2500 platform for 16S rRNA V3-V4 variable regions. The V3-V4 region of 16S rRNA was amplified using specific V3 Forward primer CCTACGGGNBGCASCAG and V4 Reverse primer GACTACNVGGGTATCTAATCC. The pre-processed consensus from v3-v4 sequences was pooled and clustered into OTUs based on their sequence similarity using the Uclust program available in QIIME software. Operational taxonomic units (OTU) were identified using the SILVA database. The alpha diversity (Shannon index) was calculated to evaluate the richness, and diversity of the microbial communities.

3.2.11. Statistical analysis

The data were subjected to one-way analyses of variance (ANOVA) using SPSS (version 20.0; IBM SPSS). Statistical significance was evaluated using Duncan's Multiple Range Test (DMRT), and a $p<0.05$ was considered to demonstrate a significant difference. Graphical representations were made through Origin Pro 8.5 software. The weather information was obtained from the Indian Meteorological Department, Ministry of Earth Sciences, Government of India.

3.3.Results

3.3.1. *In vitro* screening of selected endophytes for their plant growth promotion (PGP) traits

The selected endophytic isolates were examined for plant growth promotion traits. *Bacillus subtilis* NIIST B616 and *Bacillus subtilis* NIIST B627 have demonstrated effectiveness in producing ammonia, HCN, IAA, and VOCs. The quantification of IAA reveals that both isolates show a concentration of around 1-10 µg/mL of IAA when tryptophan is applied externally. Regarding VOC production, NIIST B616 displayed 81.17% inhibition, while NIIST B627 showed 78.82% inhibition against *R. solani* in plates. Phosphate solubilisation was observed exclusively in NIIST B616, with a phosphate solubility index of 1.82 ± 0.58, while siderophore production was solely recorded in NIIST B627 (Fig 3.1). These findings offer valuable insights into the potential of these isolates to promote plant growth.

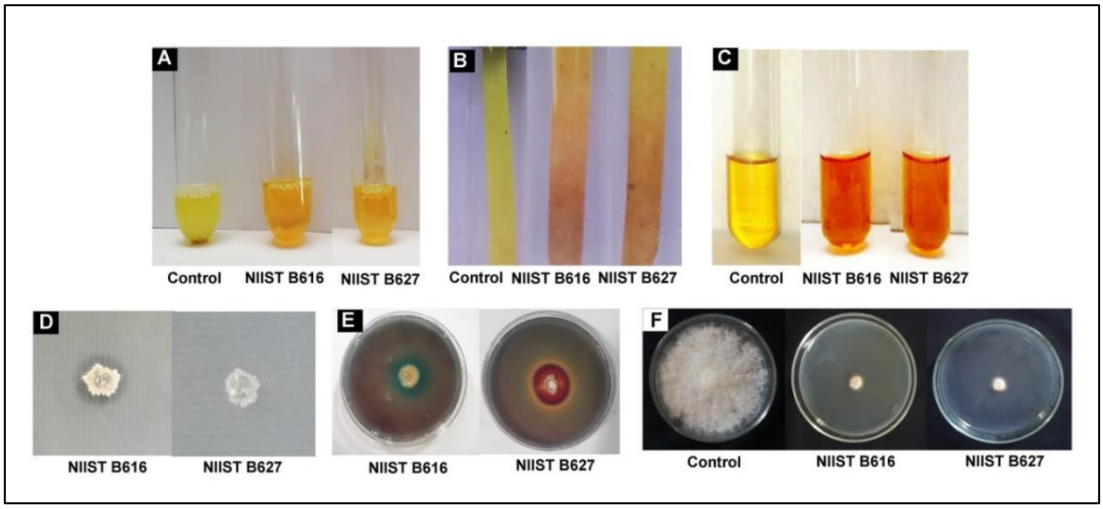


Fig 3.1 PGP traits production by *Bacillus subtilis* NIIST B616 and *Bacillus subtilis* NIIST B627- (A)ammonia production, (B) HCN production, (C) IAA production, (D) phosphate solubilisation, (E) siderophore production and (F) volatile organic compound production

3.3.2. Compatibility between selected endophytic isolates

The endophytic strains NIIST B616 and NIIST B627 were checked for compatibility using the cross-streak method and crowded plate methods. Both strains were compatible when cross-streaked on plates and did not produce any zone of inhibition (Fig 3.2A). In the crowded plate method, both strains were grown without any inhibition. The plates contain 47% colonies of

NIIST B616 and 52.99% colonies of NIIST B627 (Fig 3.2B). Therefore, there is an equal distribution of colonies from both strains.

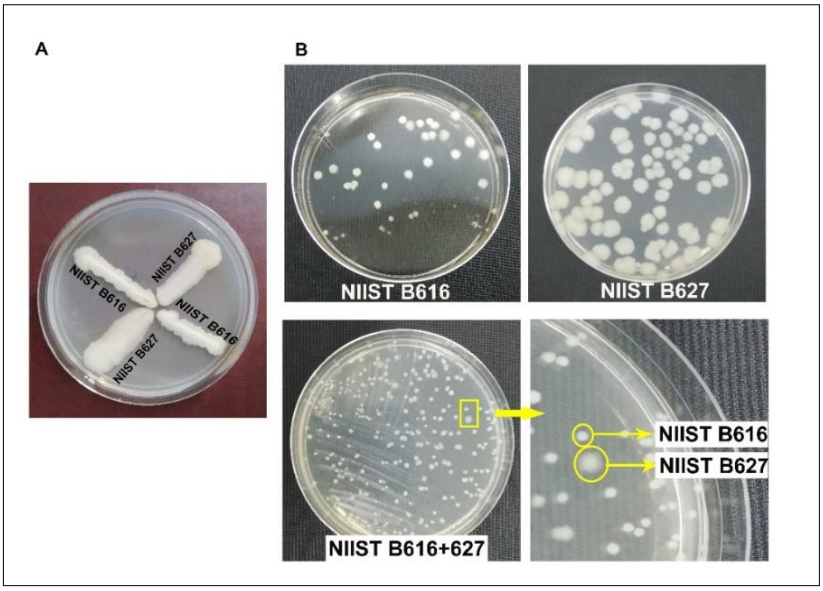


Fig 3.2 Compatibility of two strains, *Bacillus subtilis* NIIST B616 and *Bacillus subtilis* NIIST B627, on LB agar plates – (A) cross streak method, (B) crowded plate technique

3.3.3. *In vivo* screening of selected endophytes for plant growth promotion

The examination of the physicochemical attributes of the soil was conducted prior to the initiation of the experiment. The soil demonstrates a pH value of 6.5 and an electrical conductivity of 0.671 dS/m, indicating a neutrality state. Moreover, it encompasses 1.71% organic carbon content, 831.376 kg/ha of phosphorous, and 581.504 kg/ha of potassium. In subsequent steps, the soil underwent an analysis of its texture, which uncovered a composition consisting of 57.9% sand, 15.7% silt, and 26.4% clay. This composition categorises the soil as sandy loam.

Treatments with two test organisms and their combination on rice plants resulted in a statistically significant enhancement in all growth parameters. The combination of two isolates was the most effective in promoting growth across all test parameters on all data collected days, from 7 to 28 days (Table 3.1). On day 28, the treatment with the combination of two isolates resulted in a substantial increase in shoot height (38.17%), root length (23.46%), dry weight (164.71%), and total chlorophyll content (71.05%) when compared to non-treated control plants (Fig 3.3). Additionally, the yield of rice plants treated with both isolates indicated a positive effect, with the number of grains per pot (77.35%), weight of 100 grains (13.43%), number of tillers per plant (83.50%), number of panicles per plant (99.40%), dry shoot matter

(235.87%) and harvest index (7.02%), over the control plants after 120 days of growth (Table 3.2, Fig 3.3). The ANOVA results revealed that all the growth and yield parameters, except for the number of panicles per plant, exhibited statistically significant differences with all bacterial treatments over the control (p<0.05).

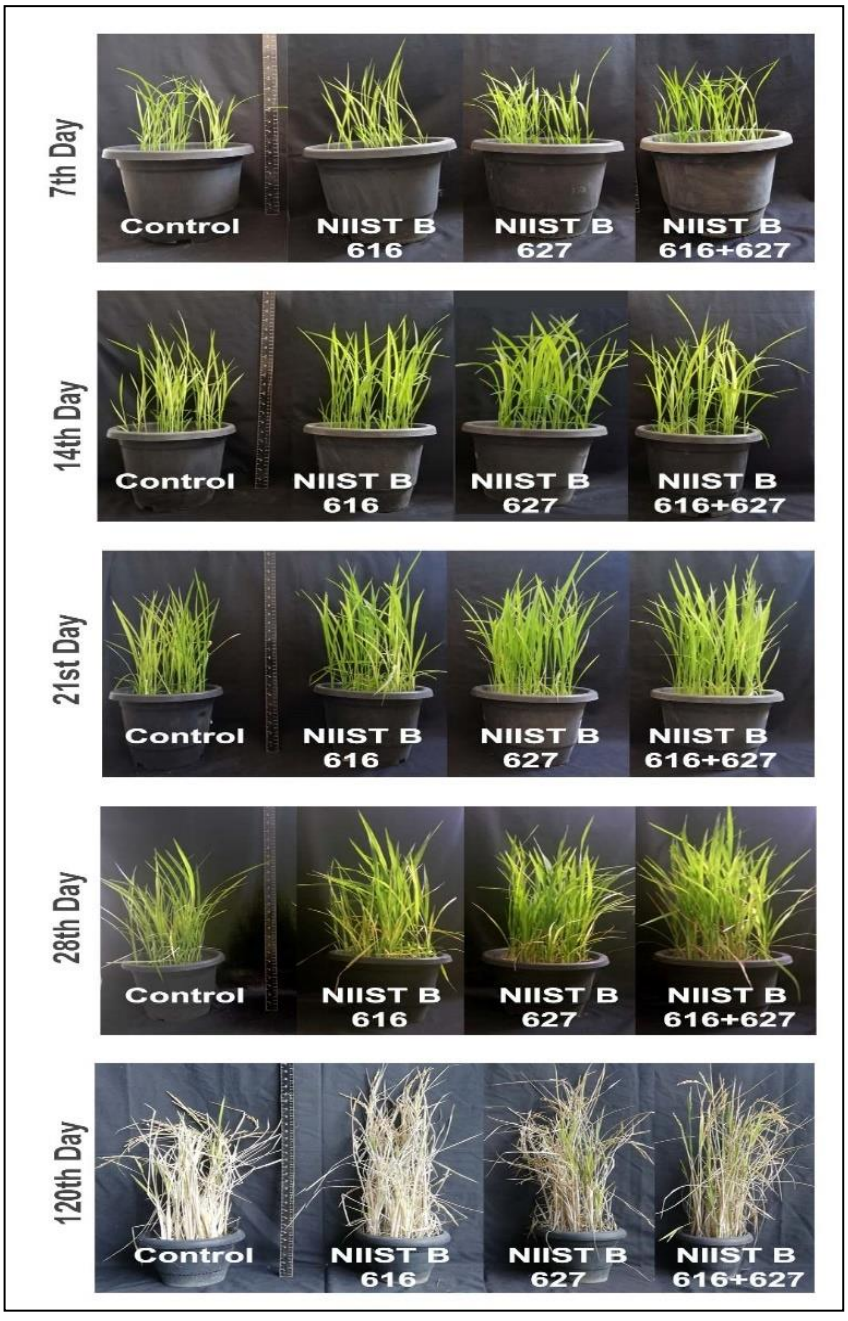


Fig 3.3 Plant growth promotion studies using selected endophytes and their combination in rice plants on different data collection days. Control - no treatment, NIIST B616 -*Bacillus subtilis* NIIST B616, NIIST B627 - *Bacillus subtilis* NIIST B627, NIIST B616+627- *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627

Table 3.1 Effect of endophytic isolates and their combination on the growth of rice plants on various days

Day	Treatments	Shoot Height (cm)	Root Length (cm)	Dry Weight (g)	Total Chlorophyll (mg/L)
7	Control	16.40±0.82[a]*	5.58±0.44[a]	0.02±0.00[a]	3.70±0.12[a]
	NIIST B616	18.56±0.25[b] (13.17)	7.60±0.80[b] (36.20)	0.03±0.00[b] (50.00)	10.84±0.53[c] (192.97)
	NIIST B627	20.32±0.18[c] (23.90)	7.66±0.51[b] (37.27)	0.03±0.00[b] (50.00)	7.98±2.00[b] (115.68)
	NIIST B616+627	23.28±0.61[d] (41.95)	8.5±0.39[c] (52.33)	0.04±0.00[c] (100.00)	12.18±0.18[d] (229.19)
14	Control	23.34±0.93[a]	8.32±0.52[a]	0.04±0.00[a]	8.44±0.36[a]
	NIIST B616	25.93±1.25[b] (11.10)	12.40±1.40[b] (49.04)	0.06±0.01[b] (50.00)	14.74±0.47[b] (74.65)
	NIIST B627	27.59±1.62[c] (18.21)	13.90±0.38[c] (67.07)	0.07±0.00[c] (75.00)	20.30±0.56[c] (140.52)
	NIIST B616+627	28.78±0.36[d] (23.31)	14.33±1.14[d] (72.24)	0.09±0.01[d] (125.00)	29.19±0.14[d] (245.85)
21	Control	29.36±1.16[a]	19.38±0.35[a]	0.12±0.00[a]	14.64±0.78[a]
	NIIST B616	30.65±0.71[b] (4.40)	20.72±0.77[b] (6.91)	0.20±0.01[b] (66.67)	22.73±0.44[b] (55.26)
	NIIST B627	31.32±0.80[b] (6.68)	22.21±1.71[c] (14.60)	0.23±0.01[c] (91.67)	28.88±0.31[c] (97.27)
	NIIST B616+627	32.48±0.72[c] (10.63)	23.20±1.39[c] (19.71)	0.25±0.03[d] (108.33)	32.10±2.20[d] (119.26)
28	Control	27.14±1.11[a]	22.42±1.68[a]	0.17±0.01[a]	22.04±1.21[a]
	NIIST B616	35.40±0.70[b] (30.43)	24.37±0.59[b] (8.70)	0.32±0.03[b] (88.24)	25.27±1.19[b] (14.66)
	NIIST B627	36.09±1.02[b] (32.98)	27.31±0.78[c] (21.81)	0.33±0.02[b] (94.12)	36.71±0.68[c] (66.56)
	NIIST B616+627	37.50±1.26[c] (38.17)	27.68±0.81[c] (23.46)	0.45±0.09[c] (164.71)	37.70±0.39[d] (71.05)

* All values are expressed as mean ± SD from ten replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses represents percentage increases over the control

Table 3.2 Effect of endophytic isolates and their combination on yield of rice plants

Treatments	No. of grains per pot	Weight of 100 grains (g)	No. of tillers per plant	No. of panicles per plant	Dry shoot matter (g)	Harvest Index**
Control	266.33±1.53^{a*}	2.16±0.00^{a}	2.00±0.00^{a}	1.67±0.58^{a}	0.92±0.00^{a}	0.57±0.00^{a}
NIIST B616	309.33±2.08^{b} (16.15)	2.33±0.00^{b} (7.87)	3.00±0.00^{b} (50)	2.33±0.58^{ab} (39.52)	2.73±0.01^{b} (193.48)	0.60±0.00^{b} (5.26)
NIIST B627	378.67±1.53^{c} (42.18)	2.41±0.00^{c} (11.57)	3.00±0.00^{b} (50)	3.00±0.00^{bc} (79.64)	2.84±0.00^{b} (208.70)	0.60±0.00^{b} (5.26)
NIIST B616+627	472.33±2.52^{d} (77.35)	2.45±0.00^{d} (13.43)	3.67±0.58^{c} (83.50)	3.33±0.58^{c} (99.40)	3.09±0.14^{c} (235.87)	0.61±0.00^{c} (7.02)

* All values are expressed as mean ± SD from three replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses represents percentage increases over the control

** Harvest index is the ratio of harvested grain to total dry shoot matter

3.3.4. Growth promotion of rice plant by endophytic isolates in the presence of *R. solani*

The investigation disclosed that rice plants subjected to the application of two isolates simultaneously (NIIST B616+627) demonstrated the utmost percentage augmentation in all growth parameters, notwithstanding the challenge posed by *R. solani*. On day 28, a substantial enhancement was observed in shoot height (38.90%), root length (42.63%), dry weight (260%), and overall chlorophyll content (133.92%) in comparison to the treatment comprising solely of *R. solani* (Table 3.3, Fig 3.4). Furthermore, the yield of rice plants confronted with *R. solani* was noted to be higher in plants treated with both isolates together, followed by plants treated with NIIST B627 and NIIST B616 (Fig 3.4). The combined treatment of endophytes on plants recorded a percentage increase in the number of grains per pot (172.98), weight of 100 grains (19.70), number of tillers per plant (50.00), number of panicles per plant (100.75), dry shoot matter (368.25), and harvest index (31.02) compared to plants treated with *R. solani* alone (Table 3.4). Statistical analysis utilising ANOVA disclosed a noteworthy disparity in all growth and yield parameters of plants treated with the combination of isolates as opposed to plants treated solely with *R. solani* ($p<0.05$).

3.3.4.1.Disease control by endophytic isolates

Development of disease symptoms in rice plants cultivated in soil infested with *R. solani* was observed over a designated time frame. Specifically, symptoms were observed after 50 days for plants grown in soil infested with *R. solani* alone, 60 days for plants grown in soil infested with NIIST B616 and *R. solani*, 65 days for plants grown in soil with NIIST B627 and *R. solani*, and 75 days for plants grown in soil with NIIST B616+627 and *R. solani*. Initially, lesions of a brownish hue were observed in the collar region of the plants. These lesions gradually expanded and lightened in colour, displaying an irregular brown border. Subsequently, these lesions became dry and changed to white, grey, or tan after 90 days, when the final data was collected. At the 90-day mark, the combination treatment and *R. solani* (NIIST B616+627+P) exhibited the lowest incidence of disease at 30%. This was followed by the treatment of NIIST B616+P (36.67%), NIIST B627+P (40%), and the *R. solani* alone treatment (85%). The combination treatment and *R. solani* (NIIST B616+627+P) displayed a relative lesion height scale of 1, while the individual treatments of NIIST B616 and NIIST B627 with *R. solani* had a scale of 3 each. The *R. solani* alone treatment exhibited the highest relative lesion height at 7 on the 90th day (Figs 3.5 and 3.6).

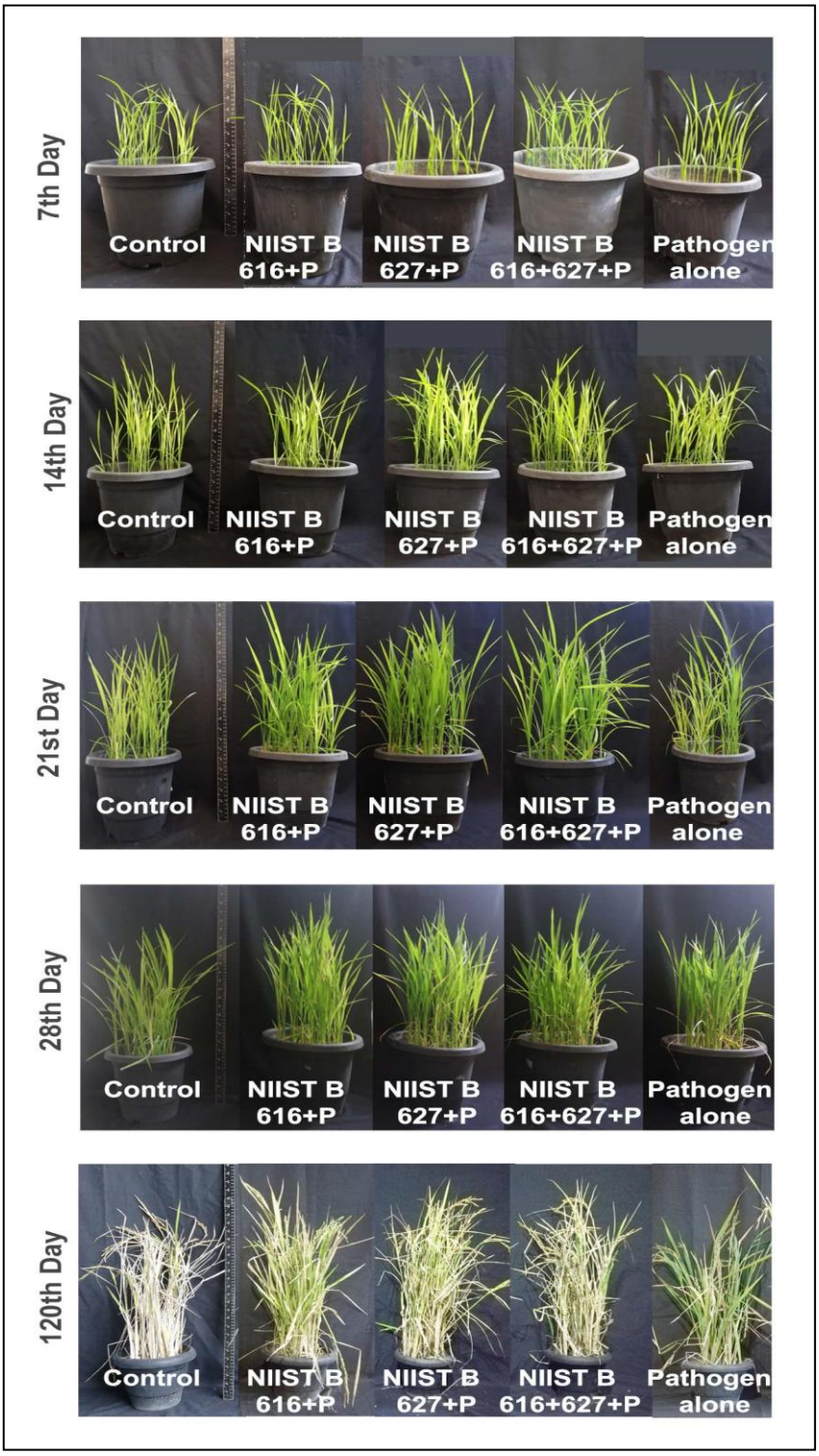


Fig 3.4 Plant growth of rice plants challenge inoculated with *R. solani* on different data collection day. Control - no treatment, NIIST B616+P -*Bacillus subtilis* NIIST B616 + *R solani*, NIIST B627+P - *Bacillus subtilis* NIIST B627 + *R solani*, NIIST B616+627+P - *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627 + *R solani*, Pathogen -*R solani* alone

Table 3.3 Effect of endophytic isolates and their combination on the growth of rice plants challenge inoculated with *R. solani* on various days

Day	Treatments	Shoot Length (cm)	Root Length (cm)	Dry Weight (g)	Total Chlorophyll (mg/L)
7	Control	16.40±0.87^{b} (8.47) *	5.58±0.44^{a} (1.82)	0.02±0.00^{a} (0.00)	3.74±0.04^{a} (6.63)
	NIIST B616+P	17.60±0.12^{c} (16.40)	6.38±0.31^{b} (16.42)	0.03±0.01^{ab} (50.00)	8.20±0.22^{c} (136.31)
	NIIST B627+P	18.98±0.30^{d} (25.52)	6.52±0.27^{b} (18.98)	0.03±0.00^{bc} (50.00)	6.94±0.37^{b} (100)
	NIIST B616+627+P	20.10±0.48^{e} (32.94)	7.18±0.48^{c} (31.02)	0.04±0.00^{c} (100.00)	8.54±0.26^{d} (146.11)
	Pathogen	15.12±0.57^{a}	5.48±0.26^{a}	0.02±0.00^{a}	3.47±0.14^{a}
14	Control	23.34±0.93^{b} (19.08)	8.32±0.52^{b} (10.64)	0.04±0.00^{b} (33.33)	8.44±0.34^{b} (33.12)
	NIIST B616+P	25.02±0.72^{c} (27.65)	10.09±0.22^{c} (34.17)	0.06±0.00^{c} (100.00)	14.13±0.48^{c} (122.87)
	NIIST B627+P	25.46±0.59^{c} (29.90)	10.39±0.10^{c} (38.16)	0.07±0.00^{d} (133.33)	14.15±0.44^{d} (123.18)
	NIIST B616+627+P	26.76±0.37^{d} (36.53)	11.02±0.61^{d} (46.54)	0.08±0.00^{e} (166.67)	17.00±0.92^{e} (168.14)
	Pathogen	19.60±0.40^{a}	7.52±0.40^{a}	0.03±0.00^{a}	6.34±0.30^{a}
21	Control	29.04 ±1.01^{b} (31.28)	19.38±0.35^{b} (41.25)	0.12±0.00^{b} (100)	14.64±0.78^{b} (47.88)
	NIIST B616+P	29.29±0.89^{b} (32.41)	20.18±0.87^{c} (47.08)	0.18±0.01^{c} (200.00)	17.71±0.43^{c} (78.89)
	NIIST B627+P	30.24±0.62^{c} (36.71)	20.83±0.38^{cd} (51.82)	0.19±0.01^{d} (216.67)	18.12±0.50^{c} (83.03)
	NIIST B616+627+P	30.78±0.77^{c} (39.15)	21.28±1.36^{d} (55.10)	0.20±0.01^{e} (233.33)	21.54±0.50^{d} (117.56)
	Pathogen	22.12±0.43^{a}	13.72±0.80^{a}	0.06±0.01^{a}	9.90±0.81^{a}
28	Control	27.14±1.11^{b} (9.97)	22.42±1.68^{b} (32.25)	0.17±0.01^{b} (70.00)	22.04±1.21^{b} (116.72)
	NIIST B616+P	32.78±0.74^{c} (32.82)	23.25±0.80^{b} (37.08)	0.30±0.01^{c} (200.00)	22.26±0.68^{b} (118.88)
	NIIST B627+P	33.14±0.62^{c} (34.28)	24.19±0.63^{c} (34.55)	0.30±0.01^{c} (200.00)	22.44±0.41^{b} (120.65)
	NIIST B616+627+P	34.28±0.36^{d} (38.90)	25.04±0.59^{c} (42.63)	0.36±0.01^{d} (260.00)	23.79±0.13^{c} (133.92)
	Pathogen	24.68±0.40^{a}	16.96±0.71^{a}	0.10±0.00^{a}	10.17±0.17^{a}

*All values are expressed as mean ± SD from ten replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses represent percentage increases over the pathogen

Table 3.4 Effect of endophytic isolates and their combination on yield of rice plants challenge inoculated with *R. solani*

Treatments	No. of grains per pot	Weight of 100 grains (g)	No. of tillers per plant	No. of panicles per plant	Dry shoot matter (g)	Harvest Index**
Control	266.33±1.53^{b} (69.99) *	2.16±0.00^{b} (6.40)	2.00±0.00^{a} (0.00)	1.67±0.58^{ab} (25.56)	0.92±0.00^{b} (46.03)	0.56±0.00^{b} (23.42)
NIIST B616+P	302.67±2.08^{c} (93.19)	2.29±0.01^{c} (12.81)	2.33±0.58^{ab} (16.5)	2.33±0.58^{ab} (75.19)	2.61±0.01^{c} (314.29)	0.59±0.00^{c} (27.98)
NIIST B627+P	303.67±2.08^{c} (93.83)	2.40±0.02^{d} (18.23)	2.33±0.58^{ab} (16.5)	2.33±0.58^{ab} (75.19)	2.66±0.02^{d} (322.22)	0.60±0.00^{d} (30.15)
NIIST B616+627+P	427.67±2.52^{d} (172.98)	2.43±0.01^{d} (19.70)	3.00±0.00^{b} (50.00)	2.67±0.58^{b} (100.75)	2.95±0.01^{e} (368.25)	0.60±0.00^{e} (31.02)
Pathogen	156.67±2.52^{a}	2.03±0.58^{a}	2.00±0.00^{a}	1.33±0.58^{a}	0.63±0.00^{a}	0.46±0.00^{a}

* All values are expressed as mean ± SD from three replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses represents percentage increases over the pathogen. The different percentage increase for same value indicates their minor variation to the next decimal point

** Harvest index is the ratio of harvested grain to total dry shoot matter

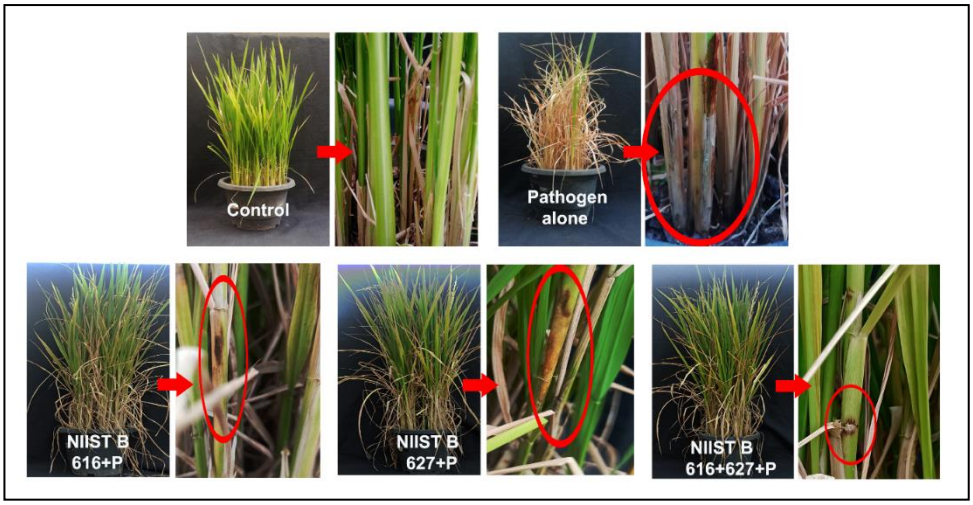


Fig 3.5 Effect of endophytic isolates and their combination on disease control in *R. solani* treated plants after 90 days**.** The arrow mark indicates the respective enlarged portion of the collar region of the plant. Control - no treatment, NIIST B616+P -*Bacillus subtilis* NIIST B616 + *R solani*, NIIST B627+P - *Bacillus subtilis* NIIST B627 + *R solani*, NIIST B616+627+P - *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627 + *R solani*, Pathogen -*R solani* alone

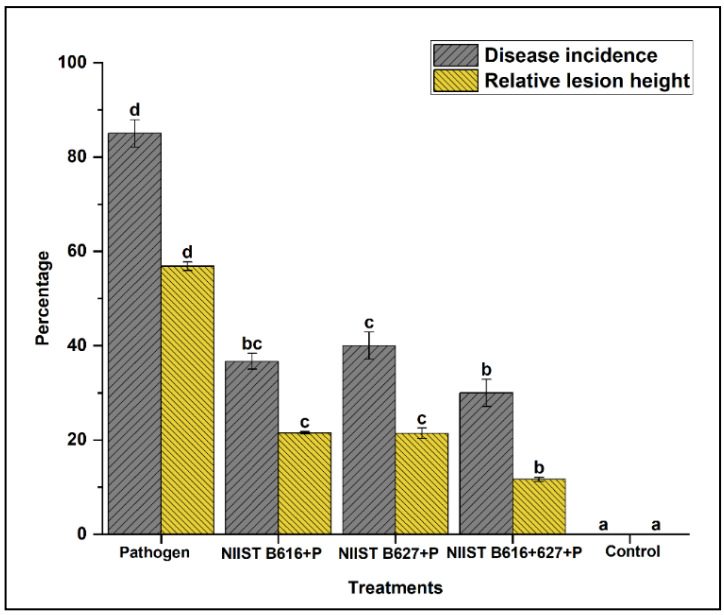


Fig 3.6 Efficacy of isolates and their combination on disease control in *R. solani* treated plants after 90 days. All values are expressed as mean ± SE from three replications. Different superscript letters indicate the different degrees of treatment at a statistical significance of 5% (α). Control - no treatment, NIIST B616+P -*Bacillus subtilis* NIIST B616 + *R solani*, NIIST

B627+P - *Bacillus subtilis* NIIST B627 + *R solani*, NIIST B616+627+P - *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627 + *R solani*, Pathogen -*R solani* alone

3.3.5. Induction of systemic resistance against *R. solani* by endophytic *Bacilli*

In general, all three enzymes exhibited a notable increase in plants subjected to endophytic isolates and *R. solani* within 30 days. This increase was followed by a slight rise up to 50 days and a decline on day 60, remaining stable for the remaining observation period. Additionally, plants solely treated with *R. solani* demonstrated a sharp rise in enzyme levels for up to 30 days, with a gradual decrease thereafter. The control plants, on the other hand, experienced a minor increase in PAL (within 40 days), POX (within 20 days), and PPO (within 20 days), which was then succeeded by a decrease, remaining constant throughout the observation period (Fig 3.7A-C).

Regarding phenolic content, all plants treated with endophytic isolates and *R. solani* displayed consistent growth for 60 days, remaining stable for the remainder of the observation period. Conversely, plants treated solely with *R. solani* demonstrated a slight increase for up to 30 days, which then gradually declined. Control plants exhibited a slight increase for up to 20 days, followed by a decrease for up to 50 days, but remained unchanged for the entire observational period (Fig 3.7D).

On day 80, the data revealed that plants treated with both isolates exhibited significantly elevated enzyme activity and phenolic content. NIIST B627+P and NIIST B616+P demonstrated slightly decreased activity levels compared to the combination treatment. In contrast, plants treated exclusively with *R. solani* displayed notably reduced activity levels.

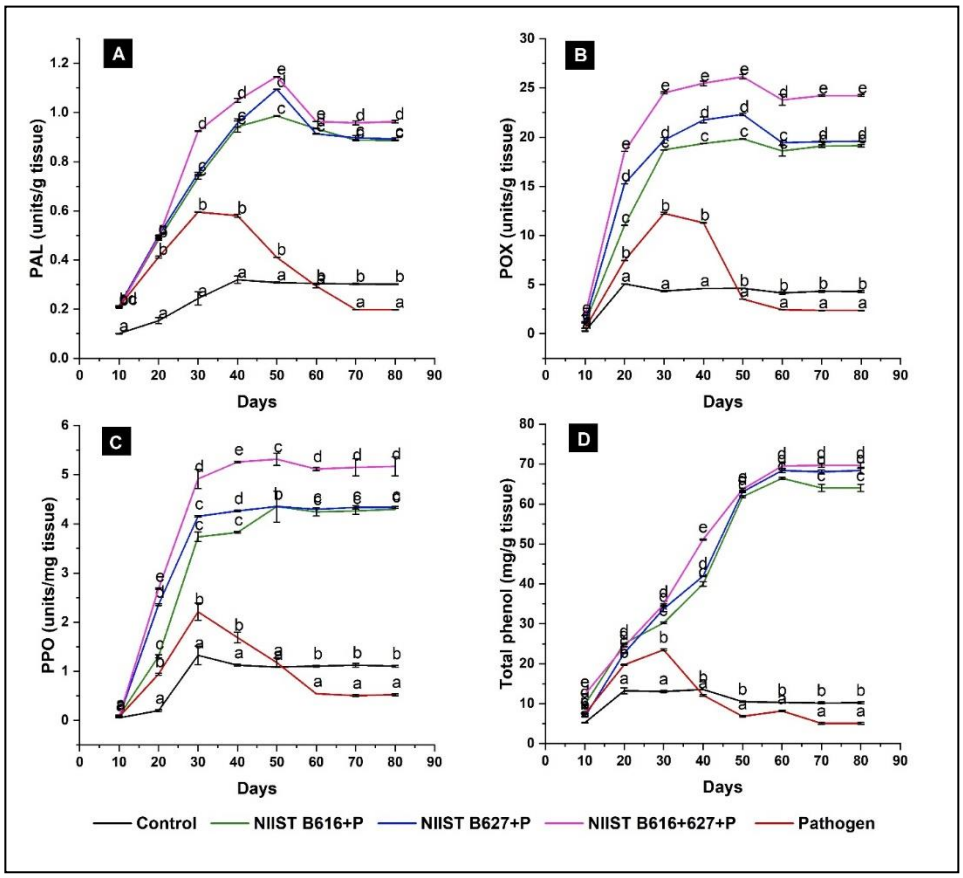


Fig 3.7 Enzyme activity of (A) PAL, (B) POX, (C) PPO, and (D) total phenol content of rice plants treated with isolates and *R. solani* on various days. All values are expressed in mean ± SE from three replications. Different superscript letters on the same day indicate the different degrees of treatment at a statistical significance of 5% (α). Control- no treatment, NIIST B616+P -*Bacillus subtilis* NIIST B616 + *R solani*, NIIST B627+P - *Bacillus subtilis* NIIST B627 + *R solani*, NIIST B616+627+P - *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627 + *R solani*, Pathogen alone-*R solani* alone

3.3.6. Validation of ISR through split-root experiment

The initial documentation of the disease symptom occurred with *R. solani / R. solani* treatment starting from the seventh day onwards, resulting in the manifestation of a brownish lesion in the collar region. Subsequently, the lesion expanded and became bleached, acquiring a greenish-white hue by day 14. The plant started displaying indications of mortality as early as day 21, and by day 28, the infected plant suffered complete death. In contrast, the remaining plants subjected to endophytic bacterial treatments and control conditions maintained their

well-being throughout the observation period, devoid of any disease symptoms (Figs 3.8 & 3.9).

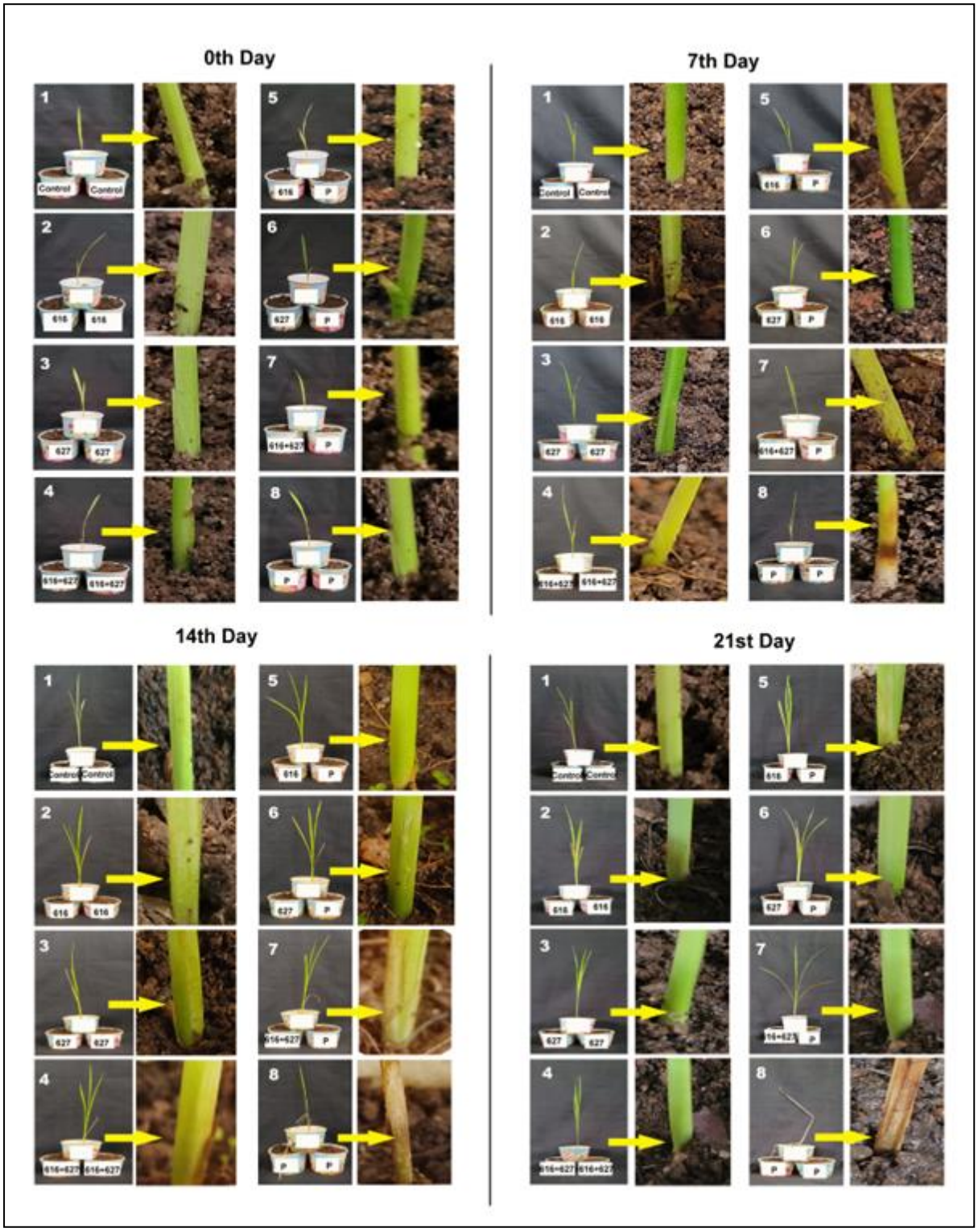


Fig 3.8 Split-root experiment on 0-21 days. The arrow mark indicates the respective enlarged portions of the collar region of the plant. **1.** Control/Control **2.** 616/616 **3.** 627/627 **4.** 616+627/616+627 **5.** 616/P **6.** 627/P **7.** 616+627/P **8.** P/P

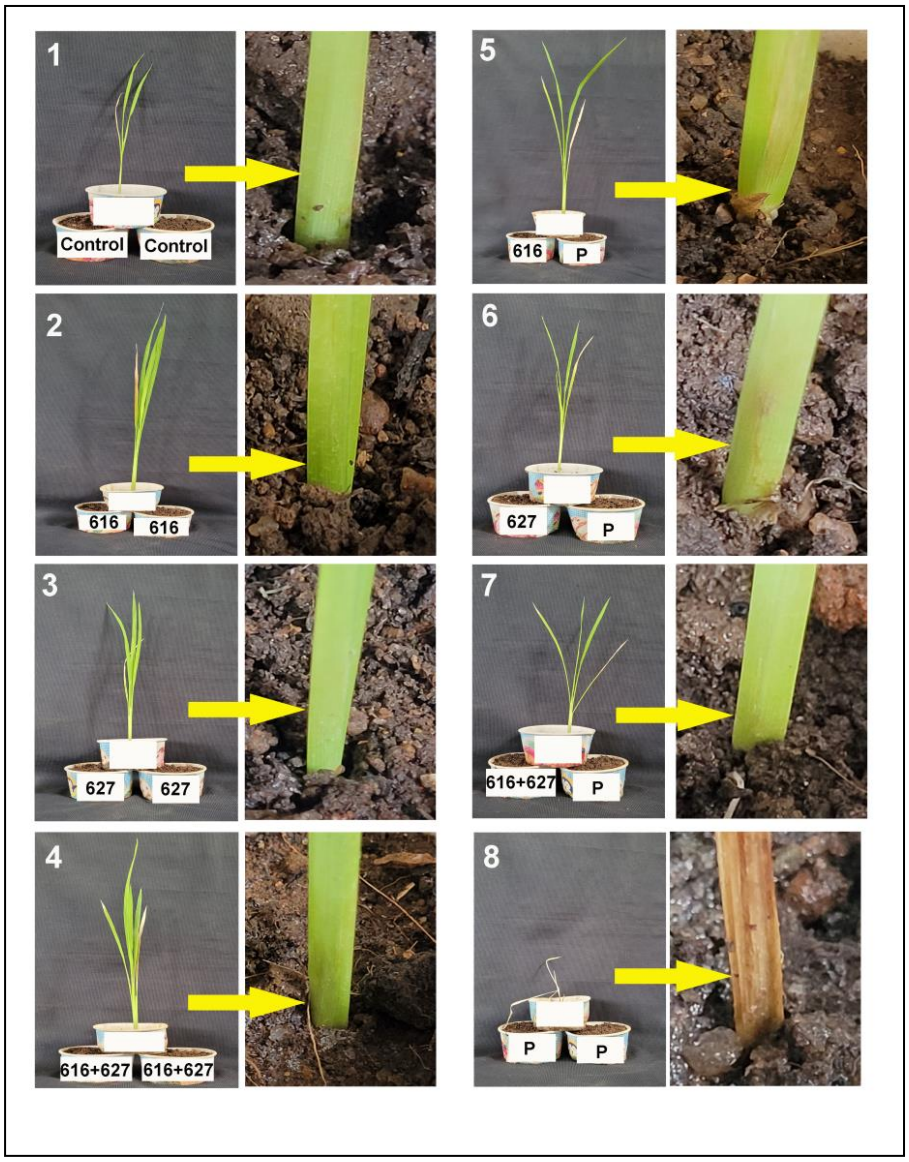


Fig 3.9 Split-root experiment with different treatments on elicitation of induced systemic resistance on day 28. The arrow mark indicates the respective enlarged portion of the collar region of the plant. **1.** Control/Control **2.** 616/616 **3.** 627/627 **4.** 616+627/616+627 **5.** 616/P **6.** 627/P **7.** 616+627/P **8.** P/P

3.3.7. Root colonization

The presence of both bacteria in the root sections was detected by SEM examination of individual treatments, confirming that both introduced strains were endophytic and colonizing the roots (Fig 3.10).

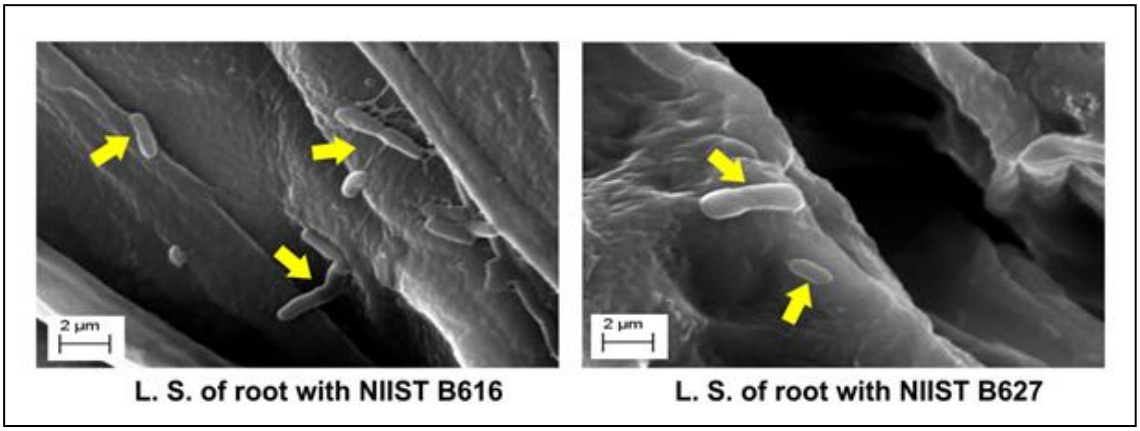


Fig 3.10 SEM images of the LS of rice plant root showing the presence of the introduced test bacterial strains- *Bacillus subtilis* NIIST B616 and *Bacillus subtilis* NIIST B627

3.3.8. Rhizospheric microbial community analysis

The microbial diversity indices revealed that all rhizospheric soil samples were diverse, with the maximum number of taxa found in NIIST B616 + 627 rhizospheric soil, followed by NIIST B627, control, while the minimum number was found in NIIST B616. The Shannon diversity index disclosed higher diversity in NIIST B627 (10.53), followed by NIIST B616 + 627 (10.34), control (10.16) and the lowest in NIIST B616 (9.25) (Fig 3.11). The community composition of annotated rhizomicrobiome indicated that there were 29 phyla in control, but there was a decrease found in the rhizospheric soil of NIIST B616 with 24 phyla, and just the opposite was found in the other two experimental groups; the rhizospheric soil of NIIST B627 and NIIST B616 + 627 had 31 phyla and 32 phyla respectively. The dominance of microbial taxa at the phylum level of all samples disclosed in similar phyla, namely Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Firmicutes, Planctomycetes, Gemmatimonadetes, Patescibacteria and Bacteroidetes. The control soil sample revealed the dominance of Proteobacteria (23.58 %), Actinobacteria (21.05%), Acidobacteria (12%), Chloroflexi

(9.74%), Firmicutes (6.18%), Unknown[11] species (6.10%), Planctomycetes (5.96%), Gemmatimonadetes (5.67%), others[12] (3.56%), Patescibacteria (3.17%), and Bacteroidetes (2.98%). In rhizospheric soil treated with NIIST B616 showed a dominance from Proteobacteria (29.62%), Actinobacteria (21.23%), Chloroflexi (10.60%), Acidobacteria (8.92%), Planctomycetes (6.63%), Gemmatimonadetes (5.96%), Bacteroidetes (5.08%), Firmicutes (4.39%), Unknown group (3.15%), others (2.43%) and Patescibacteria (1.99%) while in soil treated with NIIST B627 have Proteobacteria (29.29%), Actinobacteria (15.18%), Chloroflexi (10.45%), Planctomycetes (8.51%), Acidobacteria (7.77%), others (6.18%), Bacteroidetes (5.30%), Gemmatimonadetes (5%), Firmicutes (4.68%) Patescibacteria (4.20%), unknown (3.44%). The rhizosphere soil treated with NIIST B616 and NIIST B627 together, demonstrates the abundance percent from Proteobacteria (31%), Actinobacteria (15.31%), Chloroflexi (9.77%), Planctomycetes (8.43%), Acidobacteria (7.29%), others (5.61%), Bacteroidetes (5.34%), Gemmatimonadetes (4.91%), Firmicutes (4.75%), Patescibacteria (4.27%), and unknown (3.30%) (Fig 3.12).

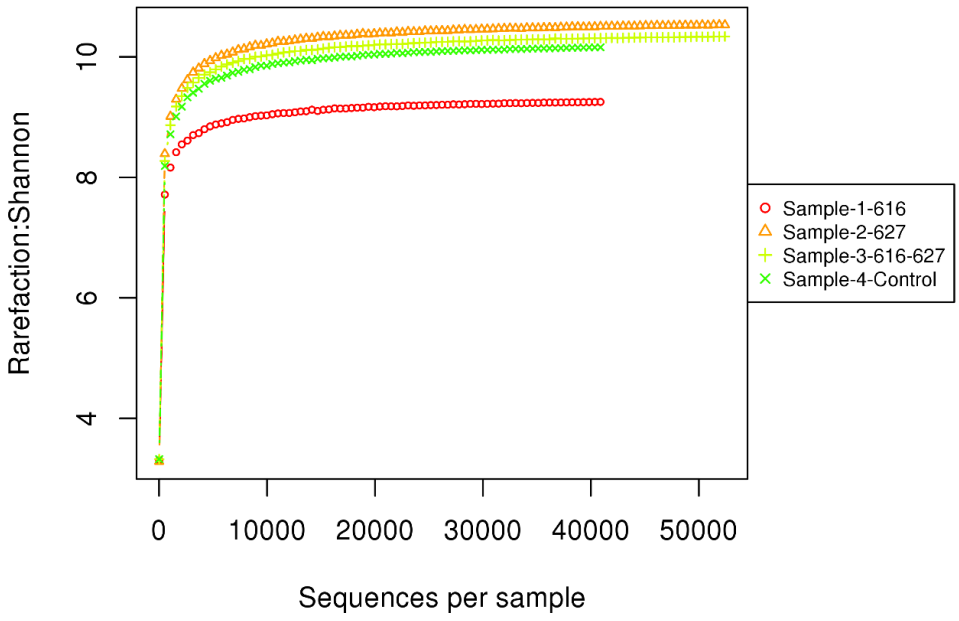


Fig 3.11 Shannon curve obtained for rhizospheric soil samples of NIIST B616, NIIST B627, NIIST B616+627 and control

[11] Sequences that do not have any alignment against the taxonomic database are categorised as Unknown

[12] The taxa other than the top 10 are categorised as Others

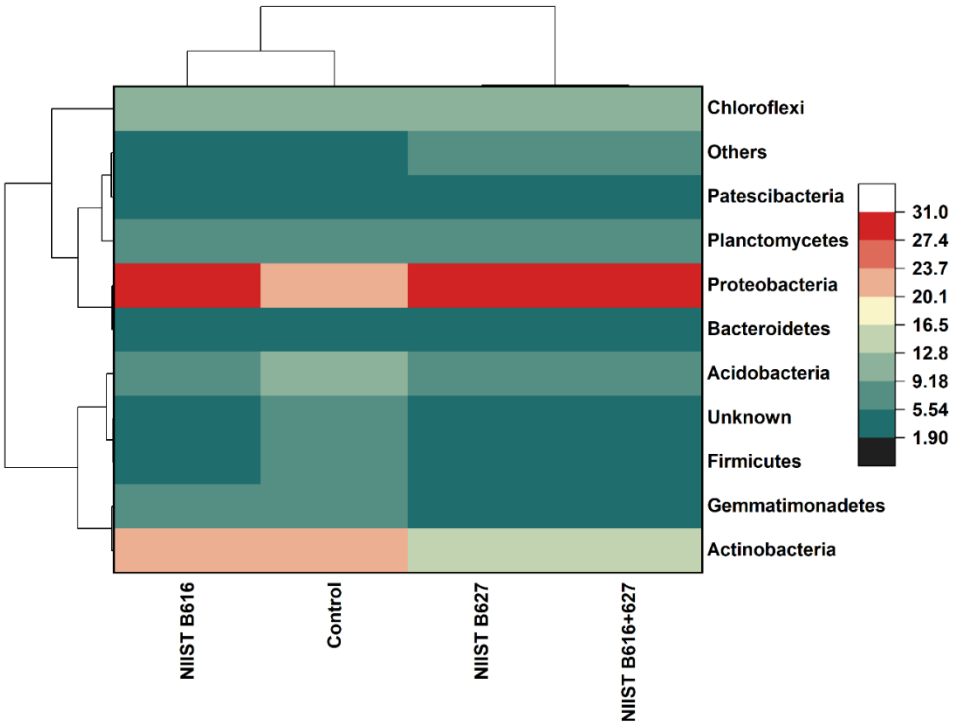


Fig 3.12 Heatmap of relative abundance at the phylum level of rhizospheric soil among experimental groups control, NIIST B616, NIIST B627 and NIIST B616+627

3.4.Discussion

The use of endophytic bacteria for environmentally friendly crop enhancement and disease control has seen a significant increase in recent times (White et al., 2019). Endophytic *Bacillus* species have received more attention among these bacteria, although considerably less than their rhizosphere counterparts. This investigation focused on endophytic *B. subtilis* strains, NIIST B616 and NIIST B627, which were isolated from Kuttanad rice plants and selected based on their antagonistic potency against *R. solani*, the causal agent of sheath blight disease. The research affirms their effective use in promoting plant growth, increasing yield, and providing resistance against sheath blight disease through ISR. Endophytes have a significant impact on promoting plant growth through various mechanisms, both direct and indirect. These mechanisms have a positive influence on plant growth and yield, which encompass actions such as phosphate and potassium solubilisation, siderophore production, as well as the synthesis of IAA, HCN, and ammonia (Rana et al., 2020). Similarly, our research reveals that

the two selected endophytic *Bacillus* strains which displayed effectiveness against *R. solani* in *in vitro* studies also showed plant growth-promoting attributes, such as phosphate solubilisation, production of HCN, IAA, siderophore, and VOCs. Their compatibility to coexist further validates the selection of these organisms. In a corresponding investigation, 32 bacterial endophytes derived from six different rice cultivars were similarly found to produce IAA and siderophore and exhibit phosphate solubilisation activity, indicating their potential to promote plant growth. Furthermore, these endophytes were found to inhibit the growth of *R. solani*, confirming their disease-resistant properties (Kumar et al., 2020).

Numerous studies have documented the positive effects of endophytic bacteria on plant growth, productivity and management of disease through ISR (Kumar et al., 2020; Nagendran et al., 2014; Safari Motlagh et al., 2022). Two endophytic *Bacillus* isolated from *Teucrium polium* and their consortia were reported to have stimulated plant growth in maize (Hassan, 2017), while diazotroph endophyte *Lysinibacillus sphaericus* protected rice plants against sheath blight disease (Shabanamol et al., 2017). Previous reports have indicated that multi-strain inoculation has more significant benefits than single inoculation on various plants, including rice, grass, corn, and douglas-fir (Khan et al., 2016). Likewise, our investigation observed a statistically significant improvement in plant growth and yield indices for individual test organisms and their combination (multi-strain inoculation) in both pathogen infested soil and pathogen devoid soil. The combined application of bacteria exhibited the most remarkable effect on plants when grown in pots in both cases. Equivalent results were also seen with several endophytic *Bacillus* species (Hassan, 2017; Sahu et al., 2020), with *B. subtilis* showing considerable plant growth promotion, yield, and suppression of sheath blight disease in nursery conditions (Durgadevi et al., 2015). Growth enhancement of rice plant, based on its root length, fresh weight, and shoot length by *B. methylotrophicus* (DD-1) (Liu et al., 2020) and dual inoculation with nitrogen-fixing bacteria and phosphate solubilising bacteria which benefits over single ones (Kumar et al., 2015), revealed the enhanced effect of plant growth and yield by these bacteria.

Another aspect of these endophytic bacteria, aside from enhancing plant growth is their ability to induce disease resistance in plants through ISR. This is particularly relevant for rice, the primary food source in Kerala is vulnerable to diseases such as sheath blight, which poses a significant challenge to rice cultivation specifically in lowland and rainfed rice fields, like the below sea level farmlands of Kuttanad (Kumar et al., 2020; Sahu et al., 2020). This disease is of great concern since Asia accounts for 90% of global paddy production and consumption,

and India is the second-largest rice producer (Shahbandeh, 2021). Consequently, sheath blight is considered a dominant disease worldwide, leading to an annual yield loss of 10-30% and potentially reaching 50% in the near future (Jamali et al., 2020; Li et al., 2021). To mitigate this problem, researchers have explored alternative solutions by investigating the use of biocontrol agents, which are advantageous due to their high multiplication and growth rates, as well as their aggressive colonization abilities (Jamali et al., 2020). In this study, endophytic bacterial isolates derived from Kuttanad were applied to rice plants challenge-inoculated with *R. solani*, resulting in a reduced incidence of the disease compared to plants inoculated solely with *R. solani*. The most favourable outcome was observed in rice plants treated with a combination of both bacterial strains (NIIST B616+627+P), which reduced sheath blight disease by 64.71%. In contrast, when the bacterial treatments were applied individually, the disease incidence was reduced by 56.86% and 52.94%, respectively.

Reduction in disease symptoms provided a vivid sign of disease suppression. The individual and combined application of two endophytic bacteria demonstrated a surge in defence related enzymes PAL, POX, PPO, and total phenol content in plants challenge inoculated with *R. solani*. However, in plants treated with *R. solani* alone, all enzyme levels rise sharply in the initial days and then gradually drop, which may be one of the main reasons for the onset of disease symptoms. Various studies back up our conclusion that a rise in enzyme levels aids plants in reducing disease symptoms. With varying quantities of endophytic *Bacillus subtilis* var. *amyloliquefaciens* (FZB24) applied to seeds, seedlings, soil, and leaves, the levels of POX, PPO, PAL, and total phenol content increased compared to untreated plants, resulting in a decrease in sheath blight disease severity by up to 55% (Nagendran et al., 2014). A further investigation by Jamali et al. (2020) has corroborated that *B. subtilis* treatment after inoculation with *R. solani* increased defence enzyme levels in rice plants, enhancing disease resistance. Similarly, Jayaraj et al. (Jayaraj et al., 2004) demonstrated that foliar application of *B. subtilis* increased PAL and POX activity, decreasing plant disease symptoms. Our research also verified the escalation of PAL, POX, PPO, and total phenolic content as a defence mechanism elicited through ISR against *R. solani*, leading to a decrease in the disease symptoms in plants that were inoculated with the endophytic bacteria. The concurrent presence of two isolates significantly increased the levels of PAL (2.8 fold), POX (7.4 fold), PPO (4.5 fold), and total phenol (8.5 fold), compared to the plants treated exclusively with *R. solani*. Consequently, our findings suggest that the use of *B. subtilis* strains potentially stimulates ISR, increases the production of polyphenolic compounds, and enhances the biosynthesis of secondary

metabolites in treated plants. A split-root experiment was conducted to support the concept of ISR. Since pathogen and endophytic isolates do not come into contact, the possibility of direct inhibition is eliminated. However, the presence of endophytic bacteria clearly induces resistance to *R. solani* in plants, as no disease symptoms were observed in the plants treated with these bacteria. Previous studies have demonstrated the development of ISR against *Fusarium udum* in pigeon peas using a split-root system with bacterial strains *B. cereus* BS 03 and *Pseudomonas aeruginosa* RRLJ 04 and in combination with the rhizobial strain RH 2 (Dutta et al., 2008). Moreover, SEM analysis confirmed the presence of two *Bacillus* species colonising the roots, providing evidence for their ability to enhance plant growth through mechanisms other than disease resistance. Similar findings were reported by Wang et al., (2019), who observed the endophytic coloniszation of *Bacillus velezensis* in sugarcane roots 14 days post-inoculation. Therefore, this characteristic of bacterial colonization is widely believed to play a crucial role in promoting plant growth (Posada et al., 2018).

The changes in the microbial community of rhizospheric soil upon the addition of endophytes were analysed through a metagenomic study. Metagenomics has opened new vistas in understanding microbial communities, unveiling a wealth of 16S rRNA gene sequences from previously uncultured bacterial species. Through this approach, diverse microbial groups have come to light (Adedayo et al., 2023). Analysis of rhizomicrobiomes across various soil samples has revealed the prevalence of major phyla such as Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Firmicutes, Planctomycetes, Gemmatimonadetes, Patescibacteria, and Bacteroidetes. Studies consistently demonstrate that Proteobacteria dominate rhizospheric communities, showcasing the selective influence of roots (González et al., 2022; Knief et al., 2012). In a comparative analysis, rhizospheric samples of rice plants, Proterobacteria emerged as the predominant taxa (Prasannakumar et al., 2021). Similar trends were observed in tomato rhizosphere soil, with elevated levels of Proteobacteria, Bacteroidetes, and Acidobacteria (Cheng et al., 2020). In our study, it was intriguing to find that when rhizospheric soil was treated with the endophytic bacteria NIIST B616 and NIIST B627 together, increased diversity was observed compared to both the controls and the individual bacterial treatments. Specifically, in soils treated with individual bacteria, NIIST B627 exhibited the highest diversity, while NIIST B616 displayed the lowest diversity, even less diverse than the control group. The decrease in the microbiome of NIIST B616 rhizospheric soil could indeed be attributed to the action of metabolites produced by the added bacteria or by the plant itself. Given that NIIST B616 is endophytic, it may infiltrate the plant, inducing physiological and

chemical alterations which lead to the secretion of substances that modulate microbial growth in the root vicinity. This process could potentially disrupt the adaptation of certain microbes, leading to a reduction in their abundance and diversity. This hypothesis finds support in Qiao's study, which compared nutrient-rich soil with bulk soil microbiomes and observed decreases in certain microbial populations alongside increases in the diversity of certain other groups, speculated to be influenced by substances secreted by plants. These substances could potentially foster the growth of specific microbes, which in turn establish competitive relations with others or produce antimicrobials, thus inhibiting other microbial communities (Qiao et al., 2017). Notably, combining both bacteria in the rhizosphere led to enhanced diversity, surpassing both the control and individual treatments, underscoring the role of endophytic bacteria in shaping microbial community structures. This microbial community within the soil significantly influences plant growth, succession, and the overall structure of plant communities (Nan et al., 2020). Further investigation is warranted to validate this hypothesis and deepen our understanding of the complex interactions between plants, bacteria, and soil microbiomes.

3.5.Conclusion

Endophytic bacteria are regarded as a promising and sustainable alternative approach for simultaneously enhancing crop productivity and managing diseases in contemporary agricultural practices. Our investigation underscores the utilisation of endophytic *Bacillus* to augment rice plant growth, yield, and disease management by stimulating induced systemic resistance against sheath blight disease. The application of endophytic *B. subtilis* strains has demonstrated significant improvements in plant growth and defence mechanisms, as evidenced by the increased activity of defence-related enzymes and total phenol content in rice plants challenged by *R. solani*. This augmentation ultimately leads to a reduction in disease incidence. Consequently, incorporating endophytic bacteria into agricultural practices will serve as an environment-friendly alternative to harmful pesticides or herbicides, thereby fostering sustainability in agriculture. Despite this, our analysis of the rhizospheric microbial community indicates minimal changes in the existing microbiome upon the introduction of these *B. subtilis* strains to the soil. Therefore, both endophytic isolates hold promise for enhancing crop productivity and effectively combating sheath blight, particularly in regions like Kuttanad. By leveraging the benefits of endophytic bacteria, we can achieve sustainable agricultural practices while addressing the challenges of disease management in rice cultivation.

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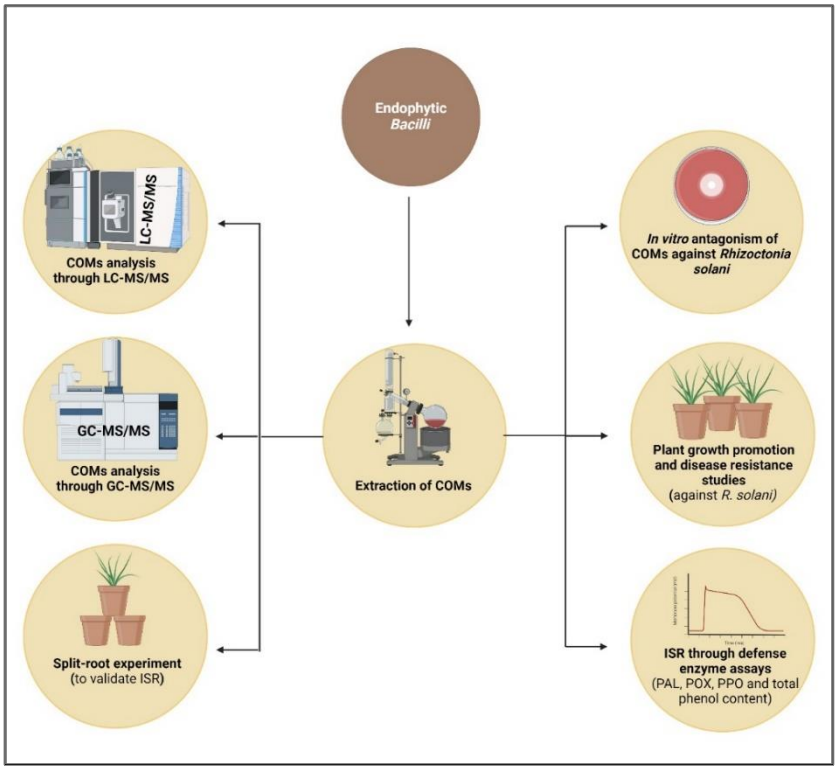
Chapter 4

Improved Plant Growth and Sheath Blight Disease Control in Rice Plant Through ISR by Endophytic Metabolite

Graphical Abstract



4.1.Introduction

Sheath blight disease is a prevalent fungal disease that has a detrimental impact on rice plants. The aetiology of this disease is linked to the pathogen *Rhizoctonia solani*, which causes significant reductions in crop yield and poses a major obstacle to crop improvement (Singh et al., 2019). Farmers across the globe commonly depend on chemical pesticides as their primary defence mechanism against the disease. Yet, the repeated use of synthetic pesticides leads to the emergence of pesticide-resistant pathogenic microbes, which negatively impact natural predators and the environment, contributing significantly to ecological imbalance (Subramaniam et al., 2016). However, various microbial species and their microbial products, particularly secondary metabolites, have been identified to exhibit antagonistic activity or induce systemic resistance (ISR) in plants against several phytopathogens.

Secondary metabolites are intricate organic compounds with a low molecular weight produced by various life forms, including microorganisms and plants. These compounds, which consist of antibiotics, ribosomal peptides, non-ribosomal peptides, polyketides, volatile organic compounds, and other types, are not utilised during the organisms' lifecycle unless certain unfavourable circumstances arise (Buddhika & Abeysinghe, 2021). Microorganisms, during their idiophase, synthesised these structurally diverse compounds (Ruiz et al., 2010) that serve a variety of functions, including antibacterial, antiviral, antifungal, enzyme inhibition, receptor antagonism and agonism, immunomodulation and also serve as antitumor agents, bio-indicators, preservatives, feed additives, growth promoters, herbicides, and pesticides (Pradeepa, 2019). Therefore, the vast reservoir of crude organic metabolites (COMs), which includes secondary metabolites, possesses immense potential for stimulating plant growth and effectively managing diseases.

Resistance against plant diseases is believed to be a complex and ever-changing phenomenon that involves several processes. It is postulated that the plant's defence response activation is contingent upon the specific recognition of certain microorganisms or microbial products by the plant. Plants exhibit varied responses to stimuli, which entail the production and cumulation of antimicrobial phytoalexins, generation of defence-associated proteins, initiation of hypersensitive response, synthesis of activated oxygen species, and alteration of cell wall structure through callose deposition. The activation of defence responses in plants is observed to be more rapid and heightened upon exposure to any bioagent (Akram et al., 2013). Presently, there is a growing emphasis on using bioagents and their derivatives that do not

cause any harm to the environment. The bioagents explicitly endophytes, which are endogenous microorganisms inhabiting the plant body, exhibit a crucial function in this context by producing specialised metabolites that contribute significantly to the growth, maturation, and protection of the host plant. Numerous accounts are surfacing that aim to develop eco-friendly remedies, such as utilising *Bacillus* for the proficient management of soil-borne pathogens and the successful induction of systemic resistance in treated plants. Several strains of *Bacillus*, particularly *B. subtilis* RB14-C, can synthesise the antibiotic iturin A, which exhibits antifungal properties against *Rhizoctonia solani* in tomato plants (Szczech & Shoda, 2006). The induction of ISR in plants is attributed to various secondary metabolites, commonly referred to as "elicitors", that are responsible for initiating distinct signalling pathways to activate plant resistance (Borriss et al., 2019). The induction by bioagents is not attributable to their antimicrobial attributes or capacity to metamorphose into antimicrobial agents. Nevertheless, antimicrobial agents can elicit ISR and afford protection from the time of administration until the complete manifestation of ISR (Ku, 2001). Hence, ISR, through bioagent products, presents a promising strategy for the sustainable safeguarding of crops and can potentially lessen our dependence on synthetic pesticides.

The objective of the current study was to assess the efficacy of crude organic metabolites recovered from the two isolated endophytic *Bacillus* (detailed in previous chapters) for enhancing the growth of rice plants and protecting against sheath blight disease through ISR. In order to scrutinise the mechanism of ISR, the activity of defence-related enzymes such as L-phenylalanine ammonia-lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO), and total phenolic content was evaluated. A split-root experiment was performed to validate the ISR. Moreover, gas chromatography-tandem mass spectrometry (GC-MS/MS) and liquid chromatography and tandem mass spectrometry (LC-MS/MS) analyses were carried out to elucidate the secondary metabolite content in the crude organic metabolites.

4.2.Materials and Methods

4.2.1. Microbial strains and culture conditions

Two endophytic *Bacillus*, namely *Bacillus subtilis* NIIST B616 and *Bacillus subtilis* NIIST B627, as detailed in the previous chapters were used for extracting crude organic metabolites.

The phytopathogen, *Rhizoctonia solani* ITCC 6882 was obtained from the Indian Type Culture Collection (ITCC), Division of Plant Pathology, Indian Agricultural Research Institute (IARI),

New Delhi, India. It was maintained in PDA (HiMedia, India) at 28±2℃ and kept at 4℃ for future investigations.

4.2.2. Experimental site

The study was conducted at the Agro-Processing and Technology Division of CSIR-NIIST in Thiruvananthapuram, Kerala, India, from 2019 to 2021. The region experiences a hot tropical climate, with heavy rainfall during the monsoon seasons. The average minimum and maximum temperatures recorded during the study range between 24.21 to 31.37℃, with an average rainfall of 10.4 mm and relative humidity of 89.17%.

4.2.3. Extraction of crude organic metabolites

One mL of 18 h old inoculum of both *Bacillus* was separately added to 100 mL of Luria Bertani (LB) broth (HiMedia, India) contained within a 250 mL Erlenmeyer flask and kept in a shaking incubator for 72 h at 28°C at 130 rpm. Following incubation, the broth was centrifuged, and the supernatant was collected for subsequent metabolite extraction using hexane, chloroform, ethyl acetate, and ethyl acetate: methanol (95:5) (Sigma-Aldrich, US). The obtained COMs were then concentrated in a rotary evaporator (Heidolph, Germany).

4.2.4. *In vitro* antagonism of crude organic metabolites

In vitro antagonism of the crude organic extract was performed using the agar well diffusion method (Sriram et al., 2019). 20 µL of 20 mg/mL crude extract from both strains were introduced separately into the well (6.0 mm in diameter) in separate PDA plates, with a 6.0 mm circular disc of actively growing mycelia being placed opposite (approximately 5 cm) to the extract. The plates without crude metabolites served as control. All plates were incubated at 28±2℃ in biological oxygen demand (BOD) incubator for 7 days, and the inhibition zone (mm) was measured. The readings were taken from three replicates. Changes in the fungal hyphae at the point of inhibition were also observed through scanning electron microscopy (SEM). For this, fungal mycelia from the point of inhibition and from the control plates were carefully collected. These mycelia were then placed on double adhesive tape on a stub. Afterwards, the sample was dried, and a thin layer of gold was applied using a sputter coater. The gold-coated metal stub was observed using the scanning electron microscope (Zeiss Evo 40 EP) with an accelerating voltage of 20 KV and a probe diameter of 102 pA to capture

secondary electron images (Fischer et al., 2012). The field was scanned to examine any variations in hyphal morphology, and suitable fields in the preparation were photographed.

4.2.5. *In vivo* screening of crude organic metabolite for plant growth promotion

4.2.5.1.Physicochemical properties of soil

The soil employed in this investigation was subjected to air-dry at room temperature. Subsequent to this, it was meticulously crushed using a mortar and pestle and passed through a stainless-steel sieve with a mesh size of 2 mm. The resultant finely ground soil sample was suitably retained in polythene bags for analytical purposes. The characteristics such as pH of the soil, electric conductivity, available organic carbon, available phosphorous, available potassium and soil texture were analysed according to the procedure as detailed in previous chapter.

4.2.5.2.Surface sterilisation of seed

The study used highly susceptible Jyothi rice seeds from the Regional Agricultural Research Station in Pattambi, Kerala. To sterilise the rice seeds, a modification of the technique described by Dileep Kumar & Dube (1992) was employed. Initially, the seeds were immersed in 70% ethanol for 2 minutes and then treated with 2.5% sodium hypochlorite solution. Following this, the seeds underwent five rinses in sterile distilled water and were dried under sterile conditions.

4.2.5.3.Impact of crude organic metabolite on plant growth

The experimental setup involved plastic pots measuring 18×22 cm, which were filled with sandy loam soil mixed with cow dung (3:1). The experimental consisted of 200 pots with four distinct sections, namely the control group, which did not receive any metabolite, a group that was exposed to the metabolite of both bacteria alone (NIIST B616 or NIIST B627), and a combined group with the metabolites of two *Bacillus* together added (NIIST B616+627). For the individual treatments, 50 mL of 1mg/mL crude metabolite was introduced per pot. On the other hand, the combination treatment involved equal amounts of both metabolites adjusted to 1 mg/mL were introduced. The uppermost layer of each pot was mixed with a sterile glass rod to a depth of approximately 2 cm. Subsequently, 20 surface sterilised seeds (Dileep Kumar & Dube, 1992) were introduced into each pot at a depth of 1 cm and grown under nursery conditions. The control group consisted of pots with only surface sterilised seeds. Experimental

procedures were conducted following a completely randomised design (CRD), with data recorded from ten replications each. Plant growth-promoting parameters, such as shoot height, root length, dry weight, and total chlorophyll content (Hiscox & Israelstam, 1979), were monitored every seven days up to 28 days. A booster dose of foliar application was administered after 30 days. The yield estimation (after 120 days) was carried out by analysing various factors, including the number of grains, the weight of 100 grains, the number of tillers and panicles per plant, dry shoot matter and harvest index. The data was recorded as outlined in the previous chapter.

4.2.6. Impact of crude organic metabolites on plant growth in the presence of *R. solani*

The study comprised five distinct sections, namely the control group, which involved no organic metabolite or pathogen *R. solani*; the pathogen alone group, which involved only *R. solani*; the NIIST B616+P group, which involved the metabolite of NIIST B616 and *R. solani*; the NIIST B627+P group which involved metabolite of NIIST B627 and *R. solani*, and the NIIST B616+627+P group which involved a combination of metabolites of NIIST B616, NIIST B627 and *R. solani*. For each treatment, 50 pots were maintained. Except for the control group, all pots were exposed to a 50 mL homogenised solution of *R. solani* (10^8 CFU/mL). A booster dose foliar application was performed after 30 days of growth, and the growth parameters and yield were assessed as previously described.

4.2.6.1.Impact of crude organic metabolite on disease resistance

The occurrence of disease symptoms and the relative height of lesions 90 days after the treatment were determined through the Rice Standard Evaluation System scale developed by the International Rice Research Institute, Philippines (IRRI, 2013). The percentage of disease incidence and the assessment of relative lesion height were carried out as described in Chapter 3.

4.2.7. Induction of systemic resistance by crude organic metabolites

For this study, five treatments were chosen: treatment with no organic metabolite or pathogen (control), *R. solani* alone (pathogen alone), the metabolite of NIIST B616 + *R. solani* (NIIST B616 + P), the metabolite of NIIST B627 + *R. solani* (NIIST B627 + P), and a combination of metabolites of NIIST B616, NIIST B627 + *R. solani* (NIIST B616 + 627+P). For a period of 80 days, three different defence enzymes such as PAL (EC 4.3.1.24), POX (EC 1.11.1.7), and

PPO (EC 1.10.3.1), as well as the total phenol content were evaluated at 10 day intervals. To conduct the assays, fresh leaf samples were collected, weighing 3 g (for PAL, POX, and PPO) and 0.5 g (for total phenol), respectively.

The leaf samples were homogenised using a pre-cooled mortar and pestle, along with 9 mL of sodium borate buffer and 0.8 mL/L of 2-mercaptoethanol to perform the PAL assay. The resulting extract was then centrifuged at 12,000 *g* for 20 min at a temperature of 5°C, after which the supernatant was utilised as the enzyme for the assay. The PAL activity was determined in accordance with the method outlined by Sadasivam & Manickam (1991). A UV-Vis spectrophotometer (Shimadzu, Japan) was employed to measure the wavelength at 290 nm, and the reaction rate was recorded as micromole trans-cinnamic acid formed (Units/g fresh weight).

The POX activity was estimated using the methodology reported earlier (Thimmaiah, 1999). In brief, leaf samples were homogenised in 9 mL of 0.1 M phosphate buffer (pH 7.0) using a pre-chilled mortar and pestle. The resulting extract underwent centrifugation at 18,000 *g* for 15 min at 5°C, following which the supernatant was utilised as the enzyme sample for the assay. The absorbance was measured at 430 nm, and the total activity of the POX enzyme was computed as units/g fresh weight of the sample, where one unit of the enzyme was considered an increase in OD by 1.0 under standard conditions.

For the analysis of PPO, the samples were homogenised in a solution of 0.1 M sodium phosphate buffer (6 mL) at pH 7.1, followed by centrifugation at 1,500 *g* for 40 min at 5°C. The resulting supernatant was utilised as the enzyme sample, and the PPO activity was determined using the method described by Sadasivam & Manickam (1991). The change in absorbance per millilitre of enzyme extract per minute was measured to determine PPO activity, which was then calculated in units/mg of fresh weight.

To determine the total phenolic content, the specimens were extracted in 80% ethanol (10 mL) and then centrifuged at 12,000 *g* for 20 min. The estimation procedure followed the protocol established by Mahadevan & Sridhar (1986). Total phenolic content was quantified using a standard graph obtained from catechol and expressed as mg/g of material (tissue weight). All readings were taken from three replicates.

4.2.8. Confirmation of ISR through split-root experiment

The objective of the split-root experiment was to confirm ISR by the COMs from endophytes against *R. solani* in rice plants. The study followed the procedure of Dutta et al., (2008) using a three-cup system with a minor alteration. The experiment was divided into eight sections, with a three-cup system (two lower cups and one upper cup). Each of the bottom cups containing either no pathogen/no metabolite (Control), *R. solani/R. solani* (P/P), metabolite of NIIST B616/metabolite of NIIST B616 (616/616), metabolite of NIIST B627/metabolite of NIIST B627 (627/627), metabolite combination/metabolite combination (616 + 627 / 616 + 627), metabolite of NIIST B616/*R. solani* (616/P), metabolite of NIIST B627/*R. solani* (627/P), and metabolite combination/*R. solani* (616 + 627/P) were taken. The seedlings cultivated in sterile soil for seven days were uprooted and carefully washed thrice with sterile distilled water to prevent any interference with the root system. A comparable plant, possessing root lengths of a similar measure, was inserted through the upper cup so that half of the root system was directed towards each cup below, each containing distinct treatments as noted previously. Particular attention was given to guaranteeing that the two cups beneath were not in contact with one another. The progression of the disease symptom caused by *R. solani* was systematically documented over the course of 28 days at intervals of 7 days. The experiment was conducted in three replications.

4.2.9. Analysis of the crude organic metabolites using GC-MS/MS and LC-MS/MS techniques

The identification of the metabolites was made through GC-MS/MS analysis using a Thermo Scientific Trace 1310 GC-MS/MS (U.S.) equipped with TSQ 8000 Mass Selective Detector (MS), GC column (TG-5MS, 30 m × 0.25 mm × 0.25 µm). A 0.5 µL sample was injected with an AIAS 1310 automated injector, and the column temperature was maintained at 40°C for the initial 5 min. The run was initiated with an increasing temperature of 10°C/ min until 200°C and followed by keeping the temperature isothermally for 5 min. The scan range was 45-600 m/z with electronic ionisation (EI) in split-less mode and compared with the National Institute of Standards and Technology (NIST) library data.

LC-MS/MS analysis of bacterial crude organic extract was performed on an LC-MS/MS system (Nexera with LCMS-8045, Shimadzu, Japan) hyphenated with HPLC (NexeraLC-30AD) equipped with temperature-controlled column oven (CTO-20AC), an

autosampler (SIL-30AC), and prominence diode array detector (SPD-M20A) coupled to triple quadrupole mass spectrometer. Samples were prepared in MS grade methanol in 1 mg/mL concentration filtered through a 0.2 µm nylon membrane filter and diluted to 50 µg/mL. From this, 10 µL was injected for the run using a mobile phase comprising 0.1% formic acid in water (solvent A) and 100% methanol (solvent B). The compounds were separated with the following linear-programmed solvent gradient: 0.01-2.00 min (10% B), 2.00-5.00 min (10% B), 5.00-8.00 min (50% B), 8.00-11.00 min (50% B), 11.00-16.00 min (90% B), 16.00-17.00 min (90% B), 17.00-22.00 min (10% B). Analysis was carried out in a 1.9 µm C18 column 2.1×150 mm (Shim-pack GISS, Japan) with a flow rate of 0.2 mL/min at a temperature of 40°C. Full scan acquisitions were set at 50 to 1500 m/z at two scans/s. The multiple reaction monitoring (MRM) positive mode was operated during LC-MS/MS with an electrospray ionisation probe (ESI). LCMS data were collected and processed using Lab Solutions software (Shimadzu, Japan). An interface temperature of 400°C was conditioned for ionisation, a desolvation line temperature of 300°C, a heat block temperature of 400°C, nebulising gas flow (nitrogen) at 3 L/min and drying gas flow (nitrogen) at 10 L/min.

4.2.10. Statistical analysis

The statistical analysis involved subjecting the data to one-way analyses of variance (ANOVA) utilising SPSS (version 20.0; IBM SPSS). Duncan's Multiple Range Test (DMRT) was employed at $p<0.05$ to ascertain statistical significance and was deemed to indicate a significant difference. The graphical representations were created using Origin Pro 8.5 software. The source of the weather information is from the Indian Meteorological Department, Ministry of Earth Sciences, Government of India.

4.3.Results

4.3.1. Extraction and i*n vitro* antagonism of crude organic metabolites

The ethyl acetate solvent was found to be the most effective for extracting the COMs from both bacteria among the various solvents tested. The NIIST B616 and NIIST B627 strains yielded 354 and 295 mg of crude organic extracts, respectively, from a 1 litre culture medium. The *in vitro* antagonism of the COMs of the two bacteria demonstrated an inhibition distance of 10.33 ± 0.58 and 7.67 ± 0.58 mm, respectively, in the ethyl acetate extract (Fig 4.1A). The scanning electron microscope images of the fungal hyphae at the point of inhibition revealed cellular damage, the formation of aberrant bulbous-like structures, and shrinkage of the mycelial

hyphae (Fig 4.1B). The crude metabolite obtained from other solvents did not exhibit any zone of inhibition.

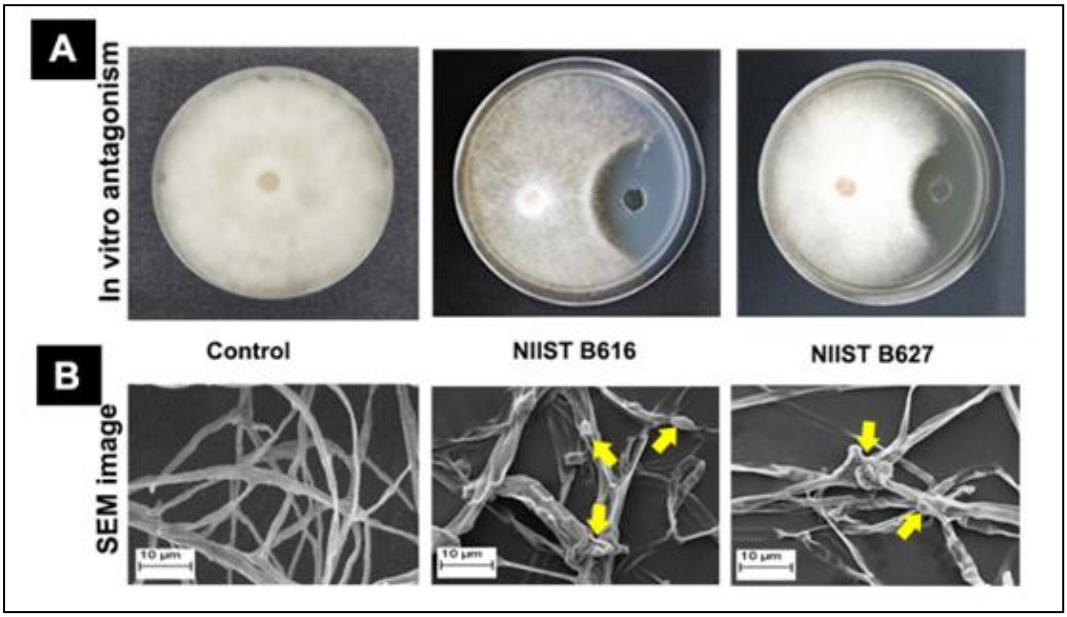


Fig 4.1 *In vitro* antagonism of (A) COMs against *R. solani* in such a way that the *R. solani* is inoculated on the left (mycelial plug) and the COMs of NIIST B616 and NIIST B627 separately on a well on the right and (B) SEM images of the fungal hyphae from the point of inhibition. Arrow marks indicate the morphological changes of the mycelial hyphae

4.3.2. Impact of crude organic metabolites on rice plant growth

Before conducting the plant growth-promoting experiment, the physicochemical characteristics of the soil were investigated. The results showed that the soil has a pH value of 6.5 and an electrical conductivity of 1.44 dS/m, indicating critical for germination. Additionally, it contains 1.95% organic carbon, 583.072 kg/ha of phosphorus, and 844.03 kg/ha of potassium. The soil's texture analysis revealed a composition of 57.8% sand, 16.2 % silt, and 25.9 % clay, classifying the soil as sandy loam.

Applying COMs to rice plants improved growth parameters like shoot height, root length, dry weight, and total chlorophyll. The highest percentage increase in all test parameters was obtained by the combination treatment (NIIST B616+627). It was followed by NIIST B627. On day 28, the plants treated with the combination of crude metabolites demonstrated a significant increase in shoot height (29.19%), root length (42.24%), dry weight (84.31%) and total chlorophyll content (46.31%) when compared to the control group (Fig 4.2 and Table 4.1).

The ANOVA analysis has validated the statistical significance of all growth parameters with all metabolite treatments over the control (p<0.05). In the case of yield, all treatments have illustrated an increase in yield on all test parameters compared with the control. The combination of the two metabolites significantly increased the yield, as evidenced by several key metrics: the number of grains per pot rose by 74.26%, the weight of 100 grains increased by 11.36%, the number of tillers per plant enhanced by 71.67%, the number of panicles per plant doubled (100% increase), dry shoot matter increased by 219.58%, and the harvest index improved by 12.72% compared to the control. Nonetheless, only the combination treatment exhibited a statistically significant impact at p<0.05, except for the number of tillers per plant (Fig 4.2 and Table 4.2).

4.3.3. Impact of crude organic metabolites on plant growth in the presence of *R. solani*

The administration of COMs to rice plants that were challenge inoculated with *R. solani* has been found to surmount the inhibitory activity of the pathogen by improving the growth parameters. A comparison of plants treated with metabolites and the pathogen alone (Fig 4.3 and Table 4.3) revealed that the former exhibited a higher enhancement percentage. Specifically, the combination of crude metabolites led to a significant increase in shoot height (32.25%), root length (46.38%), dry weight (48.89%), and total chlorophyll content (129.80%) on day 28, compared to the pathogen alone treated plants. Among the individual treatments, the NIIST B627 treated plants produced the most significant enhancement for all growth parameters. ANOVA results revealed that all growth parameters demonstrated a statistically significant difference between the metabolite treatments and the pathogen alone (p<0.05). Concerning yield estimation, the combination of metabolites resulted in a substantial increase in yield in terms of the number of grains per pot (75.16%), the weight of 100 grains (8.84%), number of tillers per plant (66.50%), number of panicles per plant (83.50%), dry shoot matter (206.10%), and harvest index of 26.09 % compared to the pathogen alone (Fig 4.3 and Table 4.4). The ANOVA analysis showed that the combination treatment had a statistically significant effect on the pathogen alone treatment, while similar results were observed in the control group except for the number of tillers per plant (p<0.05).

Fig 4.2 Plant growth promotion studies using COMs of selected endophytes and their combination in rice plants on different data collection days. Control - no treatment, NIIST B616 – COMS of *Bacillus subtilis* NIIST B616, NIIST B627 - COMS of *Bacillus subtilis* NIIST B627, NIIST B616+627- COMS of *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627

Table 4.1 Impact of COMs on promoting the growth of rice plants on various days

Day	Treatments	Shoot Height (cm)	Root Length (cm)	Dry weight (g)	Total chlorophyll (mg/L)
7	Control	22.16±0.61[a*]	4.58±0.61[a]	0.03[a]	3.70±0.11[a]
	NIIST B616	28.74±0.56[b] (29.69)	7.66±0.29[b] (67.24)	0.07±0.01[b] (133.33)	7.44±0.77[b] (101.08)
	NIIST B627	30.04±0.97[c] (35.56)	10.06±0.44[c] (119.65)	0.08±0.01[c] (166.67)	9.95±0.25[c] (168.92)
	NIIST B616+627	31.86±0.35[d] (43.77)	11.84±0.83[d] (158.52)	0.09±0.01[c] (200.00)	10.22±1.02[c] (176.22)
14	Control	29.70±1.30[a]	8.58±0.63[a]	0.06±0.01[a]	9.55±1.03[a]
	NIIST B616	35.50±0.50[b] (19.53)	11.86±0.27[b] (38.23)	0.09[b] (50.00)	16.70±0.67[b] (74.87)
	NIIST B627	36.06±0.38[b] (21.41)	13.90±1.02[c] (62.00)	0.10±0.01[c] (66.67)	17.96±0.13[c] (88.06)
	NIIST B616+627	38.18±0.54[c] (28.55)	16.32±0.52[d] (90.21)	0.12±0.01[d] (100.00)	21.43±0.49[d] (124.40)
21	Control	40.76±1.61[a]	14.26±1.21[a]	0.34±0.03[a]	12.72±0.43[a]
	NIIST B616	46.92±0.88[b] (15.11)	20.78 ±1.23[b] (45.72)	0.45±0.01[b] (32.35)	20.51±0.42[b] (61.24)
	NIIST B627	48.18±0.71[b] (18.20)	23.34±1.48[c] (63.67)	0.47±0.01[b] (38.24)	22.44±0.07[c] (76.42)
	NIIST B616+627	50.16±0.83[c] (23.06)	24.86±1.05[c] (74.33)	0.50±0.01[c] (47.06)	23.76±0.19[d] (86.79)
28	Control	44.20±1.19[a]	20.88±0.68[a]	0.51[a]	19.80±0.78[a]
	NIIST B616	53.12±1.80[b] (20.18)	26.64±1.60[b] (27.59)	0.90±0.01[b] (76.47)	25.25±0.69[b] (27.53)
	NIIST B627	55.34±1.30[c] (25.20)	28.96±0.67[c] (38.70)	0.92±0.01[c] (80.39)	27.64±1.74[c] (39.60)
	NIIST B616+627	57.10±0.87[c] (29.19)	29.70±0.49[c] (42.24)	0.94±0.01[d] (84.31)	28.97±0.94[c] (46.31)

* All values are expressed as mean ± SD from ten replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses represent percentage increases over the control

Table 4.2 Impact of COMs on rice plant yield after 120 days

Treatments	No. of grains per pot	Weight of 100 grains (g)	No. of tillers per plant	No. of panicles per plant	Dry shoot matter (g)	Harvest Index**
Control	228.00±1.00^{a*}	2.20±0.01^{a}	2.33±0.58^{a}	2.00^{a}	0.97±0.02^{a}	0.55±0.01^{a}
NIIST B616	302.67±2.08^{b} (32.75)	2.32±0.03^{b} (5.45)	3.00±1.00^{a} (28.76)	2.67±0.58^{a} (33.5)	2.95±0.03^{b} (204.12)	0.58^{b} (5.45)
NIIST B627	308.67±1.53^{c} (35.38)	2.36^{b} (7.27)	3.00±1.00^{a} (28.76)	3.00±1.00^{ab} (50)	2.99±0.02^{b} (208.24)	0.58±0.01^{b} (5.45)
NIIST B616+627	397.33±1.53^{d} (74.26)	2.45±0.04^{c} (11.36)	4.00±1.00^{a} (71.67)	4.00±0.00^{b} (100)	3.10±0.02^{c} (219.58)	0.62±0.01^{c} (12.72)

* All values are expressed as mean ± SD from three replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses represents percentage increases over the control

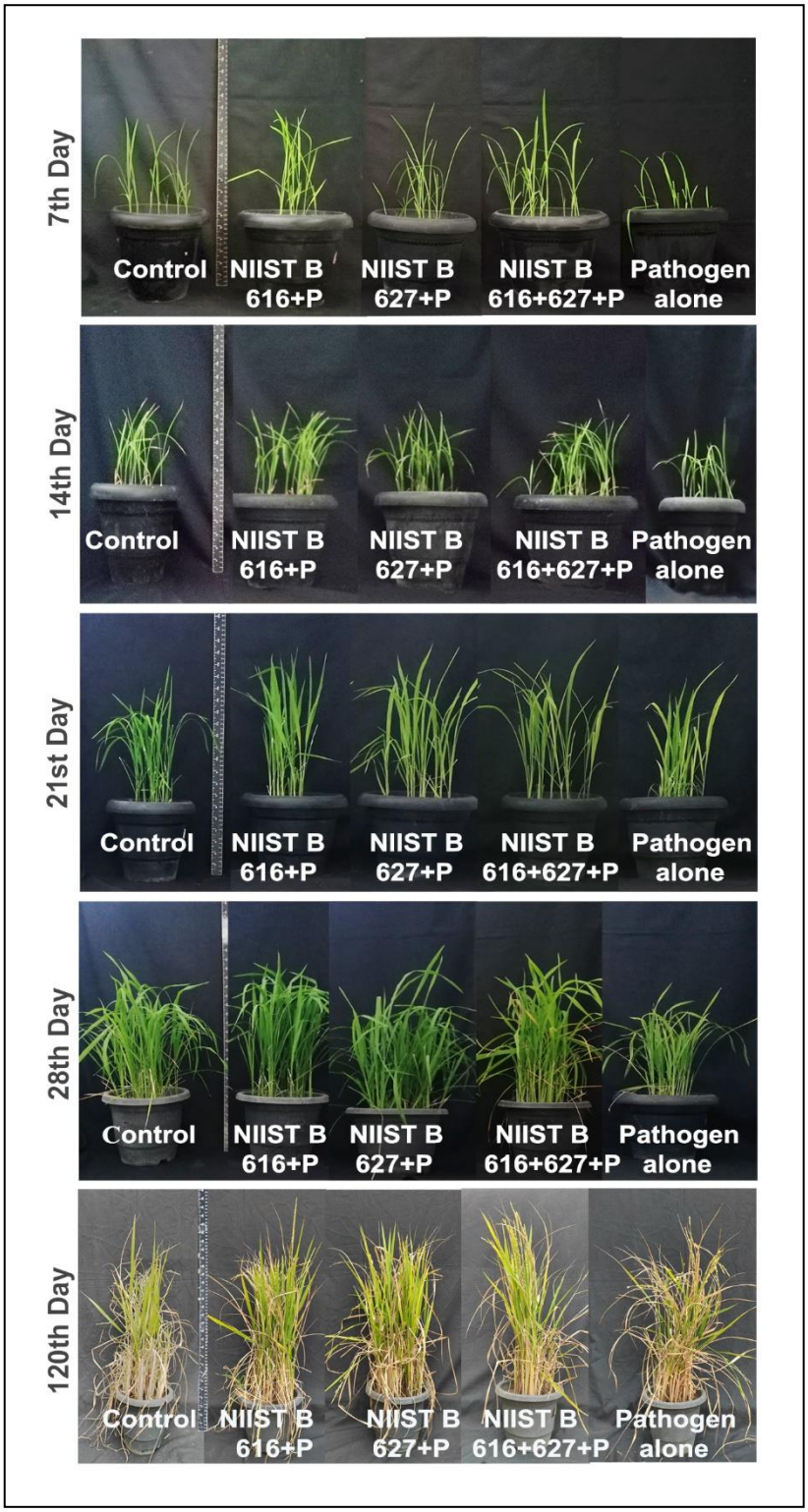


Fig 4.3 Plant growth of rice plants challenge inoculated with *R. solani* on different data collection day. Control - no treatment, NIIST B616+P -COMS of *Bacillus subtilis* NIIST B616 + *R solani*, NIIST B627+P - COMS of *Bacillus subtilis* NIIST B627 + *R solani*, NIIST B616+627+P - COMS of *Bacillus subtilis* NIIST B616+ COMS of *Bacillus subtilis* NIIST B627 + *R solani*, Pathogen -*R solani* alone

Table 4.3 Impact of COMs on promoting the growth of plants challenge inoculated with *R. solani*

Day	Treatments	Shoot Height (cm)	Root Length (cm)	Dry Weight (g)	Total Chlorophyll (mg/L)
7	Control	22.16±0.61b (28.54) *	4.58±0.61b (26.52)	0.03±0.00b (50.00)	3.70±0.11a (4.23)
	NIIST B616+P	26.48±1.13c (53.60)	5.30±0.78c (46.41)	0.06±0.01c (200.00)	5.26±0.97b (48.17)
	NIIST B627+P	27.53±0.70d (59.69)	6.3±0.84d (74.03)	0.06±0.01cd (200.00)	6.89±0.75c (94.08)
	NIIST B 616+627+P	28.26±0.70d (63.92)	7.42±0.40e (104.97)	0.07±0.01d (250.00)	8.11±1.00d (128.45)
	Pathogen	17.24±0.20a	3.62±0.65a	0.02±0.01a	3.55±0.13a
14	Control	29.70±1.23b (6.38)	8.58±0.60b (12.60)	0.06±0.01ab (20.00)	9.55±0.97b (65.51)
	NIIST B616+P	33.80±0.54c (21.06)	8.84±0.74b (16.01)	0.07±0.01b (40.00)	12.53±0.57c (117.16)
	NIIST B627+P	34.58±0.55cd (23.85)	10.70±1.03c (40.42)	0.07±0.01c (40.00)	15.32±1.60d (165.51)
	NIIST B616+627+P	35.34±0.99d (26.58)	11.06±0.67c (45.14)	0.08±0.01c (60.00)	16.11±1.36d (179.20)
	Pathogen	27.92±1.10a	7.62±1.02a	0.05±0.01a	5.77±0.60a
21	Control	40.76±1.52a (2.77)	14.26±1.46a (6.26)	0.34±0.03ab (6.25)	12.72±0.41b (34.04)
	NIIST B616+P	42.27±0.53c (6.58)	18.86±0.68c (40.54)	0.35±0.01bc (9.38)	15.90±0.87d (67.54)
	NIIST B627+P	43.74±0.41b (10.29)	20.40±0.99b (52.01)	0.36±0.01cd (12.5)	17.90±0.44c (88.62)
	NIIST B616+627+P	45.40±0.57c (114.47)	21.31±0.83c (158.79)	0.38±0.01d (118.75)	20.45±0.49e (215.49)
	Pathogen	39.66±0.88a	13.42±1.00a	0.32±0.01a	9.49±0.63a
28	Control	44.20±1.19b (14.04)	20.88±0.68b (7.85)	0.51±0.00b (13.33)	19.80±0.78b (94.12)
	NIIST B616+P	47.80±0.92c (23.32)	26.20±0.73c (35.33)	0.53±0.01c (17.78)	21.52±0.56c (110.98)
	NIIST B627+P	49.94±0.62d (28.84)	27.56±0.87d (42.36)	0.60±0.01d (33.33)	22.17±0.69c (117.35)
	NIIST B616+627+P	51.26±0.84e (32.25)	28.34±0.95d (46.38)	0.67±0.02e (48.89)	23.44±0.51d (129.80)
	Pathogen	38.76±0.95a	19.36±0.44a	0.45±0.02a	10.82±0.86a

* All values are expressed as mean ± SD from ten replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses represent percentage increases over the pathogen

Table 4.4 Impact of COMs on yield in plants challenge inoculated with *R. solani*

Treatments	No. of grains per pot	Weight of 100 grains (g)	No. of tillers per plant	No. of panicles per plant	Dry shoot matter (g)	Harvest Index**
Control	228.00±1.00^{b} (45.22) *	2.20±0.01^{b} (2.33)	2.33±0.58^{ab} (16.5)	2.00^{a} (0.00)	0.97±0.02^{b} (18.29)	0.55±0.01^{b} (19.57)
NIIST B616+P	264.00±1.00^{c} (68.15)	2.26±0.01^{c} (5.12)	2.67±0.58^{ab} (33.50)	2.67±0.58^{ab} (33.50)	2.39±0.01^{c} (191.46)	0.56^{b} (23.91)
NIIST B627+P	271.33±1.53^{d} (72.82)	2.33±0.02^{c} (8.37)	3.00±1.00^{ab} (50.00)	3.00±1.00^{ab} (50.00)	2.40±0.01^{c} (192.68)	0.57^{c} (21.74)
NIIST B616+627+P	275.00±1.00^{e} (75.16)	2.34±0.02^{c} (8.84)	3.33±0.58^{b} (66.50)	3.67±0.58^{b} (83.50)	2.51±0.01^{d} (206.10)	0.58±0.01^{d} (26.09)
Pathogen	157.00±1.00^{a}	2.15±0.01^{a}	2.00±0.00^{a}	2.00^{a}	0.82±0.02^{a}	0.46±0.01^{a}

* All values are expressed as mean ± SD from three replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses represents percentage increases over the pathogen

** Harvest index is the ratio of harvested grain to total dry shoot matter

4.3.3.1.Impact of crude organic metabolite on disease resistance

The experimental units unveiled disease symptoms when subjected to *R. solani*, with the onset of symptoms observed at varying time points for the pathogen alone (52 days), NIIST B616 + P (65 days), NIIST B627 + P (69 days), and the combination treatment (79 days). The plants treated with the combination of metabolites recorded the lowest percentage of disease incidences (33.33%), and the relative lesion height of the treated plants was observed to be on a scale of 1. In comparison, the plants treated with NIIST B616 + P and NIIST B627 + P exhibited a slightly higher percentage of disease incidences, which were recorded as 41.67% and 43.33%, respectively. Furthermore, the relative lesion height of these plants was observed to be on a scale of 3. Conversely, the plants treated with *R. solani* alone displayed a remarkably higher disease incidence of 86.67%, and the relative lesion height was observed to be at a scale of 7. Notably, all control plants showed no signs of disease incidence throughout the experiment until the last observation (Fig 4.4 and Table 4.5).

Fig 4.4 Effect of COM and their combination on disease control in *R. solani* treated plants after 90 days**.** The arrow mark indicates the respective enlarged portion of the collar region of the plant. Control - no treatment, NIIST B616+P – COM of *Bacillus subtilis* NIIST B616 + *R solani*, NIIST B627+P - COM of *Bacillus subtilis* NIIST B627 + *R solani*, NIIST B616+627+P - COM of *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627 + *R solani*, Pathogen - *R solani* alone

Table 4.5 Impact of COMs on disease resistance in plants challenge inoculated with *R. solani* after 90 days

Treatments	Disease incidence (%)	Relative lesion height (scale 0-9)
Pathogen	86.67±5.77^{d*}	7**
NIIST B616+P	41.67±2.87^{c}(51.92)	3
NIIST B627+P	43.33±2.87^{c}(50.00)	3
NIIST B616+627+P	33.33±2.87^{b}(61.54)	1
Control	0^{a}	0

* All values are expressed as mean ± SD from three replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses represent the percentage decrease over the pathogen.
Disease index scale, **0 - No infection, **1** - lesion limited to lower 20% of the plant height, **3** - 20-30%, **5** - 31- 45%. **7** - 46-65%, 9 - more than 65%.

4.3.4. Induction of systemic resistance by crude organic metabolites

The experimental groups challenged with *R. solani* exhibited disease resistance upon exposure to COMs. Analysis of defence-related enzymes, including PAL, POX, PPO and total phenol content, revealed a marked increase compared to the treatment with *R. solani* alone. In the context of the PAL assay, the individual crude metabolite treatments displayed an escalation for up to 60 days, whilst the combination of metabolite treatment experienced a rise for up to 50 days, after which the enzyme level stabilises until day 80. On the other hand, the treatment with *R. solani* alone and the control group exhibited an increase for up to 40 days, followed by a decrease by *R. solani* alone, though the control group maintained a steady level (Fig 4.5A).

Regarding the POX assay, crude metabolite treatments, whether administered individually or in combination, exhibited an increase in activity for up to 60 days, after which the enzyme levels for all treatments remained constant until 80 days. In contrast, the pathogen alone group exhibited a surge in activity for a duration of 40 days and then experienced a decline, while the control group demonstrated a rise in activity for up to 20 days and stabilised for 50 days, followed by a slight decrease in day 60, and then stabilised again until day 80 (Fig 4.5B).

The PPO assay revealed that the individual treatments of crude metabolite showcased a rise until day 50, after which NIIST B616+P maintained a constant level until day 80. However,

NIIST B627+P showed a slight decrease on day 60 before the enzyme level stabilised until the end of the experiment. The treatment with the pathogen alone exhibited an increase until day 30, which was followed by a decline. However, the control group maintained a steady enzyme level with a slight increase observed on day 30 (Fig 4.5C).

The crude metabolite treatments induced an increase in the total phenol content of individual and combination treatments of metabolites up to day 50, followed by a steady level up to day 80. The treatment with the pathogen alone resulted in an increase until day 30, followed by a decline. Nevertheless, the control group exhibited a sharp increase up to day 20, a slight increase up to day 50, a decline at day 60, and finally, a constant enzyme level up to day 80 (Fig 4.5D).

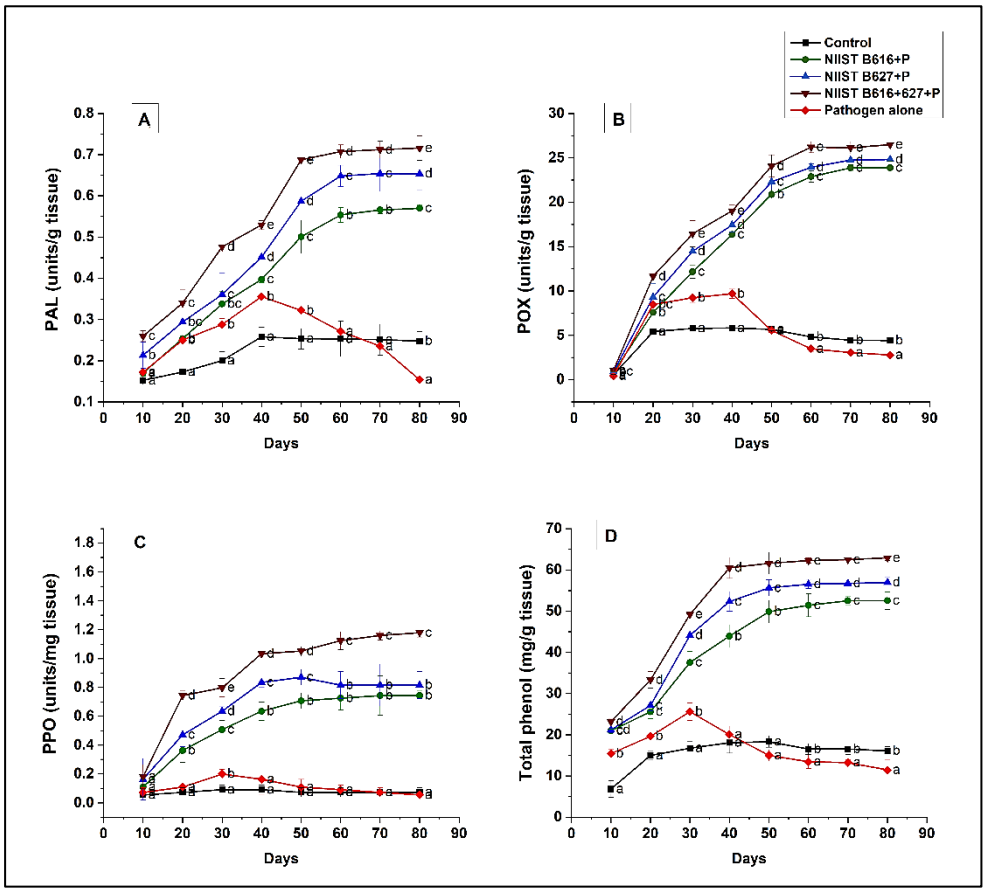


Fig 4.5 A-D Variations in enzyme activity levels of (A) PAL, (B) POX, (C) PPO, and (D) total phenol content across different treatments in host plants over time. All values are expressed as mean ± SD from three replications. Different superscript letters on the same day indicate the different degrees of treatment at a statistical significance of 5% (α). Control- no treatment, NIIST B616+P – COM of *Bacillus subtilis* NIIST B616 + *R solani*, NIIST B627+P - COM of

Bacillus subtilis NIIST B627 + *R solani*, NIIST B616+627+P - COM of *Bacillus subtilis* NIIST B616+ COM of *Bacillus subtilis* NIIST B627 + *R solani*, Pathogen alone-*R solani* alone.

4.3.5. Confirmation of induced systemic resistance through split-root experiment

This study aimed to evaluate the effectiveness of crude metabolites in inducing systemic resistance against sheath blight disease in rice plants while avoiding direct antagonism with *R. solani*. Throughout the incubation period, the development of the disease symptom was monitored, with the *R. solani* / *R. solani* treatment showing a brownish lesion on the collar region from the seventh day onwards. The disease further spread and bleached to a brownish-white colour by day 14, with signs of plant death appearing as early as day 21. Complete mortality of the infected plant was recorded on day 28 with white mycelial growth (Figs 4.6 and 4.7). The treated and control plants remained healthy throughout the observation period, without displaying any disease symptoms till the last day.

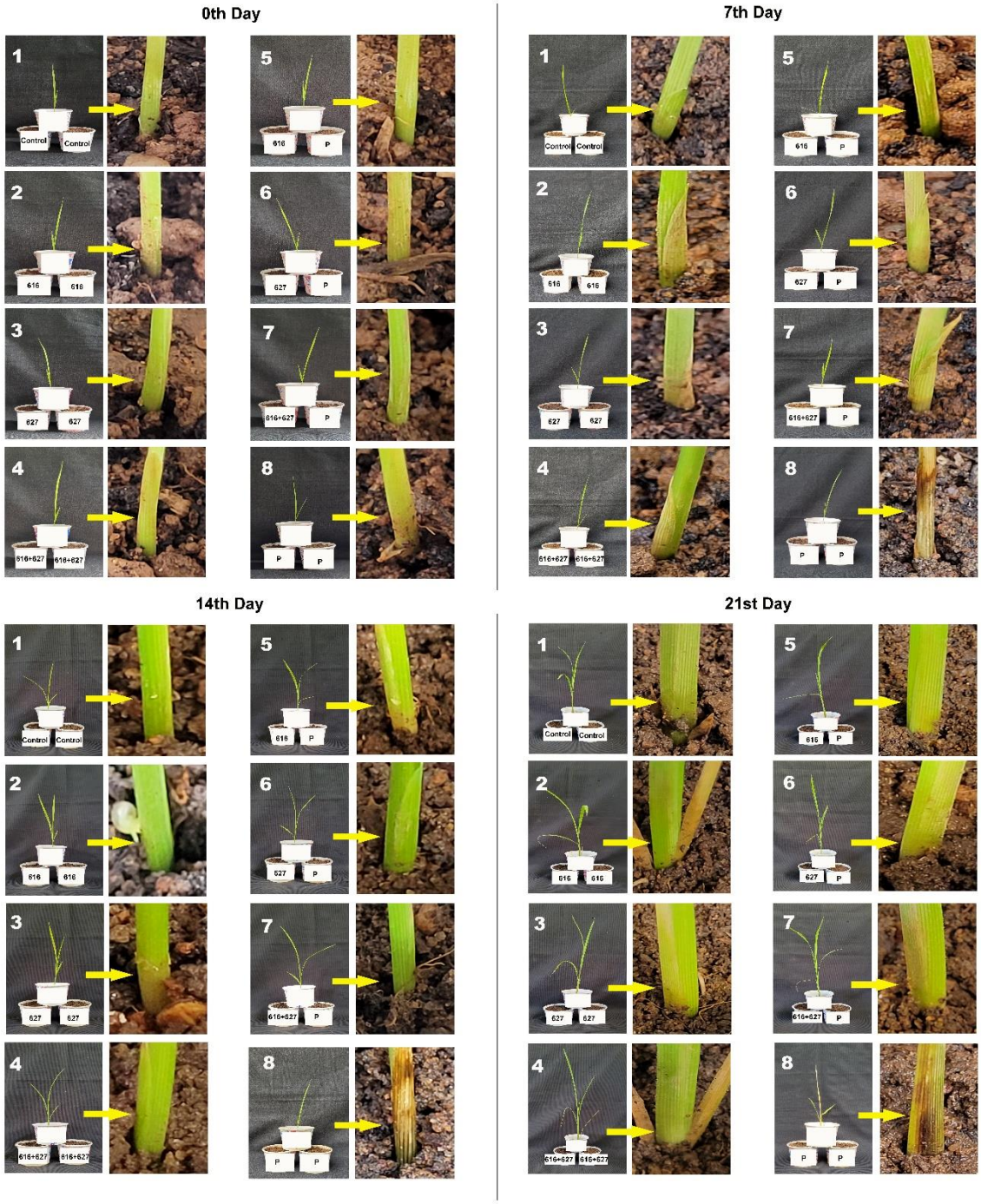


Fig 4.6 Split root experiments with COMs and *R. solani* in different combinations from day 0 to 21. The arrow mark indicates the respective enlarged portion of the collar region of the plant. 1. Control/Control 2. 616/616 3. 627/627 4. 616+627/616+627 5. 616/P 6. 627/P 7. 616+627/P 8. P/P

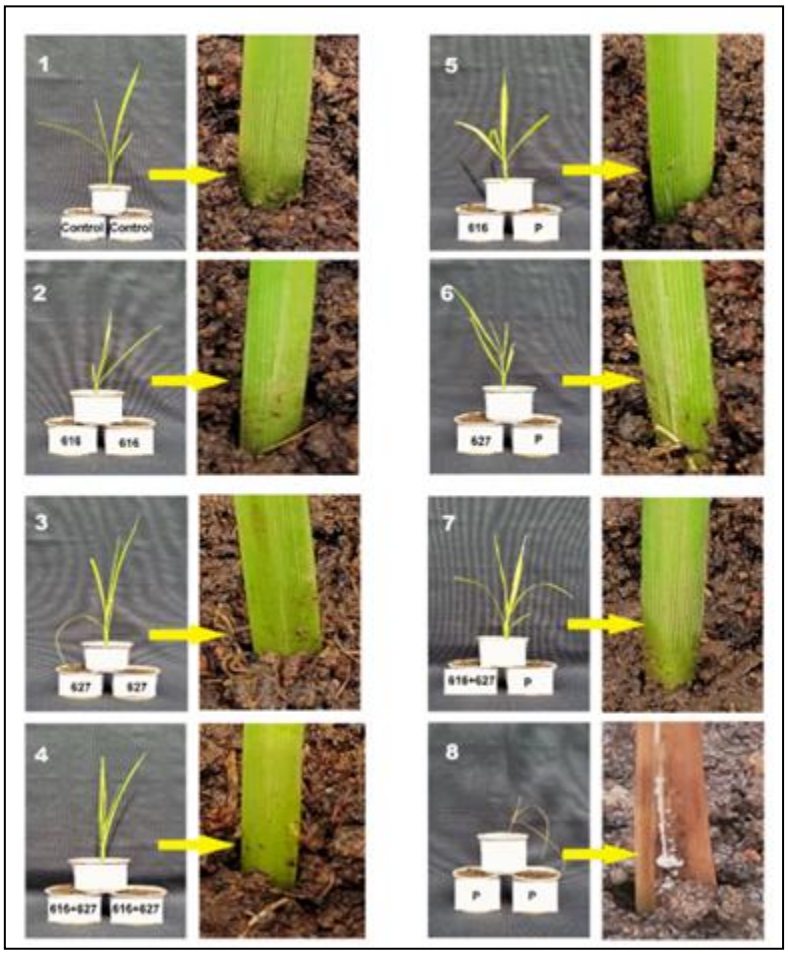


Fig 4.7 Split root experiment with different treatments on elicitation of induced systemic resistance on day 28. The arrow mark indicates the respective enlarged portion of the collar region of the plant. **1.** Control/Control **2.** 616/616 **3.** 627/627 **4.** 616+627/616+627 **5.** 616/P **6.** 627/P **7.** 616+627/P **8.** P/P

4.3.6. Analysis of the crude organic metabolites using GC-MS/MS and LC-MS/MS techniques

GC-MS/MS analysis from the crude organic extract of NIIST B616 exhibited discernible peaks with a retention time of 22.10, 23.51, 23.84, 23.97, 35.10 min with an area percentage of 19.47, 10.30, 17.52, 3.11, 4.20% respectively and NIIST B627 exhibited discernible peaks with a retention time of 23.52, 23.86, 23.99, 24.07, 34.32, 35.12 min and an area percentage of 9.56, 17.44, 4.52, 1.73, 2.29, 8.76 % respectively (Fig 4.8A and B). Subsequently, the NIST library comparative search aided in the identification of two major compounds of NIIST B616 as

pyrrolo[1,2-*a*]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl) with m/z 210.27 and pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) with m/z 244.29. The same compounds were also identified from NIIST B627.

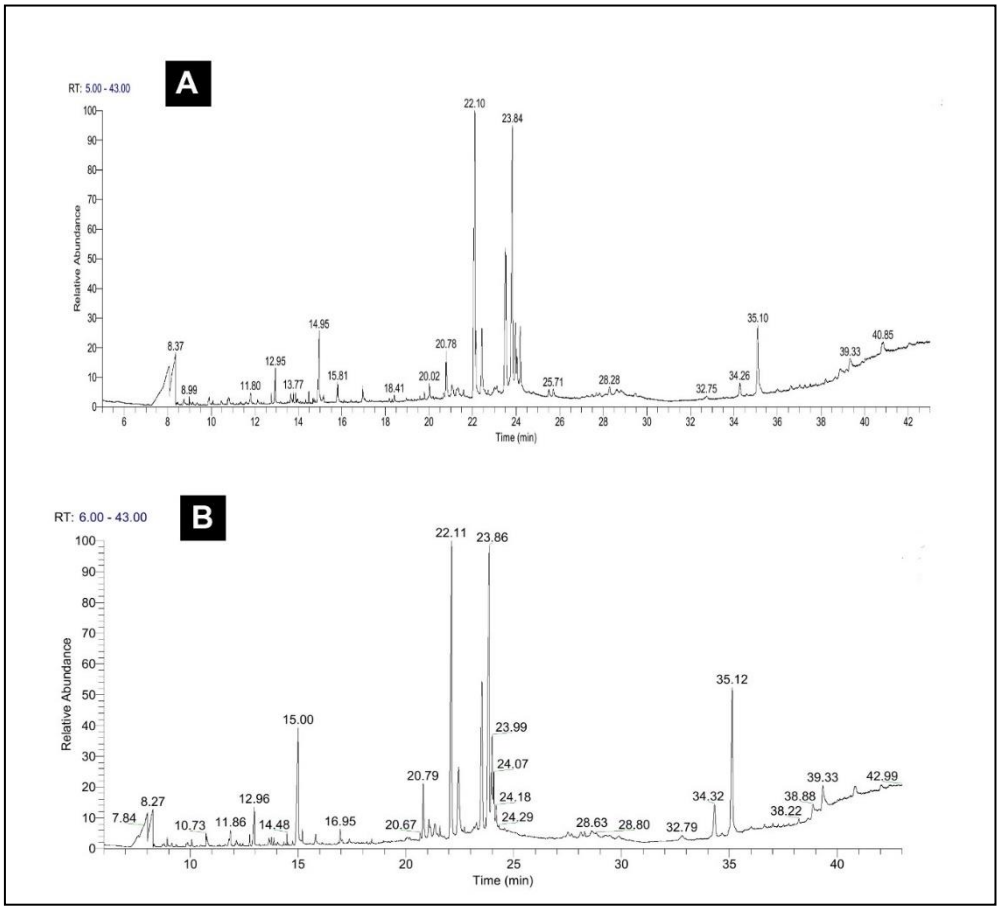


Fig 4.8 GC-MS chromatogram of the COMs of (A) *Bacillus subtilis* NIIST B616 and (B) *Bacillus subtilis* NIIST B627 showing discernible peaks and its retention time

The presence of both compounds in COMs was further confirmed through LC-MS/MS analysis. The metabolites representing *m/z* values of 210.27 and 244.29 in GC-MS/MS also substantiate its presence during LC-MS/MS analysis with *m/z* of 211 and 245, respectively (Fig 4.9A and B). MS/MS product ions of *m/z* 211 and 245 were obtained at *m/z* 70/72/86/98/114/138/154/183/211 and 70/98/103/120/154/172/217/245, respectively.

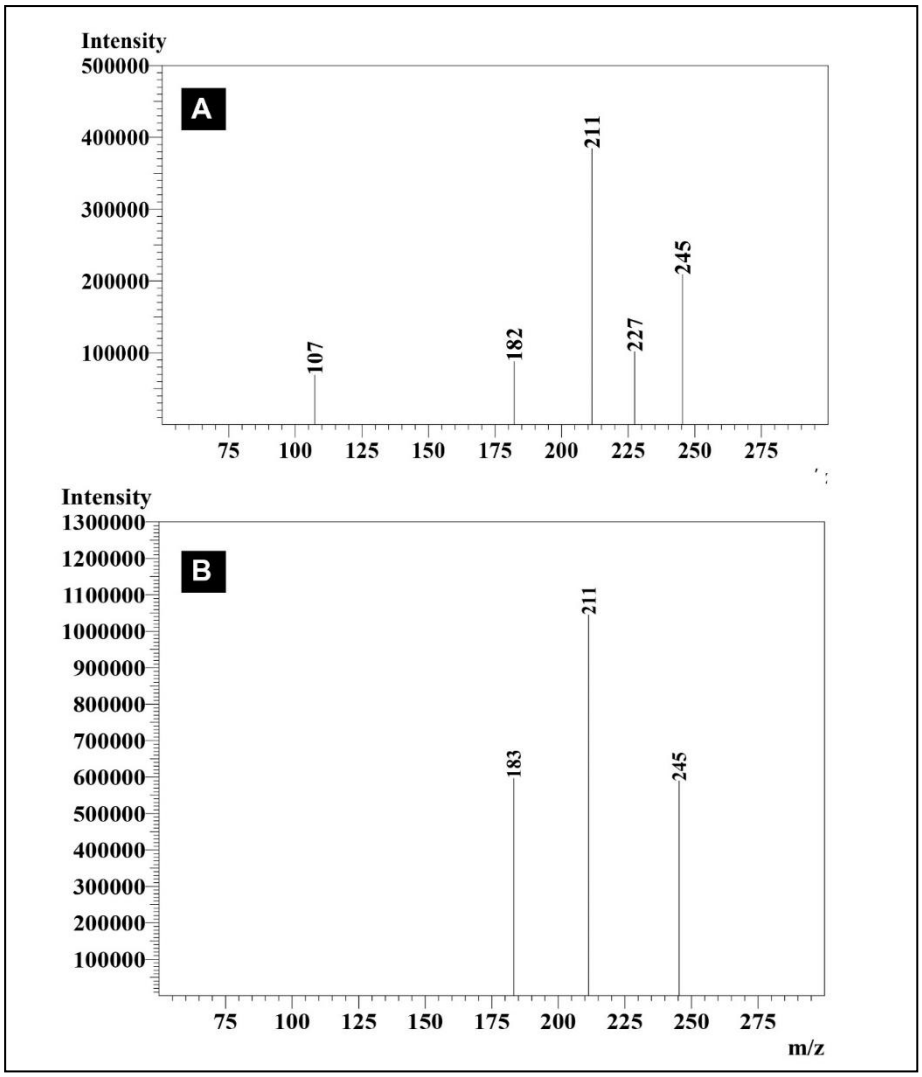


Fig 4.9 Mass spectra of COMs of (A) *Bacillus subtilis* NIIST B616 and (B) *Bacillus subtilis* NIIST B627 showing m/z 211 and m/z 245 from LC-MS/MS analysis

4.4. Discussion

Endophytes, the microorganisms associated with plants, are instrumental in enhancing plant growth and mitigating the severity or incidence of diseases by inducing systemic resistance. The use of induced systemic resistance to protect crops against diseases is a more environmentally friendly and less aggressive approach than the deployment of lethal chemicals for disease control. Some researchers have documented the utilisation of secondary metabolites from endophytes to stimulate plant growth and induce systemic resistance (Watts et al., 2023; Yang et al., 2017). An array of secondary metabolites is known to be produced by bacterial endophytes comprising terpenes, alkaloids, antibiotics such as lipopeptides, amino acid-rich peptides, cyclic cationic lipopeptides, and pigments. Besides these compounds, endophytes can

also yield substances that have both antiviral and anticancer effects (Narayanan & Glick, 2022). Certain research has indicated that the inclusion of endophytic bacteria in cucumber plants can augment or instigate specific metabolites in the plant endosphere, ultimately leading to improved plant growth (Mahmood & Kataoka, 2020). But Ismail and colleagues have established through their study that the use of endophytic microbial metabolites leads to a substantial improvement in the plant biomass, carbohydrate and protein contents, photosynthetic pigments, yield traits, endogenous hormones, and antioxidant enzyme activity of common bean plants (Ismail et al., 2021). Similarly, our study has revealed that adding endophytic bacterial metabolites enhanced the growth and yield of rice plants.

Research conducted by Gond et al. (2015) revealed that endophytic bacteria, particularly *Bacillus* spp., found in healthy maize tissues have been found to produce antifungal lipopeptides, which induce host defence gene expression in maize against *Fusarium moniliforme*. Another study has also suggested that the lipopeptide extracted from *Bacillus amyloliquefaciens* YN201732 has the potential to offer effective biocontrol against the fungal pathogen *Erysiphe cichoracearum* (Jiao et al., 2021). In the current investigation, it was observed that the introduction of the crude organic metabolite of two *Bacillus subtilis* strains, NIIST B616 and NIIST B627, separately and in combination, led to an increase in plant growth, decrease in disease occurrence and severity in rice plants exposed to the pathogen *R. solani*.

Plants have evolved various defence mechanisms to counteract the impact of pathogenic intruders. These mechanisms include the production of metabolites, pre-existing structures, and the activation of an immune response cascade. A notable instance of such a response is the generation of phenolic compounds (Lone et al., 2020). There also exist accounts concerning metabolites, such as phenazines and cyclic lipopeptides, produced by *Pseudomonas* sp. CMR12a have been shown to induce systemic resistance in rice and bean crops against blast and web blight disease, correspondingly (Ma et al., 2016). Upon the induction of ISR, various defensive enzymes such as PAL, POX, and PPO are stimulated to be synthesised in plants to protect against pathogenic organisms. PAL plays a pivotal role in the synthesis of polyphenolic compounds and the accumulation of salicylic acid (El-Gendi et al., 2022). At the same time, POX and PPO serve as reactive oxygen species (ROS) scavenging enzymes that contribute to the generation of ROS during host-pathogen interaction, which is an important component of the host's immune response against invading pathogens (Torres et al., 2006). The present investigation attributes the decrease in disease severity to the heightened levels of PAL, POX, PPO and total phenol content. These elevated enzyme levels may be due to the hypersensitive reaction frequently observed in plants during pathogenic attacks and are linked to the

enhancement of plant tolerance to the disease. The increase in the activity of defence enzymes in rice plants treated with crude metabolites demonstrates that this variation is associated with the induction of systemic resistance in treated plants. A similar reduction of disease severity coupled with a simultaneous surge in the activity of superoxide dismutase, POX and PPO was observed when crude culture filtrate of *Bacillus subtilis* HA1 was added to tomato plants (El-Gendi et al., 2022). Additionally, Ongena and colleagues demonstrated that the crude supernatant of *Pseudomonas putida* BTP1 retained its ability to elicit ISR in bean plants (Ongena et al., 2002). As a result, it was proved that the organic metabolites in their crude form contained molecules that could elicit ISR in plants.

The split-root experiment is a unique technique in plant pathology research to validate the effectiveness of various treatments in managing plant diseases. Bifurcating the root into two equal parts and administering distinct treatments to each half, researchers can scrutinise the systemic response of the plant to either the pathogen or the metabolite that instigates resistance. The experiment results demonstrated that plants treated with the crude metabolite did not contract the disease, while those treated solely with the pathogen did. Given the meticulousness with which the experiment was conducted to ensure that the treatments did not come into direct contact, the possibility of antagonism can be disregarded, indicating that the resistance developed by the plant is purely the result of ISR. Thus, ISR can be viewed as a natural approach to plant disease prevention, providing a viable alternative to harmful pesticides and herbicides.

The COMs produced by isolates were subjected to GC-MS/MS analysis, resulting in the identification of two cyclic dipeptides, viz. pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl) and pyrrolo[1,2-a] pyrazine1,4-dione,hexahydro-3-(phenylmethyl). Upon thorough investigation, these two compounds were identified as the major constituents of the bacterial metabolite. Pyrrole compounds are recognised as significant heterocyclic compounds that show a wide range of biological activities, including antimicrobial, anticancer, antiviral, and anti-inflammatory effects (Ser et al., 2015). A recent study by El-Gendi et al. (El-Gendi et al., 2022) has demonstrated that these compounds can induce ISR in tomato plants besides antimicrobial activities. Our results were consistent with the earlier reports of diverse pyrrolo[1,2-a]pyrazine-1,4-dione compounds in the culture filtrate extract of *B. velezensis* PEA1 which enhance the resistance to *Cucumber mosaic virus* in *Datura stramonium* and inhibit *Fusarium oxysporum* (Abdelkhalek et al., 2020). LC-MS/MS analysis also confirmed the presence of two cyclic dipeptides, cyclo(Pro-Leu) and cyclo(Pro-Phe), identical to those

found in GC-MS/MS analysis of metabolic extracts of NIIST B616 and NIIST B627. This finding has been further substantiated by reported MS/MS fragment data from the literature (Xing et al., 2008). As the antimicrobial activity of cyclo(Pro-Leu) and cyclo(Pro-Phe) is well-documented (Zhao et al., 2020), these molecules could be partially attributed to the antifungal activity of the endophytic *B. subtilis* NIIST B616 and NIIST B627 and the elicitation of ISR in rice plants.

4.5. Conclusion

The study elaborated in this chapter sheds light on the potency of crude organic metabolites (COMs) derived from *Bacillus* strains in fostering rice plant growth and conferring protection against sheath blight disease through ISR. The findings delineate that COMs trigger ISR in rice plants, thereby eliciting elevated activity levels of defence-related enzymes, including PAL, POX, PPO and total phenolic content in treated plants with regard to untreated counterparts. Moreover, split-root experiment validated the ISR, while GC-MS/MS and LC-MS/MS analysis unveiled the presence of cyclic dipeptides cyclo(Pro-Leu) and cyclo(Pro-Phe) within COMs, known for their antifungal attributes. These revelations underscore the potential of COMs from *Bacillus* strains as an environmentally benign alternative to synthetic pesticides for mitigating sheath blight disease and fostering sustainable crop cultivation. Nevertheless, further investigation is imperative to ascertain additional benefits of COMs, thereby curbing the reliance on harmful pesticides and herbicides prior to widespread field application or commercial utilisation.

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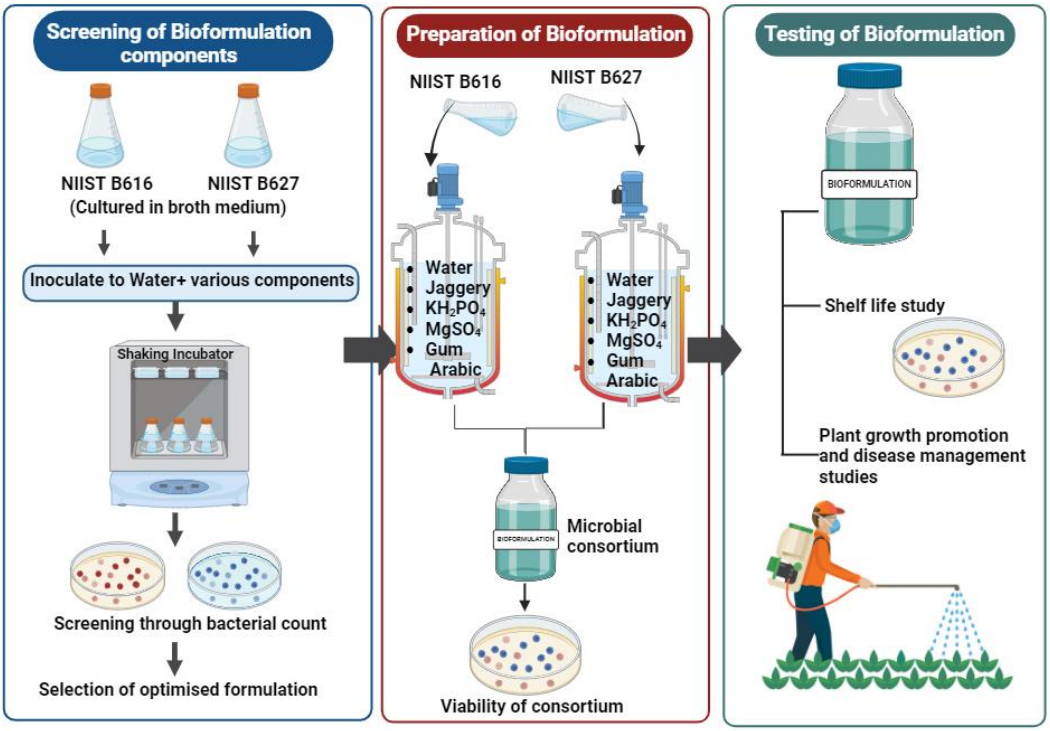
Chapter 5

Developing and Exploring the Potential of Endophytic Bioformulation for Plant Growth and Disease Resistance

Graphical Abstract



5.1.Introduction

Bioformulations are microbial based preparations that, when applied to soil, seed, or plants, promote growth, nutrient uptake, and soil fertility (Machado & Serralheiro, 2020). The core objective of using bioformulations is to enhance plant growth and yield without having a negative impact on the environment (Macik et al., 2020). However, in pursuit of elevated crop yields and disease prevention, farmers have increasingly leaned on using chemical fertilisers and pesticides since the era of the Green Revolution. Within a decade, these fertilisers have shown adverse effects on the natural microorganisms in the soil, leading to a range of consequences such as biomagnification in the food chain, eutrophication in water bodies, the release of greenhouse gases like nitrous oxide into the atmosphere causing global warming and other related issues. Thus, the agricultural sector is the leading contributor to the use of chemical pollutants, such as chemical fertilisers and plant protection chemicals, which can disrupt the agroecosystem (Maitra et al., 2021; Prakash & Arora, 2020). In this regard, biofertilisers (organic fertilisers) offer a dependable substitute for chemical fertilisers and pesticides. In addition to growth promotion and disease prevention, biofertilisers have other functions like nutrient solubilisation and mobilisation, soil decontamination or detoxification, etc. Currently, biofertilisers and biopesticides are being utilised, though on a minimal scale. These substances are commonly referred to as bioinoculants or bioformulations and are composed of either living or latent microorganisms that proliferate under favourable conditions and contribute positively to the overall health of plants. Despite their potential benefits, the use of these substances has not been fully realised due to a lack of widespread implementation (Maitra et al., 2021). Recently, microbial agents as biofertilisers have garnered widespread attention, offering hope from both economic and environmental perspectives. This approach holds promise in alleviating the burden of costly fertilisers, thus aiding in economic conservation for nations, including developing countries (Sivakumar, 2014).

Microbial bioformulation is a groundbreaking and environmentally friendly alternative to agrochemicals, leading the way toward sustainable agriculture. This approach utilises potent microbial strains and their cell-free filtrates, which possess specific capabilities such as solubilising phosphorus, potassium, and zinc, fixing nitrogen, producing siderophores, and protecting against pathogens. The use of microbial bioformulations provides numerous significant benefits, including sustainability, plant probiotic effects, and long-term viability, making it a promising innovation for the future of agriculture (Khan et al., 2023). Bioformulations containing beneficial microorganisms, such as *Bacillus, Acidothiobacillus,*

and *Trichoderma* species, have been shown to enhance plant growth and soil fertility by solubilising essential nutrients and fixing nitrogen (Kiruba N & Saeid, 2022). The addition of microbial or plant-produced secondary metabolites, such as flavonoids and phytohormones, can further augment plant growth and yield, particularly in leguminous crops (Morel et al., 2016). Additionally, bioformulations of *Xenorhabdus stockiae* have demonstrated high efficacy in controlling mushroom mites, with various formulations, including wettable powder and liquid cell pellets, maintaining their effectiveness over time, especially when stored at lower temperatures (Namsena et al., 2016). Moreover, bioformulations that combine multiple microorganisms, such as *Pseudomonas fluorescens* and *Beauveria bassiana*, have proven effective in controlling pests like leaf folder insects and diseases such as sheath blight in rice, resulting in higher crop yields (Karthiba et al., 2010).

This chapter elaborates on the development of a bioformulation, adopting the two isolated endophytic bacteria (*Bacillus subtilis* NIIST B616 and *Bacillus subtilis* NIIST B627) detailed in the previous chapters. Moreover, the bioformulation prepared was rigorously evaluated to assess its capacity to promote rice plant growth and confer resistance against sheath blight disease in rice plants.

5.2.Materials and Method

5.2.1. Microbial strains and culture conditions

Bacillus subtilis NIIST B616 (GenBank Ac. No. ON054037) and *Bacillus subtilis* NIIST B627 (GenBank Ac. No. KU577428) isolated from rice plants of Kuttanad rice fields. The isolation and preliminary studies are detailed in Chapter 2. The basis for selecting these bacteria for making the bioformulation was their ability to inhibit the pathogen *Rhizoctonia solani*, which causes sheath blight disease in rice plants.

The phytopathogen *Rhizoctonia solani* ITCC 6882 was procured from the Indian Type Culture Collection (ITCC), Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi, India. It was maintained in PDA (HiMedia, India) at 28±2°C and kept at 4°C for future investigations.

5.2.2. Experimental site

The study was conducted at the Agro-Processing and Technology Division of CSIR-NIIST, Thiruvananthapuram, Kerala, India, during the 2021-23 period. The area experiences a hot tropical climate with abundant rainfall during the monsoon seasons. The study recorded an

average temperature range of 24.37 to 31.41℃, with an average rainfall of 8.7 mm and relative humidity of 89.16%.

5.2.3. Development of the liquid bioformulation

The formulation, in general, consists of a carrier agent that aids in delivering beneficial microbes to either the soil or plants. However, in the case of liquid bioformulations, a carrier agent is not necessary; therefore, water was chosen as the medium for bacterial growth due to its convenience and accessibility. Before the initiation of the experiment, the physicochemical properties of water, including odour, colour, pH, electric conductivity (Bureau of Indian Standards, 2013), total dissolved salts (TDS) (Bureau of Indian Standards, 1984), total alkalinity (Bureau of Indian Standards, 1986), and total hardness (Bureau of Indian Standards, 2003), were examined. A blend of ingredients and water was utilised to craft the liquid bioformulation. The different ingredients comprised jaggery, potassium dihydrogen phosphate (KH_2PO_4), dipotassium phosphate anhydrous (K_2HPO_4), magnesium sulphate heptahydrate ($MgSO_4.7H_20$) and urea, each added in diverse ratios for experimentation. Five sets of formulations were designed with varying proportions of compositions, as indicated in Table 5.1. Furthermore, to enhance the stability for long-term storage, gum arabic at a concentration of 0.05% was subsequently incorporated, complementing the components above. Inoculum of endophytic *Bacilli* containing Log 8 CFU/mL (10^8CFU/mL) was individually added to the formulation and kept in a shaking incubator at 160 rpm for 96 h. The bacterial density was determined at various intervals, including 0, 24, 48, 72, and 96 h. After selecting an appropriate period with maximum growth, the formulation containing the NIIST B616 and the formulation containing the NIIST B627 were combined in equal proportions. The resulting mixtures were then stored at 28±2℃ for further investigations. Following a comprehensive analysis, the appropriate composition was ultimately chosen.

Table 5.1 Composition of different formulations used in this study

Additives	**Formulations**				
	A	B	C	D	E
Tap water (ml)	100	100	100	100	100
Jaggery (g/L)	20	18	20	25	30
KH_2PO_4 (g/L)	3	1	1	1	1
K_2HPO_4 (g/L)	1	-	-	-	-
$MgSO_4.7H_20$ (g/L)	0.1	0.1	0.1	0.1	0.1
Urea (g/L)	6	-	-	-	-
Inoculum	3%	3%	3%	3%	3%

5.2.4. Analyzing the nutrient level and shelf-life of the bioformulation

Nutrient analysis of bioformulation, such as total nitrogen, phosphorous, potassium, organic carbon, carbon: nitrogen ratio, and electric conductivity, was carried out. Nitrogen content in bioformulation was determined through the Kjeldahl digestion assembly (Miller & Miller, 1948). Phosphorous content was examined using the method of Zasoski and Burau (Zasoski & Burau, 1977) and potassium by Pickett and Koirtyohann, (1969). The electrical conductivity of the formulation was analysed using a conductivity meter (Systronics, India). Organic carbon (Ciavatta et al., 1989) and carbon: nitrogen ratio were also checked.

The shelf life of the bioformulation was evaluated by regularly collecting samples over a period of seven months, ensuring rigorous adherence to aseptic protocols throughout the testing process. One ml sample was added to 9 ml of sterile distilled water and rigorously mixed for optimal homogenization. The viable cells per mL were determined through a widely used plate count technique. Following this, the plates were incubated at 28℃ for 24 h and viable cells were calculated (Aloo et al., 2022). In addition, the pH of the resultant formulation was also analysed for seven months using a digital electronic pH meter (Eutech, India).

5.2.5. Testing the plant growth-enhancing potential of the bioformulation

5.2.5.1. *Soil physiochemical analyses*

The soil utilised in this investigation underwent air-drying at room temperature. It was then meticulously pulverised using a mortar and pestle and subsequently passed through a stainless-

steel sieve with a mesh size of 2 mm. The finely ground soil sample was appropriately stored in polythene bags for analytical purposes, specifically identifying the soil physicochemical characteristics. The characteristics such as pH of the soil, electric conductivity, available organic carbon, available phosphorous, available potassium and soil texture were analysed according to the procedure as detailed in Chapter 3.

5.2.5.2.*Impact of various dilutions of bioformulation on rice plant growth*

Various dilutions were prepared to evaluate the efficacy of the selected bioformulation for enhancing plant growth. These dilutions included BF 100% (bioformulation undiluted), BF 50% (bioformulation diluted by 50% with water), and BF 10% (bioformulation diluted by 10% with water). Three bioformulations were developed, including two individual endophytic formulations (NIIST B616 BF and NIIST B627 BF) and one consortium of two endophytic formulations (NIIST B616+627 BF). All three formulations were diluted according to the dilutions mentioned earlier. In total, ten treatments were designed, including a control group, which are as follows: (i) Control (no bioformulation), (ii) NIIST B616 BF 100%, (iii) NIIST B627 BF 100%, (iv) NIIST B616+627 BF 100%, (v) NIIST B616 BF 50%, (vi) NIIST B627 BF 50%, (vii) NIIST B616+627 BF 50%, (viii) NIIST B616 BF 10%, (ix) NIIST B627 BF 10%, (x) NIIST B616+627 BF 10%. The effect of dilutions was examined in rice plants for 28 days. Plant growth variables such as shoot height, root length, fresh weight, and dry weight were evaluated at intervals of 7 days up to day 28.

5.2.6. Impact of 10% diluted bioformulations on the growth and yield of rice plant

Among the various dilutions examined, the 10% dilution was selected for the experiment on plant growth and yield determination. This study utilised the 'Jyothi' variety of rice seeds obtained from the highly regarded Regional Agricultural Research Station, Pattambi, Kerala, India. The experiments were conducted in amply sized pots, measuring 18×22 cm and filled with sandy loam soil that was blended with cow dung (3:1). Four different groups were maintained: a control group with no bioformulation, two groups with individual formulations (NIIST B616 BF or NIIST B627 BF), and a group with a consortium of both formulations (NIIST B616+627 BF). To the respective pots, introduced a suspension of NIIST B616 BF or NIIST B627 BF (150 ml, 1×10^8 CFU/ml each) and a consortium NIIST B616+627 BF (150 ml, 1×10^8 CFU/ml). The uppermost layer was then carefully mixed up to an approximate depth of 2.0 cm using a sterile glass rod, following which 20 surface sterilised seeds were introduced into the pots at a depth of 1 cm and grown under nursery conditions (Dileep Kumar & Dube,

1992). The control group, on the other hand, consisted of pots with only surface sterilised seeds. The experiments were conducted using a completely randomised design (CRD), and data were collected from ten replications. An assessment of plant growth-promoting parameters such as shoot height, root length, fresh weight, and dry weight was recorded at intervals of 7 days for up to 28 days. After 30 days, a booster dose of foliar spray (150 mL) containing the abovementioned bioformulations was carefully applied to the appropriate section of pots, ensuring optimal plant growth potential. Furthermore, the yield was determined by the number of grains, weight of 100 grains, number of tillers per plant, number of panicles per plant, dry shoot matter, and harvest index - all of which were carefully evaluated after 120 days of growth. The data were collected, and the harvest index was calculated as described in Chapter 3.

5.2.7. Impact of 10% diluted bioformulations on growth and yield in rice plants challenge inoculated with *R. solani*

The plant growth experiments in the presence of *R. solani* were divided into five distinct sections namely Control (no bioformulation + no *R. solani)*, Pathogen alone (*R. solani* alone), NIIST B616 BF + P (NIIST B616 bioformulation + *R. solani*), NIIST B627 BF + P (NIIST B627 bioformulation + *R. solani*), NIIST B616 + 627 BF + P (NIIST B616 + 627 bioformulation + *R. solani*). The experiment was conducted similarly, as explained earlier, utilising pots infested with a 150 mL homogenised solution of *R. solani* (7 days old), except for the control group. After 30 days of growth, the booster dose was administered, and all parameters related to growth and yield were meticulously recorded, just as they had been described before.

5.2.7.1. *Impact of 10% diluted bioformulations on disease control*

The estimation of the occurrence of disease symptom and the corresponding measurement of the height of lesions after 90 days of treatment was conducted utilising the Rice Standard Evaluation System scale, a comprehensive assessment tool devised by the esteemed International Rice Research Institute, Philippines (IRRI, 2013). The percentage of disease incidence and the determination of relative lesion height were conducted as outlined in Chapter 3.

5.2.8. Impact of bioformulations on the growth of various vegetable crops

Three vegetable crops, namely cowpea (*Vigna unguiculata*), beans (*Phaseolus vulgaris*), and amaranthus (*Amaranthus cruentus*), were selected to assess the efficacy of the bioformulation

in promoting plant growth. The seeds of these crops were obtained from the College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The experimental design conformed to the aforementioned protocol. A dilution of the bioformulation (NIIST B616 + 627 BF 10%, 150 ml) was administered to all three crops except the control group (no bioformulation) for 60 days. Various plant growth parameters, including shoot height, root length, fresh weight, and dry weight, were measured at intervals of 7 days up to day 28. The data were taken from ten replications. The harvest of cowpea was recorded within 90 days after germination, while bush beans yielded within 60 days. The yield of amaranthus was documented 60 days after planting. The yield parameters assessed for cowpea and bush beans included pod count per plant, pod count per pot, and the dry weight of pods, whereas, for amaranthus, only the dry weight was measured. All data were taken from triplicates.

5.2.9. Statistical analysis

The data were subjected to one-way analyses of variance (ANOVA) using SPSS (version 20.0; IBM SPSS). Statistical significance was evaluated using Duncan's Multiple Range Test (DMRT), and a $p<0.05$ was considered to demonstrate a significant difference. Graphical representations were made using Excel 2019. The weather information is sourced from the Indian Meteorological Department, Ministry of Earth Sciences, Government of India.

5.3.Results

5.3.1. Development of the liquid bioformulation

For liquid bioformulation, water was used as the liquid medium. An analysis was conducted on tap water to evaluate its physicochemical properties. The examination revealed that the water is clear, devoid of impurities and lacks any unpleasant odour. Moreover, the pH level of the water was measured to be 6.9. Further investigation demonstrated that the water exhibits an electrical conductivity of 44.4 micromhos/cm. In addition, it contains a total dissolved solids (TDS) concentration of 26 mg/L, alkalinity of 10 mg/L, and total hardness of 20 mg/L. This water was supplemented with various substances, including jaggery, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_20$, and urea, in varying concentrations. The effectiveness of five different formulations, A, B, C, D, and E, was assessed through bacterial density. The cell density of *Bacillus subtilis* NIIST B616 and *Bacillus subtilis* NIIST B627 was evaluated for all five formulations. In the case of Formulation B, the cell density of both *Bacillus* only reached log 7 CFU/mL, whereas the other formulations achieved a cell density of log 8 CFU/mL on the final day of observation. Remarkably, all formulations remained uncontaminated. The

microbial cell density of the target bacteria met satisfactory levels in Formulations A, C, D, and E. Among these formulations, Formulation A contained urea, while Formulations D and E exhibited higher jaggery concentrations than Formulation C. As a result, the formulation with the fewest components yet demonstrating optimal bacterial density was chosen. Subsequently, Formulation C was singled out for further exploration in future studies (Fig 5.1).

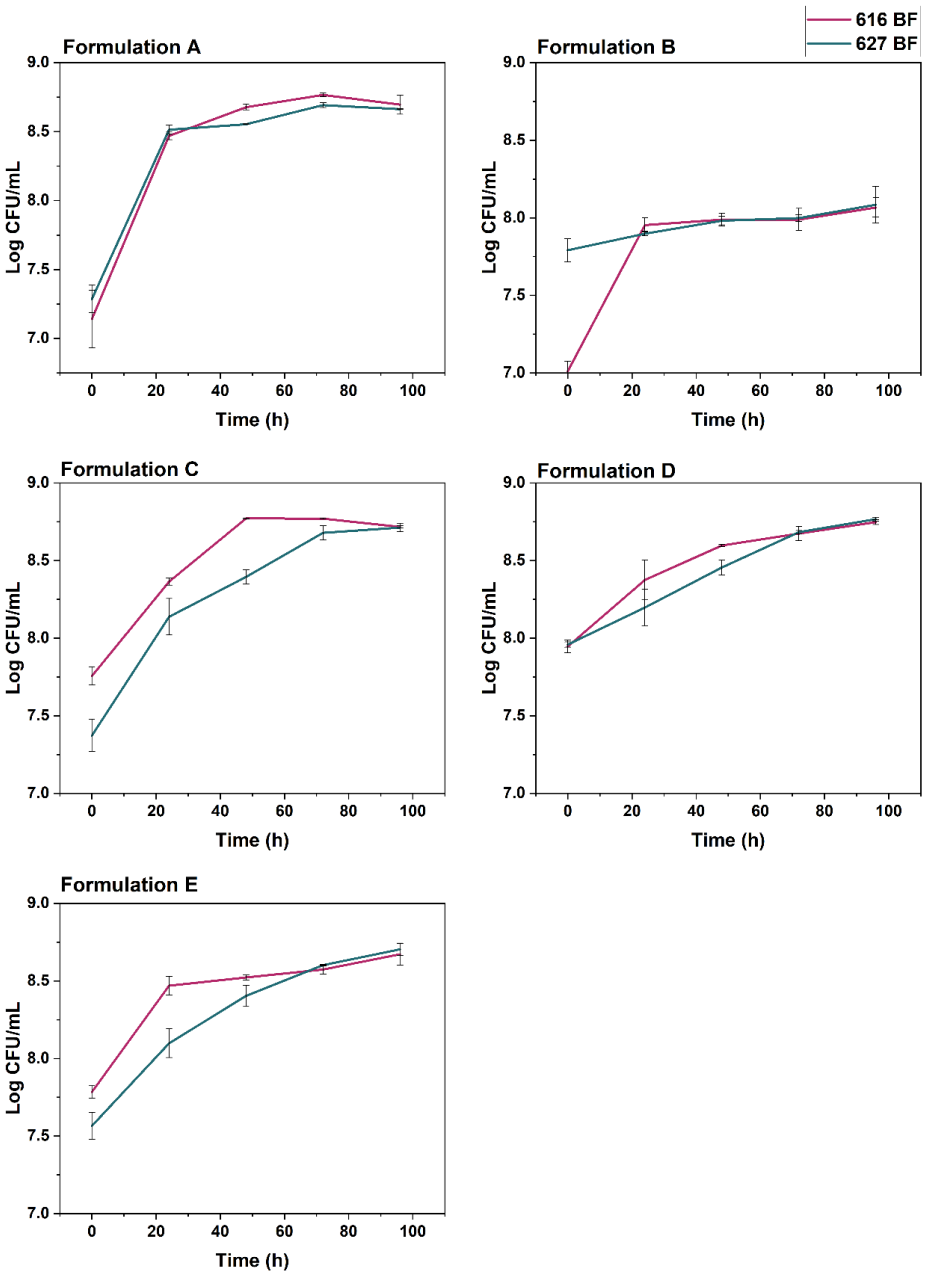


Fig 5.1 Viable count of *Bacillus subtilis* NIIST B616 and *Bacillus subtilis* NIIST B627 in different formulations at various time intervals

5.3.2. Analyzing the nutrient level and shelf-life of the bioformulation

The consortium of bioformulation contains 0.07% nitrogen, 0.078% phosphorous, 0.048% potassium, 0.812% total organic carbon, an 11.6:1% C:N ratio, as well as an electric conductivity of 1.0 dS/m. Furthermore, the consortium of two bioformulations demonstrated robust growth of bacteria within the chosen formulation throughout the incubation period at room temperature. As a result, an average viable count of approximately Log 8 CFU/mL was achieved (Fig 5.8). After a storage duration of three months, the average viable count reached Log 9.7 CFU/mL. The bioformulation effectively maintained the viable count at approximately Log 8 CFU/mL for up to six months, after which it gradually declined. By the seventh month, the viable count had decreased to Log 7.5 CFU/mL. Notably, the consortium experienced a significant decrease in pH at the end of the storage period compared to the pH recorded during the initial stage (Fig 5.2). The initial pH was 7.7 and rose to 8 by the third month. Subsequently, it declined and was measured at 6.1 by the seventh month.

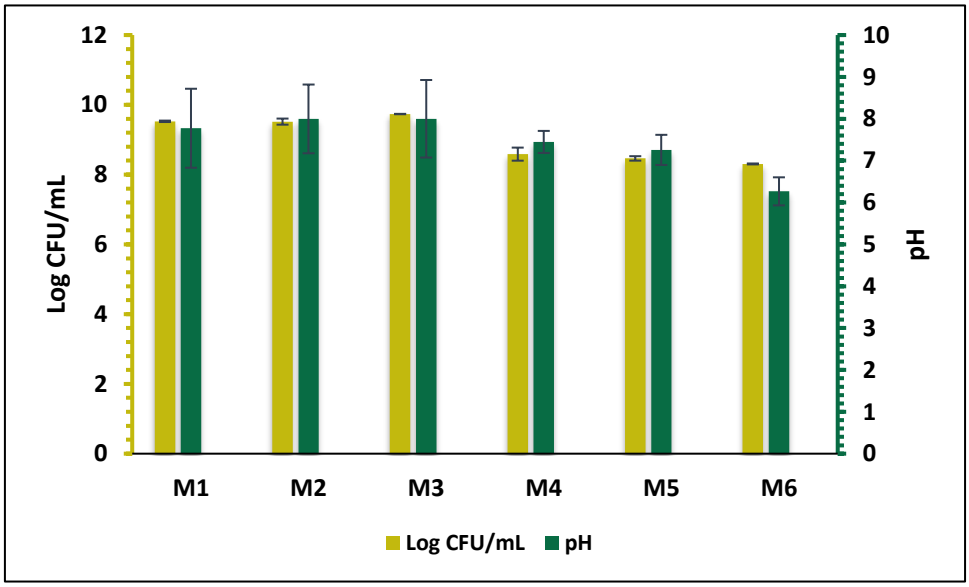


Fig 5.2 Shelf life of bioformulation through total viable count and pH of microbial consortium during the storage period at room temperature. M1 - month 1, M2 – month 2, M3 – month 3, M4 – month 4, M5 – month 5, M6 – month 6

5.3.3. Impact of bioformulation on enhancing rice plant growth

The physicochemical characteristics of the soil were assessed prior to the initiation of the experiment. The soil exhibited a pH value of 6.86 and an electric conductivity of 0.05 dS/m, both indicative of a neutral nature. Furthermore, it contained 1.4% organic carbon, 3.7 kg/ha phosphorous, and 176 kg/ha potassium. Subsequently, the soil underwent an analysis of its texture, and classified it as sandy loam soil.

Rice plant growth was assessed using the selected formulation, Formulation C with both *Bacillus*. To evaluate the impact on growth in rice plants, three distinct dilutions of the bioformulations were used: 100% (undiluted), 50%, and 10%. The results clearly showed that the 10% diluted bioformulation significantly enhanced plant growth compared to the control and other dilutions (Table 5.2, Fig 5.3). Specifically, the bioformulations NIIST B616 BF 10%, NIIST B627 BF 10%, and NIIST B616 + 627 BF 10% outperformed the 50% and 100% bioformulations, respectively. It is worth noting that the 100% bioformulations actually hindered growth compared to the control. Among the three 10% diluted bioformulations, the combination of the two endophytic formulations (NIIST B616 + 627 BF 10%) demonstrated the highest effectiveness, resulting in an 85.19% increase in dry weight on day 28 compared to the control plants. This was followed by NIIST B627 BF 10% (40.74%) and NIIST B616 BF 10% (29.63%). Consequently, the treatments can be ranked in terms of increasing dry weight as follows: NIIST B616 + 627 BF 10% > NIIST B627 BF 10% > NIIST B616 BF 10% > NIIST B 616 + 27 BF 50% > NIIST B627 BF 50% > NIIST B616 BF 50% > NIIST B616 + 627 BF 100% > NIIST B627 BF 100% > NIIST B616 BF 100%.

5.3.4. Impact of 10% diluted bioformulations on the growth and yield of rice plant

Drawing from the outcomes of the preceding experiment, it was concluded that employing a 10% dilution of the bioformulation yielded more favourable results regarding plant growth. Consequently, this identical dilution was applied to evaluate the growth and yield of rice plants. The findings of this investigation demonstrate that, on day 28, the combination of two endophytic formulations (NIIST B616 + 627 BF 10%) led to an 88.88% increase in dry weight compared to the control plants. This increase was then followed by NIIST B627 BF 10% (44.44%) and NIIST B616 BF 10% (37.03%) (Table 5.3). Additionally, the harvest index after 120 days revealed an 18.52% increase in plants treated with the consortium of formulations. Subsequently, this increase was followed by individual formulations NIIST B627 BF 10% (14.81%) and NIIST B616 BF 10% (12.96%) (Table 5.4, Fig 5.4).

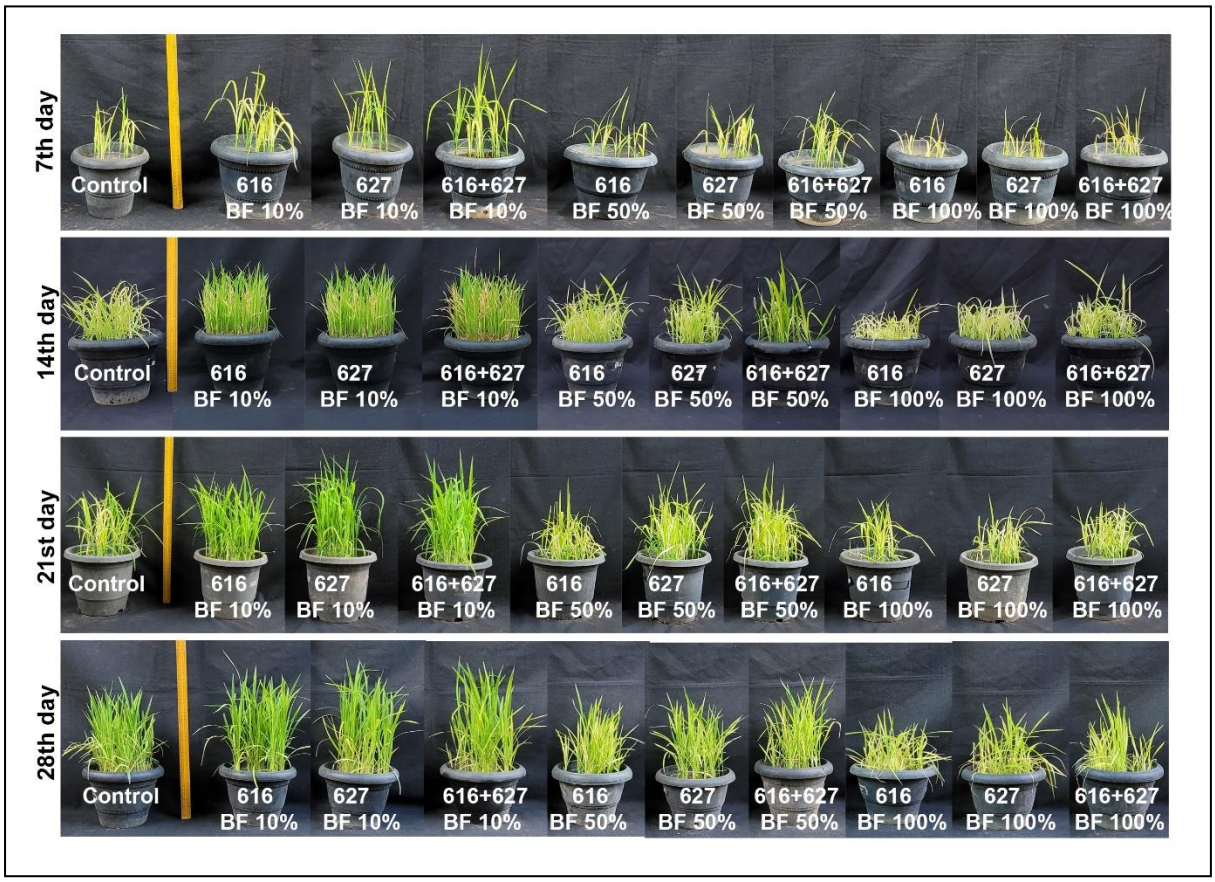


Fig 5.3 Plant growth promotion studies by different dilutions of selected bioformulation in rice plants on different data collection days. Control - no treatment, 616 BF 10% - 10% diluted bioformulation of *Bacillus subtilis* NIIST B616, 627 BF 10% - 10% diluted bioformulation of *Bacillus subtilis* NIIST B627, 616+627 BF 10% - 10% diluted bioformulation of *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627, 616 BF 50% - 50% diluted bioformulation of *Bacillus subtilis* NIIST B616, 627 BF 50% - 50% diluted bioformulation of *Bacillus subtilis* NIIST B627, 616+627 BF 50% - 50% diluted bioformulation of *Bacillus subtilis* NIIST B616+627, 616 BF 100% -100% diluted bioformulation of *Bacillus subtilis* NIIST B616, 627 BF 100% - 100% diluted bioformulation of *Bacillus subtilis* NIIST B627, 616+627 BF 100% - 100% diluted bioformulation of *Bacillus subtilis* NIIST B616+627

Table 5.2 Effect of endophytic bioformulation at different dilutions on the growth of rice plants

Days	Treatments	Shoot Height (cm)	Root Length (cm)	Fresh Weight (g)	Dry Weight (g)
7	Control	17.81±0.94^{c}	6.36±0.68^{c}	0.17±0.02^{c}	0.03±0.01^{b}
	NIIST B616 BF 100%	11.49±1.67^{a} (-35.49)	3.65±0.47^{a} (-42.61)	0.11±0.02^{a} (-35.29)	0.02±0.00^{a} (-33.33)
	NIIST B627 BF 100%	13.16±2.06^{b} (-26.11)	3.24±0.57^{a} (-49.06)	0.12±0.03^{ab} (-29.41)	0.02±0.01^{a} (-33.33)
	NIIST B616+627 BF 100%	13.54±1.72^{b} (-23.98)	4.47±0.38^{b} (-29.72)	0.13±0.01^{b} (-23.53)	0.02±0.00^{a} (-33.33)
	NIIST B616 BF 50%	18.65±1.07^{c} (4.72)	4.34±0.52^{b} (-31.76)	0.19±0.01^{de} (11.76)	0.03±0.00^{b} (0.00)
	NIIST B627 BF 50%	18.79±0.56^{c} (5.50)	4.68±0.34^{b} (-26.42)	0.19±0.01^{cd} (11.76)	0.03±0.00^{b} (0.00)
	NIIST B616+627 BF 50%	18.86±0.93^{c} (5.90)	4.85±0.57^{b} (-23.74)	0.20±0.01^{e} (17.65)	0.03±0.01^{c} (0.00)
	NIIST B616 BF 10%	21.82±0.73^{d} (22.52)	7.79±0.60^{d} (22.48)	0.22±0.02^{f} (29.41)	0.04±0.00^{cd} (33.33)
	NIIST B627 BF 10%	23.16±1.00^{e} (30.04)	7.93±0.72^{de} (24.69)	0.22±0.02^{f} (29.41)	0.04±0.01^{de} (33.33)
	NIIST B616+627 BF 10%	23.90±1.06^{e} (34.19)	8.38±0.47^{e} (31.76)	0.23±0.01^{f} (35.29)	0.04±0.01^{e} (33.33)
14	Control	23.81±0.90^{e}	10.08±0.89^{d}	0.22±0.02^{b}	0.04±0.01^{c}
	NIIST B616 BF 100%	18.39±0.93^{a} (-22.76)	4.11±0.88^{a} (-59.23)	0.15±0.01^{a} (-31.82)	0.03±0.01^{a} (-25.00)
	NIIST B627 BF 100%	19.66±1.32^{b} (-17.43)	4.05±0.63^{a} (-59.82)	0.15±0.01^{a} (-31.82)	0.03±0.01^{ab} (-25.00)
	NIIST B616+627 BF 100%	20.73±1.67^{c} (-12.94)	4.89±0.86^{a} (-51.49)	0.16±0.01^{a} (-27.27)	0.03±0.01^{ab} (-25.00)
	NIIST B616 BF 50%	22.20±0.80^{d} (-6.76)	8.39±0.66^{b} (-16.77)	0.21±0.01^{b} (-4.55)	0.03±0.02^{ab} (-25.00)
	NIIST B627 BF 50%	22.62±0.76^{d} (-5.00)	8.74±0.64^{bc} (-13.29)	0.22±0.01^{b} (0.00)	0.04±0.01^{b} (0.00)
	NIIST B616+627 BF 50%	24.00±1.40^{e} (0.80)	9.27±0.91^{cd} (-8.04)	0.23±0.01^{b} (4.55)	0.04±0.01^{c} (0.00)
	NIIST B616 BF 10%	29.55±1.12^{f} (24.11)	13.4±0.50^{e} (32.94)	0.44±0.08^{c} (100.00)	0.07±0.01^{d} (75.00)
	NIIST B627 BF 10%	31.69±0.91^{g} (33.10)	13.84±1.60^{e} (37.30)	0.47±0.07^{c} (113.64)	0.08±0.01^{e} (100.00)
	NIIST B616+627 BF 10%	34.05±0.49^{h} (43.01)	17.48±1.23^{f} (73.41)	0.50±0.06^{d} (127.27)	0.09±0.01^{f} (125.00)

21	Control	30.98±0.77^{e}	17.89±0.87^{d}	0.50±0.05^{b}	0.15±0.01^{b}
	NIIST B616 BF 100%	20.72±1.24^{a} (-33.12)	8.12±0.90^{a} (-54.61)	0.20±0.01^{a} (-60.00)	0.06±0.01^{a} (-60.00)
	NIIST B627 BF 100%	23.25±1.96^{b} (-24.95)	8.92±0.76^{ab} (-50.14)	0.21±0.01^{a} (-58.00)	0.07±0.01^{a} (-53.33)
	NIIST B616+627 BF 100%	25.57±0.99^{c} (-17.46)	9.16±0.82^{b} (-48.80)	0.23±0.04^{a} (-54.00)	0.08±0.03^{a} (-46.67)
	NIIST B616 BF 50%	27.79±1.13^{d} (-10.30)	10.94±0.49^{c} (-38.85)	0.48±0.13^{b} (-4.00)	0.14±0.02^{b} (-6.67)
	NIIST B627 BF 50%	29.96±1.18^{e} (-3.29)	11.06±0.55^{c} (-38.18)	0.50±0.06^{b} (0.00)	0.15±0.01^{b} (0.00)
	NIIST B616+627 BF 50%	31.07±0.98^{e} (0.29)	11.14±0.42^{c} (-37.73)	0.51±0.05^{b} (2.00)	0.16±0.01^{b} (6.67)
	NIIST B616 BF 10%	40.88±0.91^{f} (31.96)	20.40±0.75^{e} (14.03)	1.20±0.09^{c} (140.00)	0.24±0.02^{c} (60.00)
	NIIST B627 BF 10%	41.04±1.10^{f} (32.47)	21.25±1.12^{e} (18.78)	1.38±0.09^{d} (176.00)	0.27±0.05^{d} (80.00)
	NIIST B616+627 BF 10%	43.09±0.33^{g} (39.09)	24.02±0.70^{f} (34.26)	1.72±0.06^{e} (244.00)	0.30±0.01^{e} (100.00)
28	Control	34.45±1.20^{cd}	22.61±0.84^{d}	2.34±0.14^{c}	0.27±0.02^{c}
	NIIST B616 BF 100%	26.41±1.26^{a} (-23.34)	12.57±1.38^{a} (-44.41)	0.35±0.03^{a} (-85.04)	0.10±0.03^{a} (-62.96)
	NIIST B627 BF 100%	27.81±1.81^{b} (-19.27)	12.79±0.81^{a} (-43.43)	0.38±0.03^{a} (-83.79)	0.10±0.03^{a} (-62.96)
	NIIST B616+627 BF 100%	28.75±2.29^{b} (-16.55)	12.95±1.08^{a} (-42.72)	0.41±0.03^{a} (-82.48)	0.11±0.02^{a} (-59.26)
	NIIST B616 BF 50%	33.52±0.76^{c} (-2.70)	17.39±0.55^{b} (-23.09)	2.20±0.170^{b} (-5.98)	0.19±0.01^{b} (-29.63)
	NIIST B627 BF 50%	34.14±0.95^{cd} (-0.90)	18.02±0.39^{bc} (-20.30)	2.22±0.07^{b} (-5.13)	0.19±0.01^{b} (-29.63)
	NIIST B616+627 BF 50%	35.06±1.30^{d} (1.77)	18.60±1.09^{c} (-17.74)	2.23±0.07^{bc} (-4.70)	0.22±0.03^{bc} (-18.52)
	NIIST B616 BF 10%	44.06±0.88^{e} (27.90)	26.96±0.70^{e} (19.24)	3.11±0.11^{d} (32.91)	0.35±0.33^{d} (29.63)
	NIIST B627 BF 10%	45.06±1.07^{e} (30.80)	28.31±0.74^{f} (25.21)	3.30±0.15^{e} (41.03)	0.38±0.18^{d} (40.74)
	NIIST B616+627 BF 10%	47.57±1.07^{f} (38.08)	29.02±0.95^{f} (28.35)	3.76±0.19^{f} (60.68)	0.50±0.04^{e} (85.19)

* All values are expressed in mean ± SD from ten replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses are percentage increases over the control

Table 5.3 Effect of 10% diluted endophytic bioformulation on the growth of rice plants on various days

Days	Treatments	Shoot Height (cm)	Root Length (cm)	Fresh Weight (g)	Dry Weight (g)
7	Control	17.1±0.60^{a}	6.56±0.88^{a}	0.16±0.02^{a}	0.03±0.01^{a}
	NIIST B616 BF	22.99±1.20^{b} (34.44)	11.00±1.11^{b} (67.68)	0.22±0.02^{b} (37.50)	0.04±0.00^{b} (33.33)
	NIIST B627 BF	23.83±1.27^{bc} (39.36)	12.80±1.32^{c} (95.12)	0.22±0.01^{bc} (37.50)	0.04±0.01^{b} (33.33)
	NIIST B616+627 BF	24.16±0.82^{c} (41.29)	14.57±0.98^{d} (122.10)	0.24±0.02^{c} (50.00)	0.05±0.01^{b} (66.67)
14	Control	24.12±1.62^{a}	11.33±0.85^{a}	0.31±0.03^{a}	0.05±0.01^{a}
	NIIST B616 BF	29.17±2.10^{b} (20.94)	15.93±1.18^{b} (40.60)	0.45±0.05^{b} (45.16)	0.07±0.03^{b} (40.00)
	NIIST B627 BF	31.71±1.35^{c} (31.47)	16.09±1.64^{b} (42.01)	0.49±0.08^{b} (58.06)	0.09±0.01^{c} (80.00)
	NIIST B616+627 BF	34.34±1.28^{d} (42.37)	20.43±2.09^{c} (80.32)	0.50±0.08^{b} (61.29)	0.10±0.01^{c} (100.00)
21	Control	31.12±0.92^{a}	19.33±1.49^{a}	0.56±0.07^{a}	0.15±0.02^{a}
	NIIST B616 BF	40.83±2.03^{b} (31.20)	22.69±2.42^{b} (17.38)	1.24±0.11^{b} (121.43)	0.25±0.03^{b} (66.67)
	NIIST B627 BF	41.12±1.52^{b} (32.13)	23.25±1.3^{b} (20.28)	1.41±0.14^{c} (151.79)	0.29±0.06^{bc} (93.33)
	NIIST B616+627 BF	43.32±1.80^{c} (39.20)	24.20±0.82^{b} (25.19)	1.74±0.19^{d} (210.71)	0.30±0.06^{c} (100.00)
28	Control	35.67±2.25^{a}	26.07±2.62^{a}	2.36±0.31^{a}	0.27±0.01^{a}
	NIIST B616 BF	44.27±0.93^{b} (24.11)	29.91±1.46^{b} (14.73)	3.14±0.41^{b} (33.05)	0.37±0.06^{b} (37.03)
	NIIST B627 BF	45.42±1.74^{b} (27.33)	31.75±1.24^{c} (21.79)	3.36±0.29^{bc} (42.37)	0.39±0.13^{b} (44.44)
	NIIST B616+627 BF	48.00±1.02^{c} (34.57)	33.31±0.85^{d} (27.77)	3.78±0.83^{c} (60.17)	0.51±0.08^{c} (88.88)

* All values are expressed in mean ± SD from ten replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses are percentage increases over the control

Table 5.4 Effect of 10% diluted endophytic bioformulation on yield of rice plants

Treatments	No. of grains per pot (n)	Weight of 100 grains (g)	No. of tillers per plant (n)	No. of panicles per plant (n)	Dry shoot matter (g)	Harvest index
Control	261.00±2.65^{a}	2.26±0.09^{a}	2.33±0.58^{a}	2.00±0.00^{a}	2.07±0.14^{a}	0.54±0.01^{a}
NIIST B616 BF	422.67±2.52^{b} (61.94)	2.54±0.10^{a} (12.39)	2.67±0.58^{a} (14.59)	2.33±0.58^{ab} (16.50)	3.90±0.10^{b} (88.41)	0.61±0.02^{b} (12.96)
NIIST B627 BF	432.33±2.52^{c} (65.64)	2.67±0.14^{b} (18.14)	2.67±0.58^{a} (14.59)	2.33±0.58^{ab} (16.50)	4.06±0.06^{b} (96.14)	0.62±0.02^{b} (14.81)
NIIST B616+627 BF	607.00±2.65^{d} (132.57)	2.79±0.36^{b} (23.45)	3.00±0.00^{a} (28.76)	3.00±0.00^{b} (50.00)	4.33±0.76^{b} (109.18)	0.64±0.02^{b} (18.52)

* All values are expressed in mean ± SD from three replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses are percentage increases over the control

** Harvest index is the ratio of harvested grain to total dry shoot matter

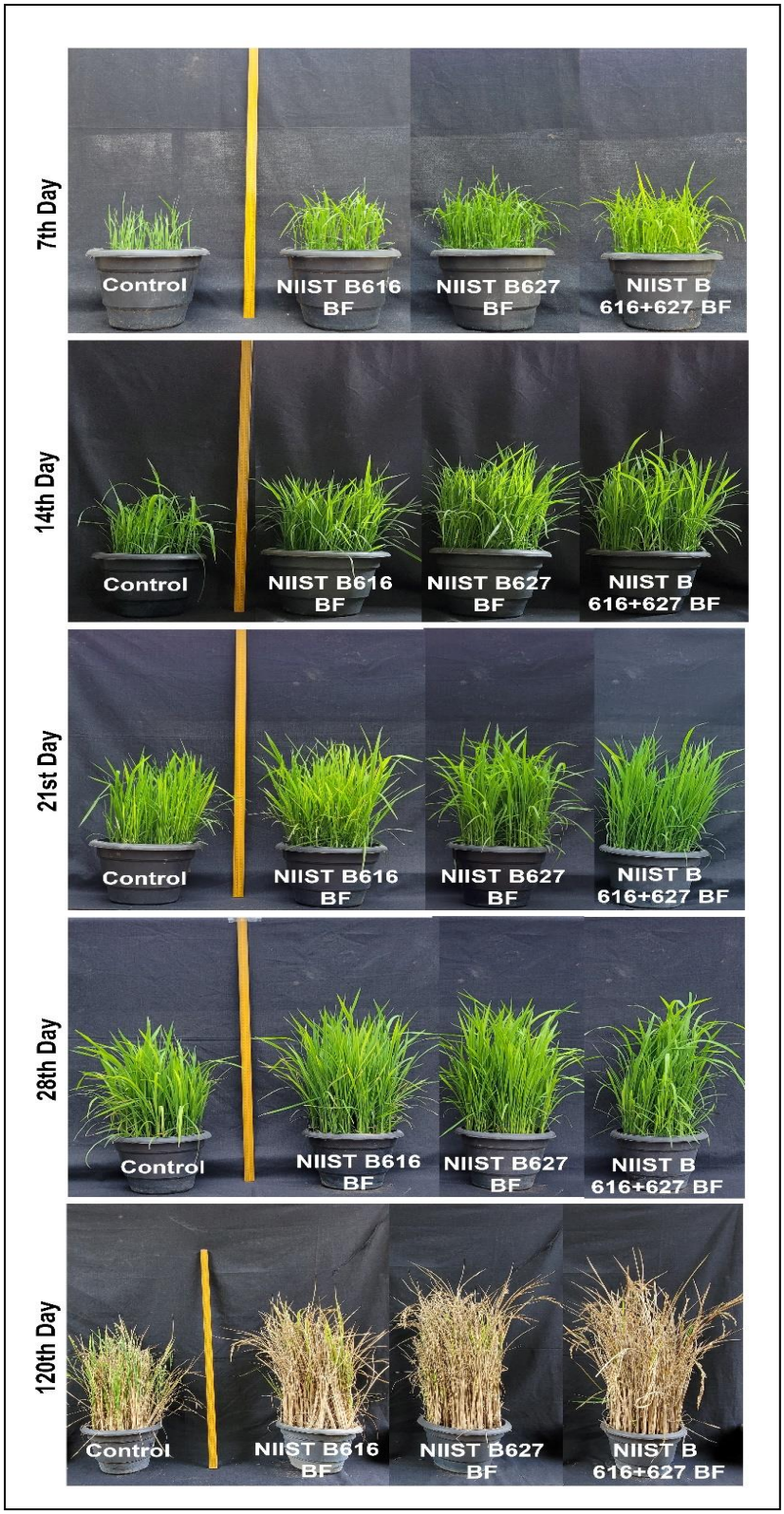


Fig 5.4 Plant growth promotion studies using 10% diluted bioformulation in rice plants on different data collection days. Control - no treatment, NIIST B616 BF – bioformulation of *Bacillus subtilis* NIIST B616, NIIST B627 BF - bioformulation of *Bacillus subtilis* NIIST B627, NIIST B616+627 BF - bioformulation of *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627

5.3.5. Impact of 10% diluted bioformulations on growth and yield in rice plants challenge inoculated with *R. solani*

The efficacy of a 10% diluted bioformulation in plants challenged with *R. solani* was assessed to determine its impact on disease resistance and growth promotion. On day 28, it was observed that the combination of formulations (NIIST B1616 + 627 BF + P) resulted in a significant increase of 161.11% in dry weight compared to plants treated only with *R. solani*. This was followed by the formulations NIIST B627 BF and NIIST B616 BF with increases of 100% and 88.89% respectively (Table 5.5). Additionally, the consortium demonstrated a notable increment of 32.61% in harvest index compared to plants treated solely with *R. solani*. This increment was also observed with the individual formulations NIIST B616 BF + P and NIIST B627 + P, respectively (Table 5.6, Fig 5.5).

5.3.5.1.Impact of 10% diluted bioformulations on disease control

The evaluation of disease control for sheath blight was carried out using endophytic bacterial formulations. The rice plants were treated with a 10% diluted formulation and subsequently exposed to *R. solani*. The symptoms started to be observed from 49 days (*R. solani* alone), 55 days (NIIST B616 BF + P), 59 days (NIIST B627 BF + P) and 65 days (NIIST B616 + 627 BF+P). It was also found that the combination of formulations (NIIST B616 + 627 BF + P) has the lowest disease incidence of 31.67%, which means it effectively reduced the disease by 64.15%. Similar reductions in disease were observed with NIIST 616 BF + P (52.82%) and NIIST B627 BF + P (56.61%). The consortium exhibited a relative lesion scale of 7, while the single formulations had a scale of 3 (Table 5.7, Fig 5.6).

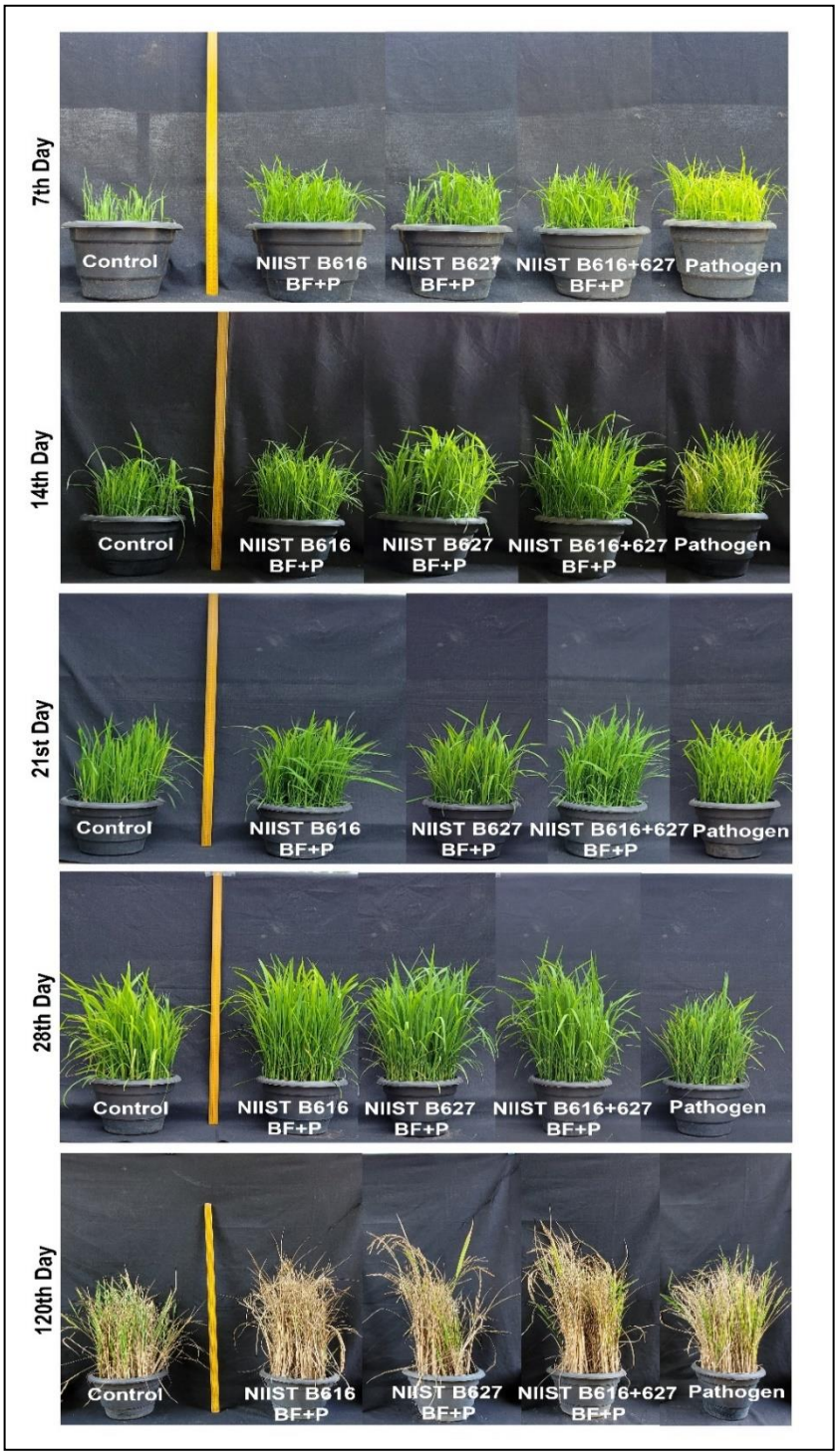


Fig 5.5 Growth promotion studies of 10% diluted endophytic formulation in rice plants challenge inoculated with *R. solani* on different data collection days. Control - no treatment, NIIST B616 BF+P – bioformulation of *Bacillus subtilis* NIIST B616 + *R solani*, NIIST B627 BF +P - bioformulation of *Bacillus subtilis* NIIST B627 + *R solani*, NIIST B616+627 BF+P - bioformulation of *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627 + *R solani*, Pathogen -*R solani* alone

Table 5.5 Effect of 10% diluted endophytic bioformulation on the growth of rice plants challenge inoculated with *R. solani* on various days

Days	Treatments	Shoot Height (cm)	Root Length (cm)	Fresh Weight (g)	Dry Weight (g)
7	Control	17.1±0.60^{a} (5.75)	6.56±0.88^{a} (4.13)	0.16±0.02^{b} (14.29)	0.03±0.01^{b} (50.00)
	NIIST B616 BF+P	22.85±1.25^{b} (41.31)	10.65±0.55^{b} (69.05)	0.20±0.01^{c} (42.86)	0.04±0.00^{c} (100.00)
	NIIST B627 BF+P	23.46±0.72^{b} (45.08)	12.54±1.32^{c} (99.05)	0.21±0.02^{cd} (50.00)	0.04±0.01^{c} (100.00)
	NIIST B616+627 BF+P	23.40±1.73^{b} (44.71)	13.15±1.43^{c} (108.73)	0.22±0.03^{d} (57.14)	0.04±0.01^{c} (100.00)
	Pathogen	16.17±1.35^{a}	6.3±0.59^{a}	0.14±0.01^{a}	0.02±0.00^{a}
14	Control	24.12±1.62^{b} (7.25)	11.33±0.85^{a} (3.85)	0.31±0.03^{a} (6.90)	0.05±0.01^{a} (25.00)
	NIIST B616 BF+P	27.63±1.17^{c} (22.85)	16.44±0.92^{c} (50.69)	0.40±0.07^{b} (37.93)	0.06±0.01^{b} (50.00)
	NIIST B627 BF+P	30.24±1.00^{d} (34.46)	13.45±1.07^{b} (23.28)	0.41±0.06^{b} (41.38)	0.06±0.01^{b} (50.00)
	NIIST B616+627 BF+P	32.32±1.42^{e} (43.71)	19.77±1.33^{d} (81.21)	0.45±0.12^{b} (55.17)	0.08±0.01^{c} (100.00)
	Pathogen	22.49±1.89^{a}	10.91±0.52^{a}	0.29±0.03^{a}	0.04±0.01^{a}
21	Control	31.12±0.92^{b} (19.37)	19.33±1.49^{b} (18.44)	0.56±0.07^{a} (43.59)	0.15±0.02^{a} (25.00)
	NIIST B616 BF+P	38.58±0.77^{c} (47.99)	22.69±2.16^{c} (39.03)	1.13±0.267^{b} (189.74)	0.24±0.04^{b} (100.00)
	NIIST B627 BF+P	39.34±1.09^{cd} (50.90)	22.92±1.99^{c} (40.44)	1.32±0.20^{bc} (235.46)	0.28±0.06^{b} (133.33)
	NIIST B616+627 BF+P	40.39±1.61^{d} (54.93)	23.14±1.14^{c} (41.79)	1.49±0.62^{c} (282.05)	0.29±0.07^{b} (141.67)
	Pathogen	26.07±1.32^{a}	16.32±1.35^{a}	0.39±0.09^{a}	0.12±0.05^{a}
28	Control	35.67±2.25^{b} (14.36)	26.07±2.62^{b} (13.45)	2.36±0.31^{b} (93.44)	0.27±0.01^{b} (50.00)
	NIIST B616 BF+P	42.96±1.67^{c} (37.74)	29.03±1.16^{c} (26.33)	2.74±0.27^{c} (124.59)	0.34±0.04^{c} (88.89)
	NIIST B627 BF+P	43.70±1.83^{c} (40.11)	30.30±2.06^{cd} (31.85)	3.02±0.31^{d} (147.54)	0.36±0.06^{c} (100.00)
	NIIST B616+627 BF+P	46.73±1.47^{d} (49.82)	31.42±1.21^{d} (36.73)	3.30±0.17^{e} (170.49)	0.47±0.08^{d} (161.11)
	Pathogen	31.19±1.91^{a}	22.98±1.91^{a}	1.22±0.09^{a}	0.18±0.06^{a}

* All values are expressed in mean ± SD from ten replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses are percentage increases over the pathogen

Table 5.6 Effect of 10% diluted endophytic bioformulation on yield of rice plants challenge inoculated with *R. solani*

Treatments	No. of grains per pot (n)	Weight of 100 grains (g)	No. of tillers per plant (n)	No. of panicles per plant (n)	Dry shoot matter (g)	Harvest index
Control	261.00±2.65^{b} (18.64)	2.26±0.09^{b} (18.32)	2.33±0.58^{ab} (39.52)	2.00±0.00^{a} (19.74)	2.07±0.14^{a} (10.11)	0.54±0.01^{b} (17.39)
NIIST B616 BF+P	390.00±2.00^{c} (77.27)	2.34±0.05^{bc} (22.51)	2.67±0.58^{ab} (59.88)	2.33±0.58^{a} (39.52)	3.00±0.62^{b} (59.57)	0.59±0.02^{c} (28.26)
NIIST B627 BF+P	397.33±2.52^{d} (80.60)	2.51±0.05^{c} (31.41)	2.67±0.58^{ab} (59.88)	2.33±0.58^{a} (39.52)	3.40±0.50^{b} (80.85)	0.59±0.01^{c} (28.26)
NIIST B616+627 BF+P	442.33±2.52^{e} (101.06)	2.52±0.04^{c} (31.94)	3.00±0.00^{c} (79.64)	2.67±0.58^{a} (59.88)	3.53±0.57^{b} (87.77)	0.61±0.2^{c} (32.61)
Pathogen	220.00±2.00^{a}	1.91±0.21^{a}	1.67±0.58^{a}	1.67±0.58^{a}	1.88±0.11^{a}	0.46±0.03^{a}

* All values are expressed in mean ± SD from three replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses are percentage increases over the pathogen

** Harvest index is the ratio of harvested grain to total dry shoot matter

Table 5.7 Efficacy of 10% diluted endophytic bioformulations on disease control in *R. solani* treated plants after 90 days

Treatments	Disease incidence (%)	Relative lesion height (scale 0-9)
Pathogen	88.33±2.89^{d}*	7**
NIIST B616 BF+P	41.67±2.89^{c} (52.82)	3
NIIST B627 BF+P	38.33±2.89^{c} (56.61)	3
NIIST B616+627 BF+P	31.67±2.89^{b} (64.15)	1
Control	0^{a}	0

* All values are expressed in mean ± SD from three replications. Different superscript letters in the same column indicate the different degrees of treatment at a statistical significance of 5% (α). Values in the parentheses are percentage decreases over pathogen alone treatment

Disease index scale, **0 - No infection, **1** - lesion limited to lower 20% of the plant height, **3** - 20-30%, **5** - 31- 45%. **7** - 46-65%, 9 - more than 65%

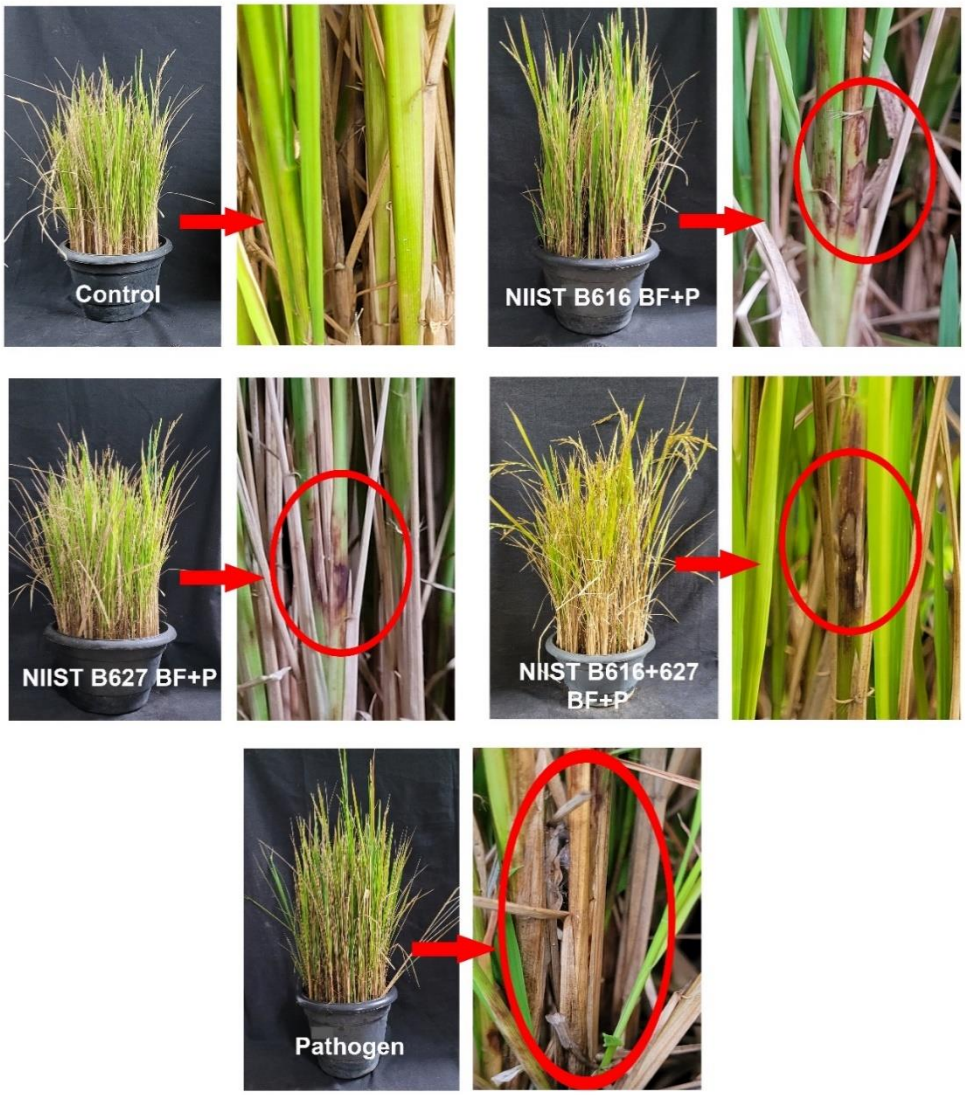


Fig 5.6 Effect of 10% diluted endophytic bioformulation on disease resistance in *R. solani* treated plants after 90 days**.** The arrow mark indicates the respective enlarged portion of the collar region of the plant. Control - no treatment, NIIST B616 BF+P – bioformulation of *Bacillus subtilis* NIIST B616 + *R solani*, NIIST B627 BF+P - bioformulation of *Bacillus subtilis* NIIST B627 + *R solani*, NIIST B616+627 BF+P - bioformulation of *Bacillus subtilis* NIIST B616+ *Bacillus subtilis* NIIST B627 + *R solani*, Pathogen -*R solani* alone

5.3.6. Impact of bioformulations on the growth of various vegetable crops

Previous experimentation has demonstrated that a consortium comprised of two bioformulations presents the ideal form. Consequently, we have chosen said consortium to evaluate the efficacy of stimulating plant growth across various types of crops. Throughout a period of 28 days, the length of the roots, the height of the shoots, as well as the weight, both fresh and dry, of three different crops, namely cowpea, bush beans and amaranthus, were measured. The findings indicated that cowpeas exhibited a significant increase of 68.94% in dry weight compared to the control plants. Similarly, bush beans displayed an increase of 66.75%, while amaranthus demonstrated a 66.63% increase compared to the control plants (Fig 5.7). In the context of yield, cowpeas demonstrated a 61.54% augmentation in the number of pods per plant, a 62.16% augmentation in the number of pods per pot, and a 116.67% augmentation in the mass of dried pods. In contrast, bush beans experienced a 66.67% augmentation in the number of pods per plant and per pot, as well as a 74.10% increase in the mass of the pods. Regarding amaranthus, there was a remarkable 76.84% increase in the mass of the plant (Fig 5.8).

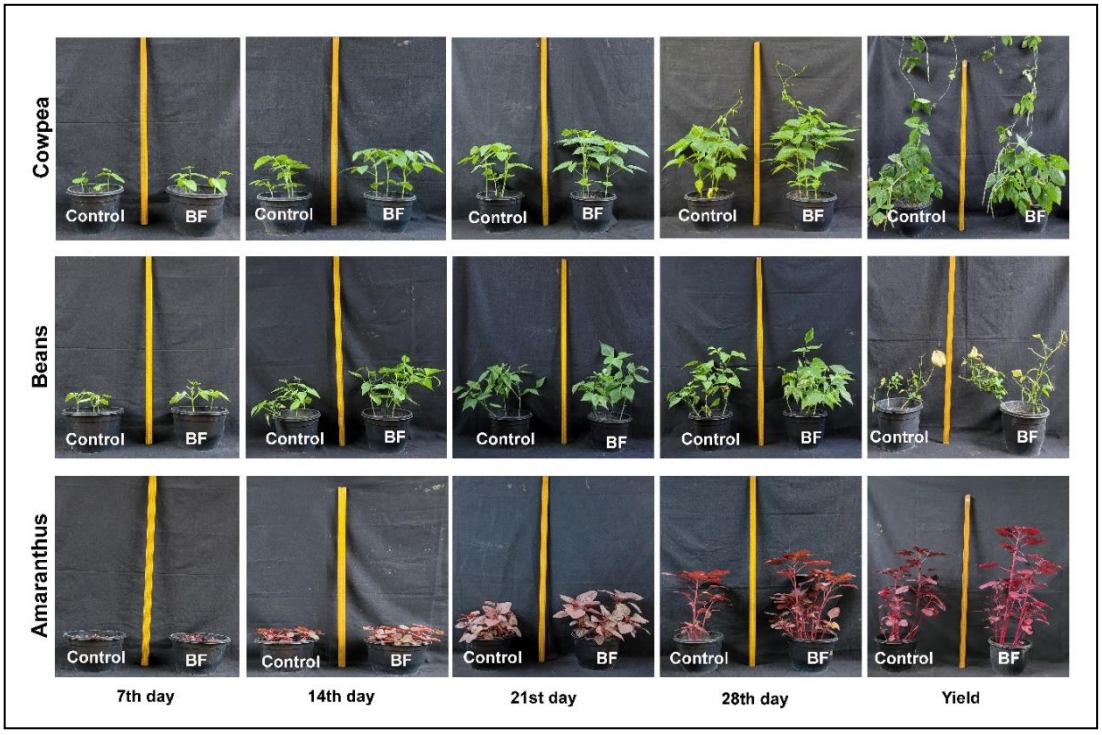


Fig 5.7 Growth promotion in three crops – cowpea, beans and amaranthus using the consortium of bioformulations on day 7, 14, 21and 28. BF -10% diluted bioformulation

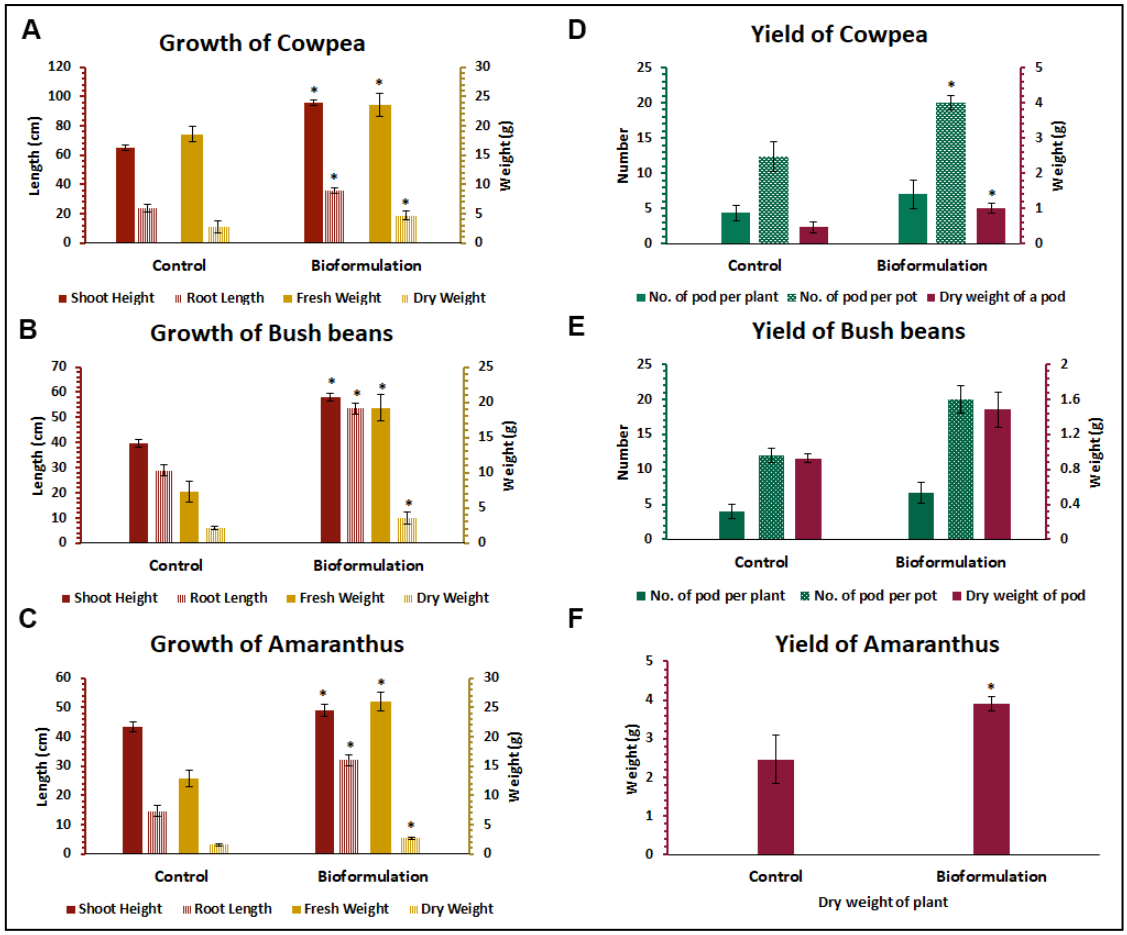


Fig 5.8 Growth promotion of three crops using 10% diluted bioformulation. A, B and C - growth parameters of cowpea, bush beans and amaranthus on day 28. D, E and F -yield parameters of the same crops. Values are expressed in mean ± SD from three replications. * in bar graph indicates the statistical significance of 5% (α)

5.4.Discussion

Microbial bioformulation technology is an ecologically conscious method that entails the preparation of biologically potent microorganisms embedded in a supportive carrier matrix (Chin & Ting, 2022). These bioformulations consist of distinct microorganisms that are environmentally safe and foster plant growth by supplying nutrients to the root system, enriching soil productivity, promoting plant health, increasing crop yield, and facilitating nutrient circulation (Rana et al., 2021). Utilizing these formulations or bioinoculants can potentially decrease the soil contamination that was once widespread due to the application of inorganic fertilisers. The employment of chemical fertiliser is motivated by various factors, such as the expanding population, the heightened need for food, and the substitution of traditional crops. One of the most pressing issues linked to the excessive use of chemical

fertilisers is the deterioration of the natural environment, which surpasses the advantages gained from the augmentation of crop yield. Consequently, this has led to a reduction in the quantity of topsoil and a decrease in the level of microbial activities (Sarad & Yadav, 2022). However, this trend can potentially be reversed through the use of bioformulations incorporating microbial consortia. The current investigation aimed to formulate such a bioformulation in the form of a liquid that contains a microbial consortium comprising two strains of *Bacillus,* namely *Bacillus subtilis* NIIST B616 and *Bacillus subtilis* NIIST B627.

The primary evaluation revolved around the compatibility of the two *Bacillus* strains, which were previously detailed in Chapter 3. Later, the two *Bacillus* strains were separately cultivated in tap water and enriched with various additives in different combinations. The selection of tap water was based on its convenience, ready availability, and compatibility with a wide range of bacterial strains, making it an ideal choice for developing liquid bioformulation. Among the different combinations of additives, a formulation (Formulation C) comprising of jaggery (20g/L), KH_2PO_4 (1g/L), $MgSO_4.7H_20$ (0.1g/L), and gum arabic (0.05%) was chosen for the development of the bioformulation, based on the bacterial growth observed up to 72 h. The bacterial strain NIIST B616 exhibited a growth of Log 8.77 CFU/mL, and NIIST B627 exhibited a growth of Log 8.68 CFU/mL at 72 h. This quantity of cells fell within the allowable limit for developing liquid bioformulations, as stated in the Fertilizer Order of 1985, amended on 1st July 2021 (Fertiliser Order, 2021). No contamination was detected at any dilution during the observation period of up to 72 h. In the formulation, jaggery served as the source of carbon and minerals such as iron and calcium, whereas KH_2PO_4 and $MgSO_4.7H_20$ provided the necessary macro and micronutrients for the growth of the bacterial strains (Arif et al., 2019). Among the various concentrations of jaggery tested, levels of 20g/L or higher were found to support higher levels of viable cells, as mentioned in the Fertilizer Order. However, our goal is to create a cost-effective bioformulation with minimal resource requirements to benefit farmers. Hence, Formulation C was chosen for bioformulation development. Liquid bioformulations containing specific additives enhance the overall quality of the final product (Macik et al., 2020). Microbes within the liquid bioformulation have a limited lifespan; hence, additional compounds like gum arabic were included to extend their shelf life. Gum arabic effectively stabilises the growth of bacterial cells within the medium during storage through colloidal stabilisation. This technique effectively prevents cell coagulation and protein precipitation (Elsakhawy et al., 2021). Gum arabic can also neutralise harmful components, improve adherence to seeds, and enhance the survivability of

microorganisms in various environments (Allouzi et al., 2022). Following 72 h harvest, equal quantities of the individual formulations were combined to form the consortium. The pH level of the consortium was measured to be 7.77, accompanied by a total count of Log 9.53 CFU/mL at the end of first month. This nearly neutral pH level promotes the thriving population of the inoculant and sustains its viability (Aloo et al., 2022). Consequently, our analysis focused on the examination of the nutrient contents of the liquid bioformulation. The consortium comprises 0.07% nitrogen, 0.078% phosphorous, 0.048% potassium, 0.812% total organic carbon, and an 11.6:1 C:N ratio. Furthermore, the electrical conductivity of the bioformulation is measured to be 1.0 dS/m. These findings are consistent with the results obtained from the liquid-based formulation of *Rhodopseudomonas palustris* PS3 (Lee et al., 2016). Likewise, it has been observed that adding gum arabic as a stabiliser in the formulation plays a critical role in extending the viability of the bacterial cells. The survival of bacterial cells within the formulation is of utmost importance as it directly impacts the quality of the final product. Therefore, to accurately assess the viability of the bioformulation, a comprehensive evaluation was carried out over seven months, with measurements taken at regular 30 day intervals. This approach facilitated a precise assessment of the bioformulation's viability and yielded significant insights into its effectiveness throughout the entire duration of the six month period (Aloo et al., 2022). The initial count of bacterial cells was recorded as Log 9.53 CFU/mL, and by the sixth month, the count had decreased to Log 8.3 CFU/mL. Subsequently, the cell density fell below the value of Log 8 in the seventh month, which is deemed to be the minimum requirement for liquid bioformulation, according to the fertilizer order. Hence, it was determined that the shelf life of the consortium of liquid bioformulations should be limited to a period of six months. Given that our bioformulation is composed of safe, low-cost materials and that the production of the inoculum is a relatively straightforward process, it is anticipated that the formulation technology will not only prove beneficial to farmers but also contribute to the advancement of the agricultural sector as a whole.

Within the two classifications of biofertilisers, specifically solid and liquid biofertilisers, the latter demonstrates more significant advantages due to its convenience, cost-effectiveness, and ease of application. The indispensability of the beneficial effects provided by liquid bioformulations is evident as they contribute to increased agricultural production while minimising environmental consequences (Dey, 2021). Previous research has examined the impact of liquid biofertilisers on various crops. Specifically, Maheswari and Elakkiya investigated the efficacy of liquid biofertilisers derived from *Azospirillum*, *Rhizobium*, and

Azotobacter on *Vigna mungo* L., revealing that the combined inoculation of all three liquid biofertilisers resulted in a better response compared to the use of individual biofertilisers (Maheswari & Elakkiya, 2014). Similarly, the effectiveness of our bioformulations was evaluated for promoting plant growth in rice using individual bioformulations (NIIST B616 BF and NIIST B627 BF) and a consortium of bioformulations (NIIST B616+627 BF), which demonstrated the effect of the consortium was superior to that of a single bioformulation. Various dilutions, such as 100% (undiluted), 50% (diluted with water), and 10% (diluted with water), were made for the application in rice plants. Among these dilutions, the 10% diluted bioformulation was found to be the most effective in terms of plant growth. However, the undiluted solution exhibited stunted growth, possibly due to its high concentration. Consequently, the 10% bioformulation was chosen for further studies on plant growth and disease management. Earlier investigations have confirmed the effectiveness of liquid bioformulation containing *Pseudomonas fluorescens* in combating sheath blight disease in paddy fields (DL et al., 2020), while the utilisation of a formulation consisting of *Bacillus subtilis* AF 1 supplemented with peat and chitin, or chitin-containing materials, on groundnut and pigeon pea seeds highlights the efficacy of biocontrol and promotion of plant growth (Manjula & Podile, 2001). Comparably, in our study, the consortium of bioformulations showed superior outcomes in terms of promoting plant growth and managing sheath blight disease in rice plants compared to the individual liquid bioformulations and control. The consortium of bioformulations increased the harvest index of rice plants by 18.52% compared to the control plants, whereas in plants challenged with the sheath blight pathogen *R. solani*, there was an increase of 32.61% in the harvest index compared to plants inoculated only with *R. solani*. These findings align with a previous study, which demonstrated that formulated inoculants yield better growth than unformulated inoculants (Lee et al., 2016). In spite of that, there are limited reports available on liquid bioformulation as a biocontrol against sheath blight disease in rice. Nevertheless, a commercial liquid formulation of *Bacillus subtilis* MBI 600 known as 'integral ®' suppresses the sheath blight disease (Kumar et al., 2012). Uniformly, our bioformulation suppressed sheath blight disease in rice plants by 64.15%. Thus, the consortium of bioformulations effectively enhances plant growth and provides resistance against sheath blight disease.

Considering the ability of a consortium of liquid bioformulations to promote plant growth in rice plants, a comprehensive analysis was conducted to assess the efficacy of this bioformulation consortium in enhancing plant growth on a diverse range of crops. Specifically,

three crops, namely cowpeas, bush beans, and amaranthus, were meticulously selected for this study. The results clearly showcased the bioformulation consortium's remarkable effectiveness in promoting plant growth across all the aforementioned crops. Hence, based on the unequivocal evidence gathered, it can be confidently concluded that the consortium of liquid bioformulations exhibits an exceedingly high level of efficiency in augmenting plant growth in a multitude of crops.

Based on the experimental outcomes, it becomes evident that the bioformulation exhibits a remarkable level of effectiveness in the crops subjected to testing, particularly in the case of rice, concerning the promotion of plant growth and the enhancement of resistance against sheath blight disease.

5.5.Conclusion

This investigation focused on developing a bioformulation composed of a bacterial consortium comprising two endophytic *Bacillus subtilis* strains, NIIST B616 and NIIST B627. The bacterial growth was facilitated through the use of a medium with specific ingredients such as jaggery, KH_2PO_4, $MgSO_4.7H_20$, and gum arabic. Regarding viability and stability, the formulated inoculation exhibited a duration of up to six months at room temperature. The physicochemical properties and viability count affirm its efficacy as a suitable biofertiliser. In addition, the diluted formulation, particularly at a concentration of 10%, demonstrated remarkable enhancement of plant growth across diverse crops and displayed effective biocontrol properties against sheath blight disease in rice plants. Therefore, this study lays a robust groundwork for future inquiries into the formulation's potential for biofertilisation in various crops under controlled and field conditions.

5.6.References

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Chapter 6

Summary, Conclusion and Future prospects

Rice is the most widely consumed cereal crop and a key source of calories for humans, making its productivity crucial. Environmental factors, both biotic and abiotic, significantly impact rice production, with diseases posing a particularly severe threat. In Kuttanad, Kerala's "rice bowl", the spread of diseases such as sheath blight, caused by the phytopathogen *Rhizoctonia solani*, leads to severe yield loss in rice cultivation. To counter this, beneficial microorganisms, particularly plant growth-promoting endophytes, can enhance soil fertility and rice production.

In this study, endophytes isolated from rice plants in various regions of Kuttanad (9° 25′ 30″ N, 76° 27′ 50″ E) were evaluated for their potential to promote rice plant growth. A total of 650 rice plant samples were collected from the Kuttanad region, and 225 endophytic bacteria were isolated from these samples. The distribution of endophytes was nearly equal within the samples' root, stem, and leaf. After preliminary screening, two endophytic bacteria, named NIIST B616 and NIIST B627, were selected for further studies based on their ability to antagonise the pathogen *R. solani* through *in vitro* conditions. In addition to that, the analysis of the mycelial hyphae at the point of inhibition revealed the occurrence of cellular extrusion, abnormal formation resembling a bulb, and shrinkage of the hyphae through scanning electron microscopy (SEM) analysis. Morphological and biochemical analyses of NIIST B616 and NIIST B627 revealed both to be Gram-positive, rod-shaped bacteria with catalase and hydrolytic activity towards cellulose and protein. Additionally, NIIST B627 demonstrated starch hydrolysis. Both strains were sensitive to antibiotics such as ampicillin, chloramphenicol, ciprofloxacin, cinoxacin, imipenem, kanamycin, nalidixic acid, and rifampicin. Molecular identification confirmed the two bacteria as *Bacillus subtilis*, with phylogenetic analysis distinguishing them as different strains.

The study further evaluated the plant growth-promoting abilities of *Bacillus subtilis* NIIST B616 and NIIST B627. Both strains were effective in producing ammonia, HCN, IAA, and VOCs. NIIST B616 exhibited phosphate solubilisation, while NIIST B627 exclusively produced siderophores. These findings highlight the potential of these isolates to promote plant growth. Compatibility testing confirmed that the two strains can be used together effectively. In plant growth experiments with rice plants, both *Bacillus*-treated plants showed significant improvements in growth parameters compared to control plants, with the best results observed when both strains were applied together. After 120 days, rice plants treated with both *Bacillus* strains showed a 7.02% increase in harvest index over control plants. Moreover, plants treated with both strains demonstrated greater resilience against *R. solani* compared to those treated

with either strain alone. Rice plants typically show disease symptoms when exposed to *R. solani*, but treatment with both endophytic *Bacillus* strains reduced disease incidence to 30%, compared to 85% in plants exposed only to *R. solani*. This represents a 64.71% reduction in disease incidence. Additionally, the presence of both *Bacillus* strains significantly elevated defence enzymes, including PAL (2.8 fold increase), POX (7.4 fold), PPO (4.5 fold), and total phenol content (8.5 fold), indicating induced systemic resistance (ISR). A split-root experiment further confirmed ISR, as no disease symptoms appeared in *Bacillus*-treated plants, despite no direct contact between the pathogen and endophytic *Bacillus*. This indicates that the presence of endophytic *Bacillus* induced resistance to *R. solani*, as evidenced by the absence of disease symptoms in treated plants. Furthermore, SEM analysis confirmed the presence of both bacterial strains in plant roots, and rhizospheric microbial community analysis revealed minimal changes between treated and untreated soils. These results strongly suggest that combining *Bacillus subtilis* strains NIIST B616 and NIIST B627 promotes plant growth and enhances resistance to *R. solani* through induced systemic resistance mechanisms.

Since both the isolated *Bacilli* demonstrated plant growth and disease resistance against *R. solani,* the effectiveness of the crude organic metabolites (COMs) from these isolates on rice plant growth and disease resistance was also conducted. Ethyl acetate was found to be the most effective solvent for extracting COMs. These extracts exhibited *in vitro* antagonism against *R. solani* and enhanced plant growth and yield while reducing disease severity in rice plants. Combining metabolites significantly increased the harvest index by 26.09% and reduced disease incidence to 33.33%. Increased levels of PAL, POX, PPO, and total phenol content indicated enhanced defence enzyme activity, suggesting induced systemic resistance (ISR). The ISR was confirmed by a split-root experiment. This suggests that ISR can be considered a natural way to prevent plant diseases. The COMs were then analysed through GC-MS/MS and LC-MS/MS, which identified two cyclic dipeptides: cyclo(Pro-Leu) and cyclo(Pro-Phe) in both strains as major constituents, which could be responsible for antifungal activity and ISR induction.

In light of the above results, a liquid bioformulation was developed using two endophytic isolates (NIIST B616 and NIIST B627) along with other supplementary ingredients such as jaggery, KH_2PO_4, $MgSO_4$, and gum arabic. After testing various combinations, an optimal formulation with jaggery (20g/L), KH_2PO_4 (1g/L), $MgSO_4$ (0.1g/L), and gum arabic (0.05%) was selected. The bioformulation remained viable for up to six months and the consortium (NIIST B616 + B627 BF) was found more effective in promoting plant growth compared to

individual isolates (NIIST B616 BF and NIIST B627 BF) in the consortium. Among different dilutions tested, the 10% bioformulation showed the best results for rice plant growth, while the undiluted (100%) formulation caused stunted growth, likely due to its high concentration. Thus, the 10% bioformulation was selected for further rice plant growth and disease management studies. The bioformulation consortium increased the harvest index of rice plants by 18.52% compared to control plants, and in rice plants challenged with the sheath blight pathogen *R. solani*, the harvest index increased by 32.61% compared to plants inoculated with *R. solani* alone. Additionally, the consortium suppressed sheath blight disease by 64.15%. Beyond rice plants, the bioformulation consortium was highly effective in promoting plant growth in other crops, including cowpeas, beans, and amaranthus. These findings establish a strong foundation for future studies on the potential of bioformulations for biofertilization across various crops, under both controlled and field conditions.

In conclusion, this study demonstrates the potential endophytic *Bacilli* capable of combating sheath blight disease in rice plants. Our research identified two promising endophytic *Bacillus subtilis*, NIIST B616 and NIIST B627, along with their metabolites, for their beneficial properties in promoting plant growth and enhancing disease resistance in rice plants. Based on these significant findings, bioformulations were developed using these *Bacilli* and tested for their efficacy in promoting plant growth and disease resistance in rice plants. Furthermore, the most effective consortium of formulations was evaluated in other crops, revealing promising results. These findings underscore the effectiveness of endophytic bioformulations in promoting plant growth and conferring disease resistance in rice plants. Consequently, they present a sustainable alternative to using chemical fertilizers and pesticides.

The future research can focus on optimizing the endophytic-bioformulation and its field application to maximize their efficacy in diverse agricultural settings. Additionally, investigating the long-term effects of these bio formulations on soil microflora, soil health and ecosystem resilience will be crucial for sustainable agricultural practices. Integrating endophytic bioformulations into integrated pest management (IPM) programs and assessing their compatibility with other sustainable agricultural practices will be pivotal for widespread adoption. Ultimately, continued exploration and innovation in this field hold the potential to revolutionize agricultural practices by offering environment friendly solutions to enhance crop productivity and mitigate disease pressures.

AcSIR Course work

Code	Course work	Credits	Status
BIO-NIIST-1-0001	Biostatistics	1	Completed
BIO-NIIST-1-0002	Computation/bioinformatics	1	Completed
BIO-NIIST-1-0004	Research Methodology/Communication/ethics	1	Completed
BIO-NIIST-1-0003	Basic Chemistry	1	Completed
BIO-NIIST-2-4101	Biotechniques and Instrumentation	1	Completed
BIO-NIIST-2-4103	Basics and Applied Microbiology	2	Completed
BIO-NIIST-2-4102	Protein Science and Proteomics	2	Completed
BIO-NIIST-3-4105	Enzymology & Enzyme Technology	2	Completed
BIO-NIIST-3-4101	Seminar course	1	Completed
BIO-NIIST-3-4103	Microbial Diversity and Ecology	2	Completed
BIO-NIIST-4-0001	Project Proposal	2	Completed
BIO-NIIST-4-0002	Review Writing	2	Completed
BIO-NIIST-4-0003	CSIR 800	4	Completed

Abstract of the thesis

Name of the student: **Gopika V. Krishnan** Registration No. **10BB17J39007**

Faculty of study: **Biological Science** Year of submission: **2024**

AcSIR academic centre/CSIR Lab: **CSIR-NIIST, Thiruvananthapuram, Kerala** Name of the supervisor(s): **Dr. Krishnakumar B.** (Supervisor) **Dr. Dileep Kumar B.S.** (Co-supervisor)

Title of the thesis**: Endophytic Bacteria and their Bioactive Metabolite for Enhanced Crop Productivity and Induction of Systemic Resistance in Rice Plant against Sheath Blight Disease**

The research study covered in this thesis focuses on the role of bacterial endophytes and their bioactive metabolite in promoting growth and inducing systemic resistance (ISR) in rice plants against sheath blight disease. As part of the study, two endophytic bacteria named NIIST B616 and NIIST B627 were isolated from rice plants in Kuttanad region of Kerala and demonstrated their significant antagonistic activity against sheath blight pathogen *Rhizoctonia solani under in vitro condition.* Through 16S rRNA sequencing and phylogenetic analysis, these bacteria were identified as distinct *Bacillus subtilis* strains, and their promising plant growth-promoting (PGP) properties were further investigated. The strains were found to reduce sheath blight severity in rice plants when applied individually or in combination, both in the presence and absence of *R. solani*. Further studies, including enzyme assays and split-root experiments, indicated that these bacteria induced ISR in rice plants. Additionally, root colonization by the strains was observed, and analysis of the rhizospheric soil showed that introducing these bacteria did not disrupt the existing microbial community. To identify active compounds, crude organic metabolites were separately extracted from each strain and assessed for their PGP and disease-suppressing abilities. These metabolites demonstrated beneficial effects on both plant growth and disease resistance. Enzyme assays and split-root experiments further confirmed the ISR-inducing properties of the metabolites. Subsequent GC-MS/MS and LC-MS/MS analyses revealed two cyclic dipeptides with known antifungal properties and ISR-inducing potential in plants. Based on these findings, a bioformulation incorporating both bacterial strains was developed. Shelf-life studies demonstrated the stability of the formulation, and further testing reconfirmed its effectiveness in enhancing rice plant growth and controlling sheath blight disease. In summary, this study brings out new insights into the application of bacterial endophytes and their metabolites as an environment friendly approach for managing sheath blight disease and promoting agricultural sustainability.

List of Publications

List of publications emanating from the thesis work

1. **Krishnan, G. V**., Abraham, B., Lankalapalli, R. S., Bhaskaran Nair Saraswathy Amma, D. K., & Bhaskaran, K. (2024). Rice sheath blight disease control by native endophytic *Bacillus subtilis* from Kuttanad, a Globally Important Agricultural Heritage System. New Zealand Journal of Botany, 1-23. https://doi.org/10.1080/0028825X.2024.2394184
2. Gopika V. Krishnan, Dileep Kumar B.S., Krishnakumar B. Crude organic metabolites: A natural approach to enhance plant growth and induce systemic resistance in rice against sheath blight disease (under review)

List of publications not related to the thesis

1. Mohan, B., Salfeena, C. T. F., Ashitha, K. T., **Krishnan, G. V**., Jesmina, A. R. S., Varghese, A. M., ... & Sasidhar, B. S. (2018). Functionalized pyrimidines from alkynes and nitriles: application towards the synthesis of marine natural product meridianin analogs. ChemistrySelect, 3(23), 6394-6398. https://doi.org/10.1002/slct.201801126
2. Jacob, J., **Krishnan, G. V.**, Thankappan, D., & Amma, D. K. B. N. S. (2020). Endophytic bacterial strains induced systemic resistance in agriculturally important crop plants. In Microbial Endophytes (pp. 75-105). Woodhead Publishing. https://doi.org/10.1016/B978-0-12-819654-0.00004-1
3. Anjali, S., Sangeetha, M., Nithya, M., **Krishnan, G. V.,** Varughese, S., Kumar, B. D., & Somappa, S. B. (2023). Pyrazole appended hetero-hybrids: Bioisosteric design, synthesis, in silico and in vitro antibacterial and anti-inflammatory evaluations. Journal of Molecular Structure, 1289, 135780. https://doi.org/10.1016/j.molstruc.2023.135780

List of conferences and seminars

1. Advanced Technologies and Innovative Practices for Climate Smart Agriculture: Bridging Academia, Industry and Society held on January 18-19, 2024 at College of Agriculture, Vellayani (Poster presentation)
2. National Seminar on “Recent Trends in Disease Prevention and Health Management” held on December 14-15, 2022 at CSIR-NIIST, Thiruvananthapuram (Oral presentation)
3. 29th APSI Scientist Meet & International Conference on “Drug Discovery and Agribiotechnology and Pharmaceutical Sciences” held on November 23-25, 2019 at Ahmedabad, Gujarat (Oral presentation)
4. International Seminar on “Life Sciences for Sustainable Development- Issues and Challenges” held on October 3-5, 2019 at University College, Thiruvananthapuram (Oral presentation)

GenBank Submission

1. ON054037: *Bacillus subtilis* NIIST B616 partial sequence; 16S ribosomal RNA sequence
2. KU577428: *Bacillus subtilis* NIIST B627 partial sequence; 16S ribosomal RNA sequence

ABSTRACTS OF PAPERS PRESENTED

A novel bio-formulation with endophytic bacteria enhancing plant growth and sheath blight resistance in paddy

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Rice (*Oryza sativa* L.) cultivation, which plays a critical role in ensuring global food security, encounters various challenges, including diseases like sheath blight. This issue is particularly prevalent in regions such as Kuttanad, a Globally Important Agricultural Heritage System (GIAHS), also known as the "rice bowl of Kerala", where rice is cultivated below sea level. The current study involved the isolation of two potent strains of *Bacillus subtilis* from rice plants in Kuttanad, Kerala, India. These strains exhibited remarkable control over sheath blight, both *in vitro* and in planta, leading to enhanced rice yield. The control of sheath blight was achieved through the mechanism of induced systemic resistance (ISR) triggered by the endophytic strains. The endophytes were found to induce ISR by enhancing the activity of defense enzymes, including phenylalanine ammonia-lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO), and total phenol content. Additionally, a split-root experiment conducted on endophyte-treated plants further confirmed the ISR induced by the endophytes. Based on these findings, the selected strains were utilized to formulate a liquid bioformulation, which was evaluated for its efficacy through physicochemical analysis and viability count. The results confirmed the bioformulation's potential as an effective growth promoter with robust biocontrol properties against sheath blight disease. This research presents a promising approach to enhance rice productivity and combat sheath blight, thereby providing a sustainable solution for rice cultivation in vulnerable regions such as Kuttanad.

Keywords: Bioformulation, endophytes, *Bacillus*, Sheath blight, rice

Plant growth promotion and disease suppression of sheath blight disease in rice plants by endophytic bacteria

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Abstract

Plant growth promoting rhizobacteria (PGPR) are becoming more and more well-known for their advantages in agriculture. This is due to the growing need for sustainable agriculture within a comprehensive vision of development and to emphasise environmental protection, as well as the emerging demand for a reduction in reliance on synthetic chemical products. In the present study, two endophytic *Bacillus* strains isolated from the Kuttanad region, designated as NIIST B616 and NIIST B627 and their crude metabolites, respectively, exhibited significant *in vitro* antagonism against *Rhizoctonia solani*, the sheath blight pathogen of rice plants. It is one of the most economically significant rice diseases worldwide. These bacteria were tested for their plant growth promotion and disease suppression studies. Treatment of individuals and their combination recorded a percentage increase in plant growth parameters in the presence and absence of *R. solani*. The combination after 120 days also recorded maximum yield in both cases. The disease incidence recorded a maximum by the pathogen alone, followed by NIIST B616+P and NIIST B627+P, whereas the combination showed the least infection. These results indicate the development of resistance in rice plants against sheath blight disease when the two endophytes were added. Both organisms' crude metabolites also showed antagonistic activity in *in vitro* analysis. So, metabolic characterisation through GC-MS/MS and LC-MS/MS was carried out, and it confirmed two identical cyclic dipeptides, Cyclo(Pro-Leu) and Cyclo(Pro-Phe), from both strains. Therefore, endophytes could provide an effective strategy for managing this disease after successful field trials.

Keywords: Endophytic bacteria, sheath blight disease, rice, plant growth promotion, Bacillus, *Rhizoctonia solani*

Endophytes mediated plant growth promotion and induction of systemic resistance against sheath blight disease in rice

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Abstract: Plant growth promoting rhizobacteria (PGPR) have gained worldwide importance and acceptance for agricultural benefits. This is due to the emerging demand for dependence diminishing of synthetic chemical products, to the growing necessity of sustainable agriculture within a holistic vision of development and to focalize environmental protection. Environment-friendly agricultural practices such as use of bio-fertilizers and bio-pesticides has become an ideal approach for many countries in the world. Besides rhizobacteria, plant growth-promoting endophytic bacteria have the ability to inhabit the plant tissues with no apparent harmful effect to the host plant and known to provide growth benefits to their host. In the present study, 225 endophytic bacterial strains were isolated from different parts of rice plant and checked their ability to control the growth of the pathogen *Rhizoctonia solani* which cause sheath blight disease in paddy. From that, two strains were selected (NIIST B 616 and NIIST B 627) and tested for plant growth promotion as well as induction of disease resistance in paddy. As these strains were shown to produce indole-3-acetic acid, hydrogen cyanide, siderophores, phosphate solubilisation activity, and the experimental findings under laboratory conditions confirmed that the endophytes treated rice plants recorded a higher increase in case of plant growth and yield as compared to control plants. As a part of induction of systemic resistance in plant, enzyme level studies were conducted and it was found that the increased activity of defense-related enzymes like L-phenylalanine ammonia lyase, peroxidase, and polyphenol oxidase were observed in rice plants treated with endophytic strains in presence of pathogen. This clearly indicate the development of resistance in plant. Therefore, the use of endophytes could provide an effective strategy in the management of this disease after successful trials in the fields.

Endophyte-Mediated Plant Growth Promotion and Systemic Resistance Induction Against Sheath Blight Disease in Rice

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Abstract: Plant Growth-Promoting Rhizobacteria (PGPR) have gained global recognition and acceptance for their agricultural benefits. This is mainly due to the increasing need to reduce dependence on synthetic chemical products, promote sustainable agriculture, and prioritize environmental protection. Eco-friendly agricultural practices, such as the use of bio-fertilizers and bio-pesticides, have emerged as ideal alternatives in many countries. In addition to rhizobacteria, plant growth-promoting endophytic bacteria can colonize plant tissues without causing any apparent harm to the host while providing significant growth benefits. In the present study, 225 endophytic bacterial strains were isolated from different parts of rice plant and checked their ability to control the growth of the pathogen *Rhizoctonia solani* which cause sheath blight disease in paddy. From that, two strains were selected (NIIST B 616 and NIIST B 627) and tested for plant growth promotion as well as induction of disease resistance in paddy. As these strains were shown to produce indole-3-acetic acid, hydrogen cyanide, siderophores, phosphate solubilisation activity, and the experimental findings under laboratory conditions confirmed that the endophytes treated rice plants recorded a higher increase in case of plant growth and yield as compared to control plants. As a part of induction of systemic resistance in plant, enzyme level studies were conducted and it was found that the increased activity of defense-related enzymes like L-phenylalanine ammonia lyase, peroxidase, and polyphenol oxidase were observed in rice plants treated with endophytic strains in presence of pathogen. This clearly indicate the development of resistance in plant. Therefore, the use of endophytes could provide an effective strategy in the management of this disease after successful trials in the fields.

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Rice sheath blight disease control by native endophytic *Bacillus subtilis* from Kuttanad, a Globally Important Agricultural Heritage System

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RESEARCH ARTICLE

Rice sheath blight disease control by native endophytic *Bacillus subtilis* from Kuttanad, a Globally Important Agricultural Heritage System

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ABSTRACT

Kuttanad in India is known as 'the rice bowl of Kerala' and renowned for its unique rice cultivation system that is below sea level. However, rice production in this wetland ecosystem is seriously threatened by sheath blight disease caused by the fungus *Rhizoctonia solani*, which has resulted in a significant decline in grain quality and a 50% loss in yield. In the present study, we isolated two endophytic *Bacillus subtilis* strains, NIIST B616 and NIIST B627, from the roots of rice plants growing in the Kuttanad region and tested their efficacy in controlling sheath blight disease. We found that rice plants inoculated with a combination of both *Bacillus* strains and *R. solani* had a 64.71% lower disease incidence and a 31.02% higher harvest index than plants treated with *R. solani* alone. An analysis of defence-related enzymes and chemicals revealed that treatment with both isolates together greatly enhanced levels of L-phenylalanine ammonia-lyase (PAL; 2.8 fold), peroxidase (POX; 7.4 fold), polyphenol oxidase (PPO; 4.5 fold), and total phenol (8.5 fold) compared with plants treated solely with *R. solani*, indicating that these isolates significantly increased plant defence responses through induced systemic resistance (ISR), and this finding was validated through a split-root experiment. More detailed analysis using gas chromatography–tandem mass spectrometry (GC-MS/MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) techniques revealed that cyclo-(Pro-Leu) and cyclo-(Pro-Phe) were the major components responsible for the antifungal properties of the two isolates and their capacity to trigger ISR in plants. Thus, the *Bacillus* strains and their metabolites may be deemed viable alternatives for controlling the prevalence of sheath blight disease in rice plants and augmenting rice production at unique locations such as Kuttanad.

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Introduction

Rice (*Oryza sativa* L.) is the third most highly produced cereal crop in the world, with almost half of the global population having adopted it as a fundamental staple food (Raman 2021). Asia accounts for 90% of total rice production, and India is the second largest rice producer in the world, cultivating the crop in nearly half of its states, including Kerala (Surendran et al. 2021a).

Kuttanad, which is known as 'the rice bowl of Kerala', is a remarkably fertile area where rice is cultivated below sea level at depths ranging from approximately 1.2 to 3.0 metres. The Kuttanad Below Sea-Level Farming System (KBSFS) has emerged as a distinctive practice that is characterised by expansive flat paddy fields covering an area of 50,000 hectares, which predominantly comprise reclaimed delta swamps (FAO 2012), and the farmers in this region have gained significant renown for their biosaline farming techniques. In recognition of its global significance, the Food and Agriculture Organisation (FAO) has designated the KBSFS as a Globally Important Agricultural Heritage System (GIAHS) (Nair 2013). However, despite its status, the rice granary of the Kuttanad wetland ecosystem faces significant threats from various diseases.

The prominent disease in this region is sheath blight, which can lead to yield losses of up to 50%, making it one of the main yield-limiting diseases in rice plants (Surendran et al. 2021b). The impacts of disease in the Kuttanad region were further exacerbated by the occurrence of sudden and intense floods in 2018, 2019, and 2020, which resulted in the significant destruction of cultivated land and affected environmental microorganisms, as well as the spread of pathogenic microbes (Divakaran et al. 2019; Surendran et al. 2021b). Consequently, disease control is essential to the sustainability of rice production in this region.

Various chemical methods have traditionally been used to improve crop growth, increase yield, and manage diseases, but these continuously deteriorate the ecological niche. To address this, the use of natural alternatives has been investigated, which holds great promise. Biocontrol agents are widely recognised for their antifungal properties and ability to induce defence mechanisms in plants, with the added benefit of being an environmentally friendly and cost-effective sustainable approach to disease management.

Several bacteria belonging to different genera (e.g. *Bacillus*, *Burkholderia*, *Pseudomonas*, *Streptomyces*) have been identified as prominent endophytes that are pivotal to improving crop productivity and controlling many plant diseases (Jasrotia et al. 2021; Ngalimat et al. 2021) via induced systemic resistance (ISR) in the host plant (Jamali et al. 2020; Sahu et al. 2020). Under conditions of abiotic stress, such as drought or salinity, and biotic stress, such as microbial infection, endophytes develop various mechanisms to counteract the stressors and enhance plant growth. Furthermore, they induce the host plants to release defence-related enzymes and chemicals, while mobilising nutrients to support their survival under unfavourable conditions (Zhang et al. 2019).

Endophytes and rhizosphere *Bacillus* have proven to be efficacious in augmenting plant growth, yield, and biotic stresses in diverse crops, such as rice, chickpea, and soybean, through a range of mechanisms (Park et al. 2017; Kumar et al. 2020; Baliyan et al. 2022), including phosphate solubilisation and the production of siderophores, antimicrobial compounds, and various phytohormones (Narayanan and Glick 2022). The

application of free-living and endophytic *Bacillus* on host plants has been observed to yield elevated levels of defence-related enzymes and other compounds, leading to ISR against many noteworthy agricultural diseases (Dutta et al. 2008; Lanna-filho et al. 2017).

The exploitation of endophytic bacteria as a biocontrol method has been shown to be more effective than the use of other free-living bacteria. Safara et al. (2022) found that endophytic bacteria help plants to fight biotrophic and necrotrophic phytopathogens by inducing their defence mechanisms. Similarly, Shabanamol et al. (2017) demonstrated that endophytic diazotrophic *Lysinibacillus sphaericus* can be used as a biocontrol agent for sheath blight disease in rice. However, the efficacy of using endophytic bacteria as a biocontrol approach for rice sheath blight disease has not been reported for special ecosystems such as Kuttanad.

The present study examined the potential of two newly isolated endophytic *Bacillus* strains and tested their potential to manage rice sheath blight disease by inducing ISR and enhancing crop production. This is the first report on ISR-based biocontrol of sheath blight disease using native endophytic *Bacillus* isolated from this unique paddy cultivation site.

Materials and methods

Isolation of the endophytic bacteria

Endophytic bacteria were isolated from the roots of rice plants growing in Kuttanad (9° 25′30″N, 76°27′50″E), Kerala, India (Figure 1). Root samples were dipped in 70% ethanol for 3 min, followed by 2.5% sodium hypochlorite solution (HiMedia, India) for 5 min. Then, the samples were thoroughly rinsed with sterile distilled water five times. The final rinse was meticulously examined for the presence of any bacteria via culturing on Luria–Bertani (LB) agar (HiMedia, India). Subsequently, the samples were macerated with a sterile mortar and pestle, plated onto LB agar plates (Elbeltagy et al. 2000), and incubated for 2–5 days at 28 ± 2°C. The individual colonies were purified and stored at 4°C.

Source of Rhizoctonia solani

The fungal pathogen *Rhizoctonia solani* (ITCC 6882) used in this study was procured from the Indian Type Culture Collection, Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi, India. It was maintained on potato dextrose agar (PDA; HiMedia, India) slants at 4°C.

Screening of antagonistic endophytes

The *Bacillus* isolates obtained were checked for *in vitro* antagonism against *R. solani* using the dual culture technique (Kumar et al. 2001) with a slight modification. A loopful of bacterial inoculum of the respective test strain (18 h old) was streaked on one side of a PDA plate approximately 2 cm from the outer edge, and an actively growing circular mycelial disc (6 mm diameter) of *R. solani* was placed opposite the bacterial streak at a distance of approximately 5 cm. PDA plates with no bacteria served as a

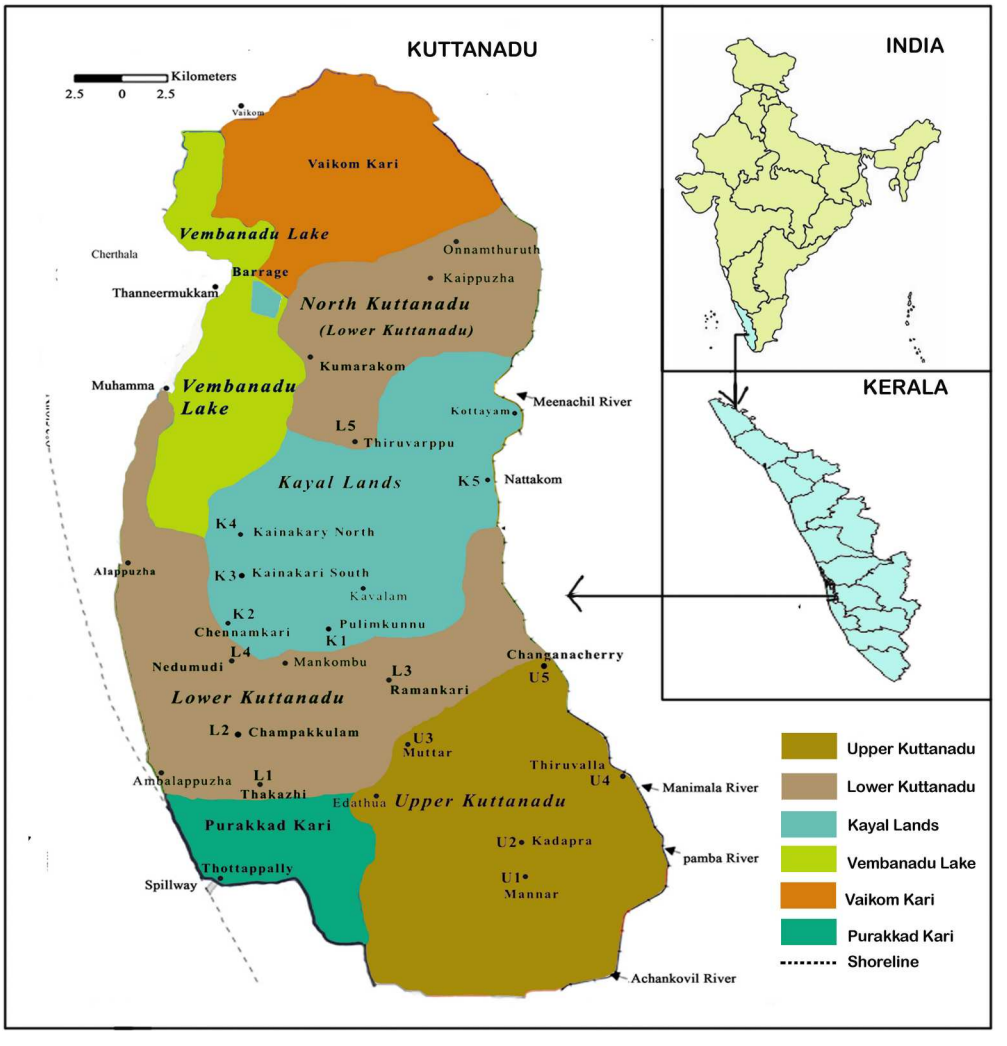

Figure 1. Map of the Kuttanad region, Kerala, India (modified from Vijayan and Ray 2015).

control. The plates were incubated in a biochemical oxygen demand (BOD) incubator (Rotek, India) at 28 ± 2°C for 7 days.

The inhibition zone was recorded as the distance (mm) between the mycelial and bacterial growth after seven days, with measurements taken from three replicates per test strain. The fungal hyphae were also analysed using scanning electron microscopy (SEM) to assess any damage resulting from bacterial inhibition. Fungal mycelia were carefully extracted from the point of inhibition and positioned on double-sided tape on the stub. The stub was then coated with gold and viewed under a scanning electron microscope (Carl Zeiss EVO 18 Research, Germany) at an accelerating voltage of 20 kV and a probe diameter of 102 pA to record secondary electron images (Fischer et al. 2012). The field was scanned and suitable areas in the preparation were photographed.

Identification and phylogenetic analysis of the endophytes

The isolates were identified through 16S rRNA sequencing using the universal primers 16S-RS-F 5′-CAGGCCTAACACATGCAAGTC-3′ and 16S-RS-R 5′-GGGCGGWGTGTACAAGGC-3′. Genomic DNA was isolated using the NucleoSpin® Tissue kit (Macherey-Nagel, Germany) following the manufacturer's instructions, and the quality of the DNA was checked using agarose gel electrophoresis. The isolated DNA was amplified in a polymerase chain reaction (PCR) thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems, USA). The purified amplicon was sequenced in an ABI 3500 Genetic Analyzer (Applied Biosystems, USA) using the Sanger DNA sequencing method. The sequence quality was checked with Sequence Scanner Software v1 (Applied Biosystems, USA), and sequence alignment and any required editing were carried out using Geneious Pro v5.1 (Drummond 2010; Kearse et al. 2012).

The DNA sequences from the 16S rRNA gene were compared with sequences held in the National Center for Biotechnology Information (NCBI) and EzBioCloud 16S databases. Sequences obtained from the NCBI Basic Local Alignment Search Tool (BLAST) results were used to construct a phylogenetic tree in Molecular Evolutionary Genetics Analysis (MEGA) 7 software using the maximum likelihood method (Hall 2013; Newman et al. 2016), and the sequences were then submitted to NCBI.

Effects of the endophytic bacteria on plant growth in the presence of R. solani

This experiment used 'Jyothi' rice seeds, which are highly susceptible to sheath blight disease. The seeds were procured from the Regional Agricultural Research Station in Pattambi, Kerala, India, and the experiment was conducted in plastic pots measuring 18 × 22 cm that were filled with sandy loam soil (pH 6.5) mixed with cow dung (3:1). A total of 250 pots were utilised in the experiment, with 50 pots per treatment. The treatments included no bacteria + no *R. solani* (control), *R. solani* alone (pathogen alone), NIIST B616 + *R. solani* (NIIST B616+P), NIIST B627 + *R. solani* (NIIST B627+P), and NIIST B616 + NIIST B627 + *R. solani* (NIIST B616+627+P).

The endophytic bacteria were cultured individually in LB broth that had been inoculated with a 24 h old inoculum of the respective strain (1% v/v). Each culture was then incubated in a shaking incubator at 130 rpm and 28 ± 2°C for 72 h. A suspension of NIIST B616 or NIIST B627 (50 mL, 1×10^8 CFU/mL each) or both strains together (50 mL, $1{\times}10^8$ CFU/mL, produced by mixing an equal volume of culture [$1{\times}10^4$ CFU/mL] for each strain) was added to the respective pots. The top layer was mixed to an approximate depth of 2 cm using a sterile glass rod, and 20 surface-sterilised seeds were planted at a depth of 1 cm in each pot and grown under nursery conditions (Kumar and Dube 1992). The control group consisted of pots containing only surface-sterilised seeds.

The pathogen *R. solani* was cultured in potato dextrose broth (PDB) by inoculating the PDB with a 96 h old fungal mycelial plug from an actively growing PDA plate. After 7 days of incubation at 130 rpm and 28 ± 2°C, the fungal mycelium was harvested and homogenised to create a uniform suspension. All pots except those in the control group were then infested with 50 mL of the homogenised *R. solani* suspension (10^8

CFU/mL). The experiment was conducted in a completely randomised design (CRD), and data were collected from 10 replicates per treatment.

Plant growth parameters including shoot length, root length, dry weight, and total chlorophyll content (Hiscox and Israelstam 1979) were recorded at 7-day intervals for up to 28 days. After 30 days, a booster dose of foliar spray (50 mL) containing the above-mentioned isolates was applied to the appropriate pots. Yield was determined after 120 days of growth by measuring the number of grains, the weight of 100 grains, the number of tillers per plant, the number of panicles per plant, and the amount of shoot dry matter. The harvest index was then calculated using the following formula:

$$\text{Harvest index} = \frac{\text{Grain yield}}{\text{Total Shoot dry matter}} \times 100$$

Effects of the endophytic bacteria on disease control

The occurrence of sheath blight disease and the lesion height were estimated on day 90 of the experiment using the Rice Standard Evaluation System scale developed by the International Rice Research Institute in the Philippines (IRRI 2013). The following formulae were used to determine the disease incidence and relative lesion height:

$$\text{Disease incidence (\%)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants}} \times 100$$

$$\text{Relative lesion height (\%)} = \frac{\text{Lesion length}}{\text{Plant height}} \times 100$$

The relative lesion heights were assessed using a scale that ranged from 0 (no infection) to 9, where a score of 1 = < 20%, 3 = 20–30%, 5 = 31–45%, 7 = 46–65%, and 9 = > 65% of the plant height. The values were obtained from three replicates per treatment.

Effects of the endophytic bacteria on the induction of systemic resistance against R. solani

ISR was analysed by examining changes in the levels of defence enzymes and the total phenol content in rice plant leaves for each of the five treatment groups described above. Three defence enzymes were investigated: L-phenylalanine ammonia-lyase (PAL; EC 4.3.1.24), peroxidase (POX; EC 1.11.1.7), and polyphenol oxidase (PPO; EC 1.10.3.1). Fresh leaf samples weighing 3 g (PAL, POX, and PPO) and 0.5 g (total phenol) were collected at 10-day intervals up to day 80 and used in the assay. Measurements were taken from three replicate leaf samples per assay

To assess the levels of PAL, the leaf samples were homogenised with 9 mL sodium borate buffer and 2-mercaptoethanol (0.8 mL/L) buffer in a pre-cooled mortar and pestle. The extract was then centrifuged at 12,000 *g* for 20 min at 5°C, and the supernatant was used as an enzyme sample for the assay. PAL activity was estimated following Sadasivam and Manickam (1991). The absorbance was measured in an ultraviolet–visible (UV-Vis) spectrophotometer (Shimadzu, Japan) at 290 nm, and the reaction rate was recorded as the micromoles of trans-cinnamic acid produced (units/g fresh weight).

POX activity was estimated according to Thimmaiah (1999). Leaf samples were homogenised in 9 mL of 0.1 M phosphate buffer (pH 7.0) in a mortar and pestle and then centrifuged at 18,000 *g* for 15 min at 5°C. The supernatant was used as an enzyme sample for the assay. Absorbance was recorded at 430 nm, and the total activity of POX was calculated as units/g fresh weight of the sample, whereby 1 unit of the enzyme was considered to be an increase in optical density (OD) of 1.0 under standard conditions.

For the PPO analysis, the leaf samples were homogenised in 6 mL of 0.1 M sodium phosphate buffer (pH 7.1) and the extract was centrifuged at 1,500 *g* at 5°C for 40 min. The supernatant was used as an enzyme sample. PPO activity was determined following Sadasivam and Manickam (1991) and was recorded as the change in absorbance per millilitre of enzyme extract per minute (units/mg fresh weight).

For the total phenol assay, the leaf samples were homogenised in 10 mL of 80% ethanol and centrifuged at 12,000 *g* for 20 min. The total phenol content was then estimated according to Mahadevan and Shridhar (1986). Total phenol was calculated using a standard graph prepared using different concentrations of catechol and expressed as mg/g of material (tissue weight).

Split-root experiment

A split-root experiment was carried out to validate the elicitation of ISR against *R. solani* in rice plants. The procedure followed Dutta et al. (2008) with a slight modification to use a three-cup system (two lower cups and one upper cup). The experiment included eight treatments, whereby the two bottom cups each contained no bacteria / no pathogen (control), *R. solani* / *R. solani* (P/P), NIIST B616 / NIIST B616 (616/616), NIIST B627 / NIIST B627 (627/627), NIIST B616 + NIIST B627 / NIIST B616 + NIIST B627 (616 +627/616+627), NIIST B616 / *R. solani* (616/P), NIIST B627 / *R. solani* (627/P), or NIIST B616 + NIIST B627 / *R. solani* (616+627/P).

Rice plant seedlings that had been grown in sterile soil were carefully uprooted at 7 days old and washed three times in sterile distilled water without disrupting their root systems. Plants with similar root lengths were then selected for use in the experiment. Each plant was inserted through an upper cup so that half of their roots went into each of the cups below. It was ensured that the two lower cups were not in direct contact with each other. The development of sheath blight disease caused by *R. solani* was then recorded at 7-day intervals up to day 28.

Extraction of crude organic metabolites from the endophytic bacteria and analysis of in vitro *antagonism*

Each of the isolated strains (18 h old, 1 mL) was inoculated separately into a 250 mL flask containing 100 mL of LB broth (HiMedia, India). Each flask was incubated in a shaker at 28°C and 130 rpm for 72 h, and the broth was then centrifuged at 10,000 *g* (Kubota Corporation, Japan) for 20 min. The resultant cell-free culture filtrate was extracted with hexane, chloroform, ethyl acetate, and ethyl acetate / methanol (95:5) and concentrated in a rotary evaporator (Heidolph, Germany) to collect the crude organic extract.

In vitro antagonism of the crude organic extracts was investigated using the agar well diffusion method (Sriram et al. 2019). For each strain, 20 µL of crude extract (20 mg/

mL) was introduced into a well (6 mm diameter) on a PDA plate, and a 6 mm circular disc of actively growing mycelia was placed opposite the extract at a distance of approximately 5 cm. PDA plates without crude metabolites served as a control. The plates were incubated at 28 ± 2°C in a BOD incubator for 7 days, and the inhibition zone (mm) was measured. Changes in the fungal hyphae at the point of inhibition were also examined using SEM as elaborated earlier. Readings were taken from three replicate plates per treatment.

Identification of the crude organic metabolites

The crude organic metabolites were identified using gas chromatography–tandem mass spectrometry (GC-MS/MS) analysis using a Thermo Scientific Trace 1310 gas chromatograph (U.S.) equipped with a TSQ 8000 mass-selective detector and a TG-5MS capillary column (30 m × 0.25 mm × 0.25 µm). A 0.5 µL sample was injected with an AIAS 1310 automated injector, and the column temperature was maintained at 40°C for the initial 5 min. The run was initiated with an increasing temperature of 10°C/min until it reached 200°C, after which the temperature was held isothermally for 5 min. The scan range was 45–600 *m/z* with electronic ionisation (EI) in split-less mode, and the data obtained were compared with the National Institute of Standards and Technology (NIST) library data.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of the bacterial crude organic extract was performed using an LC-MS/MS system consisting of a Nexera LC-30AD solvent delivery unit equipped with a CTO-20AC temperature-controlled column oven, an SIL-30AC autosampler, and an SPD-M20A prominence diode array detector, coupled to an LCMS-8045 triple quadrupole mass spectrometer (Shimadzu, Japan). Samples were prepared in 1 mg metabolite/mL methanol, filtered through a 0.2 µm nylon membrane, and diluted to 50 µg/mL. Then, 10 µL of each sample was injected with a mobile phase of 0.1% formic acid in water (solvent A) and 100% methanol (solvent B) using the following solvent gradient: 0.01–2.00 min, 10% B; 2.00–5.00 min, 10% B; 5.00–8.00 min, 50% B; 8.00–11.00 min, 50% B; 11.00–16.00 min, 90% B; 16.00–17.00 min, 90% B; and 17.00–22.00 min, 10% B. Analysis was carried out in a Shim-pack GISS C18 column (1.9 µm, 2.1 × 150 mm) with a flow rate of 0.2 mL/min at 40°C, and the data were collected and processed by Lab Solutions software (Shimadzu, Japan).

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) using SPSS (version 20.0; IBM SPSS). Statistical significance was evaluated using duncan's multiple range test (DMRT), with $p < 0.05$ considered a significant difference. Graphical representations were made using Origin Pro 8.5 software.

Results

Screening of antagonistic endophytes

Two endophytic strains, NIIST B616 and NIIST B627, were selected from the isolates (total 225 isolates) based on their *in vitro* antagonism. Both strains exhibited strong

inhibitory activity against *R. solani* (Figure 2A), with inhibition distances of 18 ± 1.00 mm for NIIST B616 and 12 ± 1.00 mm for NIIST B627. The SEM analysis of mycelial hyphae treated with each of these bacteria separately revealed cellular extrusion, abnormal bulbous-like formation, and shrinkage of the hyphae (Figure 2B).

Identification and phylogenetic analysis of the endophytes

The endophytic isolates NIIST B616 and NIIST B627 were both identified as *Bacillus subtilis* strains based on analysis of their sequences through NCBI BLAST and the EzBioCloud database. The taxonomic affiliation of the two strains was established through the construction of a phylogenetic tree (Figure 3), which revealed that they were different strains. The 16S rRNA sequences of NIIST B616 and NIIST B627 were deposited in NCBI GenBank under Accession Numbers ON054037 and KU577428, respectively. Both bacteria were compatible on mixed inoculation in an LB agar medium (Figure S1).

Effects of the endophytic bacteria on plant growth in the presence of R. solani

Rice plants treated with isolates NIIST B616 and NIIST 627 together (NIIST B616+627 +P) exhibited the highest percentage increase in all growth parameters, despite being challenged with *R. solani*. On day 28, plants in this treatment group exhibited a significant improvement in shoot length (38.90%), root length (42.63%), dry weight (260.00%), and total chlorophyll content (133.92%) compared with plants that were treated with

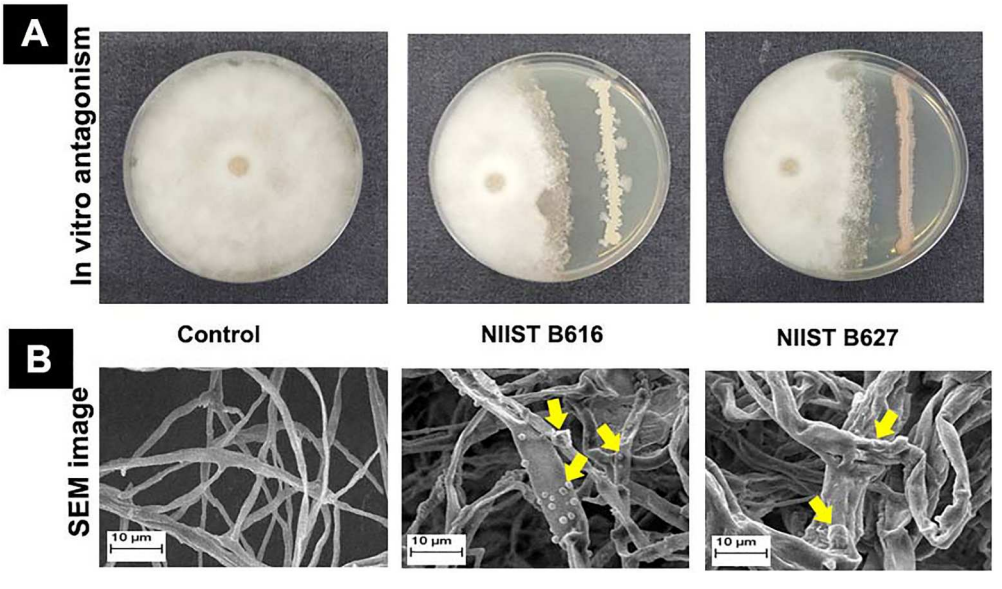

Figure 2. Screening of the antagonistic endophytes. **A,** *In vitro* antagonism of the *Bacillus subtilis* isolates NIIST B616 and NIIST B627 against *Rhizoctonia solani*, where *R. solani* was inoculated on the left (mycelial plug) and the isolates were inoculated as a vertical streak on the right. **B,** SEM images of the fungal hyphae from the point of inhibition. Arrows indicate morphological changes in the mycelial hyphae.

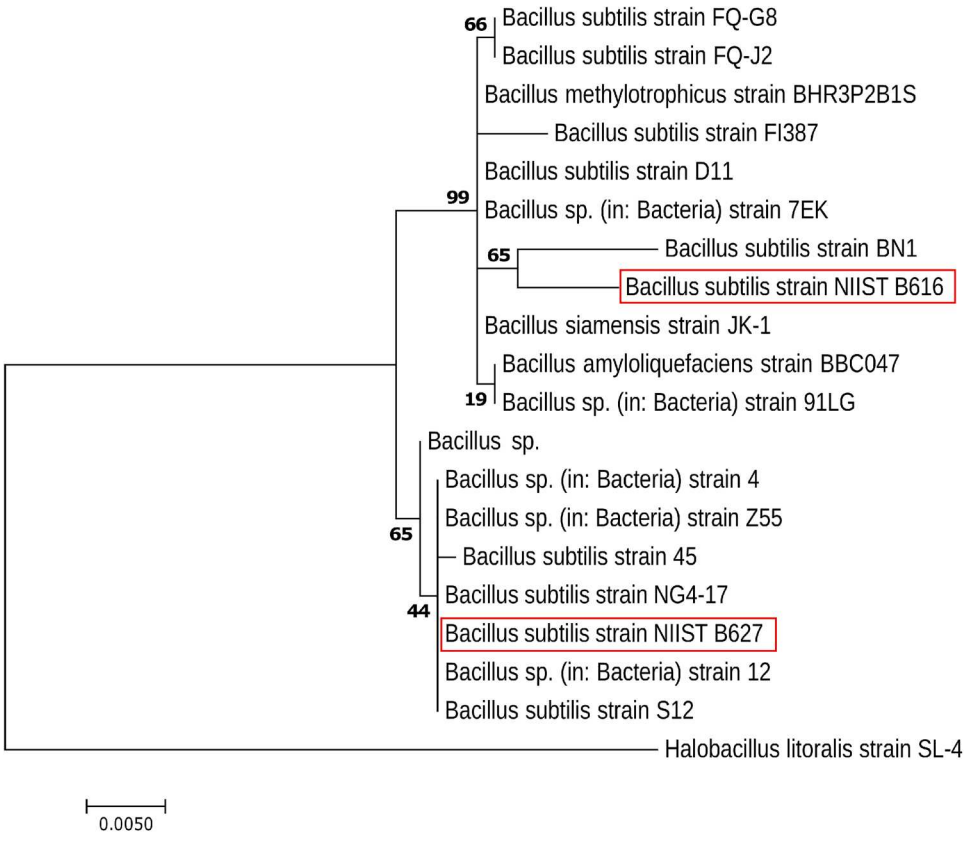

Figure 3. Phylogenetic tree constructed from 16S rRNA sequences of the two endophytic isolates using the maximum likelihood method in MEGA software version 7.0.

R. solani alone (Table 1, Figure S2). Moreover, rice plants treated with both isolates together also had the greatest yield among all the plants challenged with *R. solani*, followed by plants in the NIIST B627+P and NIIST B616+P treatment groups (Figure S2). Plants in the NIIST B616+627+P treatment group showed a 172.98% increase in the number of grains per pot, 19.70% increase in the weight of 100 grains, 50.00% increase in the number of tillers per plant, 100.75% increase in the number of panicles per plant, 368.25% increase in the amount of shoot dry matter, and 31.02% increase in the harvest index compared with plants treated with *R. solani* alone (Table 2). Statistical analysis revealed that there was a significant difference in all growth and yield parameters between plants that were treated with both isolates together and those treated with *R. solani* alone (ANOVA, $p < 0.05$).

Effects of the endophytic bacteria on disease control

Rice plants in all treatment groups that were grown in soil infested with *R. solani* developed disease symptoms over time, with symptoms being observed from day 50 for *R. solani* alone, day 60 for NIIST B616+P, day 65 for NIIST B627+P, and day 75 for NIIST B616+627+P. Initially, brownish lesions were observed in the collar region,

Table 1. Effects of the endophytic isolates *Bacillus subtilis* NIIST B616 and NIIST B627 both individually and in combination on the growth of rice plants inoculated with the pathogen *Rhizoctonia solani* at 7-day intervals up to day 28.

Day	Treatment	Shoot length (cm)	Root length (cm)	Dry weight (g)	Total chlorophyll (mg/L)
7	Control	16.40 ± 0.39^{b} (8.47)	5.58 ± 0.21^{a} (1.82)	0.02 ± 0.00^{a} (0.00)	3.74 ± 0.02^{a} (6.63)
	NIIST B616 + P	17.60 ± 0.05^{c} (16.40)	6.38 ± 0.14^{b} (16.42)	0.03 ± 0.00^{ab} (50.00)	8.20 ± 0.10^{c} (136.31)
	NIIST B627 + P	18.98 ± 0.14^{d} (25.52)	6.52 ± 0.12^{b} (18.98)	0.03 ± 0.00^{bc} (50.00)	6.94 ± 0.17^{b} (100)
	NIIST B616 + 627 + P	20.10 ± 0.21^{e} (32.94)	7.18 ± 0.22^{c} (31.02)	0.04 ± 0.00^{c} (100.00)	8.54 ± 0.11^{d} (146.11)
	Pathogen	15.12 ± 0.26^{a}	5.48 ± 0.12^{a}	0.02 ± 0.00^{a}	3.47 ± 0.06^{a}
	LSD (5%)	0.45	0.32	0.00	0.20
	CV%	2.86	5.62	15.06	3.59
14	Control	23.34 ± 0.30^{b} (19.08)	8.32 ± 0.16^{b} (10.64)	0.04 ± 0.00^{b} (33.33)	8.44 ± 0.11^{b} (33.12)
	NIIST B616 + P	25.02 ± 0.23^{c} (27.65)	10.09 ± 0.07^{c} (34.17)	0.06 ± 0.00^{c} (100.00)	14.13 ± 0.15^{c} (122.87)
	NIIST B627 + P	25.46 ± 0.19^{c} (29.90)	10.39 ± 0.03^{c} (38.16)	0.07 ± 0.00^{d} (133.33)	14.15 ± 0.14^{d} (123.18)
	NIIST B616 + 627 + P	26.76 ± 0.12^{d} (36.53)	11.02 ± 0.19^{d} (46.54)	0.08 ± 0.00^{e} (166.67)	17.00 ± 0.29^{e} (168.14)
	Pathogen	19.60 ± 0.12^{a}	7.52 ± 0.13^{a}	0.03 ± 0.00^{a}	6.34 ± 0.09^{a}
	LSD (5%)	0.58	0.38	0.00	0.49
	CV%	2.65	4.39	5.87	4.52
21	Control	29.04 ± 1.01^{b} (31.28)	19.38 ± 0.11^{b} (41.25)	0.12 ± 0.00^{b} (100)	14.64 ± 0.25^{b} (47.88)
	NIIST B616 + P	29.29 ± 0.28^{b} (32.41)	20.18 ± 0.27^{c} (47.08)	0.18 ± 0.00^{c} (200.00)	17.71 ± 0.13^{c} (78.89)
	NIIST B627 + P	30.24 ± 0.20^{c} (36.71)	20.83 ± 0.12^{cd} (51.82)	0.19 ± 0.00^{d} (216.67)	18.12 ± 0.16^{c} (83.03)
	NIIST B616 + 627 + P	30.78 ± 0.25^{c} (39.15)	21.28 ± 0.43^{d} (55.10)	0.20 ± 0.00^{e} (233.33)	21.55 ± 0.16^{d} (117.56)
	Pathogen	22.12 ± 0.14^{a}	13.72 ± 0.25^{a}	0.06 ± 0.00^{a}	9.90 ± 0.25^{a}
	LSD (5%)	0.70	0.75	0.00	0.56
	CV%	2.74	4.39	5.63	3.81
28	Control	27.14 ± 0.35^{b} (9.97)	22.42 ± 0.53^{b} (32.25)	0.17 ± 0.00^{b} (70.00)	22.04 ± 0.38^{b} (116.72)
	NIIST B616 + P	32.78 ± 0.23^{c} (32.82)	23.25 ± 0.25^{b} (37.08)	0.30 ± 0.00^{c} (200.00)	22.26 ± 0.22^{b} (118.88)
	NIIST B627 + P	33.14 ± 0.20^{c} (34.28)	24.19 ± 0.20^{c} (34.55)	0.30 ± 0.00^{c} (200.00)	22.44 ± 0.13^{b} (120.65)
	NIIST B616 + 627 + P	34.28 ± 0.11^{d} (38.90)	25.04 ± 0.19^{c} (42.63)	0.36 ± 0.00^{d} (260.00)	23.79 ± 0.04^{c} (133.92)
	Pathogen	24.68 ± 0.13^{a}	16.96 ± 0.21^{a}	0.10 ± 0.00^{a}	10.17 ± 0.05^{a}
	LSD (5%)	0.63	0.87	<0.01	0.59
	CV%	2.31	4.31	4.07	3.25

Abbreviations: LSD, least significant difference; CV, coefficient of variation.

Notes: All values are expressed as the mean ± SE of 10 replicates, and values in parentheses show the percentage increases over the pathogen treatment. Different superscript letters in the same column indicate statistically significant differences between treatments ($p < 0.05$).

Table 2. Effects of the endophytic isolates *Bacillus subtilis* NIIST B616 and NIIST B627 both individually and in combination on the yield of rice plants inoculated with the pathogen *Rhizoctonia solani* on day 120.

Treatment	No. of grains per pot	Weight of 100 grains (g)	No. of tillers per plant	No. of panicles per plant	Shoot dry matter (g)	Harvest index*
Control	266.33 ± 0.88^{b} (69.99)	2.16 ± 0.00^{b} (6.40)	2.00 ± 0.00^{a} (0.00)	1.67 ± 0.33^{ab} (25.56)	0.92 ± 0.00^{b} (46.03)	0.56 ± 0.00^{b} (23.42)
NIIST B616 + *P*	302.67 ± 1.20^{c} (93.19)	2.29 ± 0.01^{c} (12.81)	2.33 ± 0.33^{ab} (16.5)	2.33 ± 0.33^{ab} (75.19)	2.61 ± 0.01^{c} (314.29)	0.59 ± 0.00^{c} (27.98)
NIIST B627 + *P*	303.67 ± 1.20^{c} (93.83)	2.40 ± 0.01^{d} (18.23)	2.33 ± 0.33^{ab} (16.5)	2.33 ± 0.33^{ab} (75.19)	2.66 ± 0.01^{d} (322.22)	0.60 ± 0.00^{d} (30.15)
NIIST B616 + 627 + *P*	427.67 ± 1.45^{d} (172.98)	2.43 ± 0.01^{d} (19.70)	3.00 ± 0.00^{b} (50.00)	2.67 ± 0.33^{b} (100.75)	2.95 ± 0.00^{e} (368.25)	0.60 ± 0.00^{d} (31.02)
Pathogen	156.67 ± 1.45^{a}	2.03 ± 0.03^{a}	2.00 ± 0.00^{a}	1.33 ± 0.33^{a}	0.63 ± 0.00^{a}	0.46 ± 0.00^{a}
LSD (5%)	3.96	0.05	0.66	–	0.02	0.01
CV%	0.75	1.21	15.65	27.77	0.49	0.919

Abbreviations: LSD, least significant difference; CV, coefficient of variation.

Notes: All values are expressed as the mean ± SE of three replicates, and values in parentheses are the percentage increases compared with the pathogen treatment (different percentage increases for the same value indicate minor differences to the next decimal place). Different superscript letters in the same column indicate statistically significant differences between the treatments ($p < 0.05$).

* The harvest index is the ratio of harvested grain to total shoot dry matter.

which gradually enlarged and bleached with an irregular brown border. Subsequently, these lesions became dry, and they had turned white, grey or tan by day 90, when the final data were collected. On day 90, plants in the NIIST B616+627+P treatment group had the lowest disease incidence (30%), followed by those in the NIIST B616+P (36.67%), NIIST B627+P (40%), and *R. solani* alone (85%) treatment groups. Furthermore, the NIIST B616+627+P treatment group received a score of 1 on the relative lesion height scale, whereas both NIIST B616+P and NIIST B627+P received a score of 3, and *R. solani* alone received a maximum score of 7 on day 90 (Figure 4 and S3).

Effects of the endophytic bacteria on the induction of systemic resistance against R. solani

In general, plants in the NIIST B616+627+P treatment group exhibited a marked increase in all three of the defence enzymes up to day 30, followed by a slight increase up to day 50, and then a decrease to day 60, after which the level of each enzyme remained steady for the remainder of the observation period. By contrast, plants in the NIIST B616+P and NIIST B627+P treatment groups displayed a sharp increase in enzyme levels up to day 30 and a steady decline thereafter. The control plants exhibited a slight increase in PAL (up to day 40), POX (up to day 20), and PPO (up to day 20), subsequently displayed

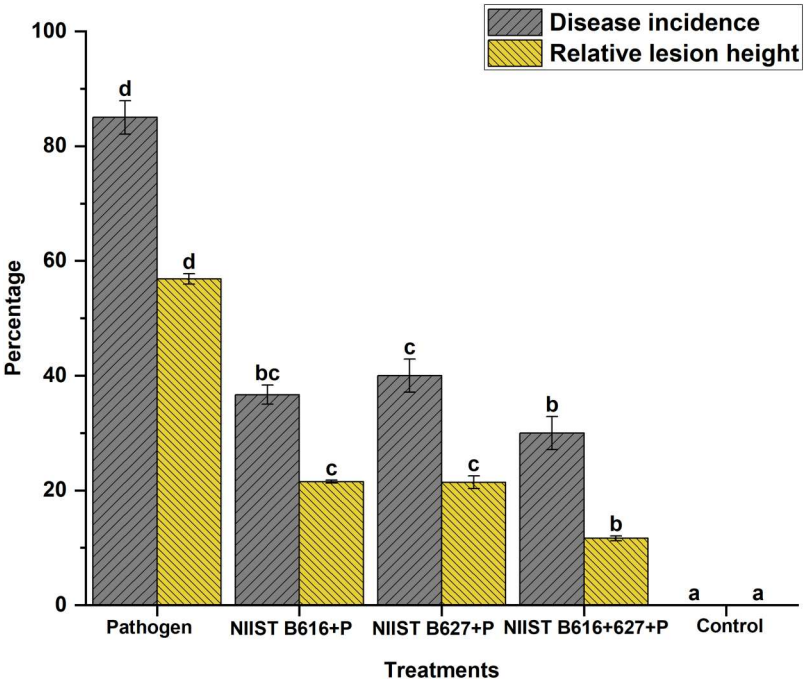

Figure 4. Efficacy of the *Bacillus subtilis* isolates NIIST B616 and NIIST B627 both individually and combined on sheath blight disease symptom development in rice plants treated with *Rhizoctonia solani* on day 90. All values are expressed as the mean ± SE of three replicates. Different superscript letters indicate significant differences between the treatments ($p < 0.05$). Control – no treatment; NIIST B616 + P – *B. subtilis* NIIST B616 + *R. solani*; NIIST B627 + P – *B. subtilis* NIIST B627 + *R. solani*; NIIST B616 + 627 + P – *B. subtilis* NIIST B616 + *B. subtilis* NIIST B627 + *R. solani*; Pathogen – *R. solani* alone.

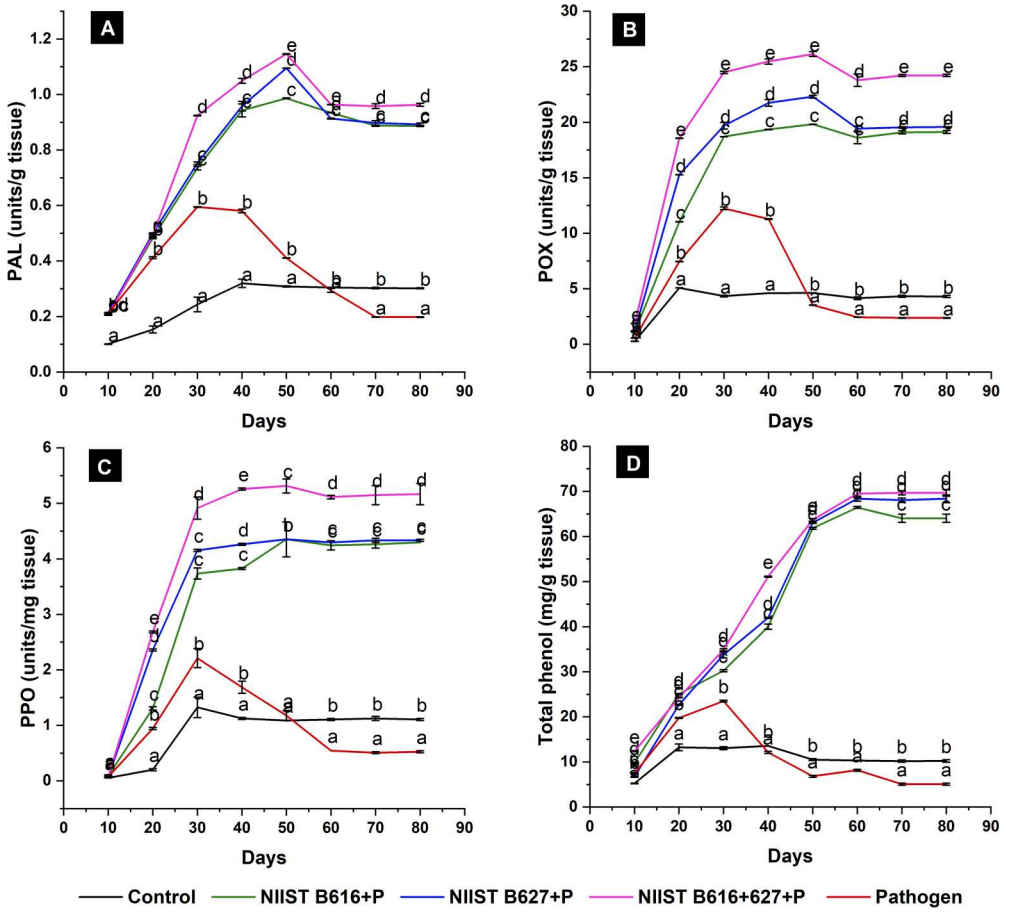

Figure 5. Enzyme activity of **A,** PAL, **B,** POX, and **C,** PPO, and **D,** the total phenol content of rice plants treated with the *Bacillus subtilis* isolates NIIST B616 and NIIST B627 and *Rhizoctonia solani* up to day 90. All values are expressed as the mean ± SE of three replicates. Different superscript letters on the same day indicate significant differences between the treatments ($p < 0.05$). Control – no treatment; NIIST B616 + P – *Bacillus subtilis* NIIST B616 + *R. solani*; NIIST B627 + P – *B. subtilis* NIIST B627 + *R. solani*; NIIST B616 + 627 + P – *B. subtilis* NIIST B616 + *B. subtilis* NIIST B627 + *R. solani*; Pathogen – *R. solani* alone.

a decrease, and then exhibited no change in levels throughout the remainder of the observation period (Figure 5A–C).

With regard to total phenol, all plants in the NIIST B616+627+P treatment group exhibited a steady increase in the total phenol content up to day 60, after which this remained at a constant level for the remainder of the observation period. By contrast, plants in the *R. solani* alone treatment group showed a slight increase in the total phenol content up to day 30 and a gradual decline thereafter. Control plants showed a slight increase up to day 20, followed by a decrease up to day 50, and then no change throughout the remainder of the observation period (Figure 5D).

The data showed that on day 80, plants in the NIIST B616+627+P treatment group had the highest enzyme activities and phenolic contents, followed by those in the

NIIST B627 and NIIST B616 treatment groups, while those in the *R. solani* alone treatment group had the lowest levels.

Split-root experiment

Plants in the *R. solani* / *R. solani* treatment group developed disease symptoms on day 7, when a brownish lesion was observed on the collar region. This lesion then spread further and bleached to a greenish-white colour on day 14. The plants started to exhibit signs of death as early as day 21, and complete mortality of the infected plants was recorded on day 28. By contrast, plants in the other treatment groups and control plants remained healthy throughout the observation period, with no signs of any disease symptoms (Figure 6 and S4).

Extraction of crude organic metabolites from the endophytic bacteria and analysis of in vitro *antagonism*

The optimum solvent for extracting the crude organic metabolites from both bacteria was determined to be ethyl acetate among the many solvents examined. The NIIST B616 and NIIST B627 strains yielded 354 and 295 mg of crude organic extracts, respectively, from 1 L of the culture medium.

The *in vitro* antagonism test using the crude organic metabolites showed an inhibition distance of 10.33 ± 0.58 for NIIST B616 and 7.67 ± 0.58 mm for NIIST B627 (Figure S5A). SEM images of fungal hyphae treated with the crude metabolites showed cellular damage, aberrant bulbous-like formation, and shrinkage of the mycelial hyphae (Figure S5B).

Identification of the crude organic metabolites

GC-MS/MS analysis showed that the crude organic extract from NIIST B616 exhibited discernible peaks with retention times of 22.10, 23.51, 23.84, 23.97, and 35.10 min, and area percentages of 19.47%, 10.30%, 17.52%, 3.11%, 4.20%, respectively (Figure 7A). Similarly, the crude organic extract from NIIST B627 exhibited discernible peaks with retention times of 23.52, 23.86, 23.99, 24.07, 34.32, and 35.12 min, and area percentages of 9.56%, 17.44%, 4.52%, 1.73%, 2.29%, and 8.76%, respectively (Figure 7B). A comparative search of the NIST library resulted in the identification of two major compounds in the NIIST B616 extract: pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) with *m/z* 210.27 and pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) with *m/z* 244.29. The same compounds were also identified from NIIST B627.

The presence of both compounds in the crude organic metabolites was further confirmed through LC-MS/MS analysis. The metabolites representing *m/z* values of 210.27 and 244.29 in the GC-MS/MS analysis were also detected in the LC-MS/MS analysis, with *m/z* values of 211 and 245, respectively (Figure 8A and B). MS/MS product ions of *m/z* 211 and 245 were obtained at *m/z* 70/72/86/98/114/138/154/183/211 and 70/98/103/120/154/172/217/245, respectively.

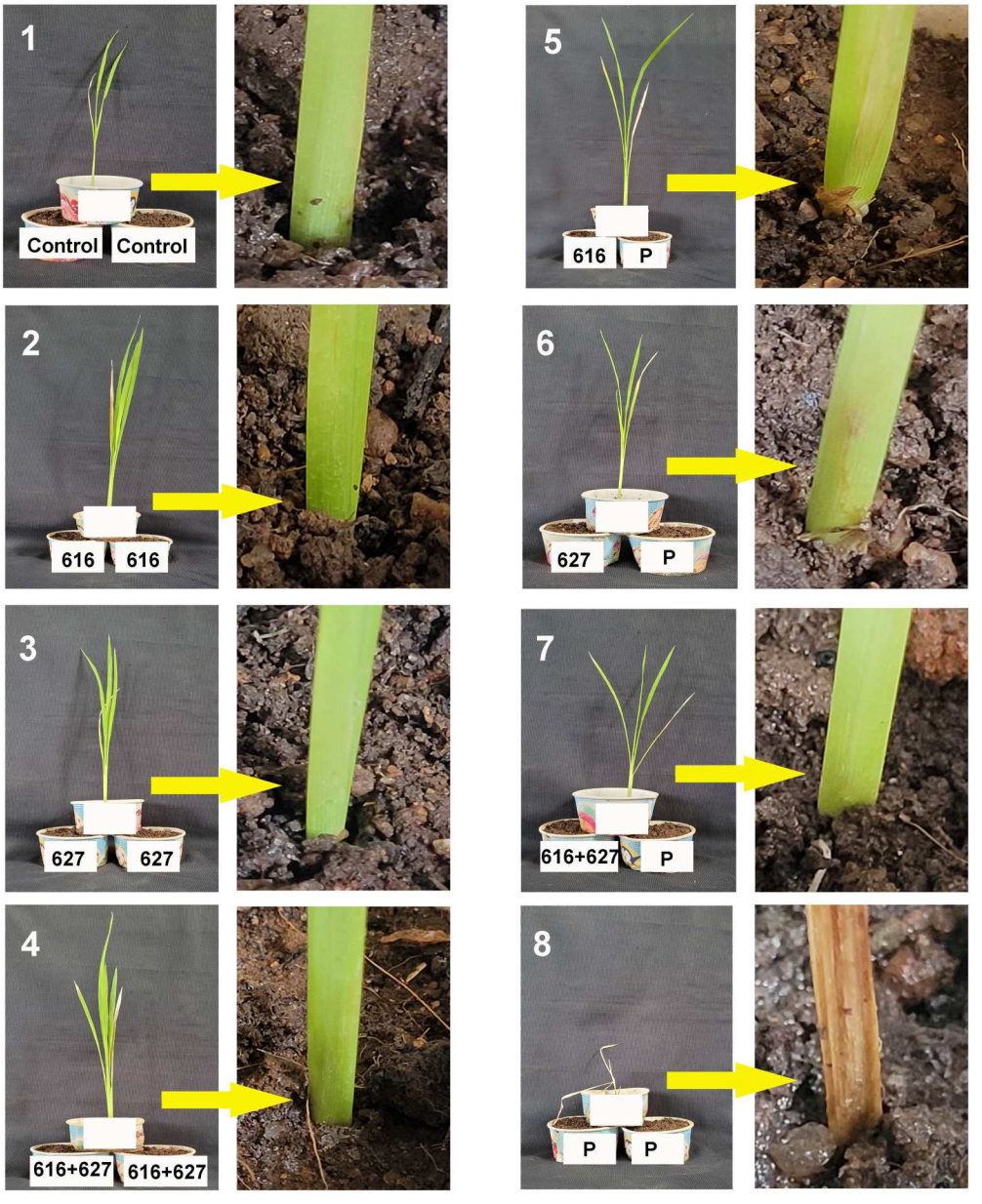

Figure 6. Result on day 28 of the split-root experiment to investigate the elicitation of ISR in rice plants. The arrows indicate enlarged portions of the collar region of the plants. **1.** No bacteria / no pathogen (control); **2.** NIIST B616 / NIIST B616 (616/616); **3.** NIIST B627 / NIIST B627 (627/627); **4.** NIIST B616 + NIIST B627 / NIIST B616 + NIIST B627 (616 + 627/616 + 627); **5.** NIIST B616 / *Rhizoctonia solani* (616/P); **6.** NIIST B627 / *R. solani* (627/P); **7.** NIIST B616 + NIIST B627 / *R. solani* (616 + 627/P); **8.** *R. solani* / *R. solani* (P/P).

Discussion

The exploitation of endophytic bacteria as an eco-friendly approach to disease management and crop improvement has experienced an upsurge in recent years (White et al.

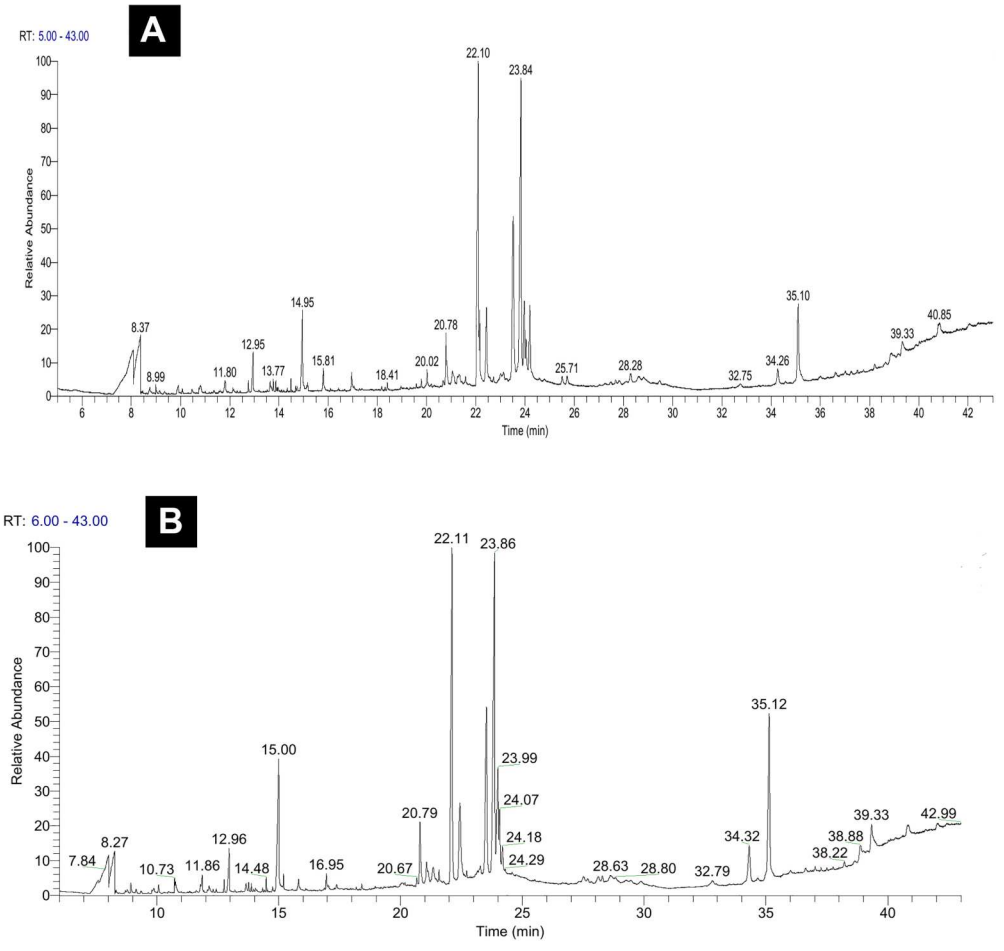

Figure 7. GC-MS chromatogram of the crude organic metabolites of **A,** *Bacillus subtilis* NIIST B616 and **B**, *B. subtilis* NIIST B627 showing discernible peaks and the associated retention times.

2019), and endophytic *Bacillus* species have received more attention than their rhizosphere counterparts. This investigation focused on two endophytic *B. subtilis* strains, NIIST B616 and NIIST B627, which were isolated from the roots of rice plants growing in the Kuttanad region of Kerala, India. These strains were selected for their potent antagonistic activity against *R. solani*, the causative agent of sheath blight disease, as well as for their demonstrated compatibility with each other.

This study confirmed the efficacy of strains NIIST B616 and NIIST B627 in enhancing the resistance of rice plants to sheath blight disease through ISR, thereby promoting plant growth and productivity. Numerous studies have documented the advantageous effects of endophytic bacteria on plant growth, productivity, and disease management through ISR (Nagendran et al. 2014; Kumar et al. 2020; Safari Motlagh et al. 2022). Similarly, our study demonstrated that the two selected endophytic *Bacillus* strains and their crude metabolites could effectively inhibit *R. solani* through *in vitro* and *in vivo* experiments.

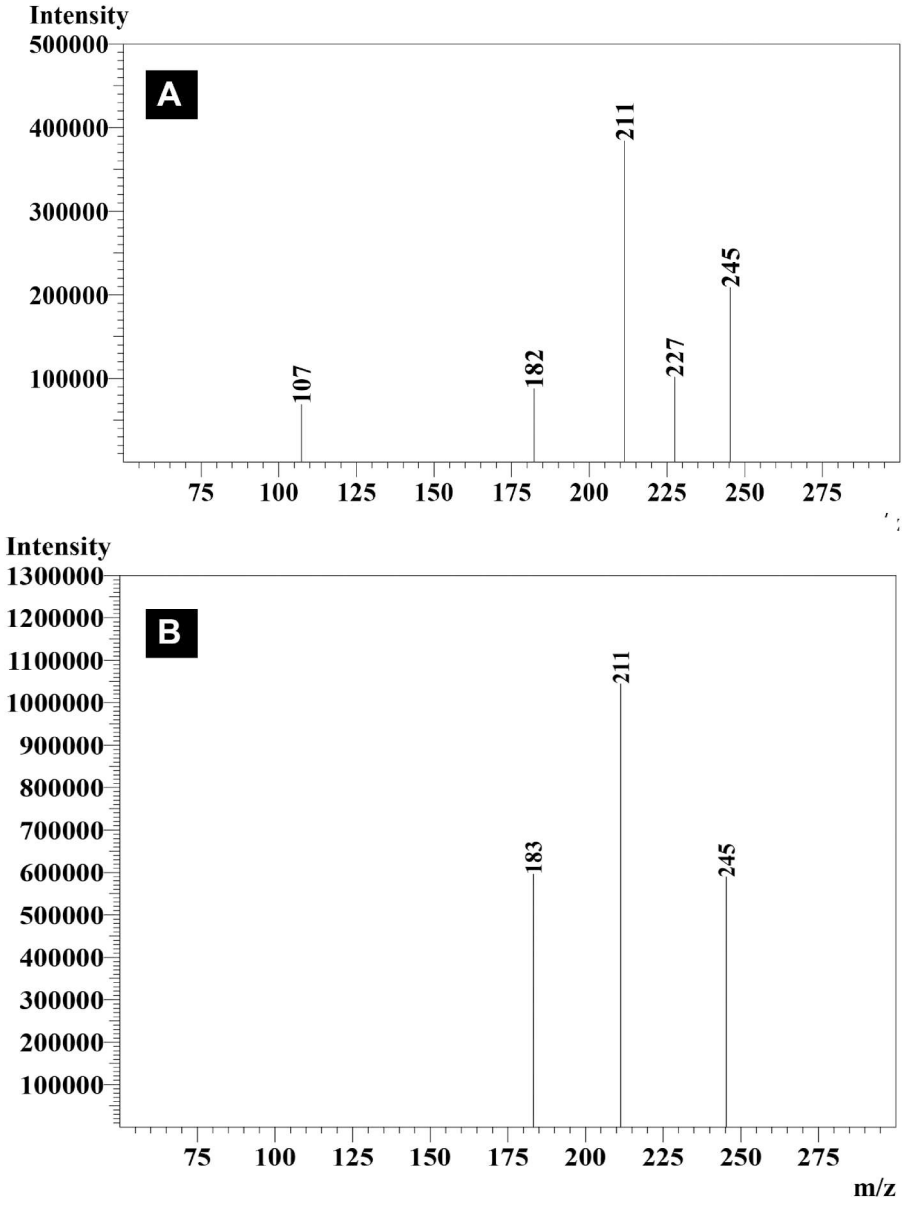

Figure 8. Mass spectra of the crude organic metabolites of **A**, *Bacillus subtilis* NIIST B616 and **B,** *B. subtilis* NIIST B627 showing *m/z* 211 and *m/z* 245 from the LC-MS/MS analysis.

Previous studies have demonstrated that endophytic bacteria isolated from wild rice can enhance the growth of perennial rice (Tian et al. 2023), while endophytic *B. subtilis* and other rhizospheric bacteria provide protection against sheath blight disease in rice (Durgadevi et al. 2021; Haque and Khan 2021, 2022; Jasrotia et al. 2021). Moreover, multi-strain inoculation has been shown to provide greater benefits than single strain inoculation in plants such as rice, grass, corn, and Douglas fir (Khan et al. 2016). Similarly, we observed a statistically significant improvement in plant

growth and yield indices for rice plants that had been grown in pots containing pathogen-infested soil and treated with NIIST B616 or NIIST B627 alone or in combination, with the combined application of these bacteria having the most pronounced effect on plants. Treatment with *Bacillus methylotrophicus* (DD-1) has also been shown to increase the root length, fresh weight, and shoot length of rice plants (Liu et al. 2020), demonstrating the positive effects of *Bacillus* bacteria on plant growth and yield.

The reduction in disease symptoms in plants treated with strains NIIST B616 and NIIST B627 provided clear evidence of disease suppression. Both the individual and combined application of these endophytes significantly increased levels of the defence-related enzymes PAL, POX, and PPO, as well as the total phenol content. By contrast, while plants treated with *R. solani* alone displayed a sharp increase in these defence enzymes and chemicals during the initial days, this was then followed by a decline, which likely contributed to the onset of disease symptoms. Several previous studies have shown that elevated enzyme levels help reduce disease symptoms (Jamali et al. 2020; Kalboush et al. 2024). For instance, Nagendran et al. (2014) showed that applying varying amounts of endophytic *B. subtilis* var. *amyloliquefaciens* (FZB24) to rice seeds, seedlings, and leaves, as well as the soil, reduced the sheath blight disease severity by up to 55% by increasing POX, PPO, PAL, and total phenol levels. Jayaraj et al. (2004) similarly demonstrated that foliar application of *B. subtilis* increased PAL and POX activity, reducing disease symptoms. In the present study, we found that the combined presence of the NIIST B616 and NIIST B627 isolates significantly increased the levels of PAL (2.8 fold), POX (7.4 fold), PPO (4.5 fold), and total phenol (8.5 fold) compared with plants treated exclusively with *R. solani*, and this was associated with reduced disease symptoms in these plants. These findings suggest that these *B. subtilis* strains stimulated ISR, increased polyphenolic compound production, and enhanced secondary metabolite biosynthesis in the treated plants.

A split-root experiment further validated the concept of ISR, as the presence of the endophytes clearly induced resistance in rice plants against *R. solani*, with no disease symptoms being observed in the treated plants, despite the pathogen and endophytic isolates not coming in contact with each other, ruling out direct inhibition. Our results align with a previous study that used a split-root system to demonstrate the development of ISR against *Fusarium udum* in pigeon pea following treatment with *B. cereus* BS 03, *Pseudomonas aeruginosa* RRLJ 04, and the rhizobial strain RH 2 (Dutta et al. 2008).

The crude organic metabolites produced by the isolates were analysed using GC-MS/MS, resulting in two major cyclic dipeptides being identified: pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) and pyrrolo[1,2-a] pyrazine1,4-dione, hexahydro-3-(phenylmethyl). These pyrrole compounds, which are recognised for their broad biological activities, including antimicrobial, anticancer, antiviral, and anti-inflammatory effects (Ser et al. 2015), were also found to induce ISR in tomato plants (El-Gendi et al. 2022). It has also previously been shown that pyrrolo[1,2-a]pyrazine-1,4-dione compounds in the culture filtrate extract of *B. velezensis* PEA1 enhance resistance to *Cucumber mosaic virus* in *Datura stramonium* and inhibit *Fusarium oxysporum* (Abdelkhalek et al. 2020). LC-MS/MS analysis also confirmed the presence of two cyclic dipeptides, cyclo(Pro-Leu) and cyclo(Pro-Phe), which matched those identified in GC-MS/MS analysis of the metabolic extracts of NIIST B616 and NIIST B627, and this confirmation was supported by MS/MS fragment data from the literature (Xing et al. 2008). Previous investigations have

documented the antimicrobial activity of cyclo(Pro-Leu) and cyclo(Pro-Phe) against the agriculturally important fungus *R. solani* (Kumar et al. 2013; Zhao et al. 2020). Consequently, these molecules could partially account for the antifungal activity of endophytic *B. subtilis* NIIST B616 and NIIST B627, as well as for their elicitation of ISR in rice plants.

Conclusion

Endophytic bacteria offer a promising sustainable and eco-friendly alternative to harmful pesticides and herbicides for managing diseases and improving crop yields in modern agriculture. This is particularly relevant for unique regions like Kuttanad, where rice cultivation requires disease-free and highly productive crops. Our study focused on the use of endophytic *Bacillus* to enhance rice growth, yield, and disease management by inducing systemic resistance against sheath blight disease. Administration of the endophytic *B. subtilis* strains NIIST B616 and NIIST B627 was found to increase plant growth, levels of the defence-related enzymes PAL, POX, and PPO, and the total phenol content in rice plants. The observed effects could be attributed to the bioactive compounds that were generated by these bacteria, specifically cyclic dipeptides that display antimicrobial properties and have the capacity to elicit the ISR phenomenon. Based on this primary analysis, both of these endophytic isolates could be used to effectively combat sheath blight disease in rice, promoting agricultural sustainability in Kuttanad and other regions.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Author contribution statement

Krishnakumar B.: Data curation, Supervision, Writing – review & editing. Dileep Kumar B.S.: Conceptualisation, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing– original draft. Ravi Shankar Lankalapalli: Formal analysis, Methodology, Supervision, Writing – review & editing. Gopika V. Krishnan: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. Billu Abraham: Methodology. NIIST publication no. NIIST/2023/Apr/439.

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