Effect of soil amendments with chitinous substrates and application of chitooligosaccharides on rice

Thesis submitted to the University of Hyderabad for the award of

Doctor of Philosophy

By

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(15LPPH06)

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This is to certify that the thesis entitled "**Effect of soil amendments with chitinous substrates and application of chitooligosaccharides on rice**" submitted by **Mr. P.V.S.R.N Sarma** bearing **Reg. No. 15LPPH06**, in partial fulfillment of the requirements for the award of Doctor of Philosophy in Plant Sciences, is a bonafide work carried out by him under the supervision and guidance of **Prof. Appa Rao Podile.**

The thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

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Further, parts of the thesis have been:

A. Published in the following publications:

Ramakrishna, B*., **Sarma, P.V.S.R.N.***, Ankati, S., Bhuvanachandra, B., & Podile, A. R. (2021). Elicitation of defense response by transglycosylated chitooligosaccharides in rice seedlings. *Carbohydrate Research*, *510*, 108459. https://doi.org/10.1016/j.carres.2021.108459 [* Equal Contribution]

Sarma, P.V.S.R.N., Srinivas, V., Anil, K., & Podile, A. R. (2012). Isolation and purification of microbial community DNA from soil naturally enriched for chitin. *Biologia*, *67*(4), 644–648. https://doi.org/10.2478/s11756-012-0059-0

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4. **Sarma PVSRN** and Appa Rao Podile. "*Enzymatic approach for production of higher chain length Chitooligosaccharides and their evaluation for elicitor respon-se in rice seedlings*" at the Young Scientist Session in the 14th International Chitin and Chitosan Conference (14th ICCC) & 12th Asia-Pacific Chitin and Chitosan Symposium (12th APCCS) that was held at Kansai University, Osaka, Japan from 27-30th August, 2018. **(Awarded CSIR International Travel grant for year 2018).**

Further, the student has passed the following courses toward the fulfillment of the course work requirement for Ph.D. degree.

Sl. No.	Course Code	Name	Credits	Pass/Fail
1.	AS-801	Analytical Techniques	4	Pass
2.	AS-802	Research Ethics, Data Analysis and Biostatistics	3	Pass
3.	AS-803	Lab Work & Seminar	5	Pass

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DECLARATION

I, **P.V.S.R.N Sarma**, hereby declare that this thesis entitled **"Effect of soil amendments with chitinous substrates and application of chitooligosaccharides on rice"** submitted by me under the guidance and supervision of **Prof. Appa Rao Podile** is a bonafide research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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2. Das SN, Madhuprakash, J, **Sarma PVSRN**, Purushotham P, Suma K, Manjeet K, Rambabu S, Gueddari NE, El, Moerschbacher BM, and Podile AR. (2015). Biotechnological approaches for field applications of chitooligosaccha-rides (COS) to induce innate immunity in plants. Critical Reviews in Biotechnology, 35(1), 29–43. https://doi.org/10.3109/07388551.2013.798255.
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These publications, constituting a part of the present thesis, have been deposited in various public repositories. All the publications related to this thesis have been appended at the end of the thesis. Only 7% similarity was identified from other sources in the present thesis. Hence, the present thesis may be considered as plagiarism-free.

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Abbreviations and Symbols

COS	Chitooligosaccharides
°C	Degree celsius (centigrade)
µg	Microgram
µL	Microlitre
µM	Micromolar
ADW	Autoclaved distilled water
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
AOS2	Allene oxide synthase 2
AT	*Arabidopsis thaliana*
BL 21	BL21(DE3) is an *E. Coli* B strain and does not contain the ion protease and the outer membrane protease ompt
BLAST	Basic local alignment search tool
BLASTN	Nucleotide BLAST
bp	Base pairs
CEBIP	Chitin elicitor-binding protein
CERK	Ceramide kinase
CFU	Colony forming unit
DHB	2, ,4-Dihydroxybutyric acid
dNTPs	Deoxynucleotide triphosphates
DP	Degree of polymerization
DP5	Pentamer
DP6	Hexamer
DP7	Heptamer
DP8	Octamer
DW	Distilled water
EC	Enzyme commission number
EDS1	*Enhanced disease susceptibility 1*
EE	Expected error
FDR	False-discovery rate
g	Gram
g	Gravity (relative centrifugal force)
GH	Glycosyl hydrolase
GlcNAc	N-acetyl-D-glucosamine;
GPS	Global positioning system
h	Hour
His- tag	Histidine tag
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin layer chromatography
ICS1	Isochorismate synthase 1
ITS	Internal transcribed spacer
IPTG	Isopropyl-β-D-1-thiogalactopyranoside;
JA	Jasmonic acid
JAMYB	*JA-inducible Myb transcription factor*
JMT1	*JA carboxyl methyltransferase 1*
kb	Kilobases
kV	Kilovolt
L	Litre
LDA	Linear discriminant analysis
LEfSe	Linear discriminant analysis effect size
LYK4	Lysm-containing receptor-like kinase
LYP	Lysin motif containing protein family
M	Molar
MALDI-TOF MS	Matrix assisted laser desorption ionization-time of flight mass spectrometry
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase

MDA	Mean decrease accuracy
mg	Milligram
Mha	Million hectares
min	Minute
mL	Millilitre
mM	Millimolar
mPNA	Mitochondria peptide nucleic acid
MS	Murashige and Skoog
Mt	Million tonnes
MW	Molecular weight
NCBI	National Center for Biotechnology Information
ng	Nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
NPR1	Nonexpressor of PR genes1
NRE	Non-rhizobial endophyte
OOB	Out-of-bag
Os	*Oryza sativa*
OTU	Operational taxonomic unit
PAD4	*Phytoalexin deficient 4*
PAL	Phenylalanine ammonia lyase
PAMP	Pathogen-associated molecular pattern
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
pET- 22b(+)	Pelb sequence for periplasmic localization Bacterial vector for expressing proteins in the periplasm
PGPR	Plant growth promoting rhizobacteria
PNA	Peptide nucleic acid
POD	Peroxidase
pPNA	Plastid peptide nucleic acid
PR	Pathogenesis-related
PRR	Pattern recognition receptor
PTI	Pattern-triggered immunity
qRT-PCR	Quantitative real-time PCR
RF	Random forest
ROS	Reactive oxygen species
rpm	Rotations per minute
rRNA	Ribosomal RNA
s	Second
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS page	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
sp.	Species
*Sp*ChiD	Chitinase D from *Serratia proteamaculans*
*Sp*ChiD-Y28A	Y28A variant of *sp*chid
TG	Transglycosylation
TLC	Thin Layer chromatography
TMV	*Tobacco mosaic virus*
var.	Variety
vs.	Verses
WRKY	WRKY transcription factors
zOTU	Zero-radius operational taxonomic unit

Chapter - I

Introduction

1.1 The plant rhizosphere

Plant roots and soil microbes interact intensively in the rhizosphere zone, the soil that is immediately surrounding the roots. The rhizosphere is the interface between plant roots and soil (Fig 1.1), where interactions between microorganisms and invertebrates influence a variety of biological and geochemical processes, which often contribute to the overall performance of the plant. Rhizosphere is home to all of the bacteria that are essential to the terrestrial biosphere. Rhizosphere separates a plant root system from the surrounding soil. With 10^{11} microbial cells per gram of root, and 10^{12} functional genes per gram of soil, it is possibly the most complex terrestrial microbial habitat on earth. The rhizosphere microbiome, which harbors plant-specific microbial communities, benefits due to rhizodeposition. The composition of the rhizosphere microbiome, which includes species richness and abundance, is, thus, frequently determined by the chemistry of root exudates. The structural and functional characteristics of the rhizosphere microbiome have a beneficial effect on plant growth and fitness. To take advantage of the dynamic rhizosphere, an integrated strategy based on multi-omics (meta-genomics, proteomics, and metabolomics) that reveals microbial activities including structure, function, and quorum sensing is required.

The rhizosphere is also a complex habitat. Understanding the ecology and evolution of rhizosphere is essential for increasing plant production and enhancing ecosystem function (Philippot et al. 2013). Integrating reductionist and systems-based techniques in both agricultural and natural settings would yield unique insights into the key factors and evolutionary processes that drive the rhizosphere microbiome. It also has extensive cross-kingdom biological interaction and geochemical response. Plant nutrient uptake, root exudates, C input hotspots, microbial community structure and abundance, plant pathogen defense, soil permeability changes, geochemical microenvironments, and fungal interaction drive these activities (Edwards et al., 2019; Edwards et al., 2015; Lundberg et al., 2012).

Each plant species exudes a unique mixture of carbohydrates, proteins, enzymes, organic acids, hormones, and other biological components that trigger the soil bacteria to migrate to root vicinity (Hassani et al. 2020). The beneficial bacteria defend plants from infections, increase the availability of nutrients, and produce organic substances for growth, health, and vitality. Aggregation, aeration, water infiltration, and water-holding capacity are among the benefits to the plant due to the increased microbial activity in the rhizosphere. Plant root exudates provide energy to microorganisms to extract minerals and trace elements. The plants, in turn, pay a carbon fee for soil organism services for receiving nutrients. Plant diversity increases microbial diversity and soil ecosystem strength. Natural systems are effective and healthy because of microbial diversity and diverse compared monoculture systems like crop land (Lemanceau et al., 2017; Özkurt et al., 2020; Sharaf et al., 2019).

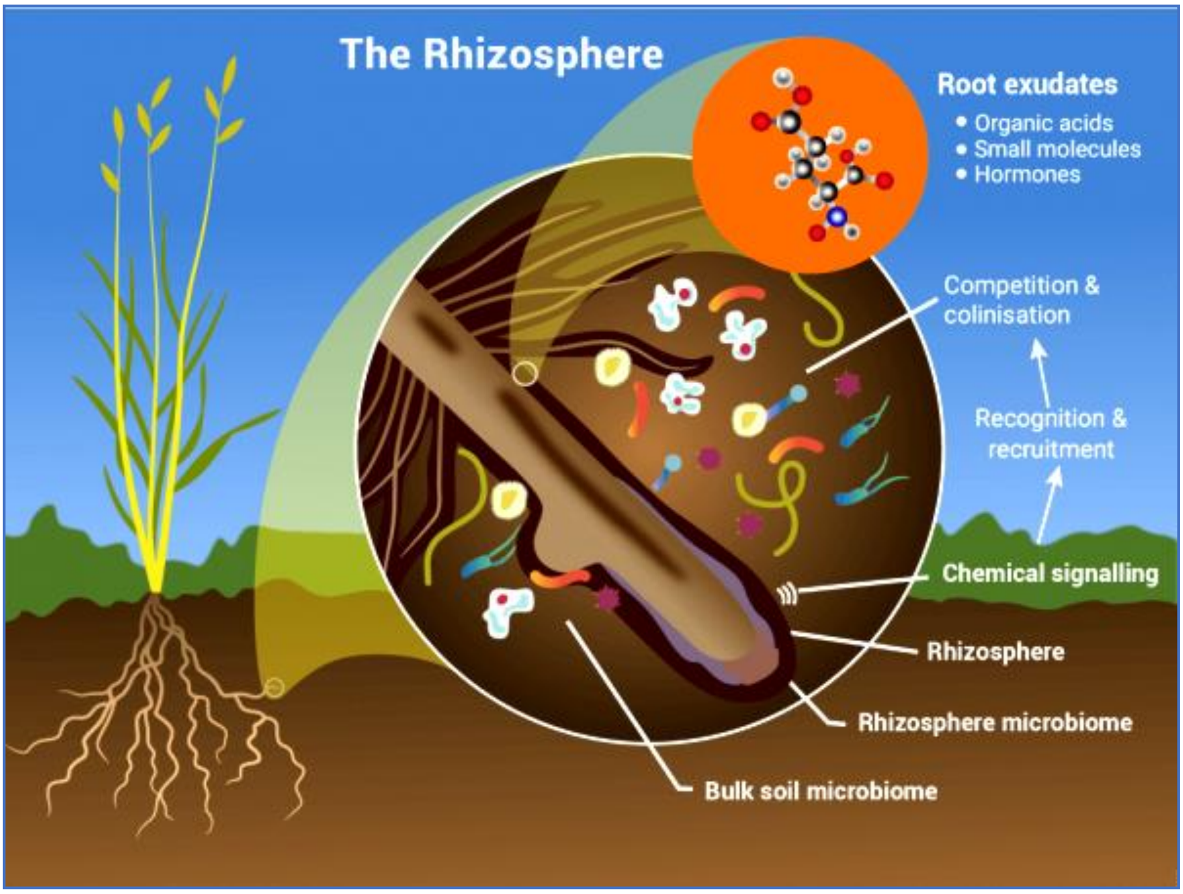


Fig 1.1: A rhizosphere microbiome overview. The rhizosphere, the zone around a plant's roots, affects both the plant and the soil health. It also has extensive cross-kingdom biological interaction and geochemical response. Plant nutrient uptake, root exudates, C input hotspots, microbial community structure and abundance, plant pathogen defense, soil permeability changes, geochemical microenvironments, and fungal interaction drive these activities (Source: Moran & McGrath, 2021)

1.2. Rice microbiome

The plant microbiome has the potential to enhance crop yield also can contribute to sustainable agriculture. Due to the relative ease of research, non-crop species have been used for plant microbiome studies, despite the need for agriculturally required microbiomes. To make plant microbiomes useful for agriculture, however, a model based on agricultural plants is essential. To elevate our understanding of microbiomes to the level of ecosystems, we need to also consider features of microbiomes not addressed by bacteria-focused studies. In addition to the microbial composition, the functional characteristics of microbiomes over time and geography will aid us in choosing the proper microorganisms by delivering stage-specific capabilities that can assist crop plants. Fungi and protist communities, which have received less attention, can provide novel insights into the functional dynamics and composition of each community, such as interrelationships between kingdoms and multitrophic interactions. An overview of the research reports on rice microbiome can be seen in Table 1.1 & 1.2.

S.No	Country	Species	Plant part	Reference
1	**USA**	*O. sativa, O. glaberrima, O. japonica*	Root	Edwards et al., 2015
2	**USA**	*O. sativa*	Seed	Eyre et al., 2019
3	**USA**	*O. sativa*	Root	Santos-Medellín et al., 2021
4	**Philippines**	Whole genome project of 3000 rice genotypes – *O. sativa*	Leaf	Roman-Reyna et al., 2020
5	**China**	*O. japonica*	Root	Xiong et al., 2021
6	**Japan**	*O. sativa*	Root	Ikeda et al., 2014
7	**India**	*O. sativa*	Root	Thapa et al., 2018
8	**India**	*O. sativa*	Root	Sahu et al., 2022
9	**India**	*O. sativa*	seed	Raj et al., 2019
10	**USA**	*O. sativa*	seed	Eyre et al., 2019
11	**China**	*O. barthii, O. nivara, O. ruffipogon, O. sativa* & *O. glaberrima*	root	Tian et al., 2021
12	**India**	Aromatic and non aromatic varieties of *O. sativa*	leaf	Kumar et al., 2021
13	**Japan**	*O. sativa*	root	Matsushima et al., 2021
14	**India**	Black rice varieties of *O. sativa*	Stems and roots	Singha et al., 2021
15	**China**	*O. sativa*	leaf	Yang et al., 2020
16	**Ghana**	*O. sativa*	root	Kanasugi et al., 2020
17	**China**	*O. sativa*	root	Wang et al., 2022
18	**China**	*O. sativa* (transgenic rice)	root	J. Wang et al., 2019
19	**USA**	*O. sativa* (62 RILs population)	root	Fernández-baca et al., 2021
20	**USA**	*O. japonica*	Seed, root	M. Wang et al., 2020
21	**Burkina Faso**	*O. sativa*	root	Barro et al., 2022
22	**Vietnam**	*O. sativa*	root	Masson et al., 2020

Table 1.1: Rice microbiome studies on diverse *Oryza* species

Fraction	Major finding	Reference
Bulk soil	Effects of chemical factors (oxygen status and inorganic matters) and cultivation practices (crop rotation and fertilization regimes) on soil microbial communities	Noll et al., 2005 Liu et al., 2016 Wang et al., 2017 Jiang et al., 2016 Yuan et al., 2019 Sun et al., 2018
Rhizosphere	Rhizosphere effects in terms of microbial communities	Hussain et al., 2012
	Effects of rice development and genotypes on soil microbial communities	Hussain et al., 2011 Shenton et al., 2016
	Biocontrol activities of rhizosphere bacteria against pathogens	Spence et al., 2014
Phyllosphere	Effects of abiotic (geographic locations, atmosphere and soil conditions, and fertilization regimes) and biotic factors (rice genotypes) on phyllosphere microbial communities	Ren et al., 2014 Venkatachalam et al., 2016
	Structure, metabolic profiles, and host factors shaping phyllosphere bacterial communities	Roman-Reyna et al., 2019
Endosphere	Effects of abiotic (graphical location, soil conditions, and cultivation practices) and biotic factors (rice genotypes and root compartment) on microbial communities	Edwards et al., 2015 Ikeda et al., 2014 Long and Yao., 2019 Santos-Medellín et al., 2017 Bertani et al., 2016 Fisher and Petrini. 1992
	Effects of a host genetic factor on the assembly and functions of root bacterial microbiomes	Zhang et al., 2019
Seed	Effects of seed compartments on composition and diversity of seed microbiota	Eyre et al., 2019
	Inheritance of seed bacterial communities	Hardoimet al., 2012
	Role of seed as a microbial pool of endophytic microbial communities of mature rice plants	Kagaet al., 2009 Wang et al., 2016
Community dynamics	Community dynamics of belowground bacterial communities (rhizosphere, rhizoplane, and root endosphere) during rice development	Edwards et al., 2018
Functional prediction	Functional characteristic (metabolic make-up) of bacterial communities associated with phyllosphere, rhizosphere, and root endosphere	Kniefet al., 2012 Okubo et al., 2014 Sessitschet al., 2012

Table 1.2: Major findings on the microbial communities associated with rice (*Oryza sativa* L.) (Source: Kim & Lee, 2019)

1.3. Importance of organic soil amendments

Application of external organic inputs to soils can be considered as one of the most ancient agricultural practices. The addition of different organic substances to agricultural soils has proved beneficial to plants and soils, in restoring soil characteristics and enhancing soil quality, and in some instances in providing plants with favorable effects (Duddigan et al. 2022). Soil amendments are used to improve the structure and biological function of soil. The physical and biological features of agricultural soils are negatively impacted by the cultivation practices. Several soil supplements, including biochar, fly ash, and compost, have been utilized to mitigate the impact of fertilizers on soil (Jin et al., 2022). Two types of soil amendments are used to improve soil fertility and stabilize site conditions: organic and inorganic supplements. Organic amendments consist of organic materials/molecules obtained from biomass or live organisms. Compost, wood chips, charcoal, animal manure, agriculture waste such as straw and husk, marine waste such as chitin are typical components of organic amendments. These substances are exceptionally rich in organic matter and macro- and micro elements, which enhance the fertility of soils by ameliorating micro-climatic conditions and may also serve as growth substrates for microorganisms (Bowles et al., 2014; Bastida et al., 2016).

Globally there is an increased interest in the use of organic matter as the primary substrate for agricultural crops, perhaps to encourage beneficial microbes. These organic inputs feed the soil with energy and nutrients, resulting in an environment that is conducive to the survival of crops and the multiplication of microorganisms (Mitter et al., 2013). In addition, the utilization of organic matter, rather than its disposal, is favored since it adds value to the market and recycles back into the soil, resulting in a more sustainable agricultural system (Wang et al., 2012). While a variety of organic materials are available, the efficacy of each of these materials varies, maybe in part because of their chemical contents, kinds, origin, and duration of decomposition. Consequently, the outcomes of these natural products vary from location to location and from field to field. Common examples include the release of pathogen-toxic chemicals, the modification of soil physicochemical properties, the enhancement of microbial activity, and the induction of host resistance against a broad spectrum of soil borne diseases (Fig 1.2).

Moreover, soil is indistinct aspect of the ecosystem which may govern the plants productivity. For sustainable agriculture, it is necessary to optimize the insufficient supply of ready-made organics. Fertile soils promote higher quality crop production, which is important to eradicate world hunger. In addition, fertile soils provide essential nutrients for

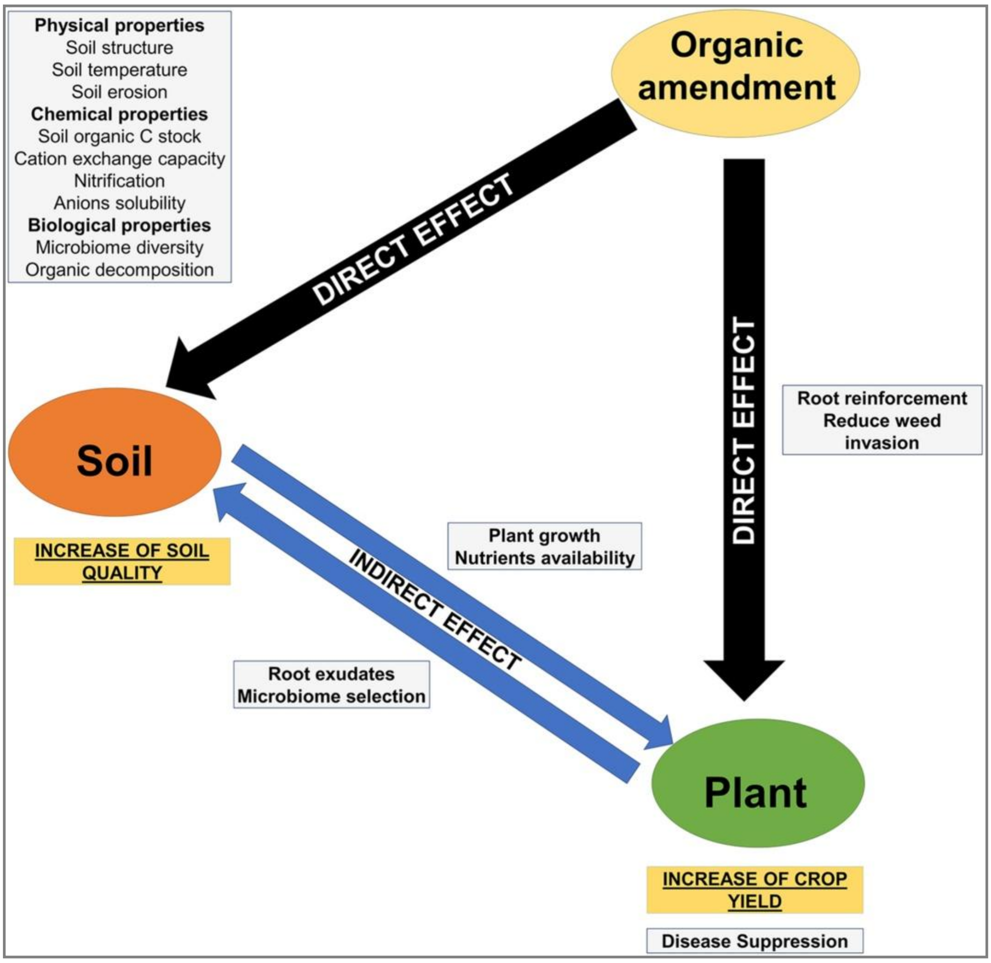


Fig 1.2: Schematic representation of the direct effects by application of organic amendments to soil and plants (black arrows). Indirect effects after organic amendment among soil and plants are also indicated (blue arrows). Beneficial properties resulting from each interaction is boxed in grey. The general effect observed on soil and plants is boxed in yellow (Source: Vida et al., 2020).

plant growth, resulting in the production of nutrient-rich food that meets all human health requirements. Effective management of soil fertility with organic amendments can increase vegetation cover, decrease soil, water, and air pollution, and regulate the availability of water resources (Chaker et al. 2023). This can be enhanced by both organic and inorganic soil fertilizers.

1.3. Chitinous substrate amendments acts as a bio-stimulant, inhibits pathogens, and stimulates plant growth

Fungal cell walls, crustacean and insect exoskeletons are rich sources of a crystalline, linear and unbranched homopolymer of β1, 4-linked N -acetyl- D –glucosamine (GlcNAc) called as chitin. In nature, chitin exists in two forms defined in terms of arrangement of individual polymeric chains in antiparallel (Alpha chitin) or parallel fashion (Beta chitin). Chitin is a highly versatile and promising biopolymer with a great variety of industrial, medical, agricultural and commercial uses. Chitin is the second most prevalent polysaccharide after cellulose; it is rich in nitrogen, calcium, magnesium, and other minerals needed for healthy microbial activity (Ravi Kumar, 2000). Chitin provides nitrogen to soil microorganisms and plants.

Chitin and its derivatives are biologically active during their interaction with plants and microorganisms (Goosen 2020). They enhance or induce natural defense mechanisms in plants and are recognized as plant growth regulators, growth stimulants, anti-stress agents, and elicitors for the production of secondary metabolites (El Hadrami et al., 2010). Application of chitin can benefit at least in four different ways viz., 1. Protect plants from pests and diseases before and after harvest, 2. Enhance the activity of antagonistic microorganisms for biological control, 3. Enhance the symbiotic plant-microorganism interactions, and 4. Regulate plant growth and development. Chitin and its derivatives are effective as fertilizers, soil conditioning agents, plant disease control agents, anti-transpirants, fruit retardants, and seed coatings (Shamshina et al. 2020) (Table 1.3).

Use	Crop	Properties	Compound	Reference
Biocontrol action enhancer	Peanut Apple	Stimulator substrate for hydrolyses enzymes	Chitin	Chien et al., 2007; Thommohaway et al., 2007
Protection after harvest	Mango Guava Tomato	Antimicrobial	Chitosan	Alimunair et al., 1994.
Retardation of fruit ripening process	Papaya	Semi permeable film formation	Chitosan	Backman et al., 2005
Nematocidal control	Tomato	Increases soil chitinolytic microbiota	Chitin	Jin et al., 2005
Mycorrhizal symbiosis stimulator	Tomato	Inducer of recognition mechanisms	Chitin	Iglesias et al., 1994
Defensive enzymes stimulation	Rice Tobacco Pea	Inducer	Chitin Chitosan	Rodrieuez et al., 2007; Falcon et al., 2002; Hadwiger et al., 1994.
Defensive enzymes stimulation	Pepper	Inducer	Chitosan	Chookhongkha et al. 2012

Table 1.3: Use of Chitin and chitosan in agriculture

Chitin undergoes depolymerization, when applied to soil, due to the chitinase activity in the soil which is linked to the presence of specific microflora. Chitin is also the principal source of carbon and nitrogen for chitinolytic organisms, which are largely marine and soil bacteria belonging to the genera of the Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes, as well as soil fungi. Chitin has also been used to enhance the efficiency of natural biological controls. Many microorganisms acting as antagonists use chitinases against plant pests and diseases (e.g., *Trichoderma* sp.). Impact of chitin amendment on actinomycetes and the tuber scab infection in potato by *Streptomyces scabies*, resulted in

9.5% disease reduction (Sharp 2013). A few months later, soil actinomycetes increased 24-30 times in chitin-amended soil and also found that some actinomycetes (i.e., *Micromonospora*) had disappeared, while others including *S. scabies* were not much detected.

Chitin and its derivatives can improve legume-Rhizobium symbiosis. Nodulation factors released by *Rhizobium* sp. are primarily lipid chitin oligosaccharides consisting of 3-to-5-units of glucosamine residues attached to fatty acid. Therefore, chitin can be provided as precursor substrate for these metabolites. Other types of interactions (e.g., mycorrhization) have benefited from adding chitin derivatives, as in tomato cultivation (Amerany et al., 2020).

Insolouble chitin can be deacetylated to more soluble biopolymer chitosans ether by enzymatic or chemical reactions, soluble in dilute acids like acetic acid and formic acid. Chitosan is a recognized antibacterial biopolymer (Kikuyama & Shibuya, 1997) and is also a source of nutrients for insects, bacteria and fungi living in the soil. Chitosan, as a soil supplement, controls *Fusarium* wilt in several plants (Lafontaine & Benhamou,1996). At optimal concentration, chitosan delays disease development by reducing plant wilting (Rabea et al., 2003). Chitosan-treated soil completely reduced *Aspergillus flavus* in field-grown corn and peanut (El Ghaouth et al., 1992). Chitosan boosts plant defenses, reducing soil-borne diseases. Further, this biopolymer encourages beneficial soil microbes like *Bacillus*, *Pseudomonas*, actinomycetes, mycorrhiza, and rhizobacteria (Bell et al,. 1998; Murphy & Cassells, 2000). This disrupts rhizosphere microbial balance, there by not favoring plant diseases. Parasitism, antibiosis, and induced resistance help beneficial organisms to outcompete pathogenic microbes (Daayf et al., 2003; Uppal et al., 2008). Assessing the changes due to the soil amendment with chitinous substrates on the rhizosphere microbiome (bacteria, fungi and eukarya) of the rice, will help to understand the contribution of such soil amendments in enhancing the microbial community in the rhizosphere.

1.4. Chitooligosaccharides act as elicitors and induce immune response in plants

Plants interact with a wide range of pathogens and have evolved mechanisms to recognize pathogen-derived molecules to elicit induced resistance. Unlike vertebrate animals, plants rely solely on innate immunity to ward off pathogenic microbes. The plants are able to sense evolutionarily conserved general elicitors of pathogens called pathogen-associated molecular patterns (PAMPs), and activate immune responses, a process that is known as pathogen triggered immunity (PTI). Chitooligosaccharides (COS), released during plant fungal interaction, elicit plant defense upon recognition by their cognate receptors.

1.4.1. Mechanism of COS-induced defense in plants

Lysine motif (LysM) receptor-like kinases (LYKs) or LysM RLPs in plants (LYPs) recognize COS as PAMPs (Fig 1.3). Rice chitin elicitor binding protein (CEBiP) was the first COS receptor (Kaku et al., 2006) identified. Subsequently, rice chitin elicitor receptor kinase 1 (*Os*CERK1; a LYK), *Os*LYP4, and *Os*LYP6 were discovered and their roles in chitin perception were revealed (Liu et al. 2012; Shimizu et al. 2010). While *At*CERK1 was required for chitin-triggered immunity in *Arabidopsis thaliana* (Miya et al. 2007; Wan et al. 2008), *At*LYK4 played an auxiliary function (Wan et al., 2012). *Os*CEBiP homologs are found in maize, sorghum, and rice (Fliegmann et al., 2011). In barley, a homolog was functionally proven to contribute to fungal resistance (Tanaka et al., 2010).Although, rice and *Arabidopsis* perceive chitin similarly, but their downstream signaling mechanisms differ (Shinya et al., 2012); (Fig 1.3). *Os*CEBiP and *Os*CERK1 form hetero-oligomer complexes in rice with chitin (Shimizu et al., 2010). In *Arabidopsis*, *At*CERK1 homodimerization promotes downstream signaling (Liu et al., 2012b). Chitin oligomers (DP1-47 or 8) stabilize the *At*CERK1 dimer as bivalent ligands (Liu et al., 2012b; Willmann & Nürnberger 2012), indicating that longer chain COS can activate innate immunity. *At*CEBiP, an *Arabidopsis* homolog of *Os*CEBiP, is biochemically functional but not necessary for chitin-induced defensive signaling (Shinya et al., 2012). Rice and Arabidopsis differ in pathogen-signal perception because to monocot-dicot distinctions (Ortmann & Moerschbacher 2006; Paulert et al., 2010; Yamada et al., 2017).

Research showed that LYKs and LYPs perceive chitin oligomers, but the intracellular transmission signal(s) are yet to be identified (Fig 1.3). The kinase activity of *At*MPK3 and *At*MPK6 was strongly induced after chitin perception (Wan et al., 2004). Pharmacological inhibitors validated protein phosphatase involvement in chitin-mediated signaling (Zhang et al., 2002). Other MAPKs, substrates, and protein phosphatases in plant chitin-mediated defensive responses are unknown. COS-specific transcription factors are yet to be identified. Chitin treatment affected the expression of 118 defense-related transcription factor genes in *Arabidopsis*, including WRKYs, AP2-ERFs, MYBs, and zinc finger proteins (Libault et al., 2007; Miya et al., 2007). Ethylene-responsive element-binding factors were crucial to plant chitin-induced innate immunity (Son et al. 2012).

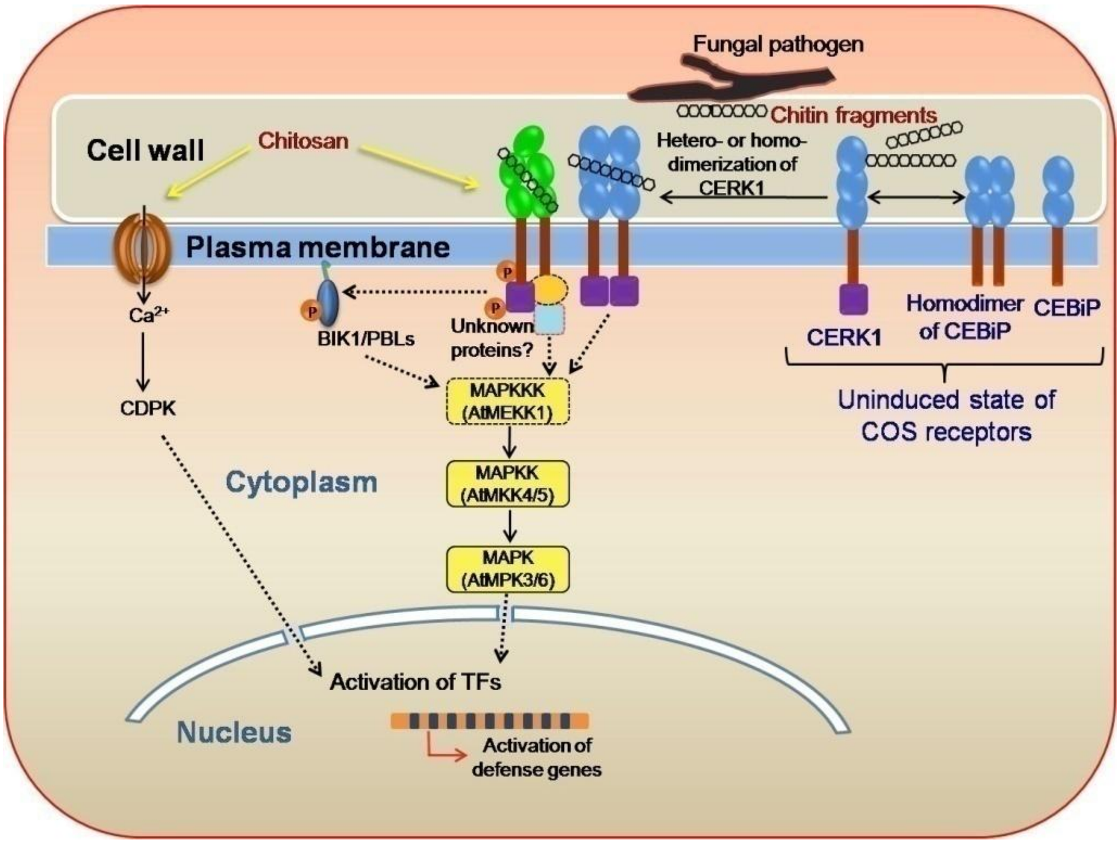


Fig 1.3: Model for COS-induced activation of chitin receptors and subsequent COS-signaling pathway in plants. During fungal infection, COS released from the fungal cell wall binds to membrane receptors: *At*CERK1 in Arabidopsis and *Os*CEBiP in rice harboring LysM domains. Binding of the ligands induces homodimerization of *At*CERK1 in Arabidopsis and hetero-oligomerization of *Os*CEBiP and *Os*CERK1 by interaction of their ectodomain in rice. The latter could form a protein complex by yet unknown proteins. Cytoplasmic receptor-like kinases, such as BIK1, could be a partner of the complex. Upon phosphorylation the BIK1 may get detached from the complex and activate other cytoplasmic proteins in a way similar to the flg22-mediated signaling (Zhang & Zhou, 2010). Activation of the complex leads to activation of a MAPK cascade which in turn phosphorylate transcription factors that regulate chitin-responsive genes. Partially deacetylated chitosan can bind to *At*CERK1 and activate the defense genes in addition to their effect on membrane destabilization. Dotted arrow and dotted circle/square denote unknown physical interaction and unidentified hypothetical molecules, respectively. Abbreviations: CDPK, calcium-dependent protein kinase; BIK1, Botrytis-induced kinase 1. (Source: Das et al., 2015)

1.5. COS production

Conventionally, the starting material for the production of COS is chitin or chitosan or fungal cell wall (Krairak & Arttisong 2007). It is attractive to use the renewable polysaccharide like chitin to generate COS to promote immunity and utilize for large-scale applications in agriculture. At present, lack of efficient large-scale production processes, high chemical synthesis costs, and lack of well-defined COS compositions limit topical application of COS. Currently COS is produced chemically. Enzymatic methods have also been used for COS production through a synthetic biology route.

1.5.1. Chemical methods

Mostly chemical methods are employed for COS production at industrial level. Acid hydrolysis was the most used industrial process for COS hydrolysis of chitin or chitosan (Thomas et al., 2015). Column chromatography fractionated polymeric chitin or chitosan after partial hydrolysis with strong hydrochloric acid. Acid hydrolysis yields lower DP COS, mostly DP1-DP4 (Jeon et al., 2000). Defaye et al., (1994) found that fluoro-hydrolysis of chitin in anhydrous hydrogen fluoride yields quantitative COS (DP 2 to 9) with DP2 and DP4 as main products. Mild acid degradation and sonolysis under ultrasound irradiation may generate COS with a high DP (Takahashi et al., 1995). Chemical hydrolysis provides more N-acetyl glucosamine than higher chain length COS (Uchida et al., 1989). Most COS synth-esis methods use GlcNAc or GlcN to produce homo-oligomers. Trombotto et al., (2008) prepared COS with different DAs from chitosan and concentrated HCl (12M) under appropriate hydrolysis conditions. With hydrolysate pH increased to 9, high DP (>15) COS precipitated. First ultrafiltration, then precipitation in pure ethanol reduced the yeild of low DP (<3) COS.

In the second phase, partial N-acetylation of the GlcN oligomer mixture in hydro-alcoholic solution generated DAs from 25 to 90%. This is the best regulated acetylation of chitosan oligomers. Low yields and time-consuming purification make such approaches unsuitable for large-scale and prolonged COS synthesis. The process may potentially yield hazardous chemicals, causing environmental pollution. Chemical synthesis of COS yields pure molecules, although most procedures are time-consuming, need a lot of organic solvents, and produce only homo-oligomers (Aam et al., (2010); Kuyama et al., (1993) synthesized fully deacetylated COS DP20 from GlcN monomers using phthalimido as the amino protecting group. The synthesis of chitotetraose and chitohexaose from GlcN monomers using dimethylmaleoyl as an amino protective group yielded completely N-acetylated COS (Aly et al., 2001).

1.5.2. Enzymatic methods

Topical application of COS for plant protection has not been realized due to the unavailability of a safer and economical procedure and a precise alternative bioprocess for large-scale production of high DP COS. Large-scale COS production would allow the use of synthetic COS of specified length and sequence as pathogen-associated molecular patterns (PAMPs) to stimulate plant immunity and growth. Enzyme-based approach can manufacture COS with appropriate DP and PA (pattern of acetylation) for an efficient and environmentally beneficial process. Chitinases (endochitinases and exochitinases), chitosanases, cellulases, lipases, pectinases, and lysozyme are used as biocatalysts (Table 1.4). The cost and availability of chitinases/chitosanases have limited the manufacturing of low DP COS, forcing the adoption of non-specific biocatalysts.

COS was made from crystalline alpha chitin and chitosan using crude *Lecanicillium fungicola* enzymes. Slightly acidified reaction media promoted the hydrolysis due to protonation of the amino groups. The enhanced electrostatic repulsion between these amino groups exposed the β-1, 4-glycosidic linkage of chitin, making it more vulnerable to enzyme recognition (Ramírez-Coutiño et al., 2006). A polygalacturonase-active *Aspergillus niger* pectinase isozyme generated COS with DP2-DP6 (Kittur et al., 2005). Endo-mode Pectinex produced 11 DP COS (Cabrera & Custem, 2005). (Zhang et al., 1999) reported COS with DP3-DP17 released by chitosan treated with cellulase, α-amylase, and proteinase. A commercial lipase degraded chitosan by cleaving glycosidic linkages in both endo and exo-mode, producing NAG and COS with DP-6 (Lee et al., 2008).

S.No	**Enzyme**	**Source**	**Substrate**	**Products (length in DP)**	**References**
1	Crude enzyme	*Lecanicillium fungicola*	Crystalline α-chitin and chitosan	NK	(Ramírez-Coutiño et al. 2006)
2	Pectinase isozyme	*Aspergillus niger*	Low molecular weight chitosan	2–6	(Kittur et al., 2003)
3	Pectinex Ultra Spl	Novozymes A/S, Bagsvaerd, Denmark	Chitosan	Up to 11	(Cabrera & Van Cutsem 2005;
4	Complex enzyme	Oriental reagent Co., China	Chitosan	3-17	(Zhang et al. 1999)
5	Lipase	Novozymes corp., China	chitosan	>=6	(Lee et al., 2008)
6	Pronase	*Streptomyces griseus*	Chitosan	2-6	(Vishu Kumar et al. 2005)
7	Papain	Sigma, USA	Chitosan	2-6	

NK-Not known

Table 1.4: Non-specific enzymes used in the production of COS with higher DP.

The specificity of chitosan-degrading enzymes has been studied conventionally by extensive enzymatic degradation of polymer, and subsequent isolation and characterization of the resulting oligomers. Enzymatic degradation of polymer and separation and characterization of oligomers have been used to study chitosan-degrading enzyme specificity. The degradation reaction kinetics changed the product profiles during hydrolysis (Brurberg et al., 1996). Reactions have multiphasic kinetics because enzymes have variable binding affinities for substrate sequences. The product mixtures at the conclusion of each phase differ greatly. Since most enzymes produce low DP COS mixtures at equilibrium, this is not conducive for commercial process development. Thus, neither chemical or biotechnological oligomerization nor physical, chemical, or enzymatical partial depolymerization can generate a variety of specified higher DP, bioactive COS needed for agricultural applications.

1.5.3. Transglycosylation (TG)

Due to the complexity of selective protection and manipulation of monosaccharide donors and acceptors, conventional chemical synthetic methods often fail to produce large amounts of oligosaccharides rapidly. Retaining glycosidases in transglycosylation (TG) processes can synthesise the glycosidic bond regioselectively and stereoselectively. Hydrolytic enzymes are run in reverse using activated glycosyl donors and acceptor alcohols, According to the mechanism, a glycosyl enzyme hydrolyzes a donor sugar with a retaining glycosidase. When this intermediate interacts with water, normal hydrolysis takes place. Glycosyl transfer, or TG, will occur if the glycosyl enzyme is blocked by an alcohol such a sugar, allowing for the formation of a new glycosidic bond while maintaining the stereochemistry (Williams & Withers 2000). Improvements in yields have been observed at reduced water concentration, performing TG reactions at higher concentrations and addition of surplus acceptor molecules.

Some glycosyl hydrolase (GH) family 18 chitinases catalyze TG, in addition to hydrolysis of chitin substrates. Since TG is kinetically regulated, efficient TG requires an enzyme with an active site layout that disfavors hydrolytic water molecule positioning and/or encourages carbohydrate molecule binding through strong contacts in positive subsites (Williams & Withers 2000). The fundamental issue with the GH that shows intrinsic TG activity of chitinase is that the outcome of the TG reaction is also serves as a substrate for the enzyme, which can reduce the yield of long chain COS. To improve TG activity and synthesize long chain COS with great biological potential, hydrolytic activity must be reduced. Chitinases can be mutated to lower hydrolytic activity. Enzyme changes should reduce hydrolytic activity and increase glycosyl donor binding in glycon subsites (Jahn et al. 2003).

Glucanase, amylase, cellulase, xylosidase, levan sucrase, dextran sucrase, and other GH enzymes that break down carbohydrates have both hydrolytic and TG activity. (Kim et al.

2000; Muzard et al. 2009). Bacteria, fungi, cycads, and plants have GH 18 family chitinases with TG (Neeraja et al., 2010; Ohnuma et al. 2011b); Purushotham & Podile 2012). Human macrophage chitotriosidase generated TG products up to DP 6–9 from DP5 (Aguilera et al. 2003). Table 1.5 List`s longer-chain COS-producing TG chitinases.

Enzyme	Source organism	Maximum length of TG products obtained	Duration of formation (min)	References
CrChi-A	*Cycas revolute*	DP9	30	(Taira et al. 2009)
NtChiV	*Nicotiana tabacum*	DP8	10	(Ohnuma et al., 2011a)
AtChiC	*Arabidopsis thaliana*	DP8	10	(Ohnuma et al,. 2011b)
AcMNPV chitinase	*Autographa californica* multiple polyhedran virus	DP8	10	(Fukamizo et al, 2011)
*Sp*ChiD	*Serratia proteamaculans 568*	DP13	45	(Purushotham and Podile 2012)
Chitinase A	*Vibrio carchariae*	DP6,DP8	30	(Suginta et al. 2005)
*Sp*ChiD mutants, M226A and Y228A	*Serratia proteamaculans* 568	DP9	360	(Madhuprakash et al. 2012)

Table 1.5: Details of transglycosylating chitinases from different biological sources (Source: (Das et al,. 2015a)

1.5.4. Enhancing TG for longer chain COS production

In an attempt to improve TG efficiency, specific mutations were introduced in ChiA and ChiB from *S. marcescens*, that were likely to disfavor correct positioning of the hydrolytic water molecule and/or favor binding of incoming carbohydrate molecules. These two family 18 chitinases have very little inherent TG for DP4 COS. The variants of ChiA and ChiB, ChiA-D313N, ChiA-D313N-F396W and ChiB-D142N, showed increased TG by forming COS with longer DP up to 8 (Zakariassen et al. 2011). Quantum mechanics/molecular mechanics have also showed that mutating Asp142 in ChiB to Asn leads to a change in active site electrostatics that could lead to lower hydrolyzability of the oxazoliniumion intermediate or an increased probability of the intermediate being attacked by an incoming sugar, perhaps due to effects on the catalytic water (Jitonnom et al., 2011). Mutation of Trp97 in the +1 subsite of ChiB and Phe396 in the +2 subsite of ChiA to Ala in *S. marcescens* led to reduced TG activity, probably due to reduced acceptor affinity. These results show that the aromatic residues that seem to determine the degree and direction of processivity also may co-determine to what extent the enzyme in question may yield TG products.

TG reaction, however, generates a mixture of long chain COS and may even synthesize large, water insoluble COS. To overcome such problems, Martinez et al., (2012) evaluated mutations on the catalytic amino acids of two family GH 18 chitinases, *Bacillus circulans* WL-12 chitinase A1 (*Bc*ChiA1) and *Trichoderma harzanium* chitinase 42 (*Th*Chit42). These mutated chitinases, where the catalytic machinery was disrupted enough to abolish hydrolytic activities, but still operational for TG reaction by providing oxazoline activated donor for synthesis of desired artificial COS, can be considered as novel ‘‘glycosynthases’’. Alteration of amino acid residues in the catalytic center, in the substrate-binding groove, and insolvent accessible regions of ChiD from *Serratia proteamaculans* (*Sp*ChiD) substantially improved the TG activity both in terms of increasing the quantity of TG products and in extending the duration of TG activity (Madhuprakash et al. 2012)

1.5.5. Enzyme-based bioprocesses for COS production

So far, no TG-based bioprocess has been described for large scale production of high DP COS. Initially, enzymatic hydrolysis was carried out in batch reactors, where chitosanase was mixed with it`s substrate and was allowed to break down glycosidic bonds of chitosan under optimum conditions (Vårum et al., 1996; Jeon & Kim 2000). However, this batch system had disadvantages such as low yields and higher cost`s associated with the use of large quantities of expensive chitosanase. The highest activity was observed when the chitosanase was immobilized on chitin rather than other carriers. The immobilized chitosanase showed a lower affinity and lower reaction rate compared to the free chitosanase. Subsequently, to improve the yield of COS, a system together with an ultrafiltration (UF) membrane reactor was developed to produce COS with relatively high DP (Jeon & Kim 2000).

However, even the UF membrane method did not allow continuous production of COS as there was increased trans-membrane pressure during the reaction due to high viscosity of the chitosan solution used as a substrate, and fouling of membrane by accumulated substrate. The enzymatic approach to generate soluble longer chain COS from chitin has even more limitations such as the crystallinity of the starting substrate, making direct usage of TG-active chitinases for the production of longer DP COS is difficult. The TG-based bioprocess may have to be combined with a pre-treatment of the starting material to break down it`s crystallinity. As an example, Matsuoka et al., (2000) described a continuous enzymatic degradation method where in N, N’-diacetyl chitobiose was prepared from colloidal rather than crystalline chitin in dialysis tubing and a chitinase recycling system.

1.6. Rationale of the study

With increasing costs of chemical fertilizers and growing environmental concerns with the excessive use of chemical fertilizers, the management of nutrients on farms has come under close scrutiny. The organic amendments on the other hand can improve soil structure, water retention capacity of the soils, and increase the carbon stocks. Little is known about how the organic amendments affect soil bacterial community composition and function. There are indications that organic additions like chitin improve soil and substrate quality, plant development, plant resistance and also boost beneficial bacteria involved in plant defense and growth (Hadrami et al., 2010). Chitin has been used to enhance beneficial plant-microbe interactions, particularly symbiotic legume-Rhizobium interaction (Brown et al. 1995). Use of chitosan treated tomato seeds show accelerated germination and produced highly vigorous seedlings and similar effect was also observed in wheat and rice (Ramirez et al., 2010). Against this background, we studied the effect of chitinous substrates amendments to rice, through a detailed analysis of rhizosphere microbiome.

COS released during plant-fungal interactions, induce plant defenses upon recognition. The COS functions as a vaccine with a wide protective range against a number of plant diseases, which makes them possible candidates for crop protection. (Yin, Zhao & Du 2010). Treatment of plants or cell suspension cultures with COS causes defense reactions like the production of ROS, temporary depolarization of membranes, extracellular alkalization, ion flow, and the production of phytoalexin (Kishimoto et al., 2010; Li et al., 2016; Madhuprakash et al., 2015; Basa et al., 2020). COS with DP>4 show biological activity. Despite their biological and agronomic interest, well-structured COS are inaccessible. Owing to the cost limitations in producing the COS from chitin through acid hydrolysis, alkaline hydrolysis, chemical synthesis, and oligosaccharide mixture generated using microbial source or by enzymatic hydrolysis, we have optimized an enzymatic process, to use the improved transglycosylating *Sp*ChiD (Madhuprakash et al., 2018). The long chain COS thus generated in the process were applied on rice crop to see the beneficial effects including defense response, growth and yield in field grown rice.

Questions raised in this study:-

- What type of microbiome changes occur in the rice rhizosphere with chitinous substrate amendments?
- How to scale up the process to produce mg quantities of higher DP COS?
- What are the suitable methods for testing the elicitor activity of COS?
- Will the COS application in rice crop enhance immunity and increase yield?

To address above questions, following approaches were tested in rice:

1. Application of chitinous substrates in rice crop under field conditions as three different soil amendments, namely chitin (alpha and beta variants) and chitosan (deacetylated chitin), applied to rice rhizosphere and examine for community dynamics of bacteria, fungi, and eukarya during crop development stages.
2. A hypertransglycosylating chitinase mutant *Sp*ChiD-Y28A was utilised to produce COS with DP6 and DP7 and applied the COS on rice.

Chapter - II

Materials and Methods

2. Materials and Methods

2.1.1. Assessment of chitinous substrates as soil amendments in a field experiment

Rice (*Oryza sativa* cv. Zordar Variety-NP9311) seeds were obtained from Nuziveedu Seeds Pvt Ltd (NSL), Hyderabad, Telangana, India for this study. This is a short-duration variety that matures in 125-130 days. In September 2019, seeds were germinated in a nursery and transplanted to field plots (strip plots of 2ft X 2ft size). Field experiments were carried out at NSL experimental field stations at Girmapur village, Medchal (Dt.), Telangana, India.

Chitinous substrates were procured from Mahtani Chitosan Pvt Ltd, Gujarat, India. Soil amendments were made using alpha-chitin, beta-chitin, chitosan, and bio-fertilizer-Azo-powder consisting of *Azotobacter* (KN Biosciences Pvt Ltd, Hyderabad). Chitinous substrates were tested at two different dosages (low-50mg/kg and high-100mg/kg of soil) and for biofertilizer, recommended dosage (RD) 5×10^7 CFU (1g/kg of soil) and ½ RD of biofertilizer 5×10^7 CFU (0.5g/kg of soil) as per manufacturer instructions were amended to soil and mixed well in the designated plot before the transplantation of rice seedlings. Control plot, with no amendments were also maintained. Other recommended management practices, such as weeding, fertilizer application and plant protection were uniformly adopted for all the experimental plots.

Plants were harvested at two developmental stages, viz. vegetative stage (one month after transplantation) and flowering stage (three months after seedling emergence). Uprooted plants were briefly shaken to remove loosely attached soil. The soil tightly bound to the roots was collected without damaging the roots by vortexing and centrifugation at 1500 x *g* for 10 min to yield the ‘rhizosphere’ fraction. Soil collected from empty plot is the bulk soil fraction. All samples were stored in deep freezer (-20^{O}C) until further processing.

2.1.2. Metagenomic DNA extraction, polymerase chain reaction (PCR) and nucleic acid sequencing

Metagenomic DNA was extracted from the bulk soil, rhizosphere (0.2-0.5 g for each) using NucleoSpin® Soil Kit (Machery Nagel, Germany). The soil samples with high humic and fulvic acids and less DNA yield, were handled using the protocol from Sarma et al., (2012). PCR was carried out for V4 hypervariable region of the bacterial 16S rRNA gene using 505F/806R primer pair (Caporaso et al., 2011), ITS1F-ITS2R (White et al., 1990) of fungi and 18S primer pair 1427F &1616R (Hannen et al., 1998) for eukarya. PCR mixture consisted of Q5 high fidelity DNA polymerase (New England Biolabs, USA), 0.2ul; 5X reaction buffer 4µl (New England Biolabs, USA); dinucleotide triphosphates (dNTPs), 0.4µl, primers 1µl of each; template DNA 1.5µl of 5ng/µl; and H_2O to 20µl. PCR conditions are represented in Table 2.1.

Steps	16S rRNA		ITS		18S	
Initial denaturation	98°C for 1 min		98°C for 1 min		98°C for 1 min	
Denauration	98°C for 30s	35 cycles	98°C for 30s	35 cycles	98°C for 30s	35 cycles
Annealing	57°C for 30s		52°C for 30s		55°C for 30s	
Extension	72°C for 45s		72°C for 60s		72°C for 60s	
Final extension	72°C for 7 min		72°C for 7 min		72°C for 7 min	

Table 2.1: PCR conditions for 16SrRNA (bacteria), ITS (fungi) and 18S (eukarya) genes amplification.

Each DNA sample was amplified in triplicate (a replicate of 20µl reactions each) and pooled products from each sample to a single volume (60µl) and observed for desired amplicons on agarose gels, followed by purification using a PCR clean-up kit (Machery Nagel, Germany) for 96 well PCR plates and DNA quality was checked using Nanodrop© (Thermofisher, USA) with A260/280 ratios between 1.8-2.0. Samples were pooled and submitted for sequencing on Illumina MiSeq platform using V3 chemistry of 300PE run at M/s. Molecular Research DNA Laboratory in Texas, USA.

2.1.3. Microbiome data analysis using bioinformatics and statistical tools

2.1.3.1. Processing of sequencing data

Initial quality filter and alignment of reads were done using Usearch 10 fastq_merge pairs with fastq_maxee using an EE score of 1. After barcode removal, only reads of the required length were used for further analysis. Plant chloroplast and mitochondrial reads were filtered using a custom-made Bash script similar to the procedure used by Chalasani et al., (2021). Reads were binned into zero-radius Operational Taxonomic Units (zOTUs), including chimera deletion according to the Usearch10 pipeline with Unoise3 (Edgar 2016). Bacterial and fungal zOTUs were annotated using the SILVA SSU132 16S rRNA database (Quast et al. 2012) and the NCBI Taxonomy database, respectively.

2.1.3.2. Bioinformatic analysis of microbiome (marker gene) data

Comprehensive statistical, visual, and comparative analysis of microbiome data, including diversity analysis, community profiling, and visualization of the data along with graphical representation, were done using MicrobiomeAnalyst (Chong et al., 2020) an R-based online tool (https://www.microbiomeanalyst.ca/). The data were filtered for low count, low variance and normalized by cumulative sum scaling for the marker gene (16S rRNA gene for bacteria and ITS gene for fungi, 18S for eukaya) analysis. Data rarefaction or transformations were not performed. The R code (Bash) used for analysis of microbiome data is enclosed as Annexure I at the end of the thesis.

2.1.3.3. Visual exploration of taxa abundance

The taxonomic composition of microbial communities in the metagenomic DNA samples were visualized through the direct quantitative comparison of abundances. This relative abundance of taxonomic composition was visualized at the level of phylum and genus, using stacked bar plots.

2.1.3.4. Alpha diversity analysis

Alpha diversity analyses were performed using the phyloseq package (McMurdie & Holmes 2013). Diversity within a sample or community is measured by Microbiome-Analyst. Shannon index was used to measure the alpha-diversity (both species richness and evenness) with the statistical method set to Mann–Whitney/Kruskal–Wallis (non-parametric tests) for significance testing (Kruskal & Wallis 1952). The Mann–Whitney-U test was used to compare two groups (Mann & Whitney 1947), while the Kruskal–Wallis H test compared more than two groups. The alpha-diversity measures were visualized as boxplots for each sample group or experimental factor.

2.1.3.4. Beta diversity analysis

Beta diversity analyses were performed using the ‘phyloseq’ package (Murdie & Holmes, 2013) of Microbiome-Analyst to compare the diversity or composition between two samples or microbial communities. For beta diversity assessment among the samples, Bray–Curtis similarity (97% DNA identity) indices were calculated and visualized as two-dimensional (2-D) Principal Coordinates Analysis (PCoA) plots. To test the statistical significance of the clustering pattern in PCoA plots, Permutational multivariate analysis of variance (PERMANOVA) was used.

2.1.3.5. Core microbiome analysis

Core microbiome analysis was performed with Microbiome Analyst, based on the core function in R-package microbiome. The core taxa (phyla and genera) that remain unchanged in their composition across the whole microbial community were visualized in the form of heatmaps of compositional (relative) abundance.

2.1.3.6. Biomarker identification and classification

The unique and/or predictive features (biomarkers) were identified and classified using Linear Discriminant Analysis (LDA) Effect Size (LEfSe) and Random Forests (RF) analysis. For LEfSe, the non-parametric factorial Kruskal–Wallis sum-rank test was performed to detect features with significant differential abundance with respect to the class of interest, followed by LDA to estimate the relevance or effect size of differentially abundant features 390. Features were considered to be significant based on their false discovery rate (FDR)-adjusted p-value ($p<0.05$) and log LDA score cut-off of 2 (Segata et al. 2011). Random Forests (RF) analysis, a classification algorithm approach based upon a collection of unpruned decision trees (classification trees or forests, each built using a bootstrap sample of training data using a randomly selected subset of OTUs), was performed using the Random Forest package (Liaw & Wiener 2002) of Microbiome-Analyst to identify the most indicative microorganisms characterizing each of the microbial communities. The RF classifier was built by growing 5,000 trees, and the class prediction was performed based on the majority vote of the individual trees. Each RF model is validated with the estimation of the classification (out-of-bag [OOB]) error (Bylander 2002). The percent mean reduction in accuracy of the value matrix was used to determine the top 15 taxa (at the genus level) that were most predictive of each microbiome assemblage.

2.1.3.7. Statistical analysis

PERMANOVA (Anderson and Braak 2003), unconstrained PCoA, and analysis of similarity (ANOSIM) (Clarke, 1993) were based on Bray–Curtis similarity matrices (Bray and Curtis 1957) and calculated from standardized, square-root transformed abundance data and calculated and/or visualized in Primer v7 software (PRIMER-E; Quest Research Ltd., Auckland, New Zealand). Factors influencing the microbial community were statistically assessed using permutation of residuals under a reduced model, the sum of squares type III (partial) with 9,999 permutations using unrestricted permutation of the raw data model of PERMANOVA. We considered pseudo-F values as proxies of a given factor's importance for sample separation and are based on the beta-diversity ratio (difference between two or more sample groups) to alpha-diversity (difference between individual samples within each group). The pseudo-F values for each set of factors were plotted and visualized in Prism 9 (GraphPad, San Diego, USA). PCoA plots are designed to visualize distance matrices with maximum sample separation along multiple axes (however, for clarity, only the first two axis are shown) without prior factorial description. Difference (the ratio of beta- to alpha-diversity) between each set of data for a particular factor is calculated using one-way ANOSIM tests.

2.2. Production of COS

Expression of the mutant chitinase *Sp*ChiD-Y28A, its purification and the transglycosylation (TG) were essentially same as described by Ramakrishna et al. (2021). Similarly, testing of the COS on rice seedlings and the assay of the enzymes involved in plant strengthening including defense related enzymes and qRT-PCR analyses of the plant genes were also described in detail by Ramakrishna et al. (2021).

2.2.1 Bacterial strains, plasmids, and chemicals

The plasmid pET-22b (+) and *Escherichia coli* strain BL21 (DE3) (Novagen, Madison, USA) were used for the heterologous expression of *Sp*ChiD-Y28A. Ampicillin, Isopropyl-β-D-1-thiogalacto-pyranoside (IPTG), and all other chemicals were purchased from Calbiochem or Merck (Darmstadt, Germany). Ni-NTA-His bound resin was procured from Novagen (Madison, USA) for protein purification. COS with DP5 was obtained from Bio-Base Europe Pilot Plant (BBEPP) (Desteldonk, Belgium) as a part of the European Union's Nano3Bio consortium project.

2.2.2 Expression and purification of *Sp*ChiD-Y28A

BL21 (DE3) cells (*E. coli* strain) harboring the plasmid pET-22b (+) – *Sp*ChiD-Y28A was grown in of LB broth containing ampicillin (100μg mL^{-1}) at 37°C to a cell density of 0.5 OD at 600 nm. The temperature was lowered to 18°C and protein expression was induced by the addition of 0.5 mM IPTG for 24 h. Cells were then collected by centrifugation at 10000×*g* for 10 min at 4°C followed by cell lysis using sonication. After centrifugation at 25000×*g* for 30 min at 4°C, *Sp*ChiD-Y28A was purified using Ni-NTA affinity chromatography and eluted as described earlier (Purushotham & Podile 2012). 12% SDS-PAGE was used to analyse fractions containing *Sp*ChiD-Y28A. The fractions were collected, concentrated, and buffer exchanged with 20 mM potassium phosphate buffer, pH 8.0, and quantified by BCA protein assay kit.

2.2.3. Analysis by thin-layer chromatography (TLC) and high-pressure liquid chromatography HPLC

Aliquots (20µl) of the reaction mixtures were chromatographed on a silica gel plate (TLC Silica Gel60; MerckCo., Germany) with methanol, n-butanol, 25% ammonia solution, and water (5:4:2:1 [vol/vol/vol/vol]). The products were detected by spraying the plate with aniline-diphenylamine reagent (aniline - 400µl, diphenylamine - 400mg, acetone 20ml, 85% phosphoric acid- 3ml) and baking it at 180°C using a hot air gun (Black & Decker, Idstein, Germany) for 3 min.

For high-pressure liquid chromatography (HPLC), aliquots (20µl) of the reaction mixture sample preparation and analysis of reaction products formed by *Sp*ChiD from DP5 substrate were same as described for TLC analysis. However, various concentrations of *Sp*ChiD (560 nM unless stated otherwise) and COS (3.5 mM unless stated otherwise) were used for HPLC analysis. 75µl of the reaction mixture was transferred to an eppendorf tube containing an equivalent volume of 70% acetonitrile to stop the reaction. Using a Hamilton syringe, twenty microliters of the reaction mixture were injected into an HPLC (Shimadzu, Tokyo, Japan) fitted with a Shodex-Asahipack NH2P-50 4E column (4.6mm (id) by 250 mm; Showa Denko K.K) (Hamilton Bonaduz, Switzerland). At 25°C, reaction mixtures were examined. The flow rate was set to 0.7 ml/min, the mobile phase was composed of 67% acetonitrile and 33% MilliQ H_2O, and the eluted COS were monitored at 210 nm. For the creation of standard graphs, a COS HPLC mixture containing DP1 through DP6 oligomers in equal amounts was utilized. Separate calibration curves of COS were developed for each oligosaccharide. These data points revealed a line with r curve for each standard sugar with r2 values ranging from 0.997 to 1.0, enabling the accurate determination of COS molar concentrations.

2.2.3. Product analysis by MALDI –TOF-MS

Matrix-assisted laser desorption ionization-time of flight mass spectrometry was used to examine reaction mixture/ purified COS (MALDI-TOF-MS). Sample (40µl) was concentrated under reduced pressure at 25°C until the solvent was completely evaporated and dissolved in 4µl of HPLC- grade MilliQ H_2O (Merck, Mumbai, India). 2µl of a 9 mg/ml mixture of 2, 5 –dihydroxy benzoic acid (DHB) in 30% acetonitrile were applied to a ground-steel target plate (MTP-384- TF; Bruker Daltonics, Bremen, Germany), and 2µl sample was mixed into the DHB droplet and dried under a stream of air before being analysed using Ultraflex MALDI-TOF/TOF (Bru-The Flex Control 3.0 software package was used to control the instrument in positive acquisition mode. All spectra were obtained in the positive ion mode using the reflectron mode with voltage of 26, acceleration voltage of 25kV, and a pulsed ion extraction time of 40 ns. The acquisition range was 50 to 3000 m/z. Bruker Flex analysis software was used to generate peak lists from the MS spectra (version 3.0).

2.2.4. Optimization of conditions for bulk preparation of COS

*Sp*ChiD Y28A, a hyper TG chitinase was chosen for the production of COS>5. Following optimizations were carried out before bulk reaction was set up to obtain more COS and avoid precipitation. Initially, 2ml reaction volumes were set up using *Sp*ChiD Y28A enzyme and following reaction components at were tested, Tris-HCl buffer (7.0- 9.0) at different pH range, NaCl addition (10-50 mM), addition of Bovine Serum Albumin –BSA (100ug, 200ug) and substrate concentrations DP5 (1mg/ml to 5mg/ml) Incubated at at 40˚C. A 25µl of reaction mixture was withdrawn at different time intervals up to 6h, followed by inactivation of enzyme by boiling at 85^{O}C for 15 min. HPLC quantification for TG products is performed as described earlier.To obtain higher chain length COS in good quantity bulk reaction was set up at 250 & 500 ml volume using *Sp*Chi D Y28A enzyme (6mg/ml) with 3mg/ml substrate (DP5) in 50 mM Tris-HCl buffer pH 7.0, 50mM

NaCl and 100µg/ml BSA at $37^{O}C$ for 90 min. Reaction was terminated by boiling the solution at $85^{O}C$ for 15 min. The products were quantified using HPLC.

2.2.5. Purification of COS by semi-preparative HPLC

COS with different degree of polymerization (DP) in the reaction mixture were purified by semi preparative HPLC (Shimadzu, Tokyo, Japan) equipped with SHODEX Asahipak NH2P-50-10E column (10 ID × 250 mm, Resonac Europe GmbH, Germany) with suitable guard column. Mobile phase (Acetonitrile and water at 67:33 (v/v)) at a flow rate of 0.7 mL/min was used. The COS eluted were monitored at 210 nm and collected using FRC-10A SHIMADZU fraction collector. Fractions having COS with DP5-7 were pooled separately and lyophilized to a powder form using Scanvac cool safe freeze dryer (Labogene, Denmark).

2.3. Elicitor treatments to rice plants under greenhouse conditions

Rice (*Oryza sativa* cv. BPT 5204) seeds were obtained from the Indian Institute of Rice Research (IIRR), Hyderabad, Telangana, India. Surface sterilization of seeds was carried out using 2% (v/v) sodium hypochlorite for 5 min followed by 5 times washing with deionized water. Sterilized seeds were soaked in distilled water for 24h in dark. Soaked seeds were germinated in petri plates lined with moistened filter paper and incubated at 27 ± 2°C in dark. Uniformly germinated seedlings were carefully transferred to 50 mL tubes (five seedlings per tube) containing half-strength Murashige and Skoog (MS) media. Seedlings were grown till the 3rd leaf stage in a growth chamber with 12h light (300 µmol $m^{-2}s^{-1}$, 25°C) and 12h dark (21°C) with 50% relative humidity. Half strength MS media solution was renewed every 3 days. Based on the mode of treatment, rice seedlings at the 3rd leaf stage were divided into two groups for root dip and foliar treatment, separately. COS with DP5-7 and SA (positive control) were used at a concentration of 10µg mL^{-1}. In the root dip treatment, respective test solutions were added to the hydroponic solution, and care was taken to ensure all roots were completely immersed. For foliar application, each tube was sprayed with test solutions thrice at an interval of 5 min, so that the leaves were drenched completely. After the treatment, the rice seedlings were harvested at different time points in liquid N_2 and stored at - 80°C till further processing.

2.4. Bioassays for elicitor activity

2.4.1. Hydrogen peroxide (H_2O_2) assay

The H_2O_2 levels were determined according to (Velikova et al., 2000). Rice seedlings (0.5 g) were homogenized in the ice bath with 5 mL 0.1% (w/v) TCA solution. The homogenate was centrifuged at 12000×*g* for 15 min. The supernatant (0.5 mL) was added to 0.5 mL of 10 mM potassium phosphate buffer, pH 7.0 and 1 mL of 1 M KI, and the absorbance was measured at 390 nm. The content of H_2O_2 was calculated based on the standard curve.

2.4.2. Phenylalanine ammonia lyase (PAL: EC 4.3.1.5) activity

The PAL activity was determined according to Lisker et al., (1983). Seedlings were homogenized in 25 mM sodium borate buffer, pH 8.8 containing 32 mM β-mercapto ethanol. The reaction mixture comprised 50 mM L-phenylalanine in 100 mM sodium borate buffer, pH 8.8 and 0.1 mL crude extract and incubated at 40◦C for 2h. The reaction was arrested by the addition of 80 µL of 5 N HCl. The absorbance at 290 nm was read against the same volume of the reaction mixture without L-phenylalanine. The enzyme activity was expressed as µmol of trans-cinnamic acid mg^{-1} protein h^{-1}.

2.4.3. Peroxidase (POD: EC 1.11.1.7) activity

The POD activity was measured as per the procedure of Putter (1974). Seedlings were homogenized in 5 mL of 0.1 M potassium phosphate buffer, pH 7.0 (with 0.1 mM EDTA, 1% PVP) and centrifuged at 12000×*g* for 15 min at 4°C. The supernatant was used as the enzyme source. The reaction mixture comprised 0.1 M potassium phosphate buffer, 18 mM guaiacol, and 8 mM hydrogen peroxide. Enzyme extract was added to initiate the reaction and recorded the rate of increase in absorbance at 436 nm. Enzyme activity was calculated using an extinction coefficient of 25.5 μM^{-1} cm^{-1}.

2.5. Total RNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from the shoot of COS treated rice seedlings using the NucleoSpin total RNA Kit (Macherey-Nagel, Germany). Approximately 2.0 µg of the purified RNA was reverse transcribed to cDNA using the cDNA synthesis kit (DSS Takara bio, India). Further, cDNA was used for amplification of few defense candidate genes of rice viz., plant innate immunity-related genes, Salycylic Acid (SA) biosynthesis genes (PAL1 and ICS1), SA signaling genes (EDS1 and PAD4), Mitogen-activated protein kinases (MAPK5a and MAPK6), Jasmonic acid (JA) biosynthesis genes (*JA carboxyl methyltransferase*-1 JMT1, Allene oxide synthase 2, AOS2 and), JA response gene (*JA-inducible Myb transcription factor*, JAMYB), transcription factor genes (NPR1, WRKY13, and WRKY45), pathogenesis-related genes (PR1a, PR1b, PR4, and PR10), and defense response genes. (Chitinase-*I*, peroxidase (POD22.3), *and* β *-1,3*-glucanase). The qRT-PCR experiments were carried out in an ABI-7500 Real Time PCR System (Applied Biosystems, USA) using SYBR Green Supermix Kit (DSS Takara bio, India). Respective primer sets for defense related genes (Table 2.2) mentioned above were procured commercially and used in this study (Kumari et al. 2016). PCR reaction mixture for each sample contained 5μL of SYBR Green PCR Master mix (DSS Takara, India), 0.2 μL of each primer, and 2μL of diluted cDNA in a total volume of 10 μL. The following cycling conditions were used: a hot start of 50°C for 20s, 95°C for 10 min, 95°C for 15s, annealing temperature 45–65°C for 30s, and 72°C for 30s. The rice ubiquitin gene was used as the internal control. The reactions were performed in triplicates. To determine the relative gene expression, mean Ct values were obtained, and fold change values were calculated using the 2-ΔΔCT method (Schmittgen & Livak, 2008). Results are shown as the mean of three technical replicates with their standard error. The significant difference between treatments with respect to controls and among the time points was determined using Turkey's post hoc multiple comparison test.

Gene	**Forward primer (5′-3′)**	**Reverse primer (5′-3′)**
Os18srRNA	CGCGCAAATTACCCAATCCTGACA	TCCCGAAGGCCAACGTAAATAGGA
Os-actin	CTCTCAGCACATTCCAGCAG	AGGAGGACGGCGATAACAG
OsMAPK5a	GTCTGCTCCGTGATGAAC	TGATGCCTATGATGTTCTCG
OsMAPK6	GATACATTCGCCAACTTCC	CAGTGATGCCAGGTAAGG
OsPAL1	TGTGCGTGCTTCTGCTGCTG	AGGGTGTTGATGCGCACGAG
OsICS1	TGTCCCCACAAAGGCATCCTGG	TGGCCCTCAACCTTTAAACATGCC
OsEDS1	CAGGAGAGGCAGTGTTAATCG	GCAAGCGGAGTAAGTGGTATG
OsPAD4	TCAGAGGCAAGGCAGTAGTG	ACCGCTCACGCAGGATAG
OsNPR1	AGAAGTCATTGCCTCCAG	ACATCGTCAGAGTCAAGG
OsAos2	GCGAGAGACGGAGAACCC	CGACGAGCAACAGCCTTC
OsJMT1	CACGGTCAGTCCAAAGATGA	CTCAACCGTTTTGGCAAACT
OsJAMYB	GAGGACCAGAGTGCAAAAGC	CATGGCATCCTTGAACCTCT

OsPR1a	AACTTCGTCGGCCAATCTC	CATGCATAAACACGTAGCATAGC
OsPR1b	TACGACTACGCCTCCAACA	CCGGCTTATAGTTGCATGTGA
OsPR10	ACGCCTAAGATGAAGAGGAATAC	CTCAAACGCCACGAGAATTTG
OsWRKY13	GCCAGCGGAGAACGAATC	CTCCTCCTGCTTCACAACC
OsWRKY45	AATTCGGTGGTCGTCAAGAA	AAGTAGGCCTTTGGGTGCTT
POX22.3	CAGGCAGCTAATCAGTAGTAG	ACCATGTCGGTTGCGTCGAG
*Os*Gns1	TACCGCTCCAACGGCATC	GGATGTTGTTCCGCACCC
*Os*Cht-1	TCGGCTCCAACCTGCTGA	CACTGCCCTGTCATCACCG
*Os*PR4	TGGGACCTGAACAAAGTGAGC	TGGATACACTTGCCACACGAG

Table 2.2: List of primers targeting for gene expression studies in rice plants treated with COS. Primer sets related to plant innate immunity-related genes, SA biosynthesis genes, SA signaling genes, JA biosynthesis genes, JA response gene, transcription factor genes, pathogenesis-related genes and plant defense response genes were used to monitor gene expression studies and elucidate the effect of COS treatments.

2.6. Purified COS treatment on rice

Rice (*O. sativa* cv. Zordar Variety-NP9311, Nuziveedu Seeds Pvt Ltd, Hyderabad) seeds used in the present study. Seeds were germinated in nursery and transplanted to the pots prepared for the experiment purpose in August 2018. All experiments were carried out at Nuziveedu rice experimental fields at Girmapur village, Medchal (dt), Telangana.

Different treatments with purified COS are as follows: T1) DP5, T2) DP6, T3) DP7, T4) Salicylic acid (positive control) concentrations of 10µg/ml (Low) and 20µg/ml (High) concentrations in triplicates. Seedlings were allowed to grow up to 4th leaf stage and each treatment was given as foliar spray for pots by dissolving each COS in distilled water mixed 0.01% tween 20 (surfactant) to ensure adsorption of test solution to leaves effectively. Negative control pots were sprayed with water alone. Mode of application for all above mentioned treatments is by foliar spray with fine mist (spraying bottle) until plant is completely drenched. Booster treatment was given by the same method during the flowering stage of crop following the same concentrations. After harvest, yield parameters like seeds per panicle, grain yield (total seed weight per panicle), and 100 seed weight were analyzed. Further data was analyzed by one way ANOVA and represented in results.

Results & Discussion

Chapter - III

Chitinous substrate amendments to rice – a microbiome approach

Chapter 3: Chitinous substrate amendments to rice –a microbiome approach

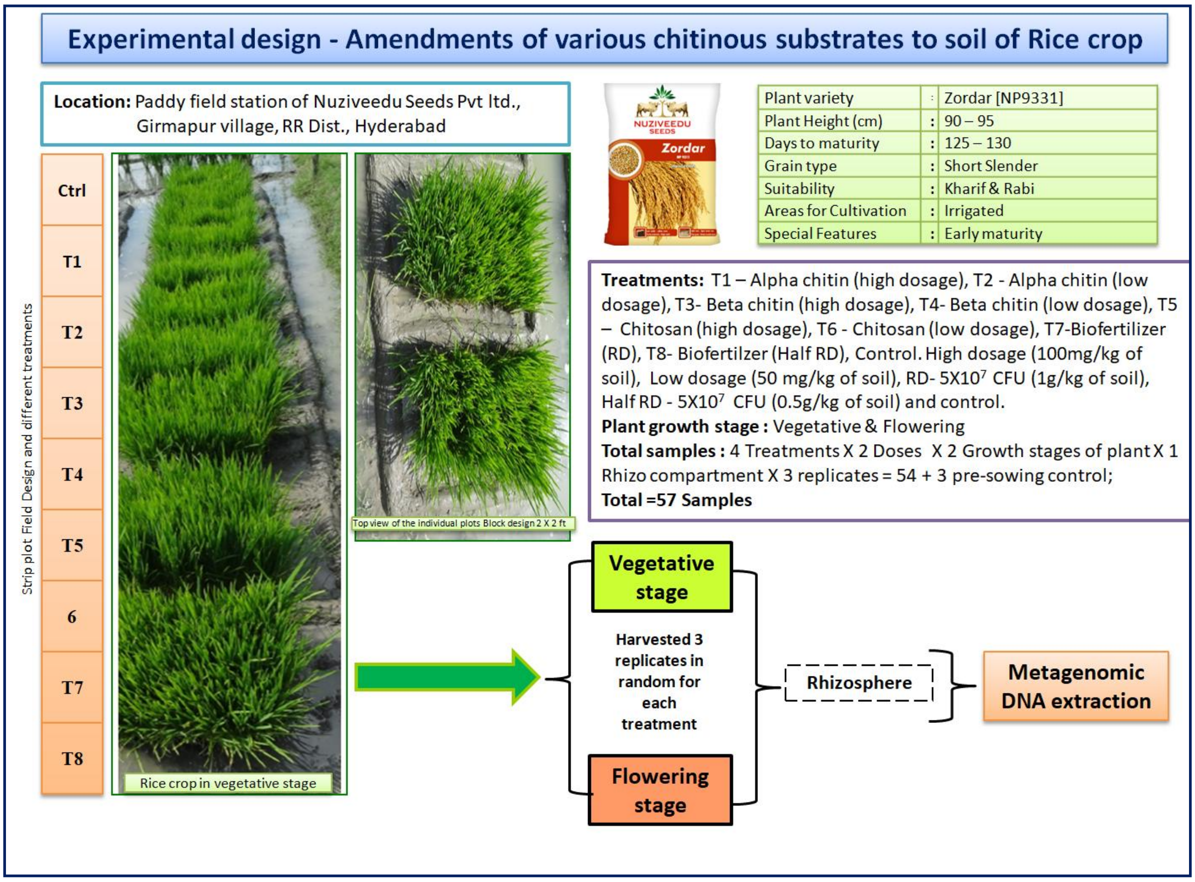
3. Introduction

Chitin is the second most abundant naturally occurring amino-polysaccharide, next only to cellulose that provides rigidity to the cell walls of the crustaceans, insects, and fungi due to its crystallinity. Chitosans can be derived from chitin by chemical or enzymatic deacetylation, which is relatively soluble. Several metric tons of chitin is extracted, through simple but harsh chemical processes from shrimp and crab shells. Chitosans are derived from chitin, primarily by chemical processes for industrial applications. Chitin and chitosans are relatively less expensive, naturally occurring biologically synthesized polymers, for a wide range of applications including agriculture.

Soil amendments with chitinous substrates enhanced plant defense and also contributed to better growth of crops. Extracellular chitinases are produced by chitinolytic microorganisms to hydrolyze chitin-rich tissues of other species. Chitinolytic organisms are pathogenic or parasites, but a majority are also saprotrophic/necrotrophic feeding on dead material or live in a mutualistic relationship with plants. Thus, chitinolytic microbes play a key role in plant-related ecosystem, particularly with reference to the health and nutrition of plants. The effect of the externally added chitin has been studied intensively on the microbial species that act as antagonists of pathogens causing diseases of plants. Manjula and Podile (2001) showed that chitin-supplemented formulations of *Bacillus subtilis* AF1 improved both the biocontrol and plant growth-promoting activities on pigeonpea and groundnut. Similarly, Kishore et al., (2005) showed improved control of *Phaeoisariopsis personata* (causes late leaf spot in groundnut), by a chitinoytic *Serratia marcescens* with chitin amendment. In addition to direct antibiosis, Kishore et al., (2005) found that, chitin supplementation increased the activity of key plant defense enzymes. The beneficial effect with chitin amendment, to the soil may promote the growth of antagonistic microbes. The complexity of the soil microbial system made it extremely difficult to precisely monitor the changes in the microbial populations, and lead to a reductionistic approach to monitor the population of culturable antagonists in such treatments.

Plant strengthening activities of chitin/chitosan as soil supplements, seed coating, and foliar spray were studied. Several plant species, including coffee (Dzung et al., 2011), soybean, mini-tomato, lettuce, and rice, have shown improved growth after being treated with chitosan (Chibu and Shibayama, 1999). Boonlertnirun et al., (2008) reported the use of polymeric chitosan to soak rice seeds before planting, followed by soil amendment that enhanced the yield. Adding shrimp waste derived chitin and/or chitosan to soil can temporarily improve the root growth and reduce the rate of nematode infection in tomato plant (Radwan et al., 2012). It was assumed that soil suppressiveness can be enhanced by chitin and or chitosan. While there could be a debate on the contribution of bacteria and fungi to the chitinolytic process in the soil, microcosm experiments demonstrated the role of particular members of the Gamma- and Beta proteobacteria were dominant after the addition of chitin (Keilac et al., 2013). Similarly, Das et al., (2010) reported that Gammaproteobacteria and *Bacilli* dominated chitin-enriched soils (dumping yards for shrimp waste).

In this study, an experiment was designed to monitor the microbiome changes with respect to chitin amendments to the soil, for rice crop, under field conditions. Soil amendment was made with alpha chitin, beta chitin, chitosan, biofertilizer for rice [KN Biosciences, Hyderabad] compared with negative control that received no treatment. Rhizosphere samples were collected at vegetative and flowering stages (Fig 3) for metagenomic DNA isolation. 16S rRNA gene, ITS and 18S rRNA genes were amplified and sequenced by Miseq Illumina sequencing at Mr.DNA technologies, Texas, USA. Data analyses using appropriate statistical tools were carried out from the sequencing data using a bioinformatic pipeline (Chalasani et al. 2021).



Experimental design - Amendments of various chitinous substrates to soil of Rice crop

Location: Paddy field station of Nuziveedu Seeds Pvt ltd., Girmapur village, RR Dist., Hyderabad

Plant variety	Zordar [NP9331]
Plant Height (cm)	90 – 95
Days to maturity	125 – 130
Grain type	Short Slender
Suitability	Kharif & Rabi
Areas for Cultivation	Irrigated
Special Features	Early maturity

Treatments: T1 – Alpha chitin (high dosage), T2 - Alpha chitin (low dosage), T3- Beta chitin (high dosage), T4- Beta chitin (low dosage), T5 – Chitosan (high dosage), T6 - Chitosan (low dosage), T7-Biofertilizer (RD), T8- Biofertilzer (Half RD), Control. High dosage (100mg/kg of soil), Low dosage (50 mg/kg of soil), RD- 5X10^7 CFU (1g/kg of soil), Half RD - 5X10^7 CFU (0.5g/kg of soil) and control.

Plant growth stage : Vegetative & Flowering

Total samples : 4 Treatments X 2 Doses X 2 Growth stages of plant X 1 Rhizo compartment X 3 replicates = 54 + 3 pre-sowing control;

Total =57 Samples

Fig 3: Experimental flowchart for chitin substrate amendments to rice crop.

3.1 Results

3.1.1. Influence of alpha chitin on rice rhizosphere bacteriome

Alpha chitin, at high dosage, during flowering stage has highest alpha-diversity (Shannon diversity index), followed by other treatments (Fig 3.1A). Using PCoA plots, the response of bacterial communities to alpha chitin was shown as separate clustering of sample groups without any overlaps (Fig 3.1B). PERMANOVA revealed substantial variations in zOTU assemblage between bacterial communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for bacterial communities in different fractions is 0. 818 (81.8%) (Fig 3.1C).

In all the treatments, the relative abundance of Proteobacteria is higher, including alpha chitin, biofertilizer, vegetative stage control, flowering stage control and pre-sowing control of rice rhizosphere (Fig 3.2A). Other dominant phyla include Chloroflexi, Myxococcota, Bacteroidota and Planctomycetota. Uncultured Sutterellaceae, Uncultured Planctomycetota, *Flavisolibacter*, Uncultured Chloroflexi, Uncultured Nitrosomonadaceae, Uncultured Anaerolineaceae, *Longilinea*, Uncultured Vicinamibacterales, Uncultured Steroidobacteraceae, Uncultured Burkholderiales, Uncultured Myxococcota and *Caenimonas* were top abundant genus in alpha chitin treatment (Fig 3.2B).

Core microbiome calculated using sample prevalence (10%) with relative abundance of 0.01 % for taxa at phylum and genus level is presented in Fig 3.3A and Fig 3.3B. Core microbiome at phylum level comprises of Proteobacteria, Planctomycetota, Myxococcota, Gemmatimonadota, Desulfobacterota, Chloroflexi, Bacteroidota, Acidobacteriota, Actinobacteriota, Crenarchaeota, Firmicutes, Verrucomicrobiota, Nitrospirota and Patescibacteria (Fig 3.3A). Uncultured Vicinamibacterales, Uncultured Sutterellaceae, Uncultured Steroidobacteraceae, Uncultured Planctomycetota, Uncultured Nitrosomonadaceae, Uncultured Gemmatimonadota, Uncultured Chloroflexi, Uncultured Anaerolineaceae, *Sphingomonas, Longilinea*, *Flavisolibacter* were top genus detected in core microbiome during Alpha chitin amendments to rice rhizosphere (Fig 3.3B).

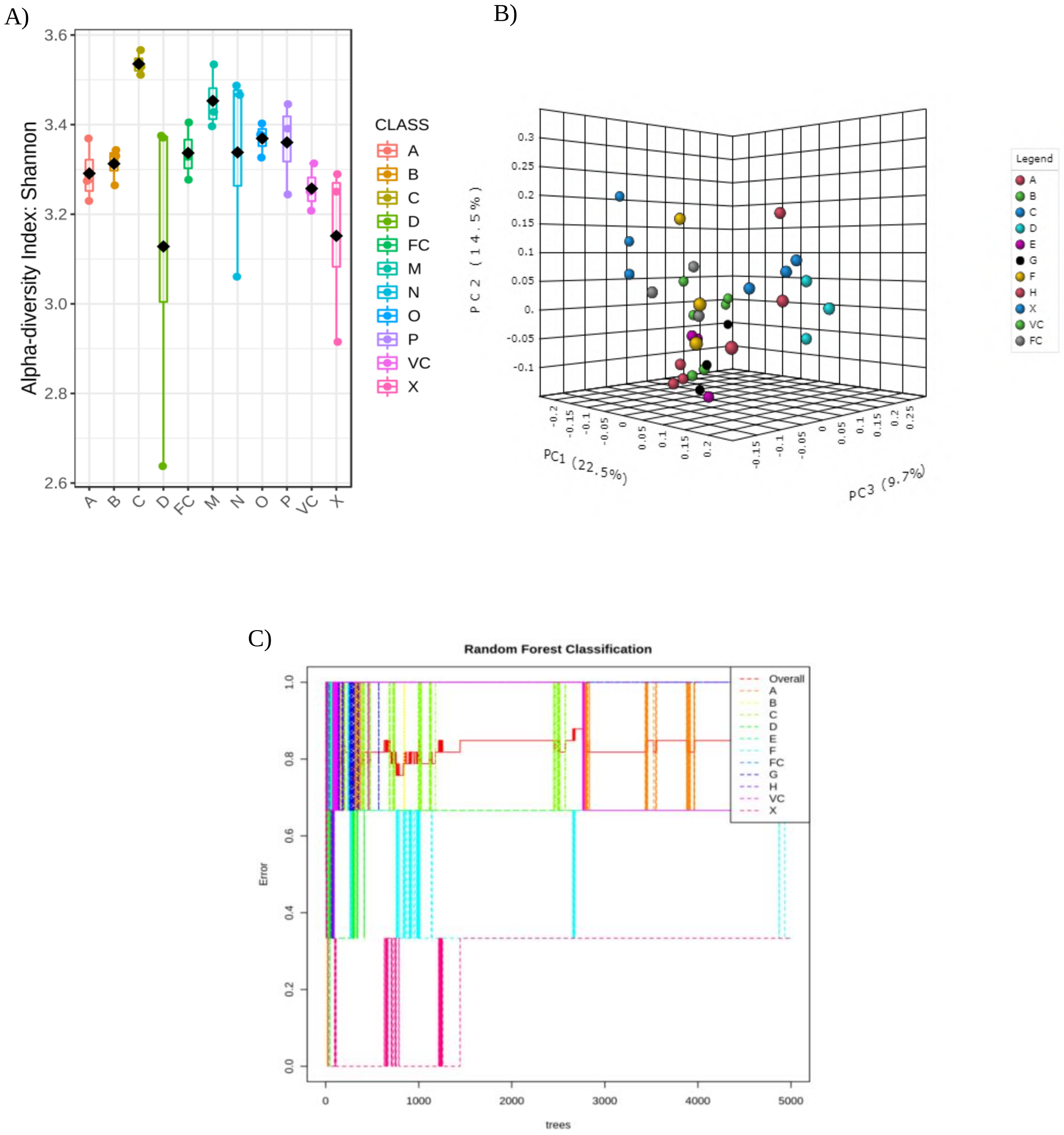


Fig 3.1: Influence of alpha chitin on rice rhizosphere for bacteriome. (A) Alpha-diversity measure using Shannon index at Genus level represented as box plots. Each box plot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.10253; [Kruskal-Wallis] statistic**:** 15.9. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [22. 5%] and the Y-axis representing the second-highest dimension of variation [9.7%] and Z- axis represents third highest degree of variation [9.7%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 3.1289; R^2**:** 0. 58716; *p* <0. 001. (C) Cumulative OOB error rates by RF classification. The overall error rate 81.8% (0.818) is shown as the red line; lines of other colors represent the error rates for each class. (Alpha Chitin-High Dosage-Vegetative – A, Alpha Chitin-Low Dosage-Vegetative – B, Alpha Chitin-High Dosage-Flowering – C, Alpha Chitin-Low Dosage-Flowering – D, Bio fertilizer-RD-Vegetative – E, Bio fertilizer-Half RD-Vegetative – F, Bio fertilizer-RD-Vegetative – G, Bio fertilizer-Half RD-Flowering – H, Pre treatment – X, Vegetative control – VC, Flowering control – FC).

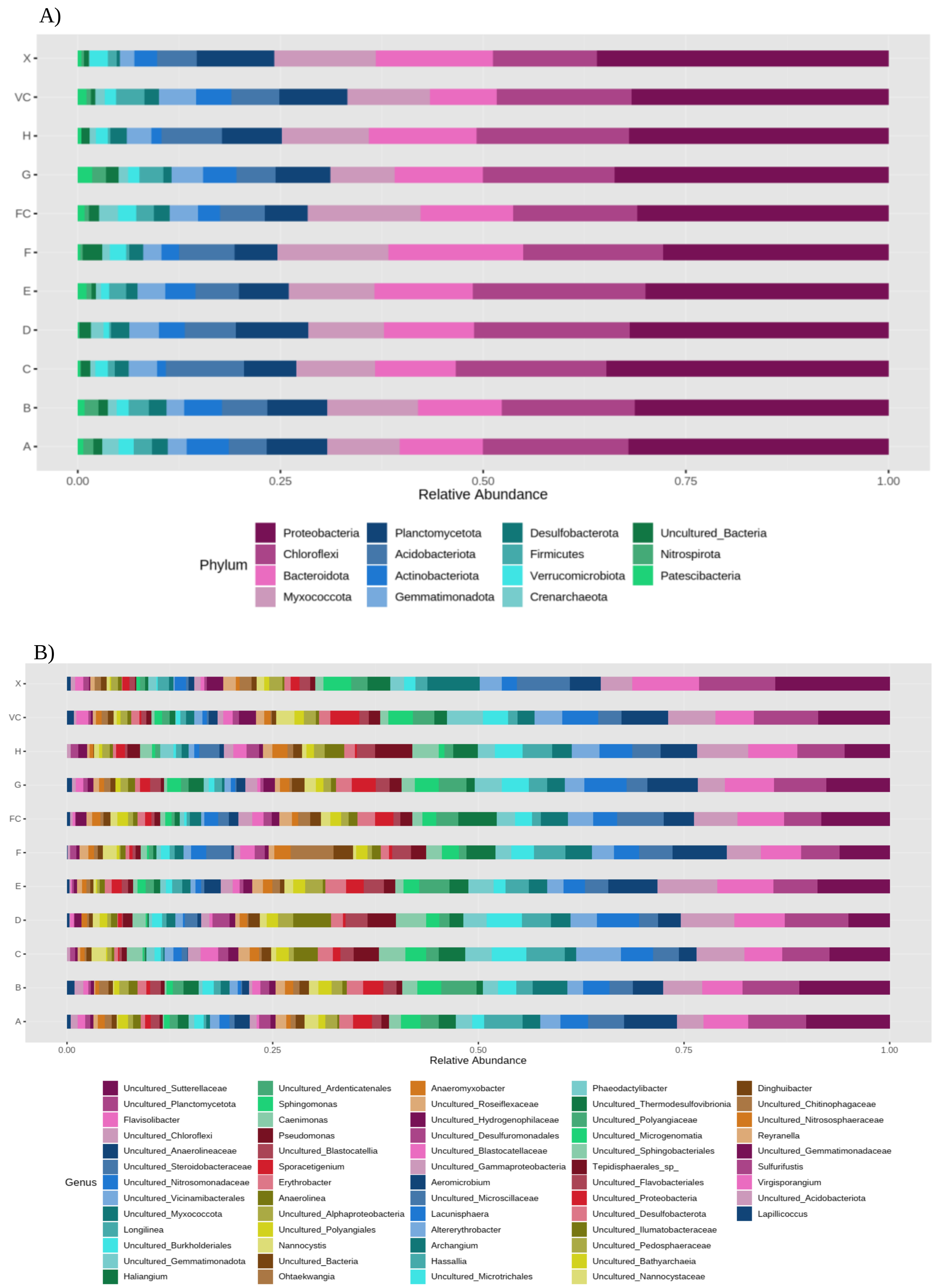


Fig 3.2: Influence of alpha chitin on the taxonomic composition of rice rhizosphere bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa at Phylum (A) and Genus level (B).‘Uncultured’ taxa labels in the figures represent unclassified bacterial taxa.(Alpha Chitin-High Dosage-Vegetative – A, Alpha Chitin-Low Dosage-Vegetative – B, Alpha Chitin-High Dosage-Flowering – C, Alpha Chitin-Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Bio fertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre treatment – X, Vegetative control – VC, Flowering control – FC).

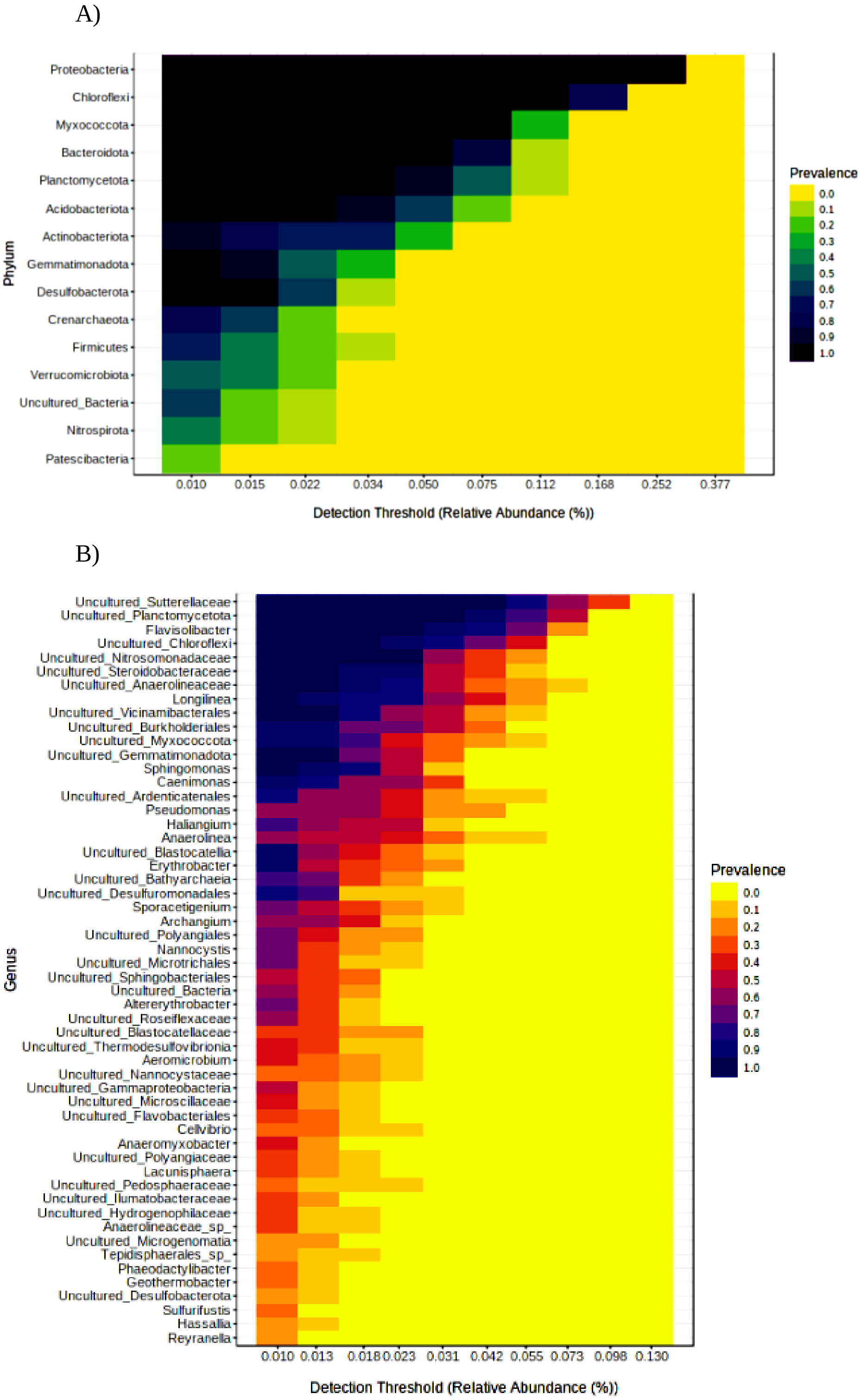


Fig 3.3: Heatmaps representing the core microbiome of alpha chitin on rice rhizosphere bacteriome at Phylum (A) and Genus levels (B). The Y-axis represents the prevalence level of core fungal taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased).

3.1.2. Influence of alpha chitin on rice rhizosphere mycobiome

Alpha chitin at high dosage has highest alpha-diversity (Shannon diversity index) at flowering stage, followed by other treatments (Fig 3.4A). Using PCoA plots, the response of fungal communities to alpha chitin treatment was shown as separate clustering of sample groups without any overlaps (Fig 3.4B). PERMANOVA revealed substantial variations in zOTU assemblage between fungal communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for fungal communities in different fractions is 0.545(54.5%) (Fig 3.4C). LEfSe analysis identified *Mortierella* and *Clonostachys* as the biomarkers associated with rice rhizosphere with alpha chitin amendment (Fig 3.4D).

The relative abundance of Uncultured Fungi was greater in alpha chitin followed by Ascomycota, Mucoromycota, Basidiomycota and Chytridiomycota of rice rhizosphere (Fig 3.5A). The abundant taxa include Uncultured Fungi, *Mortierella, Cordyceps, Hygroaster, Aspergillus, Sodiomyces alcalophilus, Clonostachys*, Uncultured Sordariaceae, *Metarhizi, Sebacina, Stachybotrys elegans, Kochiomyces, Pluteus*, Uncultured Talaromyces, *Fragosphaeria, Didymella, Rhizophlyctis, Chaetomium, Cephalotheca, Colletotrichum, Panaeolus*, Uncultured Tremellales, *Telasphaerula* and *Scedosporium* for in with alpha chitin treatement (Fig 3.5B).

Core microbiome was calculated using sample prevalence (10%) with relative abundance of 0.01 % for taxa at phylum and genus level and presented in Fig 3.6A, and Fig 3.6B. Core microbiome at phylum level comprised of Uncultured Fungi, Ascomycota, Mucoromycota, Basidiomycota, Chytridiomycota, Blastocladiomycota, Uncultured *Cladosporium*, Uncultured Cryptomycota, Uncultured Basidiomycota, Uncultured Ascomycota (Fig 3.6A).

The core microbiome at genus level consisted of Uncultured Fungi, *Cordyceps, Mortierella, Aspergillus, Sodiomyces alcalophilus, Metarhizi*, Uncultured Sordariaceae, *Clonostachys, Stachybotrys elegans, Sebacina, Rhizophlyctis, Pluteus, Fragosphaeria*, Uncultured Talaromyces, *Hygroaster, Chaetomium, Cephalotheca, Sodiomyces, Panaeolus, Nephromais, Kochiomyces, Gaertneriomyces, Didymella, Catenaria, Ascosphaerator* (Fig 3.6B).

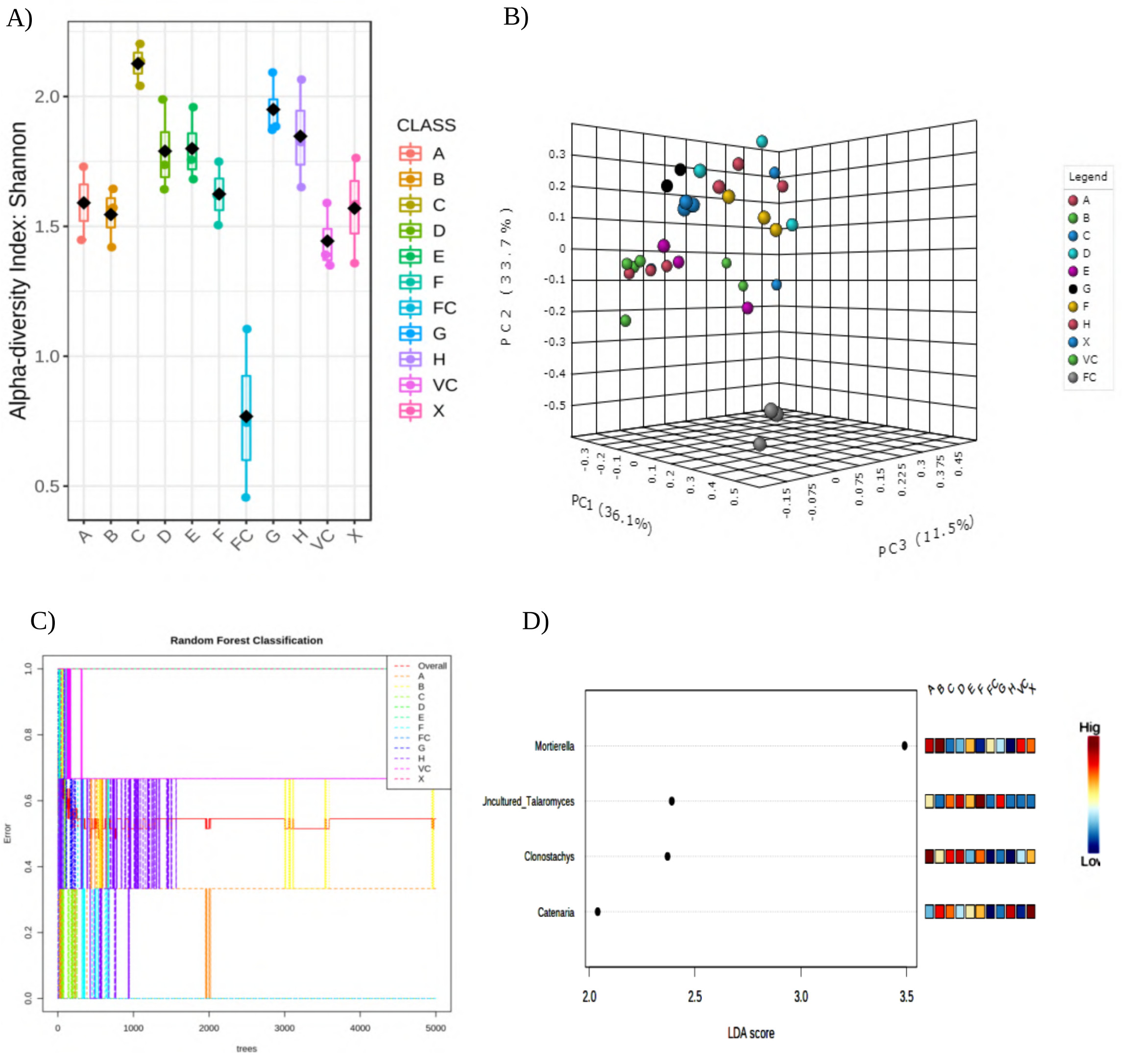


Fig 3.4: Influence of alpha chitin on rice rhizosphere mycobiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.00470; [Kruskal-Wallis] statistic**:** 25.362. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [36.1 %] and the Y-axis representing the second-highest dimension of variation [33.7%] and Z- axis represents third highest degree of variation [11.5%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 7.882; R^2**:** 0.78192; *p* <0. 001. (C) Cumulative OOB error rates by RF classification. The overall error rate 54.5% (0.545) is shown as the red line; lines of other colors represent the error rates for each class. D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off**:** 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. 'Uncultured_' taxa labels in the figures represent unclassified fungi taxa. (Alpha Chitin-High Dosage-Vegetative – A, Alpha Chitin-Low Dosage-Vegetative – B, Alpha Chitin-High Dosage-Flowering – C, Alpha Chitin-Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre-treatment – X, Vegetative control – VC, Flowering control – FC).

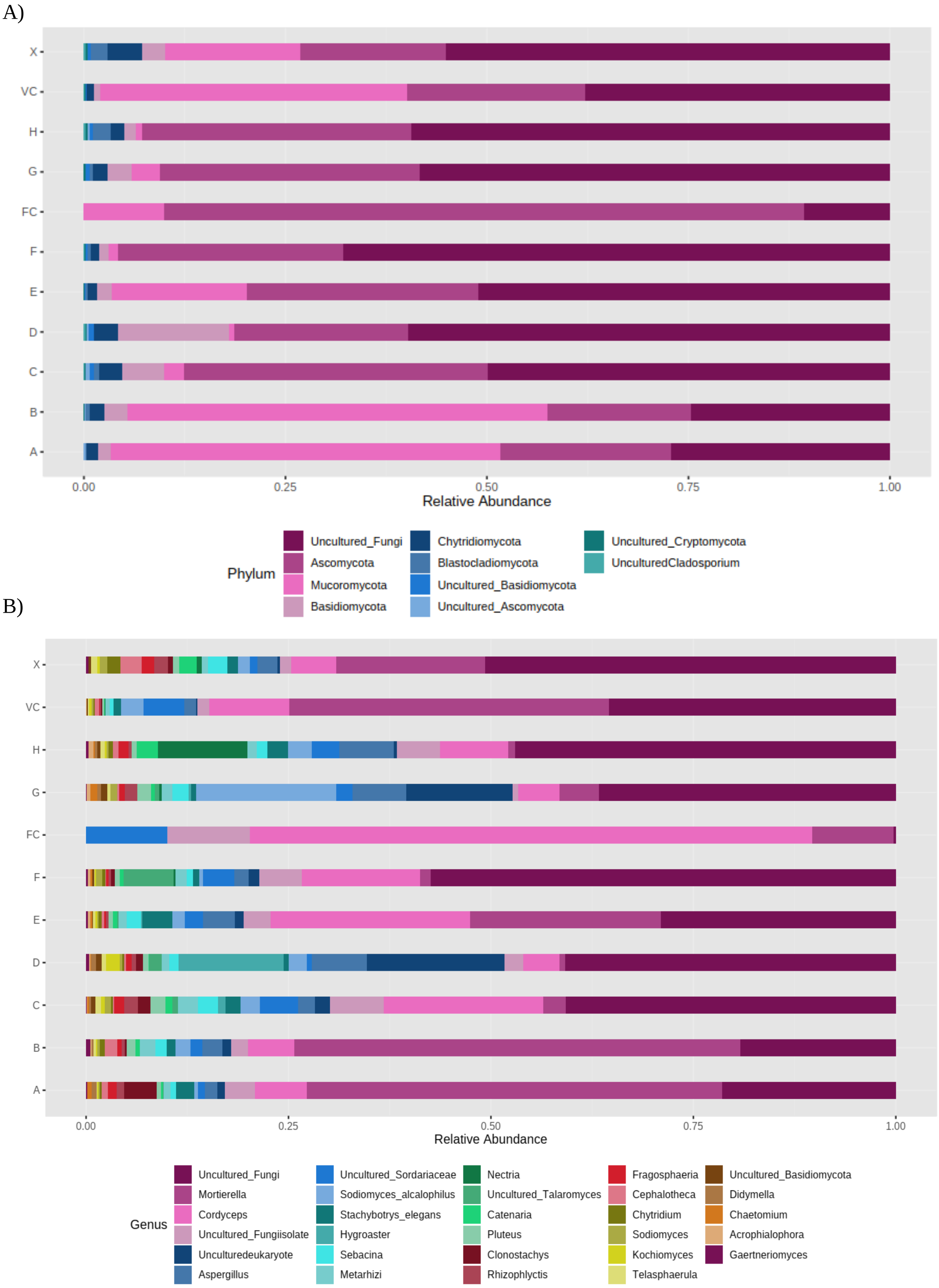


Fig 3.5: Influence of alpha chitin on the taxonomic composition rice rhizosphere fungal community. Stacked bar plots represent the relative abundance of fungal taxa at Phylum (A) and Genus level (B). ‘Uncultured’ taxa labels in the figures represent unclassified fungal taxa. (Alpha Chitin-High Dosage-Vegetative – A, Alpha Chitin-Low Dosage-Vegetative – B, Alpha Chitin-High Dosage-Flowering – C, Alpha Chitin-Low Dosage-Flowering – D, Bioertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre-treatment – X, Vegetative control – VC, Flowering control – FC).

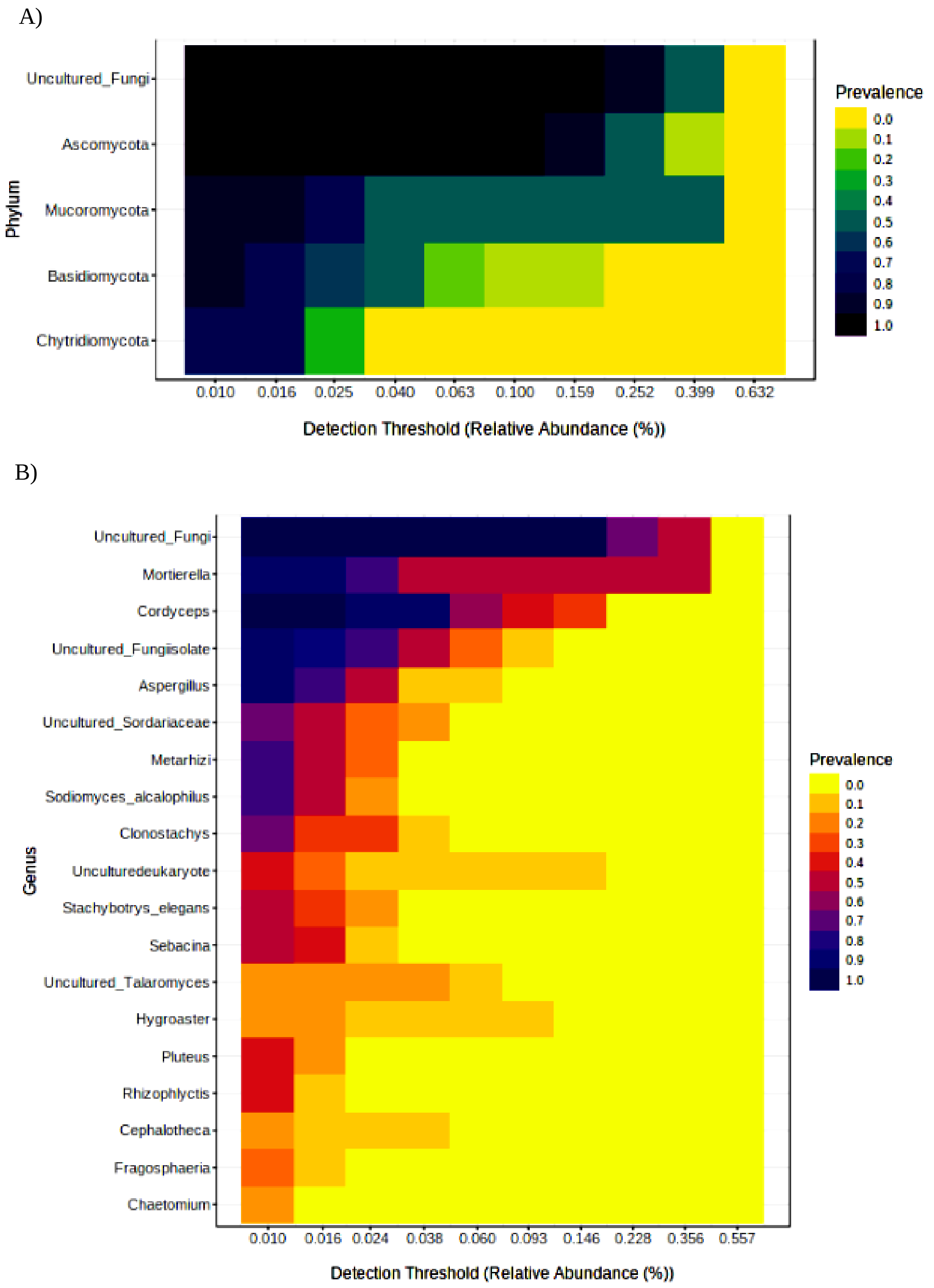


Fig 3.6: Heatmaps representing the core microbiome of alpha chitin on rice rhizosphere mycobiome at Phylum (A) and Genus levels (B). Y-axis represents the prevalence level of core fungal taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified fungal taxa.

3.1.3. Influence of alpha chitin on rice rhizosphere eukaryome

High dose of alpha chitin, at flowering stage, has highest alpha diversity (Shannon diversity index), followed by other treatments (Fig 3.7A). Using PCoA plots, the response of eukaryote communities to alpha chitin treatment was shown as separate clustering of sample groups without any overlaps (Fig 3.7B). PERMANOVA revealed substantial variations in zOTU assemblage between communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for eukaryote communities in different fractions is 0.212 (21.2%) (Fig 3.7C). LEfSe analysis identified *Cochliopodium* and Unclassified Bodonidae of eukaryota as the biomarkers associated with rice rhizosphere with alpha chitin amendment (Fig 3.7D).

The relative abundance of Uncultured Eukaryote, Amoebozoa, Fungi incertae sedis, Arthropoda, Discosea, Ciliophora, Euglenozoa, Apicomplexa were higher at phylum level (Fig 3.8A), where as top genus during alpha chitin to rice rhizosphere comprised of Uncultured Eukaryote, Uncultured Cryptomycota, *Cochliopodium, Tyrophagus putrescentiae, Eimeriidae,* Uncultured Micro Eukaryote, *Pseudoplatynematum denticulatum*, Protacanthamoeba, *Aristerostoma marinum*, Uncultured Rozellomycota, and *Neobodo saliens* (Fig 3.8B).

The core microbiome, calculated using sample prevalence (20%) with relative abundance of 0.01% for taxa, at phylum level and genus level is presented in Fig 3.9A and Fig 3.9B, respectively. The core microbiome at phylum level comprised of Uncultured Micro Eukaryote, Uncultured Eukaryote, Heterolobosea, Fungi incertae sedis, Euglenozoa, Discosea, Ciliophora, Arthropoda, Apicomplexa, Amoebozoa, Uncultured Fungi, Rozellomycota, Nematoda, Cercozoa, Tubulinea, Eukaryota incertae sedis, Ascomycota, Cnidaria, Oomycota, Hapista, Evosea, Bacillariophyta, Streptophyta, Platyhelminthes, Ochrophyta, Mollusca, Loukozoa (Fig 3.9A).

The core microbiome at genus level consisted of Uncultured Micro Eukaryote, Uncultured Eukaryote, Uncultured Cryptomycota, *Tyrophagus putrescentiae*, *Pseudoplatynematum denticulatum*, *Protacanthamoeba*, *Eimeriidae*, *Aristerostoma marinum*, Uncultured Rozellomycota, Uncultured Fungi, *Cochliopodium*, *Rhopalosiphum padi*, *Kuklikophrya ougandae*, *Acanthamoeba*, *Laimydorus*, Unclassified Bodonidae, *Neobodo saliens*, *Vexillifera armata*, *Vahlkampfia*, Uncultured Amoebozoa, *Phialina salinarum* and Cercomonas (Fig 3.9B).

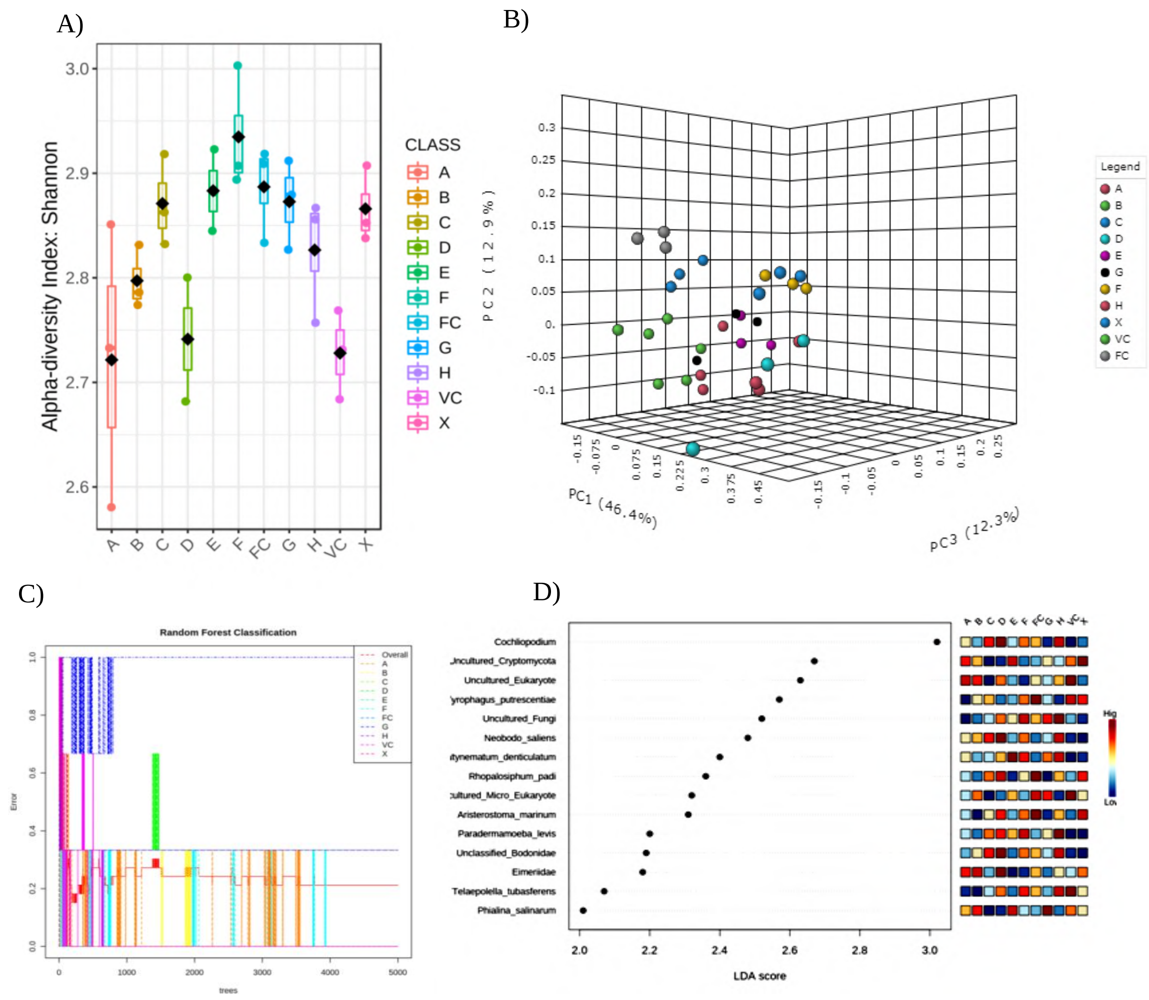


Fig 3.7: Influence of alpha chitin on rice rhizosphere eukaryome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.01848; [Kruskal-Wallis] statistic**:** 21.398. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [46.4 %] and the Y-axis representing the second-highest dimension of variation [12.9%] and Z- axis represents third highest degree of variation [12.3%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 11.513; R^2**:** 0.83957; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate 21.2% (0.212) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off**:** 0.05]. The mini heat map to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. ‘Uncultured’ taxa labels in the figures represent unclassified eukarya taxa. (Alpha Chitin-High Dosage-Vegetative – A, Alpha Chitin-Low Dosage-Vegetative – B, Alpha Chitin-High Dosage-Flowering – C, Alpha Chitin-Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Bio fertilizer-Half RD-Flowering – H, Pre-treatment – X, Vegetative control – VC, Flowering control – FC).

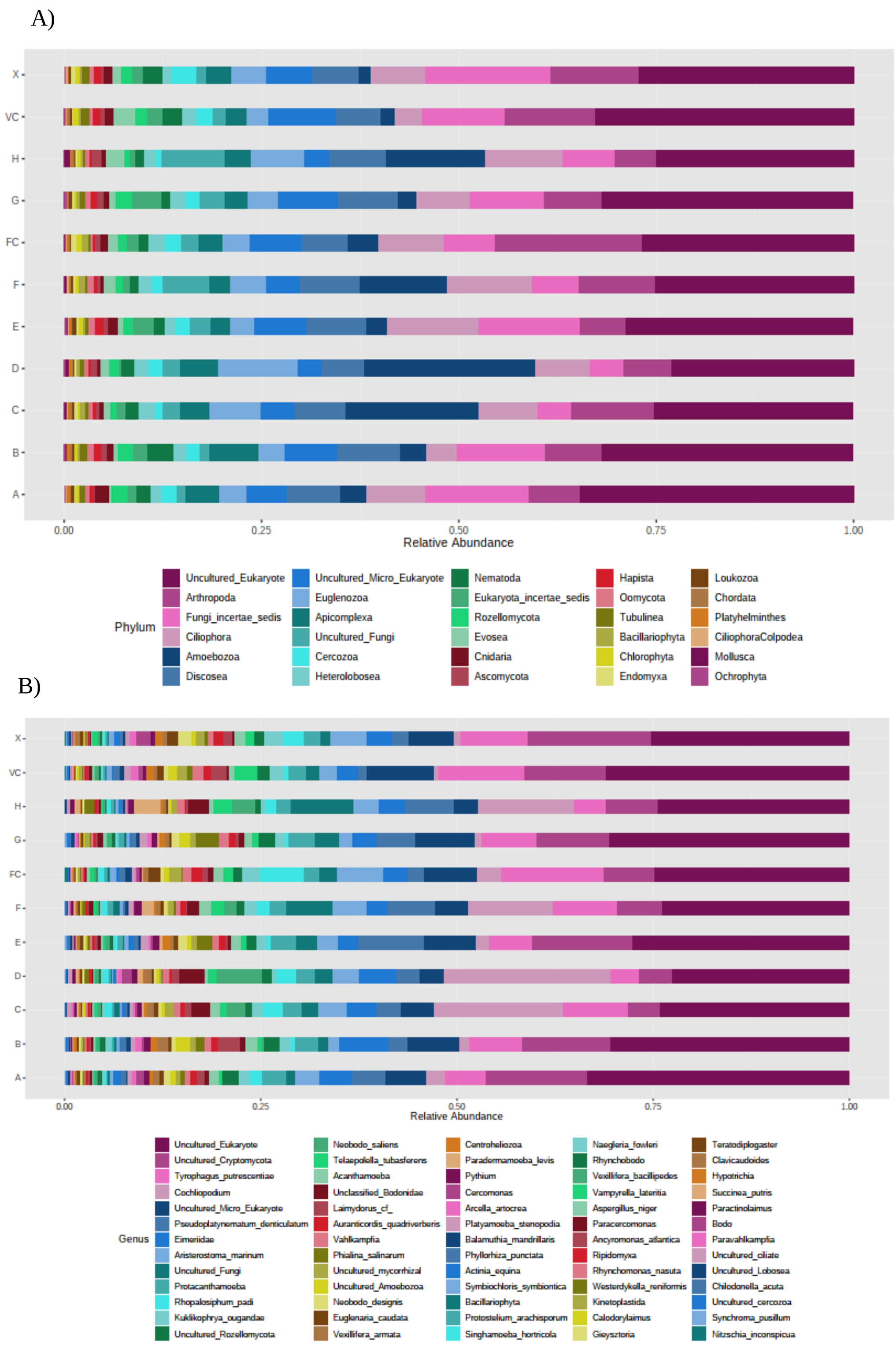


Fig 3.8: Influence of alpha chitin on the taxonomic composition rice rhizosphere eukaryote community. Stacked bar plots represent the relative abundance of eukaryote taxa at Phylum level (A) and Genus level (B) ‘Uncultured’ taxa labels in the figures represent unclassified eukaryote’s taxa. (Alpha Chitin-High Dosage-Vegetative – A, Alpha Chitin-Low Dosage-Vegetative – B, Alpha Chitin-High Dosage-Flowering – C, Alpha Chitin-Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Bio fertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre-treatment – X, Vegetative control – VC, Flowering control – FC).

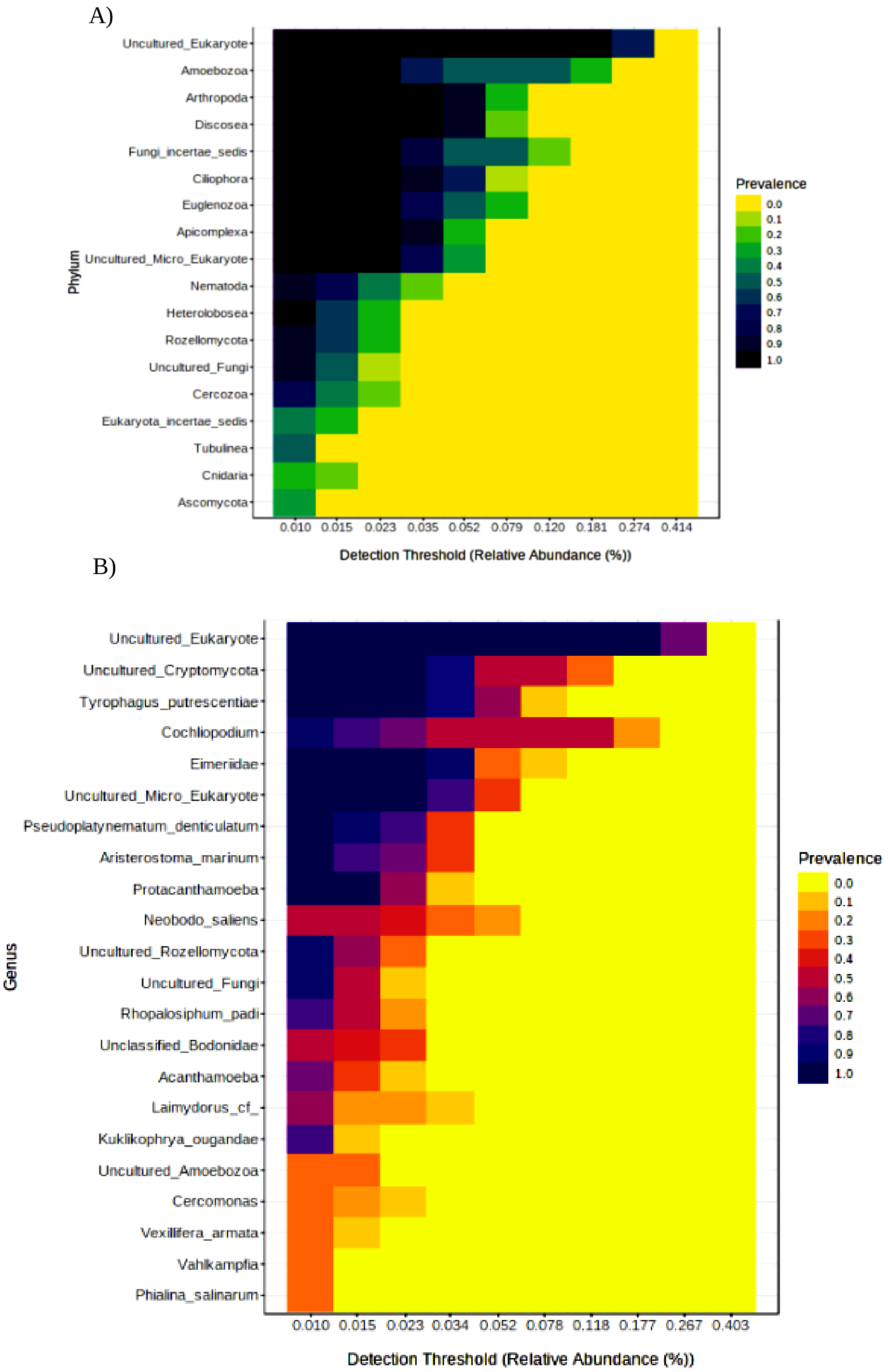


Fig 3.9: Heatmaps representing the core microbiome of alpha chitin on rice rhizosphere eukaryome at Phylum (A) and Genus levels (B). Y-axis represents the prevalence level of core fungal taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified eukaryote taxa.

3.1.4. Influence of alpha chitin on rice rhizosphere microbiome

The alpha chitin at high dosage has highest alpha-diversity (Shannon diversity index) at flowering followed by other treatments (Fig 3.10A). Using PCoA plots, the response of microbial communities to alpha chitin treatment is shown as separate clustering of sample groups without any overlaps (Fig 3.10B). PERMANOVA revealed substantial variations in zOTU assemblage between microbial communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for microbial communities in different fractions is 0.182 (18.2%) (Fig 3.10C). LEfSe analysis identified *Mortierella* and *Clonostachys* of fungi, *Cochliopodium* and Unclassified Bodonidae of eukaryota are indicator features for alpha chitin treatment to rice rhizosphere, as the biomarkers associated with rice rhizosphere (Fig 3.10D).

The relative abundance of Uncultured Fungi, Uncultured Eukaryote, Ascomycota, Mucoromycota, Amoebozoa, Fungi incertae sedis, Arthropoda, Proteobacteria, Discosea, Ciliophora, Basidiomycota, were higher at phylum level in rice rhizosphere (Fig 3.11A) whereas taxa abundance profile of genus comprises of Uncultured Eukaryote, Uncultured Fungi, *Mortierella*, Uncultured Cryptomycota, *Cochliopodium*, *Tyrophagus putrescentiae*, Uncultured Micro Eukaryote, *Cordyceps*, *Eimeriidae*, *Hygroaster*, *Pseudoplatynematum denticulatum*, *Aspergillus*, *Protacanthamoeba* and *Aristerosto mamarinum* (Fig 3.11B).

Core microbiome, calculated using Sample prevalence (20%) with relative abundance of 0.01% for taxa, at phylum (Fig 3.12A) and genus level (Fig 3.12B). Core microbiome at phylum level comprises of Uncultured Fungi, Uncultured Eukaryote, Proteobacteria, Euglenozoa, Ciliophora, Chloroflexi, Ascomycota, Amoebozoa, Uncultured Micro Eukaryote, Myxococcota, Fungi incertae sedis, Discosea, Arthropoda, and Apicomplexa (Fig 3.12A).

The core microbiome at genus level comprised of Uncultured Fungi, Uncultured Eukaryote, Cordyceps, Uncultured Cryptomycota, *Tyrophagus putrescentiae*, *Protacanthamoeba*, *Eimeriidae*, *Pseudoplatynematum denticulatum* and *Cochliopodium* (Fig 3.12B).

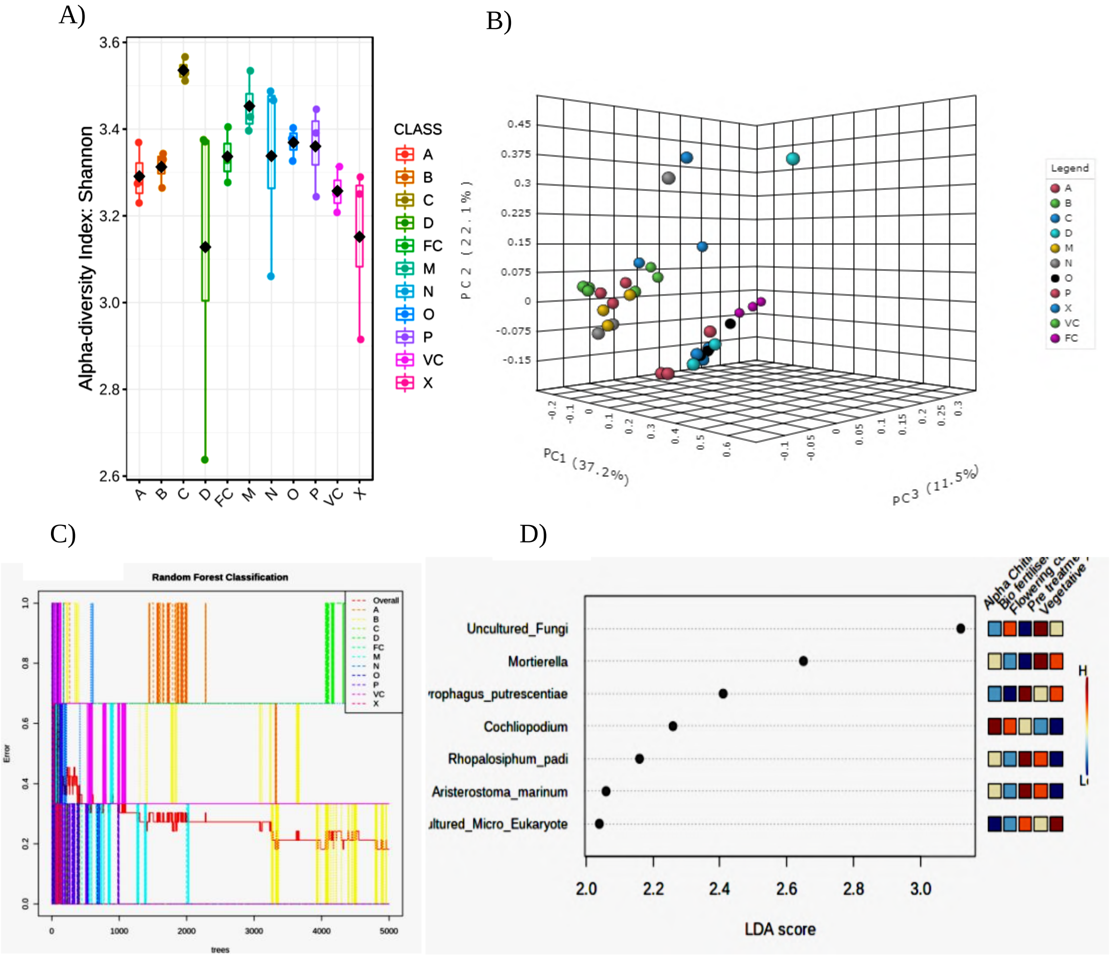


Fig 3.10: Influence of alpha chitin on rice rhizosphere. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.05723; [Kruskal-Wallis] statistic**:** 17.868. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [37.2%] and the Y-axis representing the second-highest dimension of variation [22.1%] and Z- axis represents third highest degree of variation [11.5%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 14.419; R^2**:** 0.86762; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate 18.2% (0.182) is shown as the red line; lines of other colours represent the error rates for each class. D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off**:** 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. 'Uncultured' taxa labels in the figures represent unclassified taxa belongs to microbiome. (Alpha Chitin-High Dosage-Vegetative – A, Alpha Chitin-Low Dosage-Vegetative – B, Alpha Chitin-High Dosage-Flowering – C, Alpha Chitin-Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre treatment – X, Vegetative control – VC, Flowering control – FC).

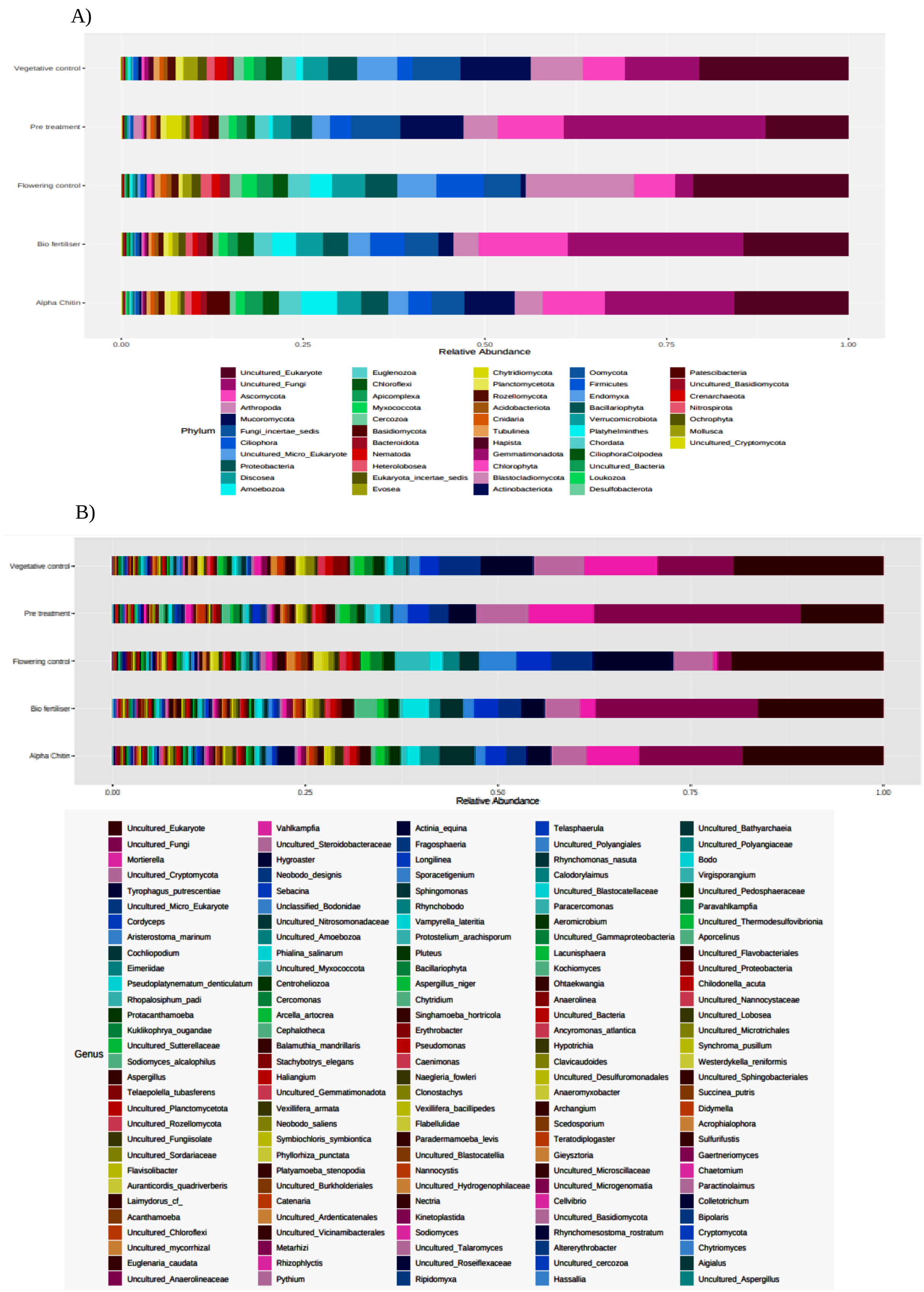


Fig 3.11: Influence of alpha chitin on the taxonomic composition of rice rhizosphere microbiome. Stacked bar plots represent the relative abundance of microbiome taxa at Phylum level (A) and Genus level (B). ‘Uncultured’ taxa labels in the figures represent unclassified microbiome taxa.

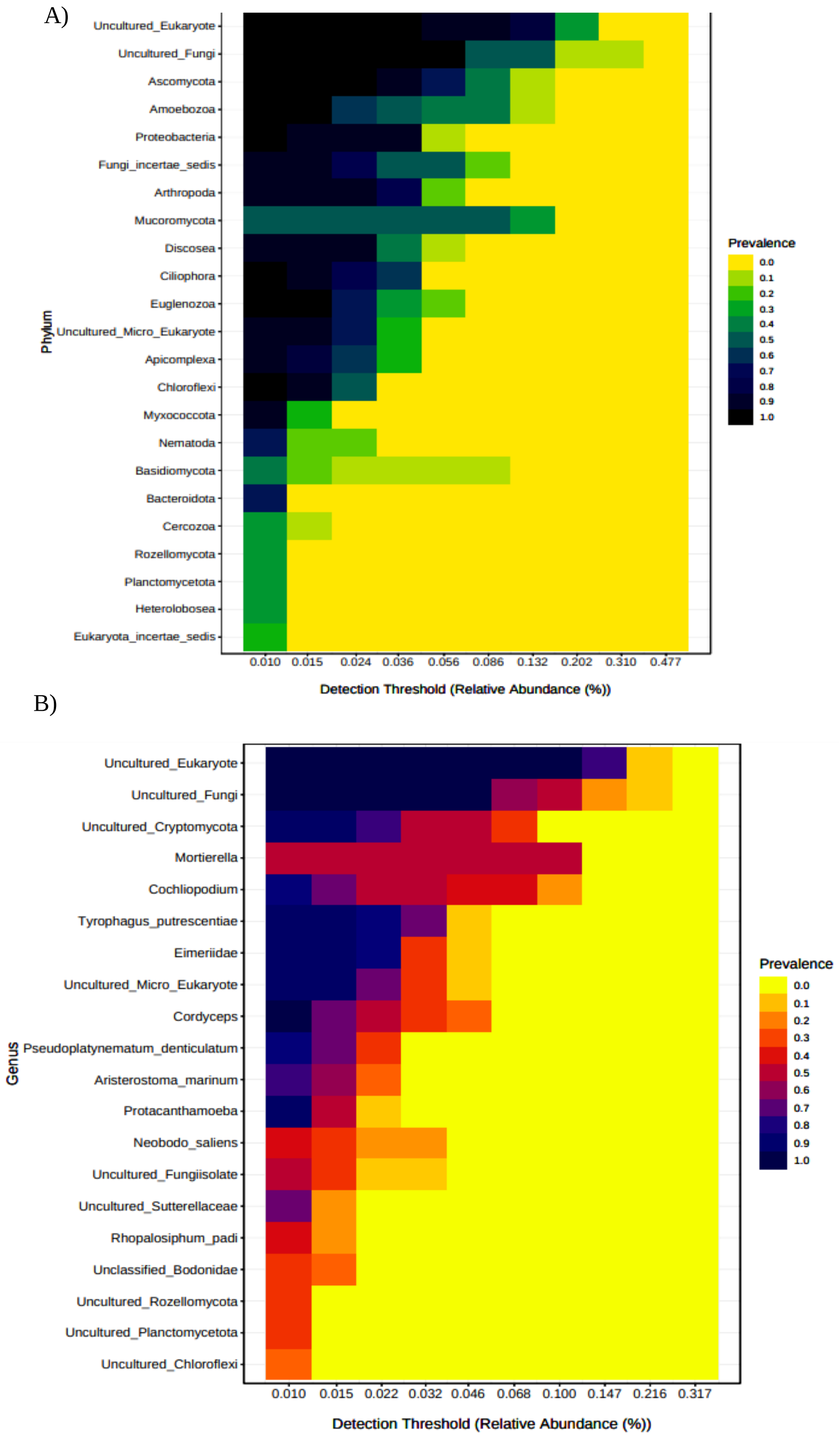


Fig 3.12: Heatmaps representing the core microbiome of alpha chitin on rice rhizosphere Phylum (A) and Genus levels (B). Y-axis represents the prevalence level of core microbiome taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified microbiome taxa.

3.1.5. Influence of beta chitin on rice rhizosphere bacteriome

High dose of Beta-chitin has highest alpha-diversity (Shannon diversity index) at flowering stage, followed by other treatments (Fig 3.13A). Using PCoA plots, the response of bacterial communities to beta chitin treatment is shown as separate clustering of sample groups without any overlaps (Fig 3.13B). PERMANOVA revealed substantial variations in zOTU assemblage between bacterial communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for bacterial communities in different fractions is 0.818 (81.8%) (Fig 3.13C). Proteobacteria, Chloroflexi, Myxococcota, Bacteroidota, Planctomycetota, Acidobacteriota, Desulfobacterota, Actinobacteriota, Gemmatimonadota and Firmicutes were top abundant phyla during beta chitin treatment to rice rhizosphere (Fig 3.14A), where as top abundant genus includes Uncultured Planctomycetota, Uncultured Sutterellaceae, Uncultured Chloroflexi, Uncultured Nitrosomonadaceae, *Flavisolibacter*, Uncultured Anaerolineaceae and Uncultured Burkholderiales (Fig 3.14B).

The core microbiome was calculated using sample prevalence (75%) with relative abundance of 0.01% for taxa at phylum level and genus level and is presented in Fig 3.15A and Fig 3.15B, respectively. The core microbiome at phylum level comprised of Proteobacteria, Planctomycetota, Myxococcota, Gemmatimonadota, Desulfobacterota, Chloroflexi, Bacteroidota, Acidobacteriota, Actinobacteriota, Firmicutes, Verrucomicrobiota and Crenarchaeota (Fig 3.15A). Core microbiome at genus level comprises of Uncultured Vicinamibacterales, Uncultured Steroidobacteraceae, Uncultured Planctomycetota, Uncultured Nitrosomonadaceae, Uncultured Desulfuromonadales, Uncultured Chloroflexi, Uncultured Burkholderiales, Uncultured Anaerolineaceae, *Flavisolibacter*, Uncultured Gemmatimonadota, *Pseudomonas* and *Caenimonas* (Fig 3.15B).

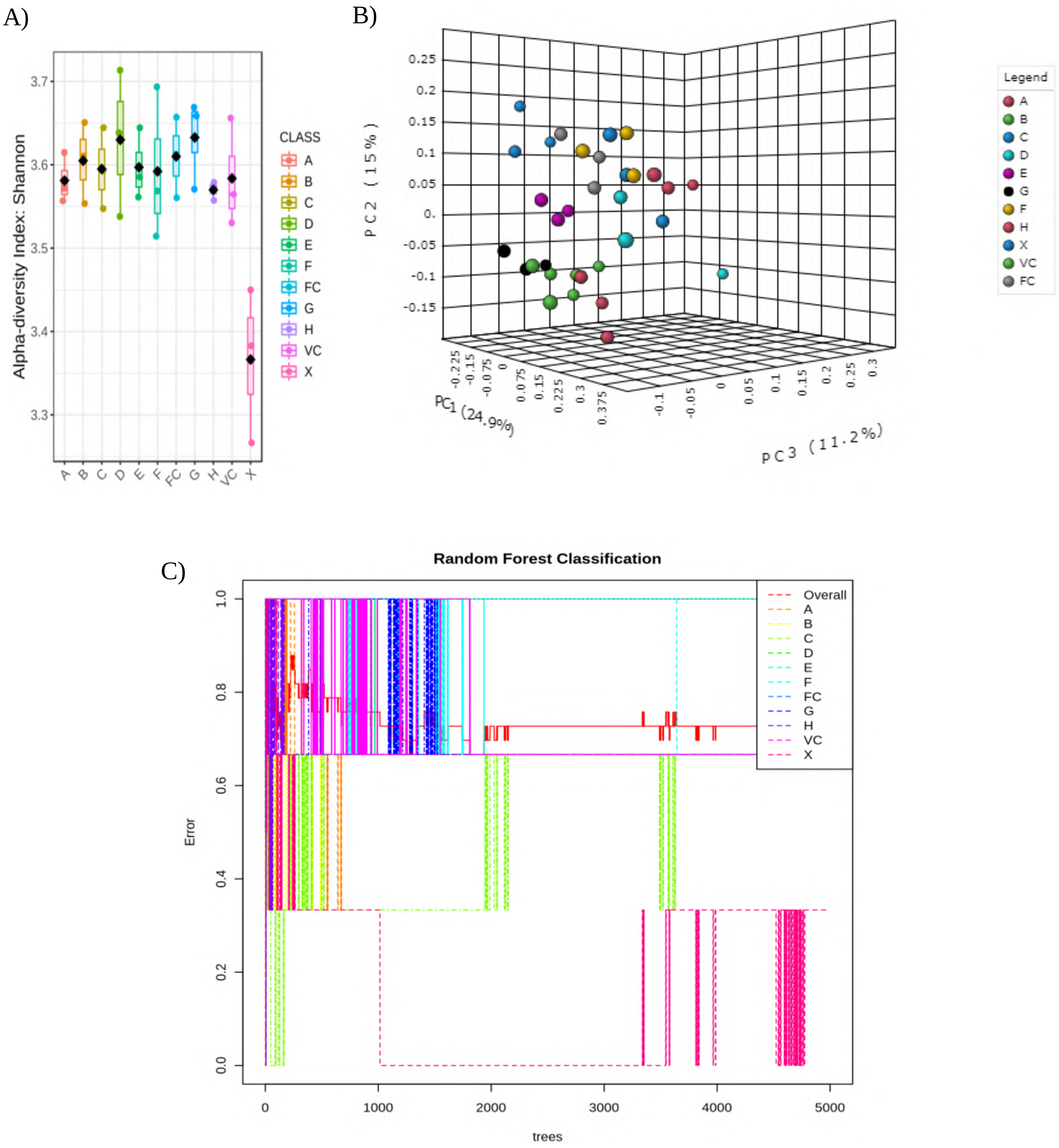


Fig 3.13: Influence of beta chitin on rice rhizosphere bacteriome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.3702; [Kruskal-Wallis] statistic**:** 10.838. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [24.9%] and the Y-axis representing the second-highest dimension of variation [15%] and Z- axis represents third highest degree of variation [11.2%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 3.0749; R^2**:** 0.58293; $p<0.001$. (C) Cumulative OOB error rates by RF classification. The overall error rate 87.9% (0.879) is shown as the red line; lines of other colours represent the error rates for each class. (Beta Chitin-High Dosage-Vegetative – A, Beta Chitin-Low Dosage-Vegetative – B, Beta Chitin-High Dosage-Flowering – C, Beta Chitin-Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Bio fertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre- treatment – X, Vegetative control – VC, Flowering control – FC).

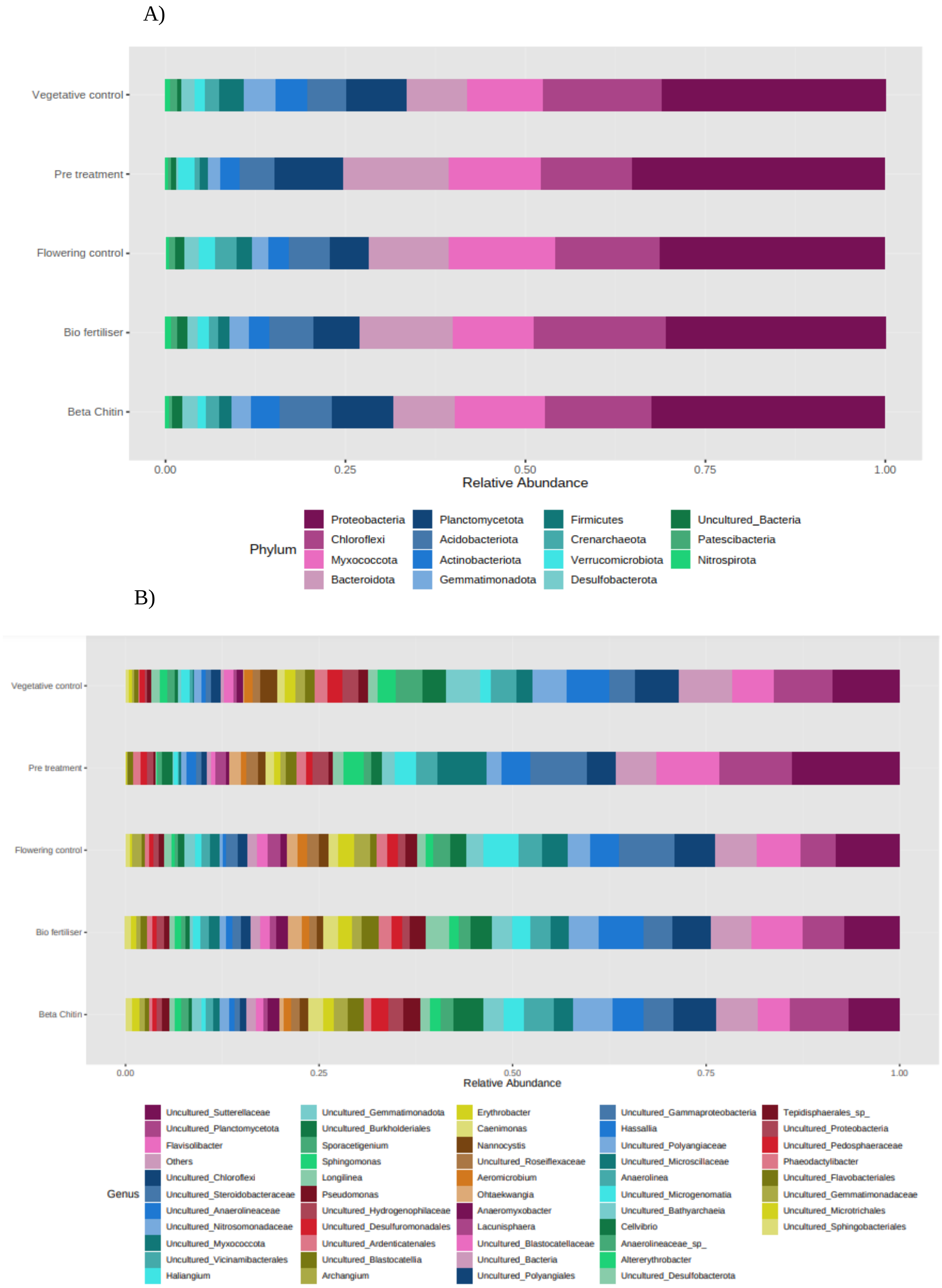


Fig 3.14: Influence of beta chitin on the taxonomic composition of rice rhizosphere bacterial community. Stacked bar plots represent the relative abundance of bacterial taxa at Phylum level (A) and Genus level (B). ‘Uncultured’ taxa labels in the figures represent unclassified bacterial taxa.

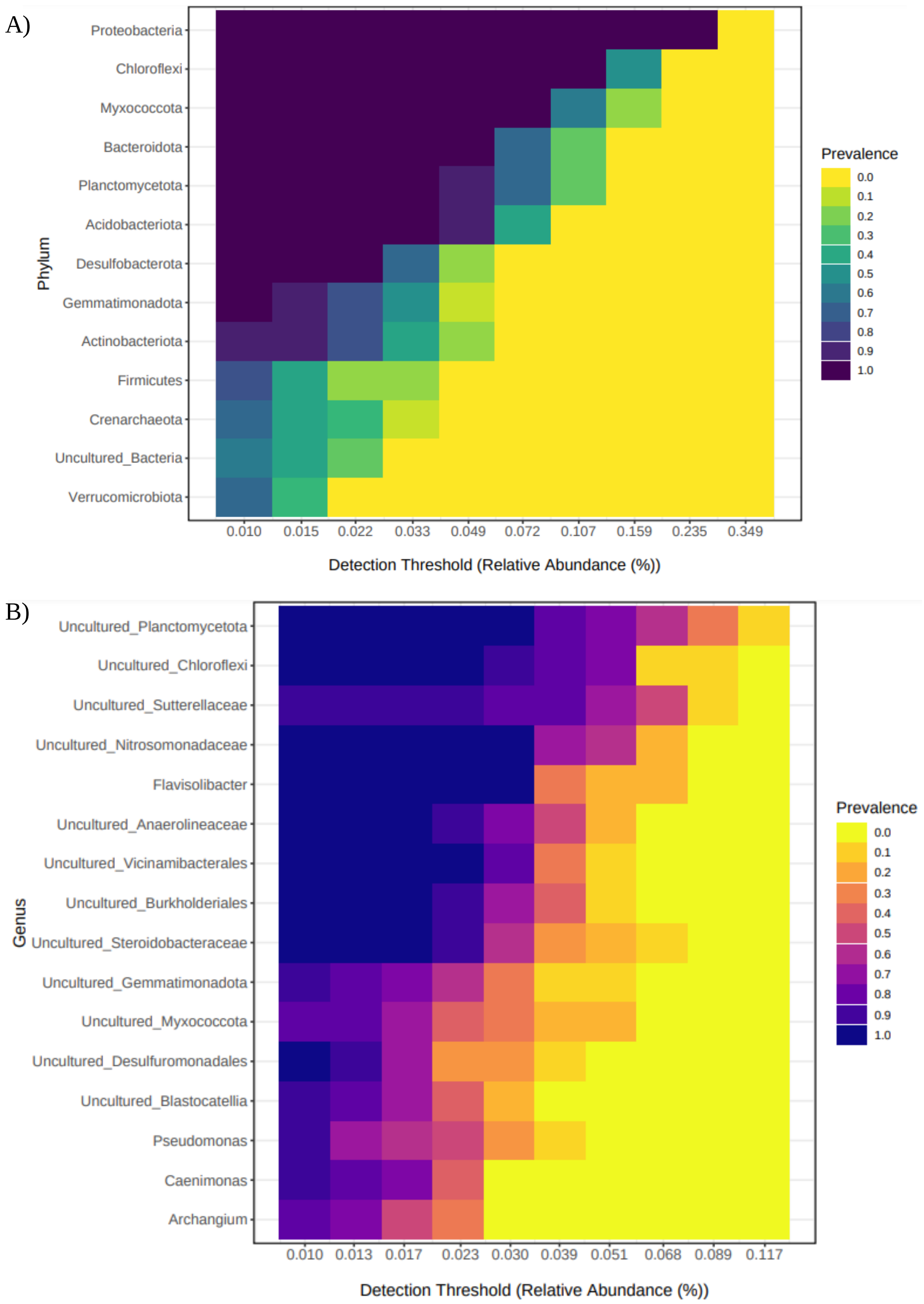


Fig 3.15: Heatmaps representing the core microbiome of beta chitin on rice rhizosphere bacteriome of Phylum (A) and Genus levels (B). Y-axis represents the prevalence level of core fungal taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified bacterial taxa.

3.1.6. Influence of beta chitin on rice rhizosphere mycobiome

A low dose of beta chitin at flowering stage has highest alpha-diversity (Shannon diversity index), followed by other treatments (Fig 3.16A). Using PCoA plots, the response of fungal communities to alpha chitin treatment is shown as separate clustering of sample groups without any overlaps (Fig 3.16B). PERMANOVA revealed substantial variations in zOTU assemblage between fungal communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for fungal communities in different fractions is 0.636 (63.6%)(Fig 3.16C). LEfSe analysis identified *Clonostachys, Didymella* and *Sebacina*as the biomarkers associated with rice rhizosphere with beta chitin amendment (Fig 3.16D).

The relative abundance of Uncultured Fungi was greater in beta chitin followed by Mucoromycota, Ascomycota, Basidiomycota, Chytridiomycota, and Blastocladiomycota (Fig 3.17A) where as top abundant taxa at genus level includes Uncultured Fungi, *Mortierella, Cordyceps, Aspergillus, Sebacina, Stachybotrys elegans, Clonostachys, Pluteus* and *Sodiomyces alcalophilus* (Fig 3.17B).

The core microbiome was calculated using sample prevalence (75%) with relative abundance of 0.01% for taxa and is presented at phylum level (Fig 3.18A) and genus level (Fig 3.18B). The core microbiome at phylum level comprised of Uncultured Fungi, Basidiomycota, Ascomycota, Mucoromycota, Chytridiomycota, Uncultured Cryptomycota, Uncultured Basidiomycota and Blastocladiomycota (Fig 3.18A). Core microbiome at genus level includes Uncultured Fungi, *Cordyceps, Mortierella, Aspergillus, Stachybotrys elegans, Sodiomyces alcalophilus, Sebacina, Clonostachys,* Uncultured Talaromyces, Uncultured Sordariaceae, *Pluteus, Scedosporium* and *Metarhizi* (Fig 3.18B).

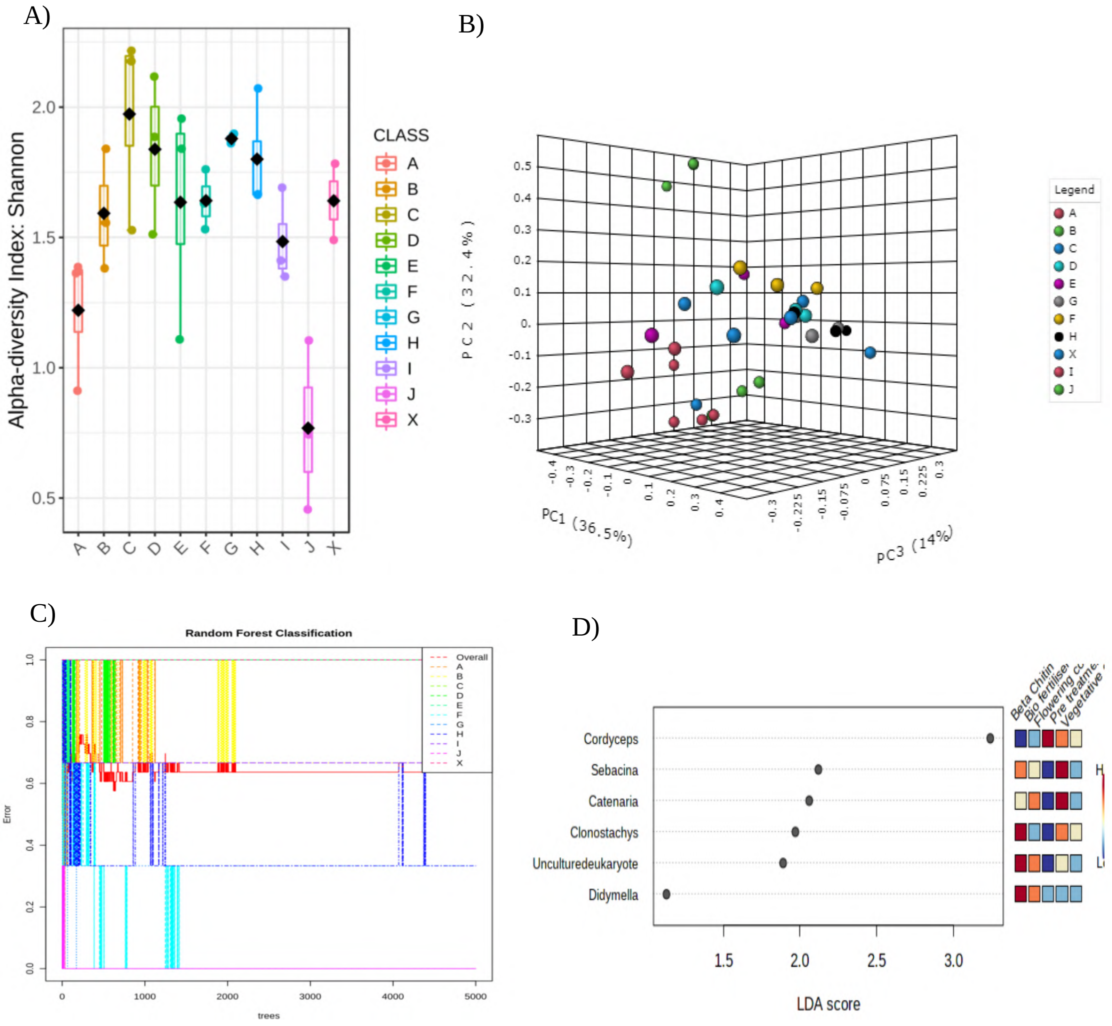


Fig 3.16: Influence of beta chitin on rice rhizosphere mycobiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.03760; [Kruskal-Wallis] statistic**:** 19.216. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [36.5 %] and the Y-axis representing the second-highest dimension of variation [32.4%] and Z- axis represents third highest degree of variation [14%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 8.7221; R^2**:** 0.79857; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate 63.6% (0.636) is shown as the red line; lines of other colours represent the error rates for each class. D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off**:** 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. 'Uncultured' taxa labels in the figures represent unclassified fungi taxa. (Beta Chitin-High Dosage-Vegetative – A, Beta Chitin-Low Dosage-Vegetative – B, Beta Chitin-High Dosage-Flowering – C, Beta Chitin-Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Bio fertilizer-Half RD-Flowering – H, Pre- treatment – X, Vegetative control – VC, Flowering control – FC).

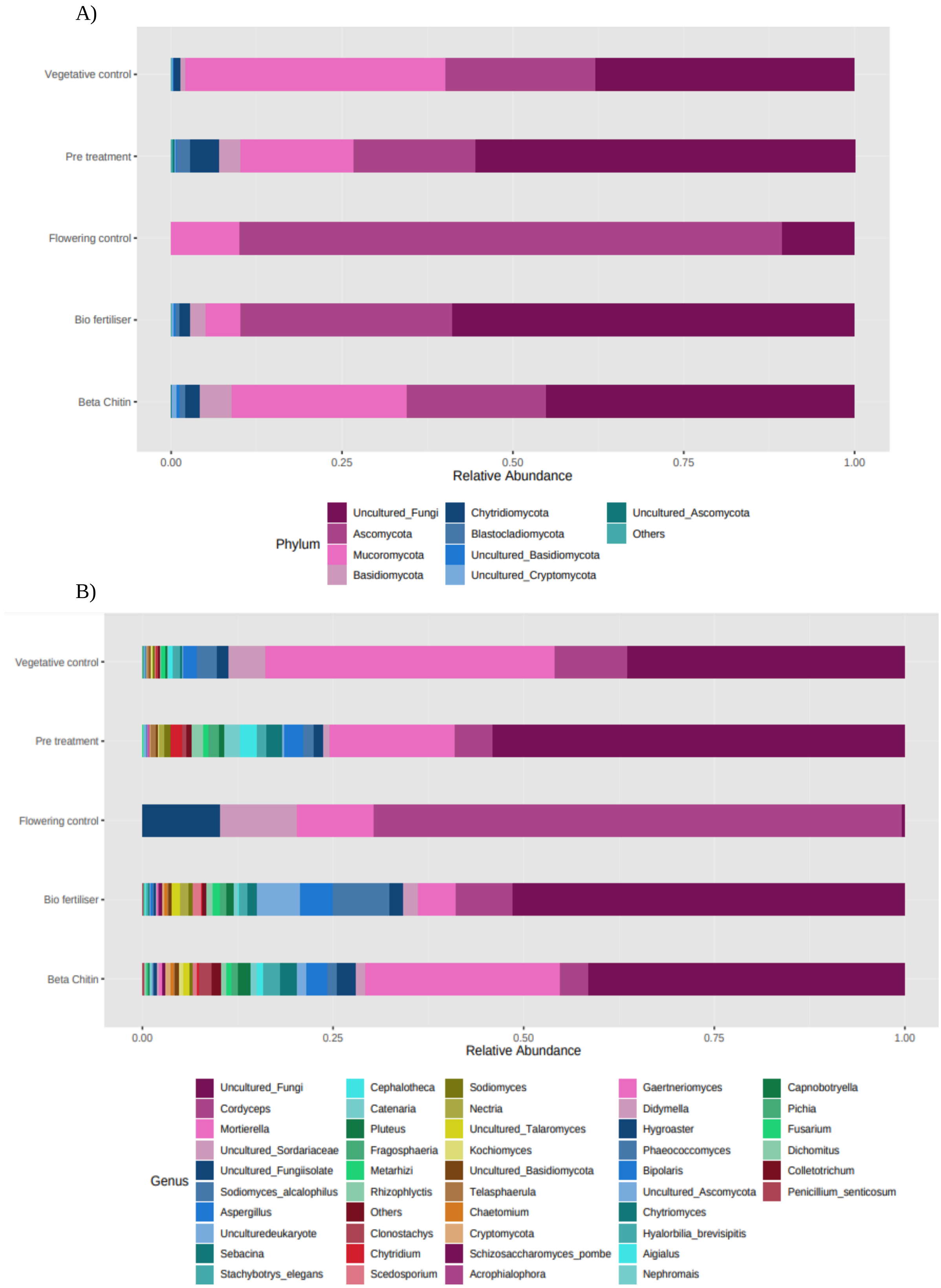


Fig 3.17: Influence of beta chitin on the taxonomic composition of rice rhizosphere fungal community. Stacked bar plots represent the relative abundance of fungal taxa at Phylum level (A) and Genus level (B). 'Uncultured' taxa labels in the figures represent unclassified fungal taxa.

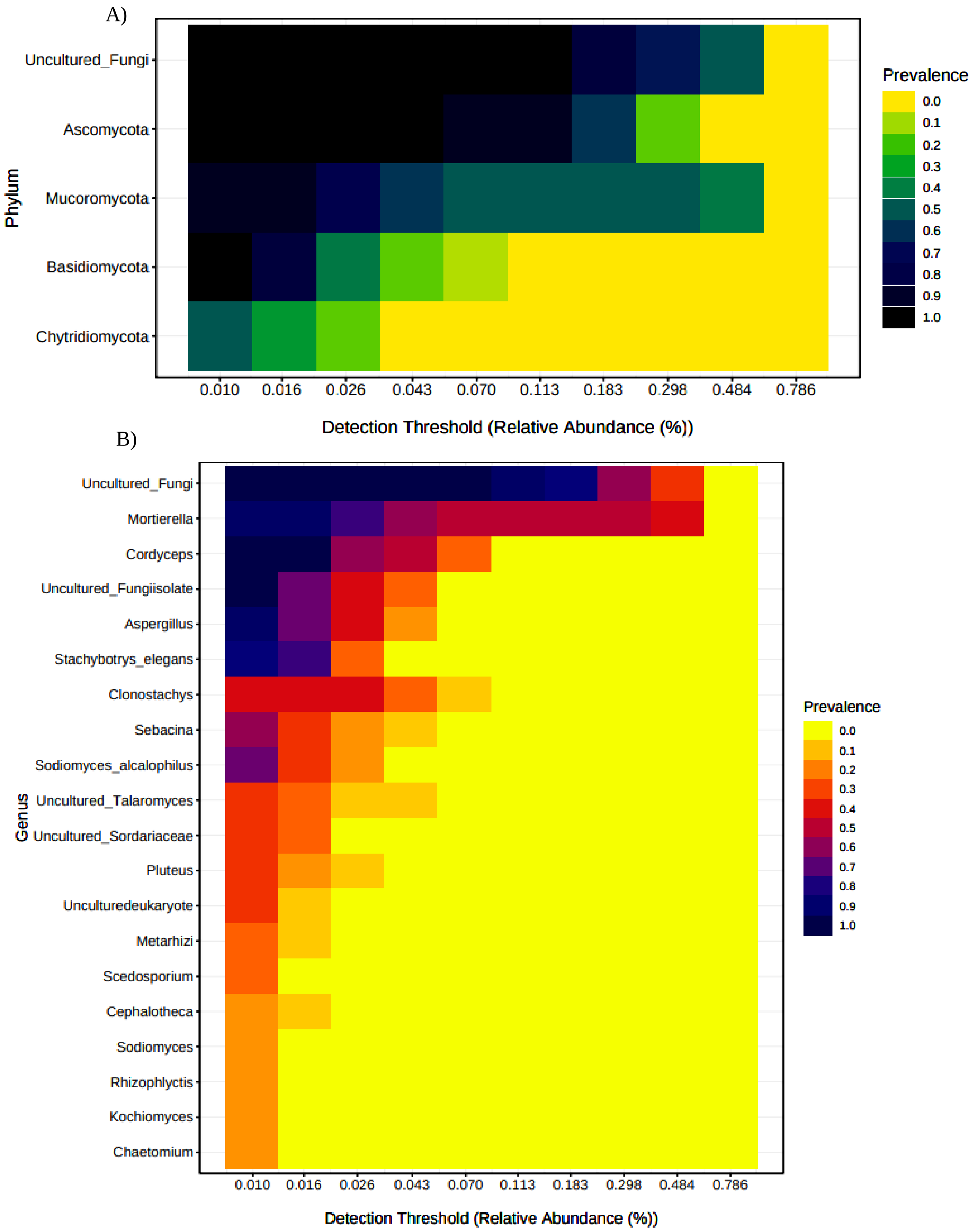


Fig 3.18: Heatmaps representing the core microbiome of beta chitin on rice rhizosphere mycobiome at Phylum (A) and Genus levels (B). Y-axis represents the prevalence level of core fungal taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified fungal taxa.

3.1.7. Influence of beta chitin on rice rhizosphere eukaryome

The beta chitin treatment, at low dosage, has lowest alpha-diversity (Shannon diversity index), followed by other treatments (Fig 3.19A) during vegetative stage. Using PCoA plots, the response of eukaryote communities to beta chitin treatment was shown as separate clustering of sample groups without any overlaps (Fig 3.19B). PERMANOVA revealed substantial variations in zOTU assemblage between fungal communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for fungal communities in different fractions is 0.303 (30.3%) (Fig 3.19C). LEfSe analysis identified Uncultured Cryptomycota and *Cochliopodium* as the biomarkers associated with rice rhizosphere with beta chitin amendment (Fig 3.19D).

The relative abundance of Uncultured Eukaryote, Fungi incertae sedis, Amoebozoa, Ciliophora, Uncultured micro Eukaryote were higher at phylum level (Fig 3.20A), whereas other abundant genera in beta chitin-amended rice rhizosphere comprised of Uncultured Eukaryote, Uncultured Cryptomycota, *Cochliopodium*, Uncultured micro Eukaryote, *Eimeriidae, Tyrophagus putrescentiae, Aristerostoma marinum, Pseudoplatynematum denticulatum*, *Protacanthamoeba* and *Telaepolella tubasferens* (Fig 3.20B).

Uncultured micro Eukaryote, Uncultured Eukaryote, Nematoda, Fungi incertae sedis, Euglenozoa, Discosea, Ciliophora, Arthropoda, Apicomplexa, Amoebozoa, Rozellomycota, Uncultured Fungi, Heterolobosea, Evosea and Cercozoa were core microbiome for beta chitin amendment to rice rhizosphere (Fig 3.21A). Uncultured micro Eukaryote, Uncultured Eukaryote, Uncultured Cryptomycota, *Tyrophagus putrescentiae*, *Pseudoplatynematum denticulatum, Eimeriidae, Cochliopodium*, Uncultured Rozellomycota, *Protacanthamoeba, Aristerostoma marinum*, Uncultured Fungi, *Telaepolella tubasferens, Rhopalosiphum padi*, Unclassified Bodonidae, *Phialinasalinarum, Laimydorus, Acanthamoeba, Vahlkampfia, Neobodo saliens* and *Neobodo designis* are part of core microbiome of eukaryota for beta chitin amendment to rice rhizosphere (Fig 3.21B).

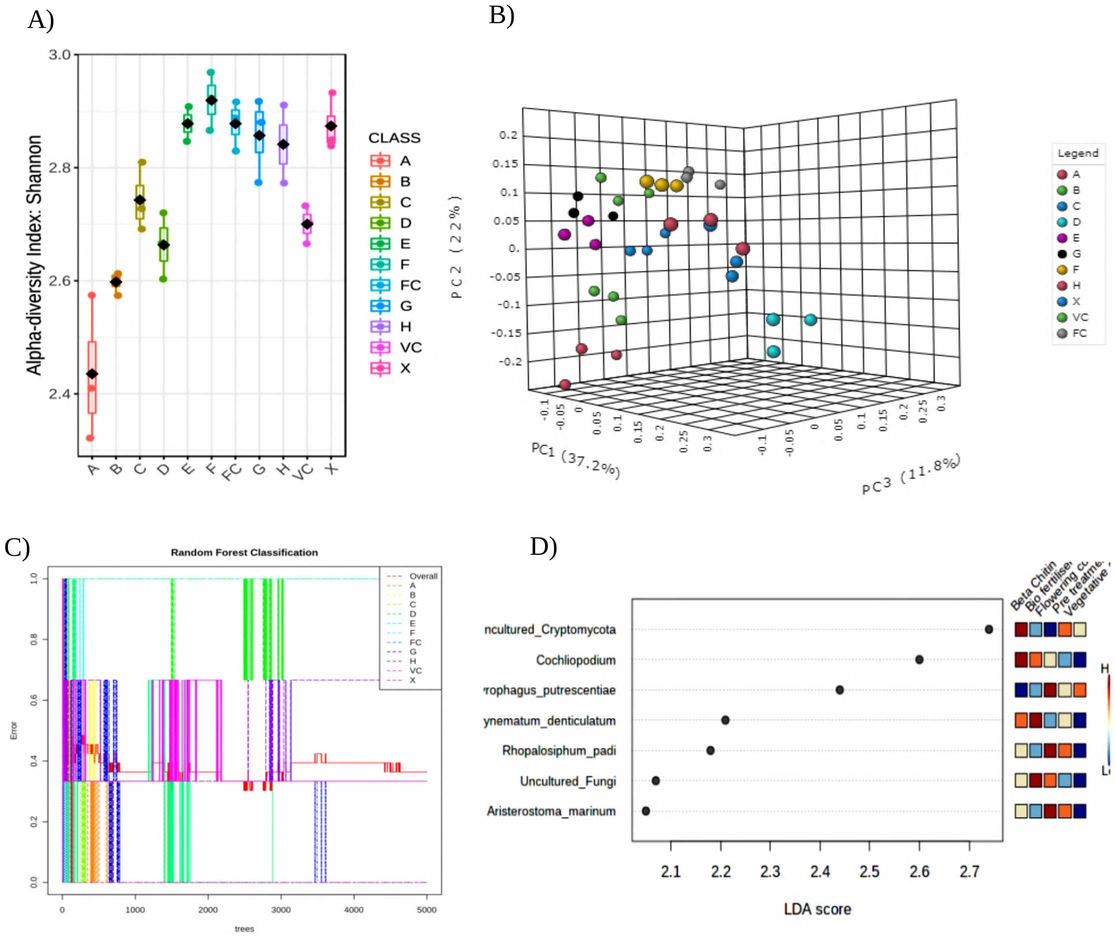


Fig 3.19: Influence of beta chitin on rice rhizosphere eukaryome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.00293; [Kruskal-Wallis] statistic**:** 26.667. (B) 2-D PCoA plots based on Bray–Curtis similarity [n= 57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [37.2 %] and the Y-axis representing the second-highest dimension of variation [22%] and Z- axis represents third highest degree of variation [11.8%].Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 14.658; R^2**:** 0.86949; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate 30.3% (0.303) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off**:** 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. 'Uncultured' taxa labels in the figures represent unclassified eukarya taxa**.** (Beta Chitin-High Dosage-Vegetative – A, Beta Chitin-Low Dosage-Vegetative – B, Beta Chitin-High Dosage-Flowering – C, Beta Chitin-Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Bio fertilizer-Half RD-Flowering – H, Pre treatment – X, Vegetative control – VC, Flowering control – FC).

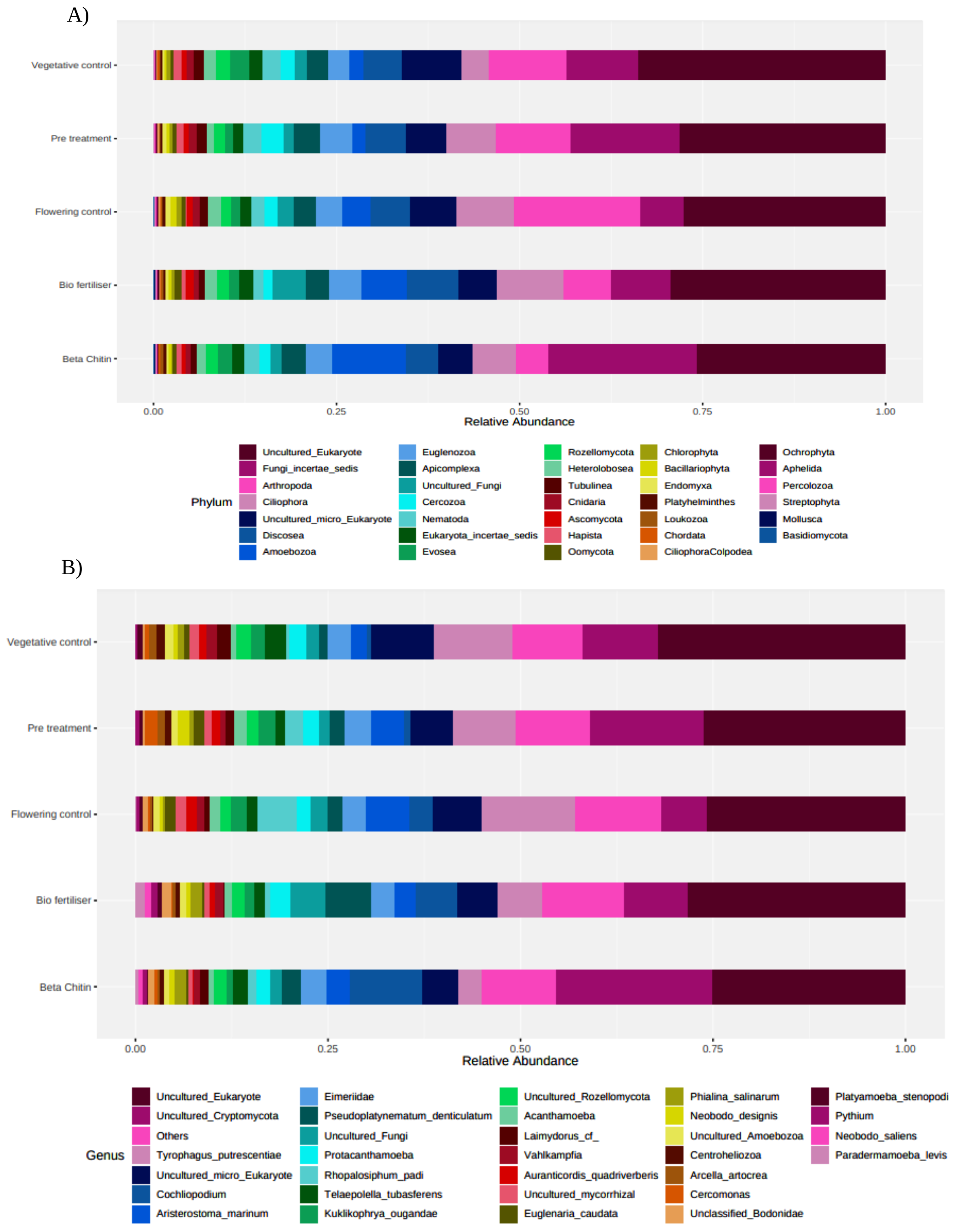


Fig 3.20: Influence of beta chitin on the taxonomic composition of rice rhizosphere eukaryote community. Stacked bar plots represent the relative abundance of eukaryote taxa at Phylum (A) and Genus level (B). ‘Uncultured’ taxa labels in the figures represent unclassified eukaryote taxa.

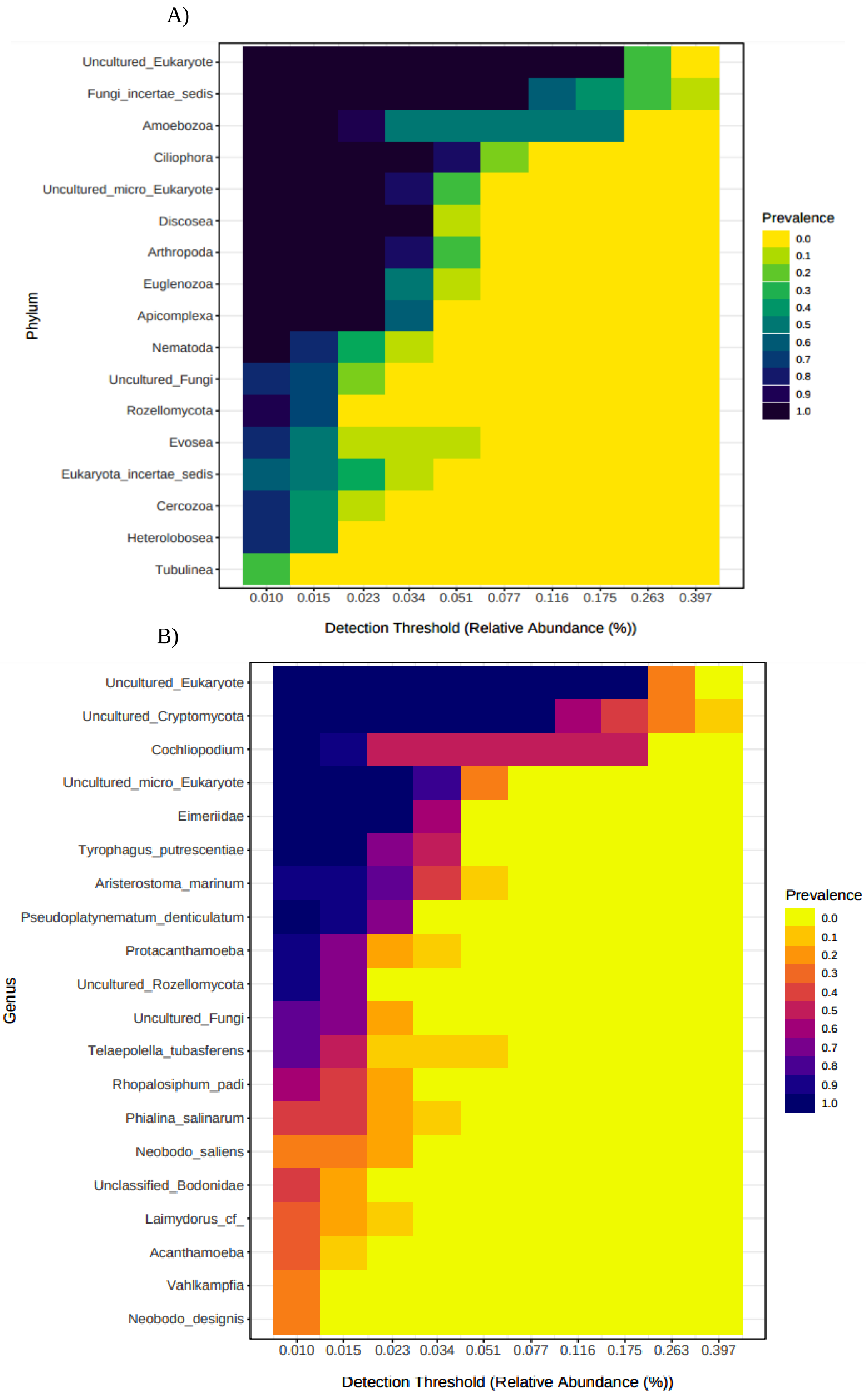


Fig 3.21: Heatmaps representing the core microbiome of beta chitin on rice rhizosphere eukaryome Phylum (A) and Genus levels (B). Y-axis represents the prevalence level of core fungal taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). 'Uncultured' taxa label in the figure represents unclassified eukaryotal taxa.

3.1.8. Influence of beta chitin on rice rhizosphere microbiome

The beta chitin treatment, at vegetative and flowering stages, showed lowest alpha-diversity (Shannon diversity index), followed by other treatments (Fig 3.22A). Using PCoA plots, the response of microbiome communities to beta chitin treatment was shown as separate clustering of sample groups without any overlaps (Fig 3.22B). PERMANOVA revealed substantial variations in zOTU assemblage between microbiome communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for microbiome communities in different fractions is 0.364 (36.4%) (Fig 3.22C). LEfSe analysis identified *Mortierella and Clonostachys of fungi, Cochliopodium* of eukaryota are indicator features for beta chitin treatment to rice (D) rhizosphere are indicator features as the biomarkers (Fig 3.22D).

The relative abundance of Uncultured Fungi, Uncultured Eukaryote, Fungi Incertae sedis, Mucoromycota, Ascomycota, Amoebozoa, Proteobacteria and Ciliophora were higher at phylum level (Fig 3.23A), whereas top genus during beta chitin to rice rhizosphere comprises of Uncultured Fungi, Uncultured Eukaryote, Uncultured Cryptomycota, *Mortierella* and *Cochliopodium* (Fig 3.23B).

The core microbiome was calculated using sample prevalence (50%) with relative abundance of 0.01% for taxa at phylum level (Fig 3.24A) and genus level (Fig 3.24B). The core microbiome at phylum level comprised of Uncultured Micro Eukaryote, Uncultured Fungi, Uncultured Eukaryote, Proteobacteria, Fungi incertae sedis, Discosea, Ciliophora, Ascomycota, Arthropoda, Myxococcota, Euglenozoa, Chloroflexi, Apicomplexa and Amoebozoa (Fig 3.24A). The core microbiome at genus level comprised of Uncultured Micro Eukaryote, Uncultured Fungi, Uncultured Eukaryote, Uncultured Cryptomycota, *Tyrophagus putrescentiae, Aristerostoma marinum, Eimeriidae, Cordyceps, Pseudoplatynematum denticulatum, Protacanthamoeba, Cochliopodium, Mortierella,* Uncultured Sutterellaceae, *Telaepolella tubasferens, Rhopalosiphum padi,* Uncultured Fungiisolate, Uncultured Planctomycetota, *Kuklikophrya ougandae, Aspergillus, Phialinasalinarum* and Uncultured Sordariaceae (Fig 3.24B).

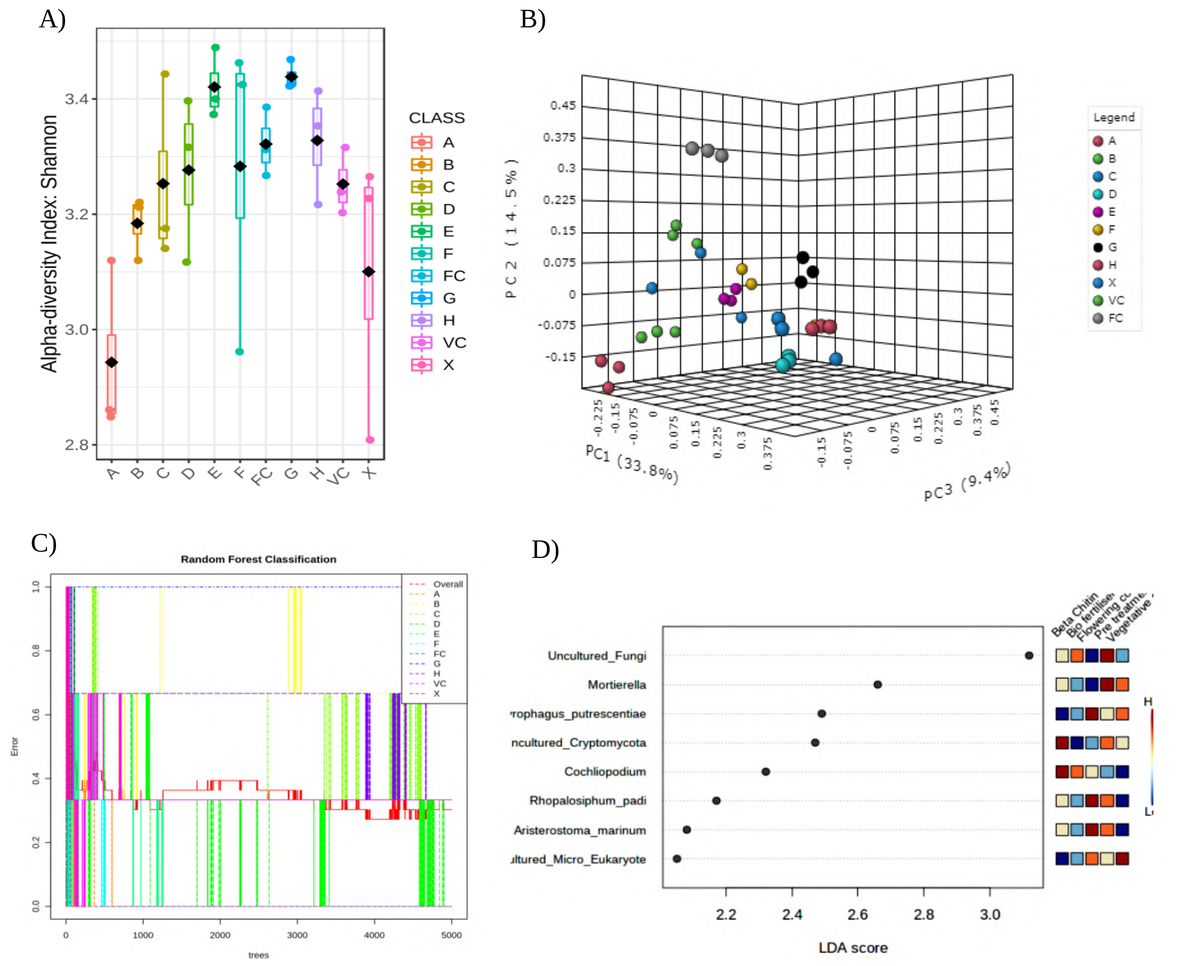


Fig 3.22: Influence of beta chitin on rice rhizosphere. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.0654; [Kruskal-Wallis] statistic**:** 17.426. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [33.8%] and the Y-axis representing the second-highest dimension of variation [14.5%] and Z- axis represents third highest degree of variation [9.4%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 5.2331; R^2**:** 0.70403; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate 36.4% (0.182) is shown as the red line; lines of other colours represent the error rates for each class. (D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off**:** 0. 05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. 'Uncultured' taxa labels in the figures represent unclassified taxa belongs to microbiome. (Beta Chitin-High Dosage-Vegetative – A, Beta Chitin-Low Dosage-Vegetative – B, Beta Chitin-High Dosage-Flowering – C, Beta Chitin-Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Bio fertilizer-Half RD-Flowering – H, Pre treatment – X, Vegetative control – VC, Flowering control – FC).

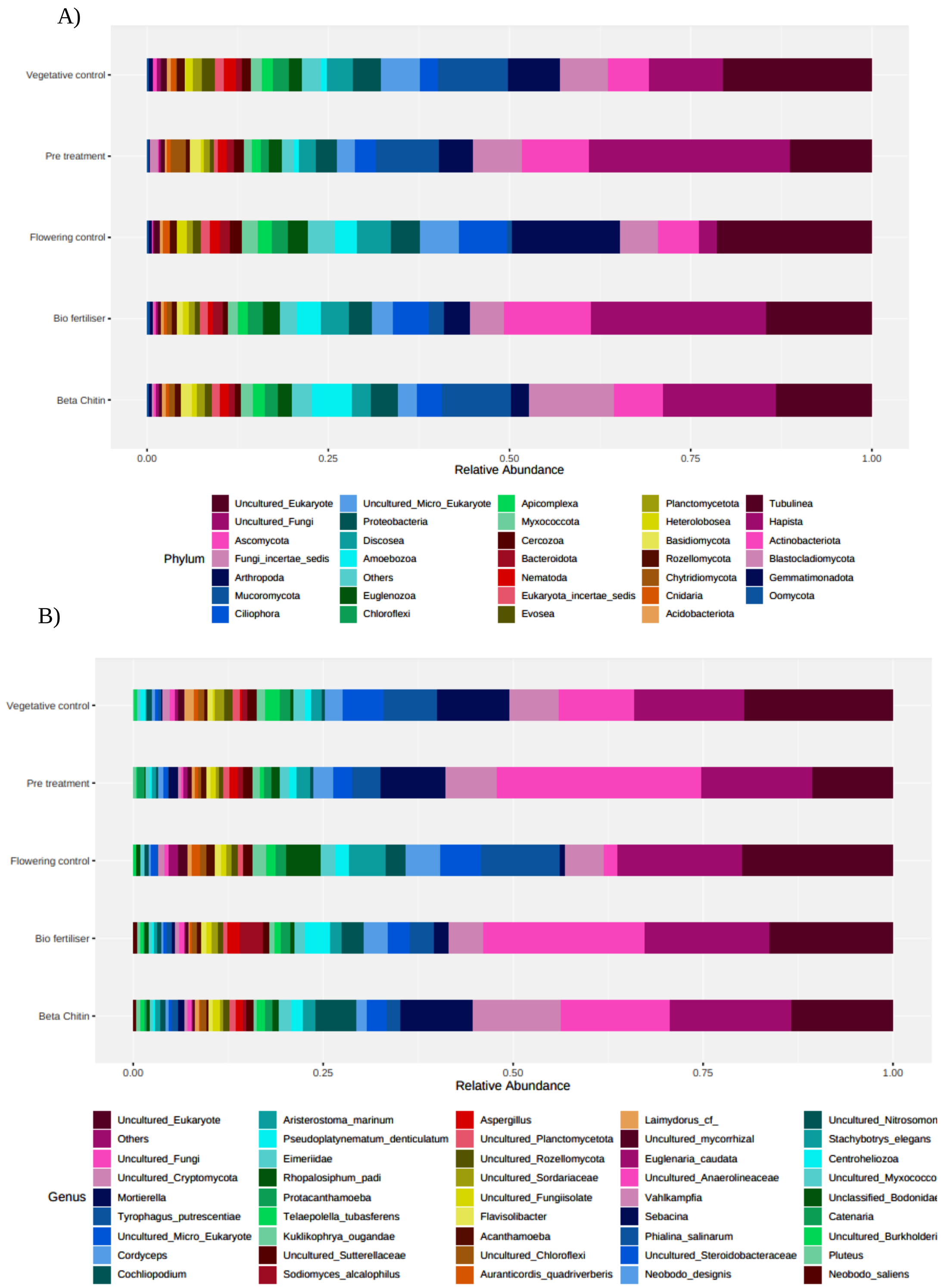


Fig 3.23: Influence of beta chitin on the taxonomic composition of rice rhizosphere. Stacked bar plots represent the relative abundance of eukaryote taxa at Phylum level (A) at the Genus level (B). ‘Uncultured’ taxa labels in the figures represent unclassified microbiome taxa.

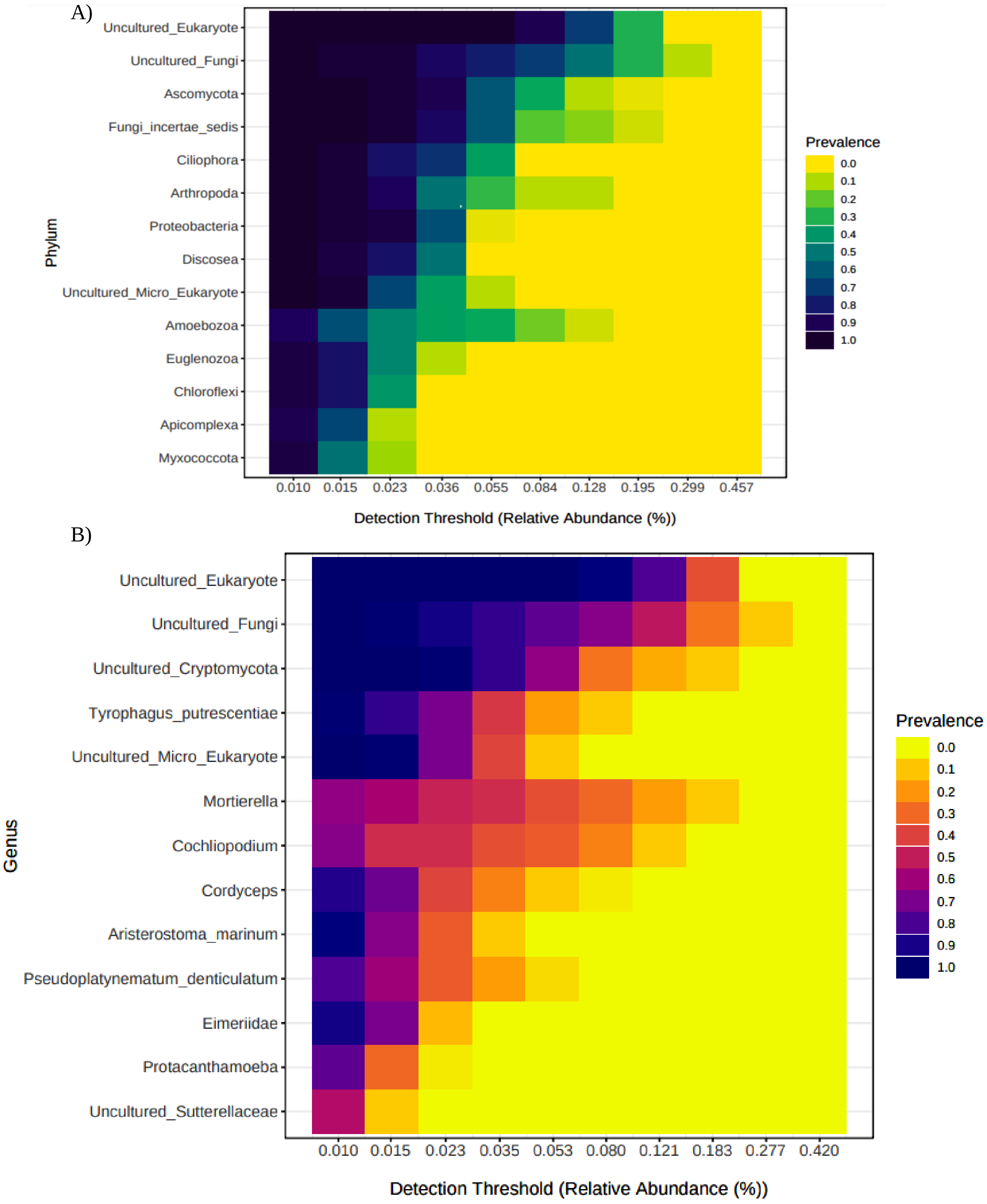


Fig 3.24: Heatmaps representing the core microbiome of beta-chitin on rice rhizosphere at Phylum (A) and Genus levels (B). Y-axis represents the prevalence level of core microbiome taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). 'Uncultured' taxa label in the figure represents unclassified microbiome taxa.

3.1.9. Influence of chitosan on rice rhizosphere bacteriomes

A low dose of chitosan has highest alpha-diversity (Shannon diversity index) at flowering than vegetative and flowering control, but lower when compared to biofertilizer treatments (Fig 3.25A). Using PCoA plots, the response of bacterial communities to chitosan treatment was shown as separate clustering of sample groups without any overlaps (Fig 3.25B). PERMANOVA revealed substantial variations in zOTU assemblage between bacterial communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for bacterial communities in different fractions is 0.818 (81.8%) (Fig 3.25C). The relative abundance of Proteobacteria, Chloroflexi, Myxococcota, Bacteroidota, Planctomycetota and Acidobacteriota were detected taxa abundant during chitosan treatment to rice rhizosphere at phylum level (Fig 3.26A). Uncultured Sutterellaceae, Uncultured Chloroflexi, Uncultured Anaerolineaceae, Uncultured Planctomycetota, Uncultured Nitrosomonadaceae, *Haliangium* and *Flavisolibacter* were the abundant taxa at genus level (Fig 3.26 B).

(D)

The core microbiome was calculated using sample prevalence (50%) with relative abundance of 0.01% for taxa at phylum level (Fig 3.27A) and genus level (Fig 3.27B) for chitosan treatment to rice rhizosphere. The core microbiome at phylum level comprised of Proteobacteria, Planctomycetota, Myxococcota, Gemmatimonadota, Chloroflexi, Bacteroidota, Acidobacteriota, Actinobacteriota, Desulfobacterota, Verrucomicrobiota, Firmicutes and Crenarchaeota (Fig 3.27A). The core microbiome at genus level comprises of Uncultured Sutterellaceae, Uncultured Steroidobacteraceae, Uncultured Planctomycetota, Uncultured Nitrosomonadaceae, Uncultured Chloroflexi, Uncultured Anaerolineaceae, *Flavisolibacter*, Uncultured Vicinamibacterales, Uncultured Gemmatimonadota, Uncultured Burkholderiales, Uncultured Myxococcota, *Haliangium, Sphingomonas, Longilinea*, *Pseudomonas*, *Caenimonas*, *Sporacetigenium*, *Uncultured Blastocatellia*, *Archangium*, *Erythrobacter*, Uncultured Hydrogenophilaceae and *Nannocystis* (Fig 3.27B).

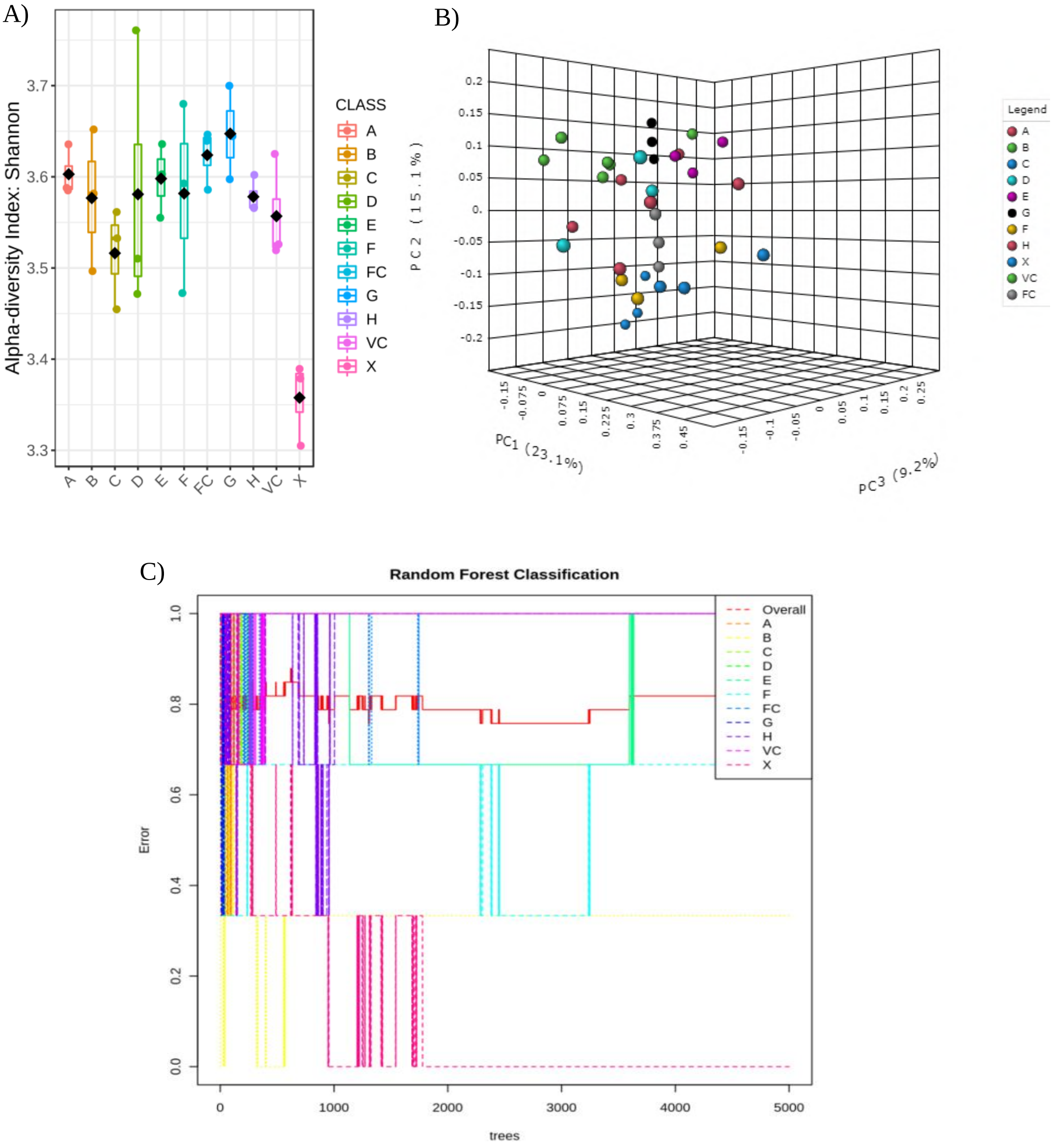


Fig 3.25: Influence of chitosan on rice rhizosphere bacteriome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.15931; [Kruskal-Wallis] statistic**:** 14.31. (B) 2-D PCoA plots based on Bray–Curtis similarity [n= 57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [23.1%] and the Y-axis representing the second-highest dimension of variation [15.1%] and Z- axis represents third highest degree of variation [9.2%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 3.3472; R^2**:** 0.60341; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate 86.8% (0.868) is shown as the red line; lines of other colours represent the error rates for each class. (Chitosan - High Dosage-Vegetative – A, Chitosan - Low Dosage-Vegetative – B, Chitosan - High Dosage-Flowering – C, Chitosan - Low Dosage-Flowering – D, Bio fertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Bio fertilizer-Half RD-Flowering – H, Pre -treatment – X, Vegetative control – VC, Flowering control – FC).

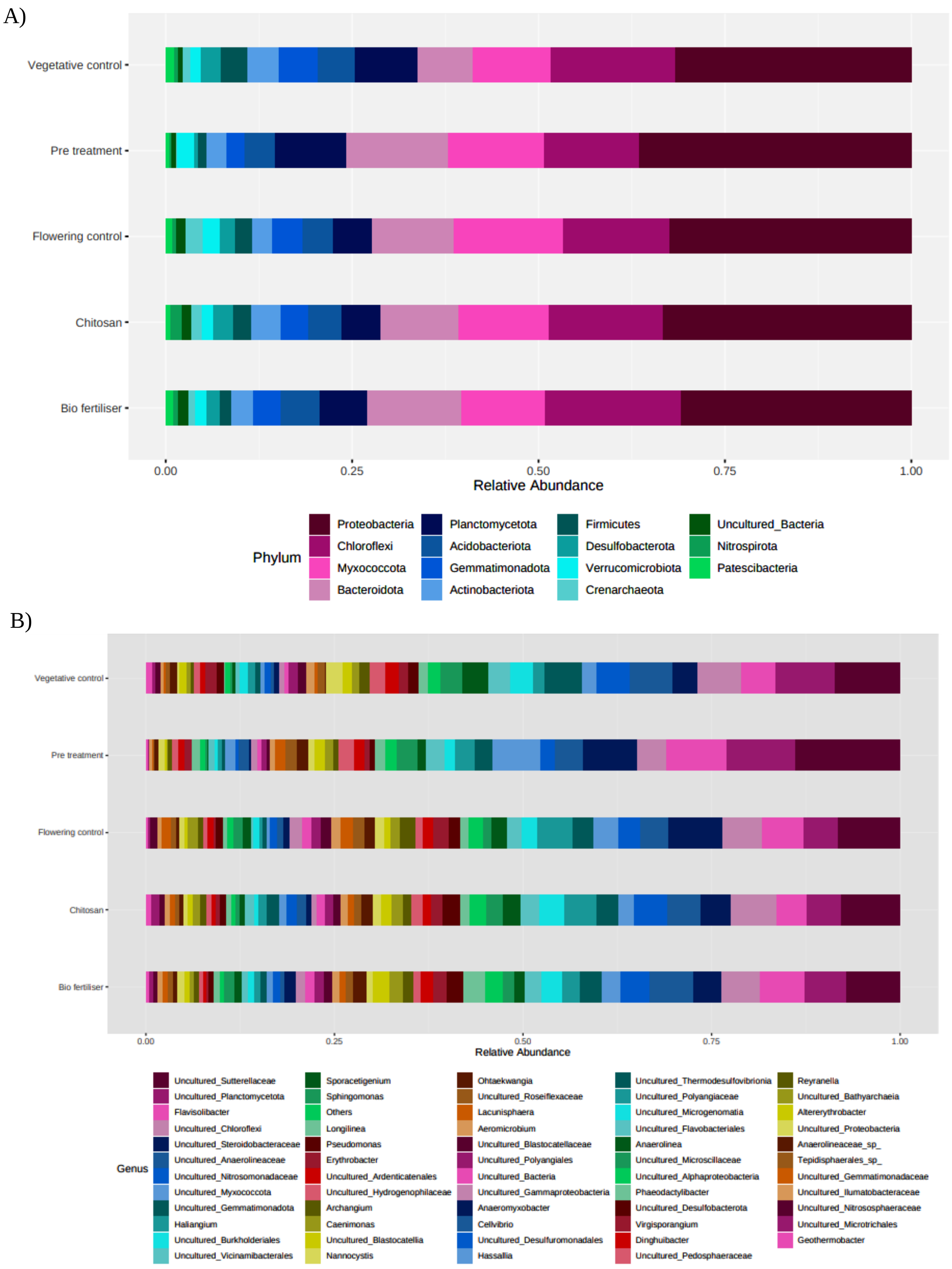


Fig 3.26: Influence of chitosan on the taxonomic composition of bacterial community of rice rhizosphere. Stacked bar plots represent the relative abundance of eukaryote taxa at Phylum level (A) and Genus level (B). ‘Uncultured’ taxa labels in the figures represent unclassified bacterial taxa

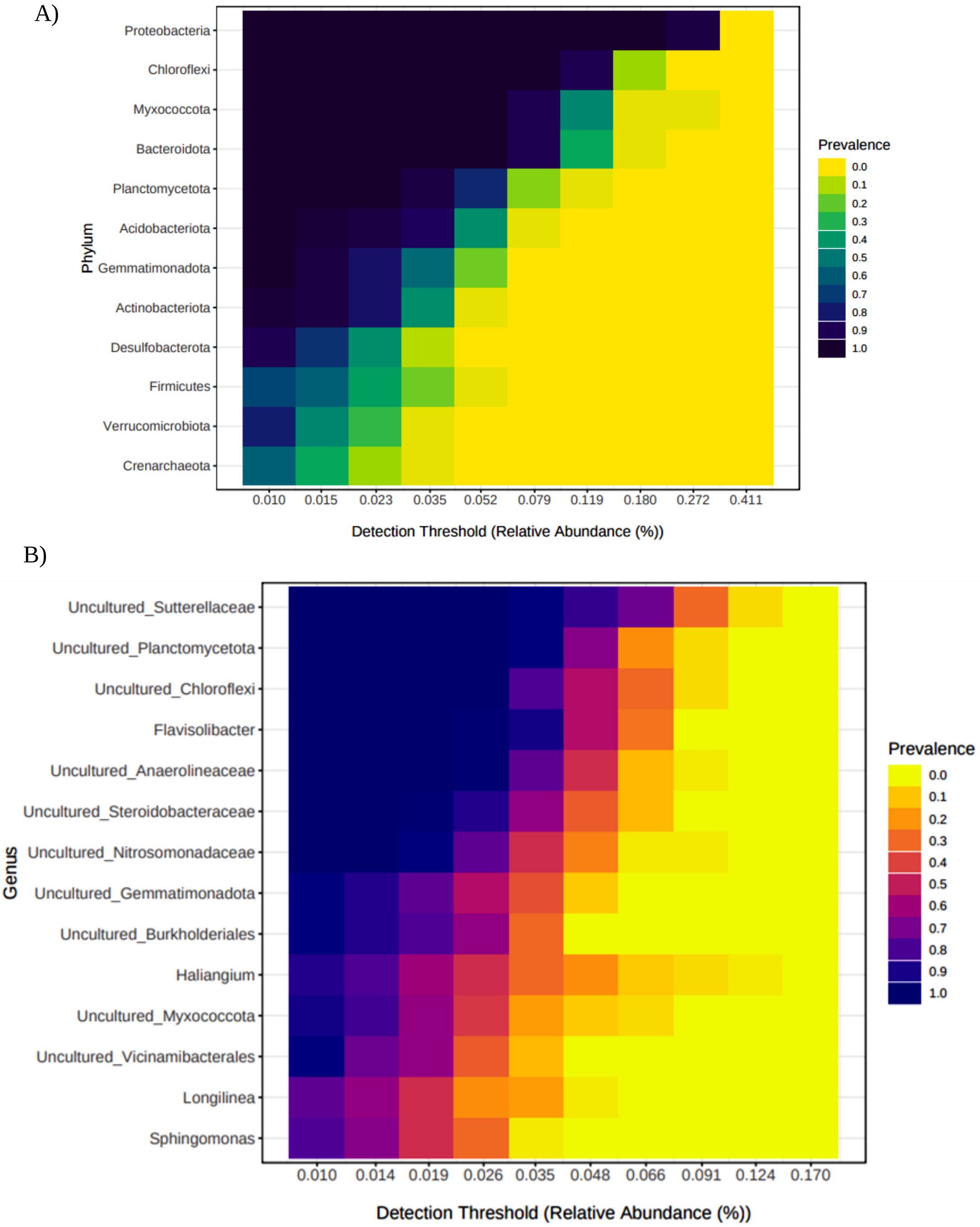


Fig 3.27: Heatmaps representing the core microbiome of chitosan on rice rhizosphere bacteriome at Phylum (A) and Genus levels (B). The Y-axis represents the prevalence level of core microbiome taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified microbiome taxa

3.1.10. Influence of chitosan on rice rhizosphere mycobiome

Chitosan treatment at, low dosage has highest alpha-diversity (Shannon diversity index), followed by other treatments (Fig 3.28A) at flowering. Using PCoA plots, the response of fungal communities to chitosan treatment is shown as separate clustering of sample groups without any overlaps (Fig 3.28B). PERMANOVA revealed substantial variations in zOTU assemblage between fungal communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for fungal communities in different fractions is 0.697 (69.7%) (Fig 3.28C). LEfSe analysis identified *Scedosprium* as the biomarkers associated with rice rhizosphere with chitosan amendments (Fig 3.28D).

The relative abundance of Uncultured Fungi, Ascomycota, Mucoromycota, Basidiomycota, Chytridiomycota and Blastocladiomycota was detected in chitosan-treated rice rhizosphere at phylum level (Fig 3.29A). Uncultured Fungi, *Mortierella, Scedosporium, Cordyceps, Aspergillus* and *Aigialus* were highly abundata taxa at genus level, with respect to chitosan treatment to rice rhizosphere (Fig 3.29B).

The core microbiome was calculated using sample prevalence (50 %) with relative abundance of 0.01 % for taxa at phylum and genus level and presented in Fig 3.30A, Fig 3.30B, respectively for chitosan treatment to rice rhizosphere. The core microbiome at phylum level comprised of Uncultured Fungi, Mucoromycota, Ascomycota, Basidiomycota and Chytridiomycota (Fig 3.30A). The core microbiome at genus level comprised of Uncultured Fungi, *Mortierella, Cordyceps, Aspergillus, Scedosporium*, *Stachybotrys elegans, Pluteus, Metarhizi, Sebacina*, Uncultured Talaromyces, *Sodiomyces alcalophilus*, *Chytriomyces* and *Catenaria* during chitosan treatment to rice rhizosphere (Fig 3.30B).

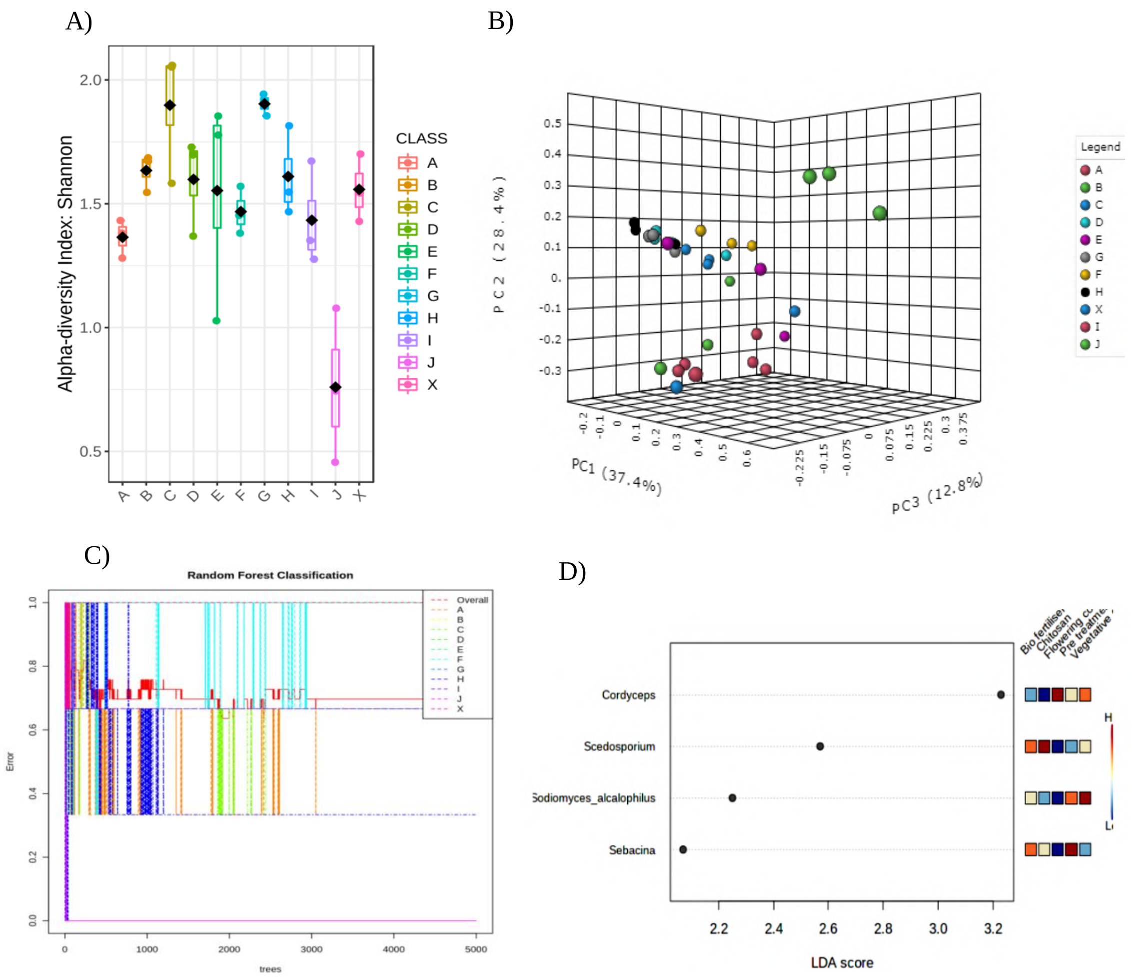


Fig 3.28: Influence of chitosan on rice rhizosphere mycobiome. (A). Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.297; [Kruskal-Wallis] statistic**:** 19.943. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [37.4 %] and the Y-axis representing the second-highest dimension of variation [28.4%] and Z- axis represents third highest degree of variation [12.8%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 5.5417; R^2**:** 0.71583; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate 69.7% (0.697) is shown as the red line; lines of other colors represent the error rates for each class.(D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off**:** 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. 'Uncultured_' taxa labels in the figures represent unclassified taxa belongs to microbiome. (Chitosan -High Dosage-Vegetative – A, Chitosan - Low Dosage-Vegetative – B, Chitosan - High Dosage-Flowering – C, Chitosan - Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Bio fertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre-treatment – X, Vegetative control – VC, Flowering control – FC).

Fig 3.29: Influence of chitosan on the taxonomic composition of rice rhizosphere fungal community. Stacked bar plots represent the relative abundance of eukaryote taxa at Phylum (A) and Genus level (B). Uncultured' taxa labels in the figures represent unclassified fungal taxa

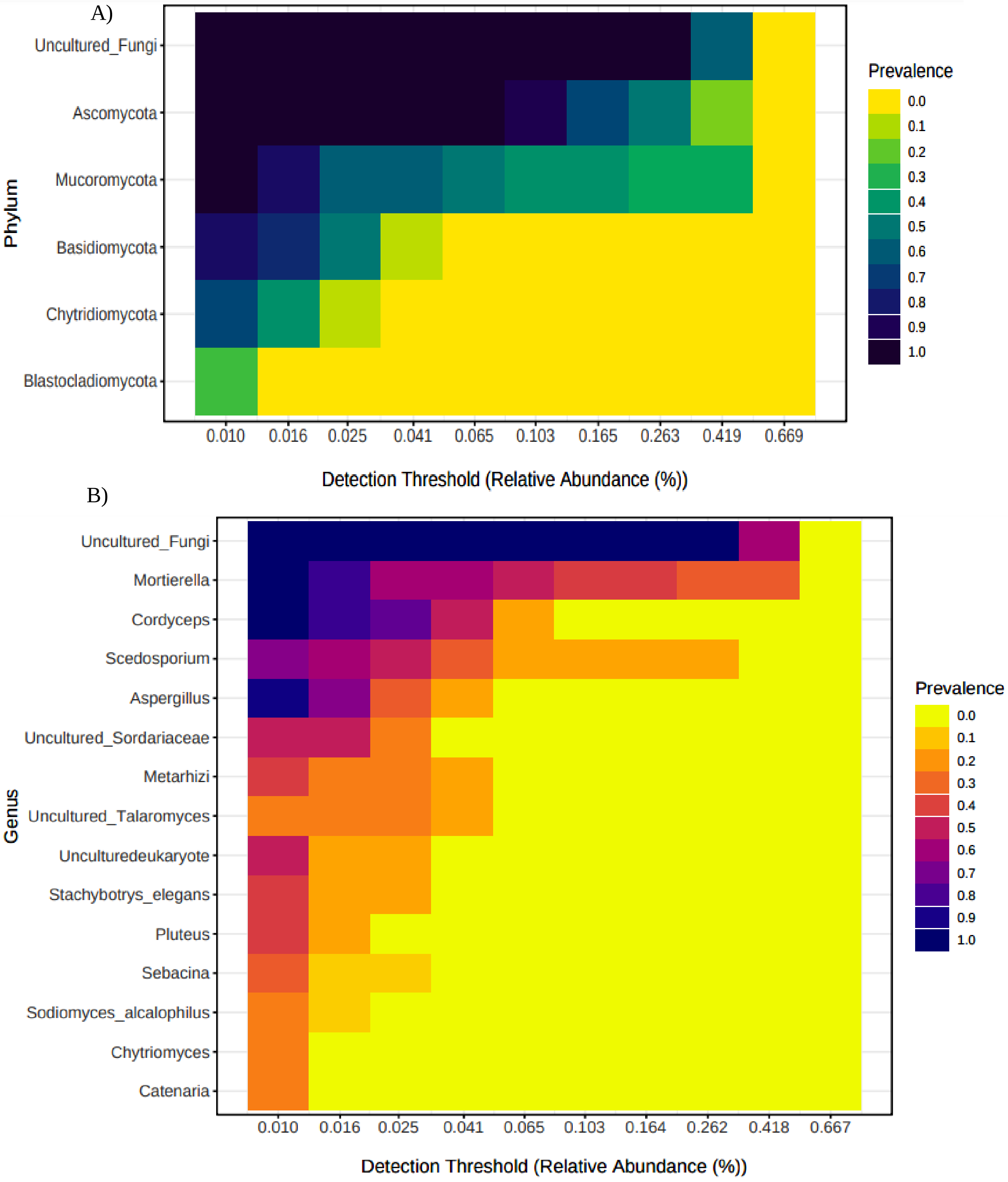


Fig 3.30: Heatmaps representing the core microbiome of chitosan on rice rhizosphere mycobiome at phylum (A) and genus levels (B). The Y-axis represents the prevalence level of core microbiome taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified microbiome taxa

3.1.11. Influence of chitosan on rice rhizosphere eukaryome

The chitosan treatments resulted in lowest alpha-diversity (Shannon diversity index), followed by other treatments (Fig 3.31A) at vegetative and flowering stages of rice. Using PCoA plots, the response of eukaryote communities to chitosan treatment was shown as separate clustering of sample groups without any overlaps (Fig 3.31B). PERMANOVA revealed substantial variations in zOTU assemblage between eukaryote communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for eukaryote communities in different fractions is 0.333 (33.3%) (Fig 3.31C). LEfSe analysis identified *Acanthamoeba* as the biomarkers associated with rice rhizosphere with chitosan amendment (Fig 3.31D).

The relative abundance of Uncultured Eukaryote, Fungi incertae sedis, Discosea, Ciliophora, Arthropoda, Amoebozoa, Uncultured micro Eukaryote, Euglenozoa were detected taxa abundant during chitosan treatment to rice rhizosphere at phylum level (Fig. 3.32A). Uncultured Eukaryote, Uncultured Cryptomycota, *Tyrophagus putrescentiae*, Uncultured micro Eukaryote, *Cochliopodium, Acanthamoeba, Eimeriidae, Aristerostoma marinum* were top abundant genus in rice rhizosphere amended with chitosan (Fig 3.32B).

The core microbiome was calculated using sample prevalence (50%) with relative abundance of 0.01% for taxa at phylum level (Fig 3.33A) and genus level (Fig 3.33B) for chitosan treatment. The core microbiome at phylum level comprised of Uncultured micro Eukaryote, Uncultured Fungi, Uncultured Eukaryote, Nematoda, Heterolobosea, Fungi incertae sedis, Euglenozoa, Discosea, Ciliophora, Arthropoda, Apicomplexa, Amoebozoa, Evosea, Rozellomycota, Cercozoa, Tubulinea, Oomycota, Cnidaria, Eukaryotaincertae sedis, Loukozoa and Hapista (Fig 3.33A). Uncultured micro Eukaryote, Uncultured Fungi, Uncultured Eukaryote, Uncultured Cryptomycota, *Tyrophagus putrescentiae*, *Pseudoplatynematum denticulatum*, *Protacanthamoeba*, *Eimeriidae*, *Aristerostoma marinum*, *Telaepolella tubasferens*, *Acanthamoeba*, Uncultured Rozellomycota, *Cochliopodium* and *Kuklikophrya ougandae* were core eukayote taxa at genus level (Fig 3.33B).

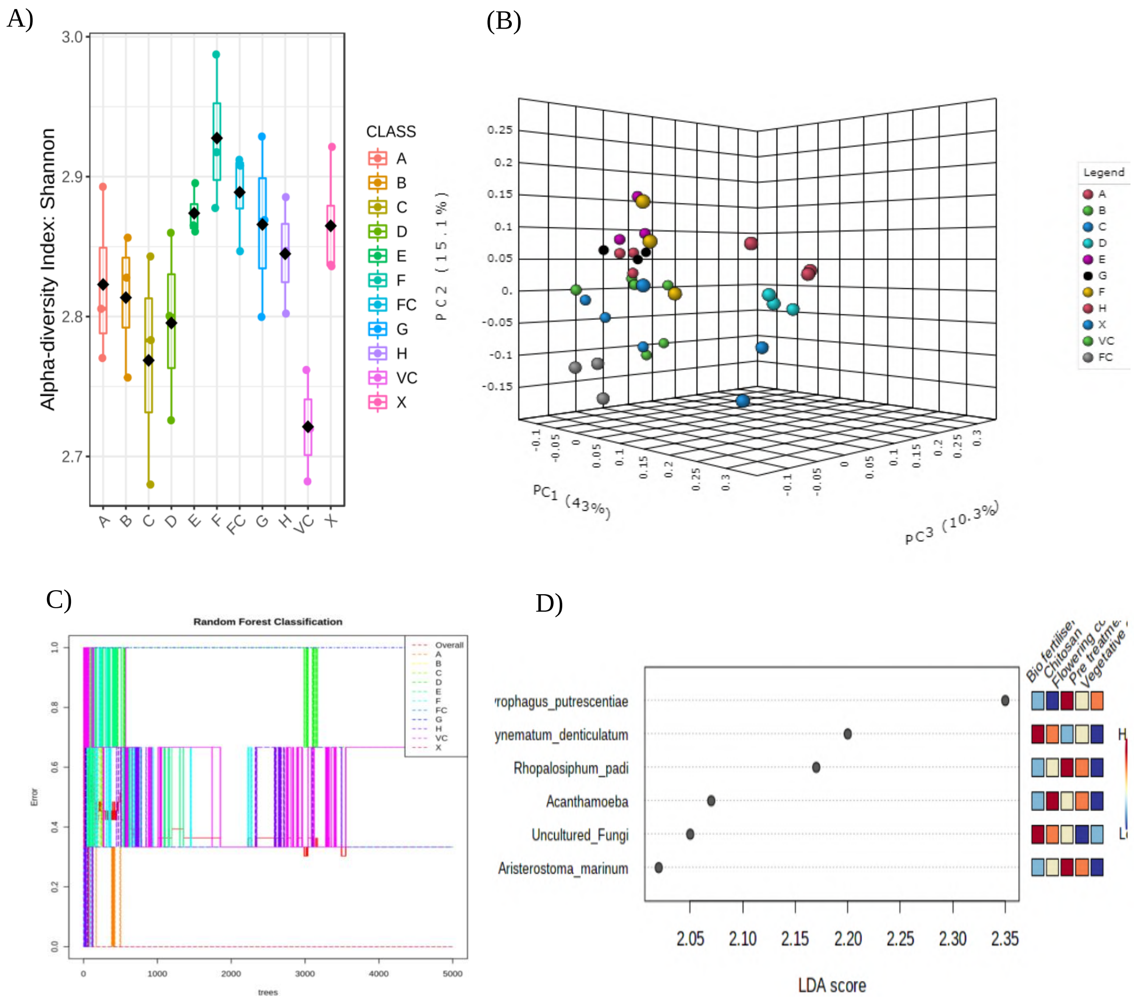


Fig 3.31: Influence of chitosan on rice rhizosphere eukaryome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.06029; [Kruskal-Wallis] statistic**:** 17.697. (B) 2-D PCoA plots based on Bray–Curtis similarity [n= 57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [43 %] and the Y-axis representing the second-highest dimension of variation [15.1%] and Z- axis represents third highest degree of variation [10.3%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 10.89; R^2**:** 0.83194; *p* <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate 33.3% (0.333) is shown as the red line; lines of other colors represent the error rates for each class. D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off**:** 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. 'Uncultured' taxa labels in the figures represent unclassified taxa belongs to microbiome (Chitosan -High Dosage-Vegetative – A, Chitosan - Low Dosage-Vegetative – B, Chitosan - High Dosage-Flowering – C, Chitosan - Low Dosage-Flowering – D, Bio fertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Bio fertilizer-Half RD-Flowering – H, Pre-treatment – X, Vegetative control – VC, Flowering control – FC).

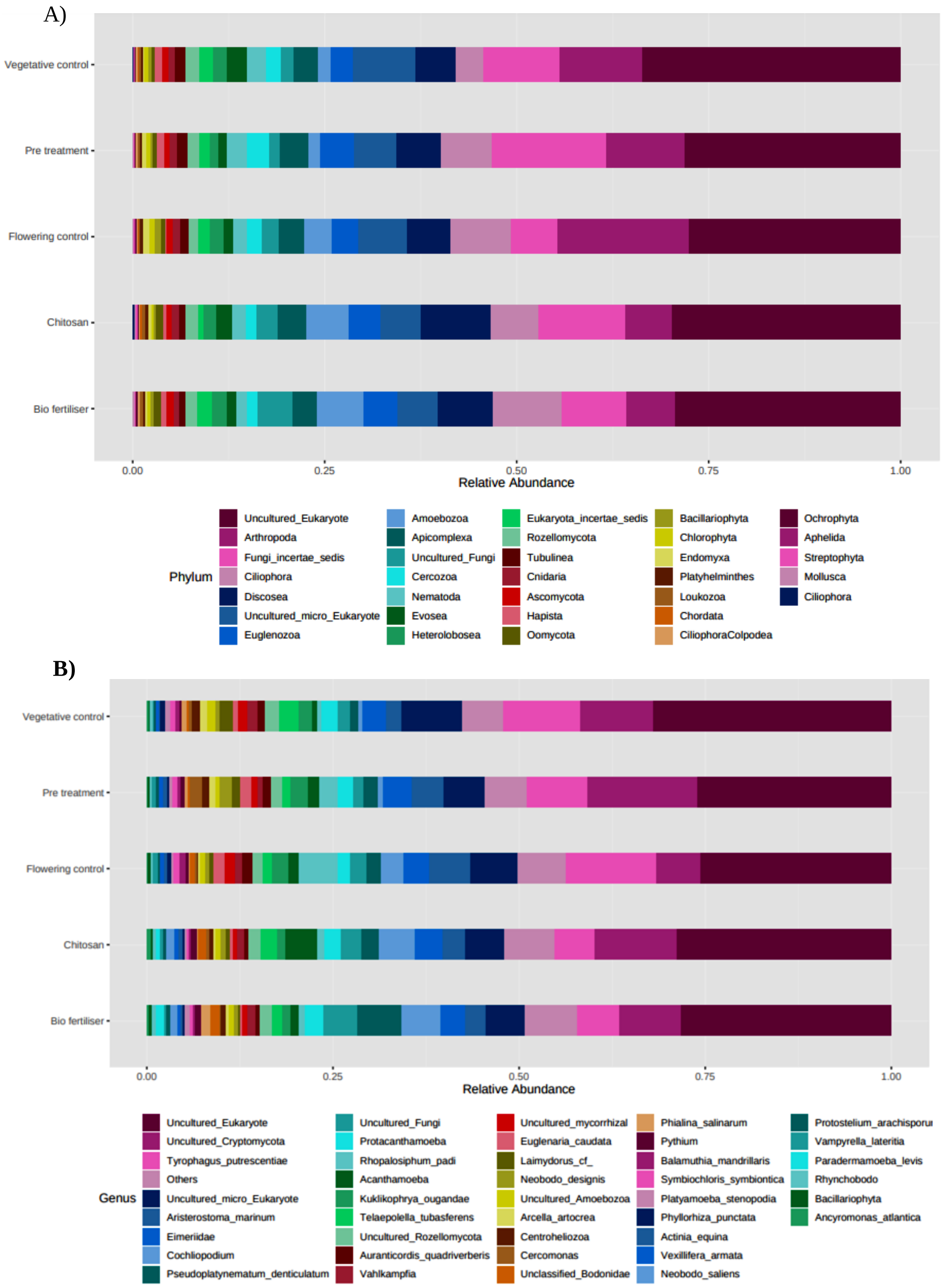


Fig 3.32: Influence of chitosan on the taxonomic composition of rice rhizosphere eukaryote community. Stacked bar plots represent the relative abundance of eukaryote taxa at Phylum level (A) and at Genus level (B). The Uncultured’ taxa labels in the figures represent unclassified eukaryote taxa.

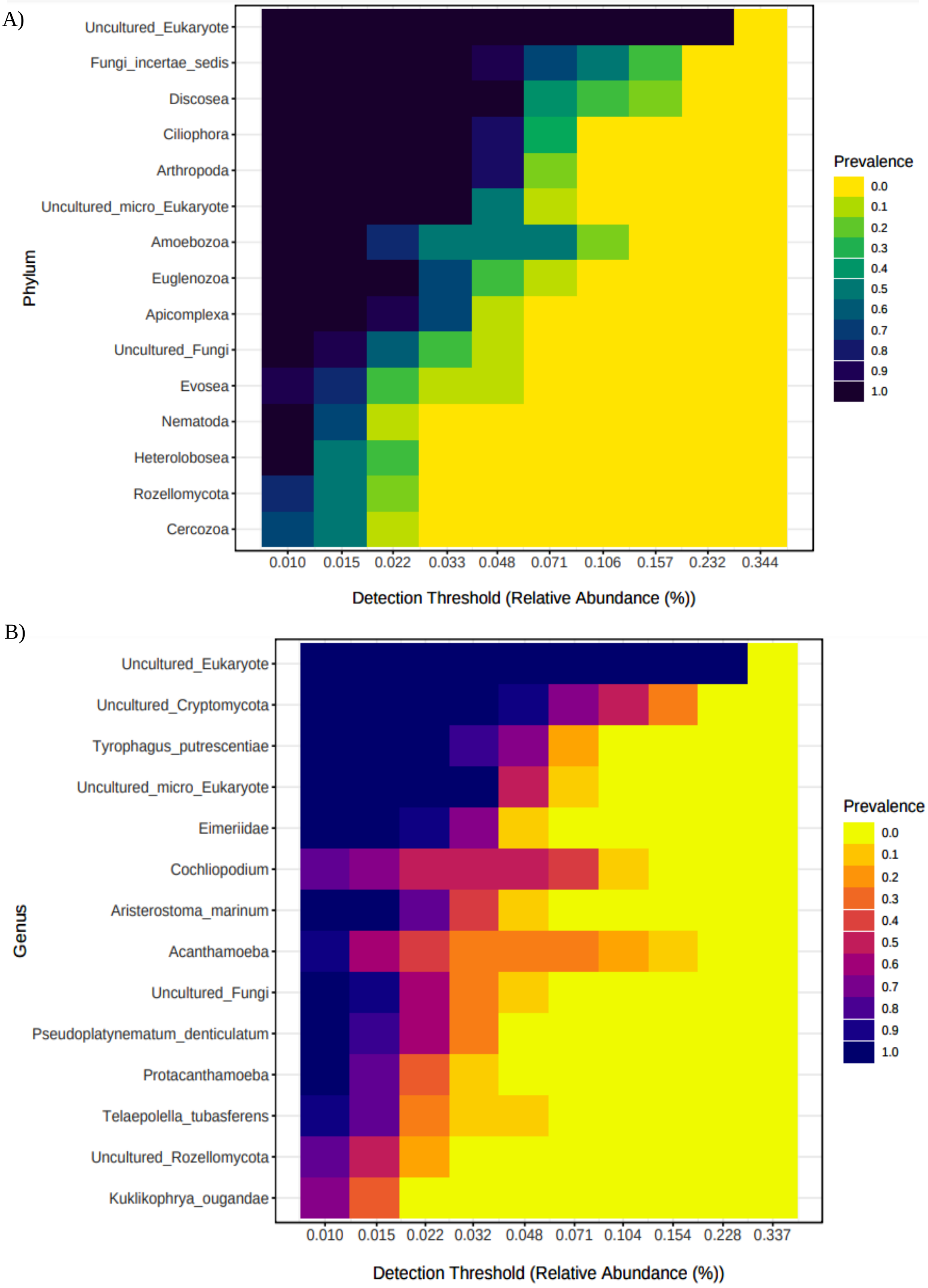


Fig 3.33: Heatmaps representing the core microbiome of chitosan on rice rhizosphere eukaryome at phylum (A) and genus levels (B). Y-axis represents the prevalence level of core microbiome taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified microbiome taxa

3.1.12. Influence of chitosan on rice rhizosphere microbiome

The chitosan at low and high dosages and at different plant development stages has lowest alpha-diversity (Shannon diversity index) when compare to other treatments i.e. biofertilizer, vegetative and flowering control (Fig 3.34A). Using PCoA plots, the response of microbiome communities to chitosan treatment was shown as separate clustering of sample groups without any overlaps (Fig 3.34B). PERMANOVA revealed substantial variations in zOTU assemblage between microbiome communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for microbiome communities in different fractions is 0.333(33.3%) (Fig 3.34C). *Sporacetigenium* sp. of bacterial genus, *Mortierella* and *Scedosporium* of fungi, *Acanthamoeba, Cochliopodium* of eukaryota are indicator features observed for chitosan treatment to rice rhizosphere (Fig 3.34D).

The relative abundance of Uncultured Eukaryote, Fungi Incertae sedis, Discosea, Ciliophora and Arthropoda were detected taxa abundant during chitosan treatment to rice rhizosphere at phylum level (Fig 3.35A). Uncultured Eukaryote, Uncultured Cryptomycota, *Tyrophagus putrescentiae*, Uncultured micro Eukaryote, *Cochliopodium* and *Acanthamoeba* were highly abundant taxa at genus level (Fig 3.35B).

The core microbiome, calculated using sample prevalence (50 %) with relative abundance of 0.01 % for taxa, is presented at phylum level (Fig 3.36A) and genus level (Fig 3.36B) for the chitosan treatment. The core microbiome at phylum level comprised of Uncultured Micro Eukaryote, Uncultured Fungi, Uncultured Eukaryote, Proteobacteria, Fungi incertae sedis, Discosea, Ciliophora, Chloroflexi, Ascomycota, Arthropoda, Apicomplexa, Euglenozoa, Amoebozoa, Myxococcota, Bacteroidota, Evosea, Nematoda, Mucoromycota, Heterolobosea, Cercozoa, Rozellomycota and Cnidaria. Uncultured micro Eukaryote, Uncultured Fungi, Uncultured Eukaryote, Uncultured Cryptomycota, *Tyrophagus putrescentiae, Pseudoplatynematum denticulatum, Protacanthamoeba, Eimeriidae, Aristerostoma marinum, Telaepolella tubasferens, Acanthamoeba*, Uncultured Rozellomycota, *Cochliopodium, Kuklikophrya ougandae*, Unclassified Bodonidae, *Rhopalosiphum padi, Neobodosaliens, Vahlkampfia, Pythium, Paradermamoeba levis, Neobodo designis* and *Actinia equine* were core microbiome taxa at genus level (Fig 3.37B).

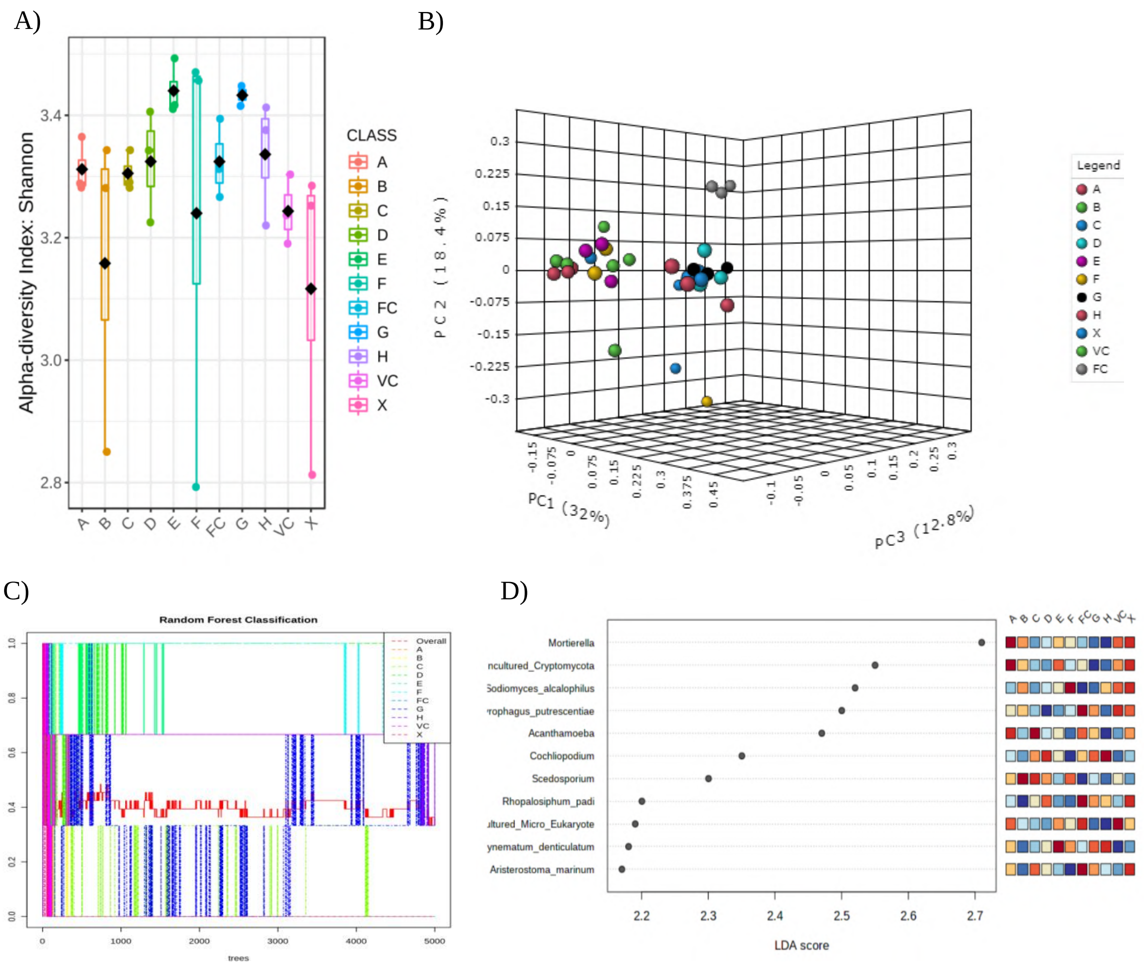


Fig 3.34: Influence of chitosan on rice rhizosphere microbiome (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.1121; [Kruskal-Wallis] statistic**:** 15.586. (B) 2-D PCoA plots based on Bray–Curtis similarity [n= 57]. The explained variances were shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [32%] and the Y-axis representing the second-highest dimension of variation [18.4%] and Z- axis represents third highest degree of variation [12.8%]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 53.8314; R^2**:** 0.63524; *p* <0. 001. (C) Cumulative OOB error rates by RF classification. The overall error rate 33.3 % (0.333) is shown as the red line; lines of other colors represent the error rates for each class. D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off**:** 0.05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. 'Uncultured' taxa labels in the figures represent unclassified taxa belongs to Microbiome (Chitosan -High Dosage-Vegetative – A, Chitosan - Low Dosage-Vegetative – B, Chitosan - High Dosage-Flowering – C, Chitosan - Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre-treatment – X, Vegetative control – VC, Flowering control – FC).

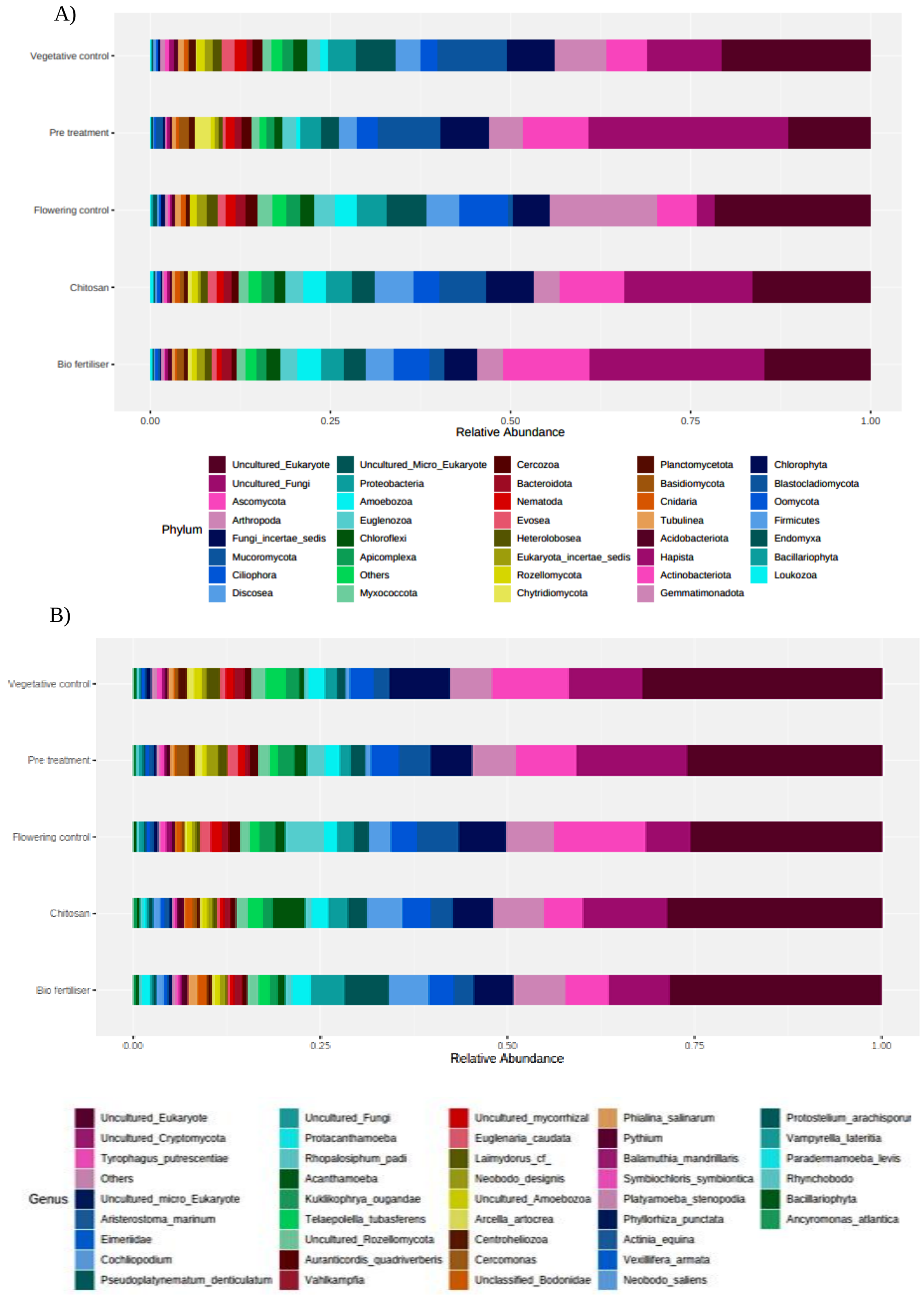


Fig 3.35: Influence of chitosan on the taxonomic composition of rhizosphere microbiome in rice. Stacked bar plots represent the relative abundance of eukaryote taxa at Phylum level (A) and Genus level (B). Uncultured' taxa labels in the figures represent unclassified microbiome taxa.

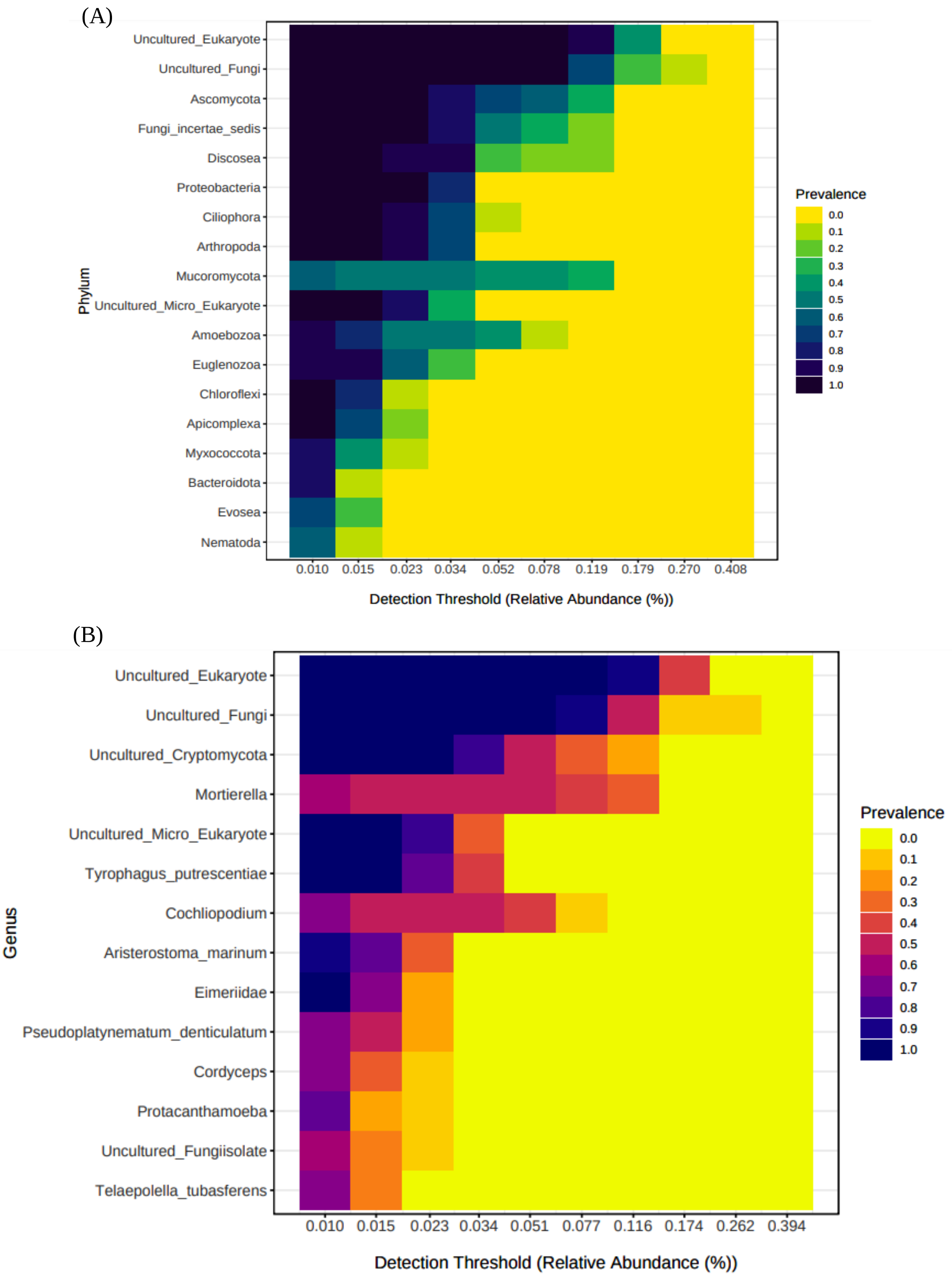


Fig 3.36: Heatmaps representing the core microbiome of chitosan on rice rhizosphere at Phylum (A) and Genus levels (B). Y-axis represents the prevalence level of core microbiome taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified microbiome taxa.

3.1.13. Influence of plant developmental stage on rice rhizosphere

The flowering stage has highest alpha-diversity (Shannon diversity index) compared to the vegetative stage (Fig 3.37A). Using PCoA plots, the response of microbiome communities to beta chitin treatment was shown as separate clustering of sample groups without any overlaps (Fig 3.37B). PERMANOVA revealed substantial variations in zOTU assemblage between microbiome communities of the different fractions. RF analysis (made by growing 5,000 decision trees) with cumulative OOB error rate for microbiome communities in different fractions is 0.526 (52.6%) (Fig 3.37C). Uncultured fungi, *Mortierella* of fungi, and *Cochliopodium* of eukaryota are the indicator features obtained for different plant developmental stages of rice rhizosphere (Fig 3.37D).

The relative abundance of Uncultured Fungi, Uncultured Eukaryote, Ascomycota, Amoebozoa, Arthropoda, Ciliophora, Discosea, Fungi incertae sedis, Euglenozoa and Proteobacteria were the abundant taxa observed during developmental stages in rice rhizosphere at phylum level (Fig 3.38A). Uncultured Fungi, Uncultured Eukaryote, *Cochliopodium*, Uncultured Cryptomycota, *Tyrophagus putrescentiae*, *Cordyceps* spp. and *Aristerostoma marinum* are majorly observed genus of rice rhizosphere (Fig 3.38B).

The core microbiome, calculated using sample prevalence (50 %) with relative abundance of 0.01 % for taxa, is presented at phylum level (Fig 3.39A) and genus level (Fig 3.39B) for vegetative and flowering stages of rice rhizosphere. The core microbiome at phylum level comprised of Uncultured Eukaryote, Proteobacteria, Fungi incertae sedis, Euglenozoa, Discosea, Ciliophora, Ascomycota and Arthropoda (Fig 3.39A). Uncultured Eukaryote, Uncultured Cryptomycota, Uncultured Micro Eukaryote, Uncultured Fungi, *Tyrophagus putrescentiae*, *Eimeriidae*, *Aristerostoma marinum*, *Pseudoplatynematum denticulatum*, Protacanthamoeba, *Cordyceps*, *Cochliopodium*, *Mortierella*, Uncultured Sutterellaceae, *Rhopalosiphum padi*, *Neobodo saliens*, *Telaepolella tubasferens*, *Acanthamoeba*, Uncultured Chloroflexi, *Aspergillus*, Uncultured Planctomycetota, Unclassified Bodonidae, *Kuklikophrya ougandae*, Uncultured Sordariaceae, *Phialina salinarum* and *Flavisolibacter* were core microbiome taxa at genus level (Fig 3.39B). Pseudo-F was calculated using PERMENOVA, and it was used as a proxy to identify key factors shaping rice rhizosphere microbiome and represented in Fig 3.40.

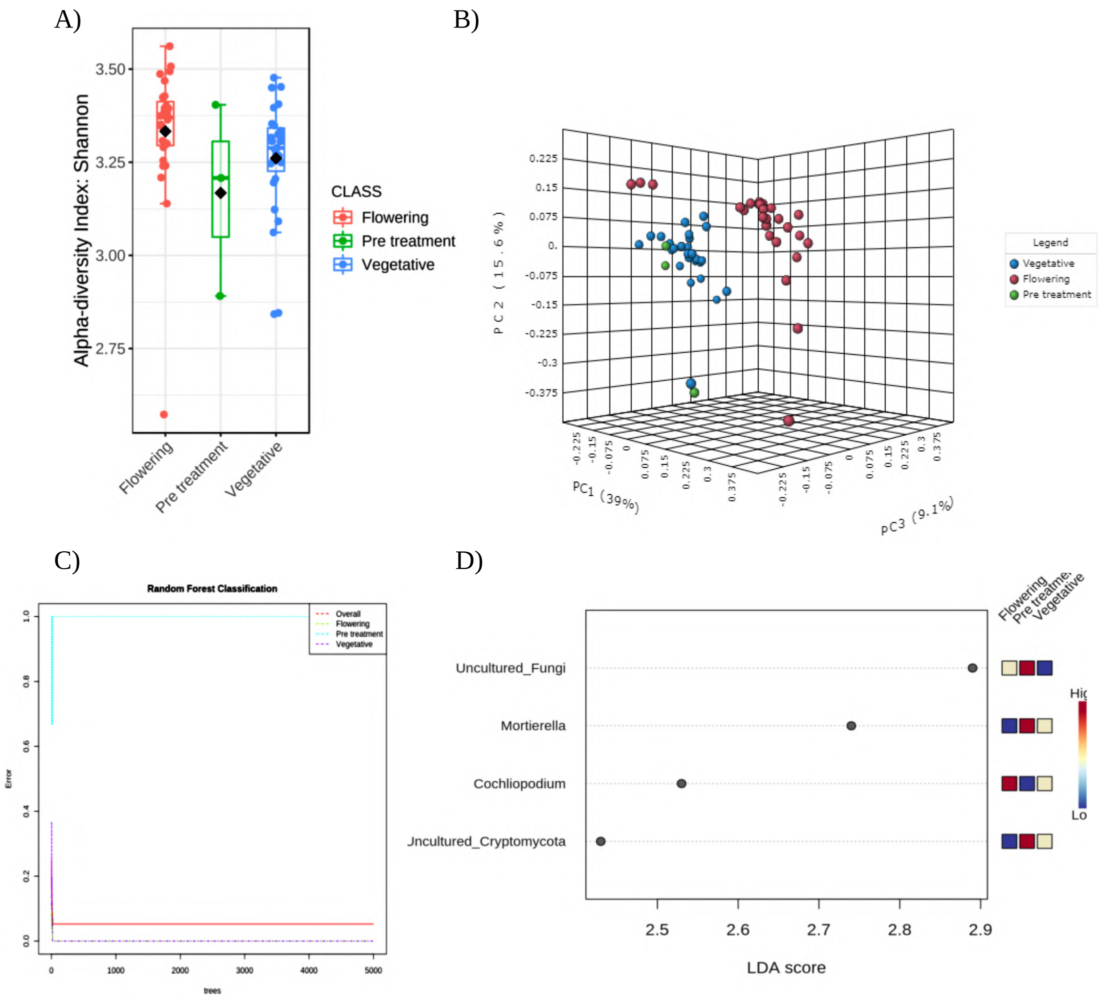


Fig 3.37: Influence of plant developmental stages on rice rhizosphere microbiome. (A) Alpha-diversity measure using Shannon index at Genus level represented as boxplots. Each boxplot represents the diversity distribution of a sample group. The sample groups are represented on the X-axis and their estimated diversity on the Y-axis. Statistical significance**:** *p***:** 0.060223; [Kruskal-Wallis] statistic**:** 5.6194. (B) 2-D PCoA plots based on Bray–Curtis similarity [n=57]. The explained variances are shown in brackets. Each axis reflects the percentage of the variation between the samples, with the X-axis representing the highest dimension of variation [39%] and the Y-axis representing the second-highest dimension of variation [15.6%] and Z- axis represents third highest degree of variation [9.1 %]. Statistical significance**:** [PERMANOVA] *pseudo-F* value**:** 9.4961 R^2**:** 0.38766; p <0.001. (C) Cumulative OOB error rates by RF classification. The overall error rate 5.26% (0.0526) is shown as the red line; lines of other colors represent the error rates for each class. D) Graphical summary of important features (differentially abundant taxa) identified by LEfSe at the Genus level. Taxa with significant differential abundance are ranked in decreasing order of their logarithmic LDA scores (Effect Size) on the X-axis. Features are considered to be significant based on their FDR-adjusted p-value [cut-off**:** 0. 05]. The mini heatmap to the right of the plot indicates whether the taxa are enriched (red) or depleted (blue) in each group. ‘Uncultured’ taxa labels in the figures represent unclassified taxa belongs to microbiome.

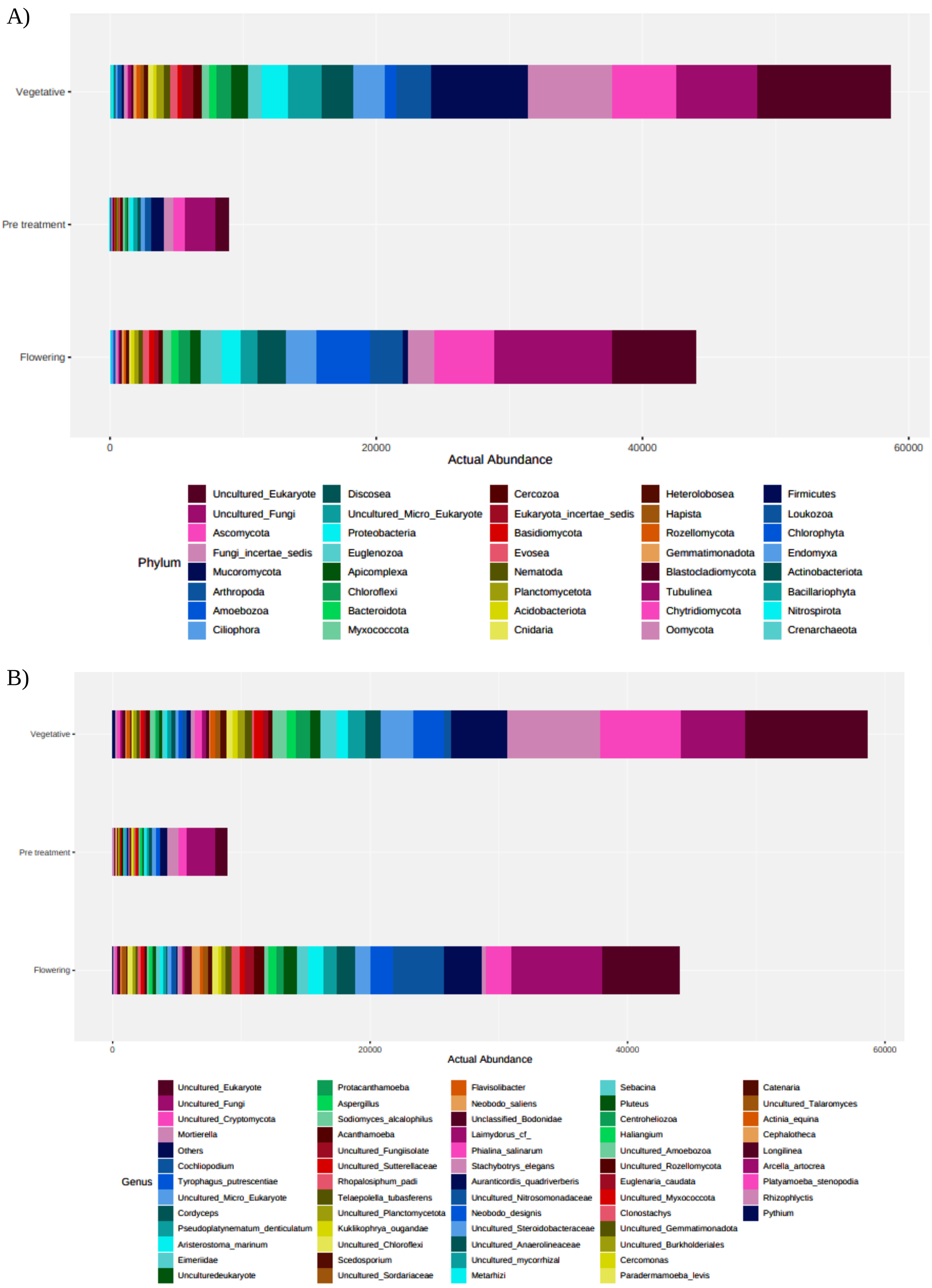


Fig 3.38: Influence of plant developmental stages on taxonomic composition of rice rhizosphere microbiome. Stacked bar plots represent the relative abundance of eukaryote taxa at Phylum level (A) and Genus level (B). ‘Uncultured’ taxa labels in the figures represent unclassified microbiome taxa

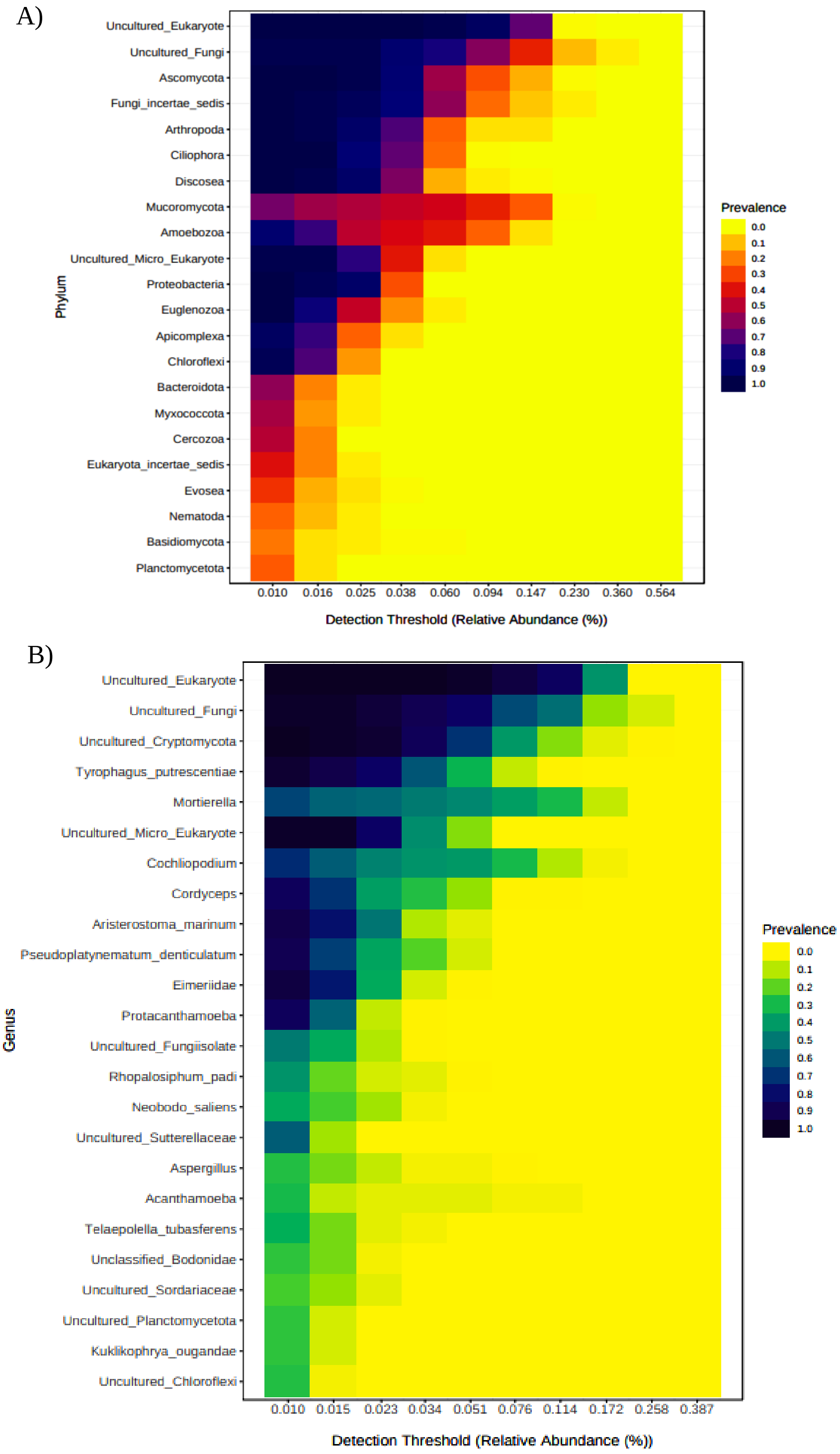


Fig 3.39: Heatmaps representing core microbiome of rice rhizosphere at Phylum (A) and Genus levels (B). Y-axis represents the prevalence level of core microbiome taxa across the detection threshold (relative abundance) range on the X-axis. The variation of prevalence of each phylum/genus is indicated by a colour gradient from blue/yellow (decreased) to red/topo blue (increased). ‘Uncultured’ taxa label in the figure represents unclassified microbiome taxa.

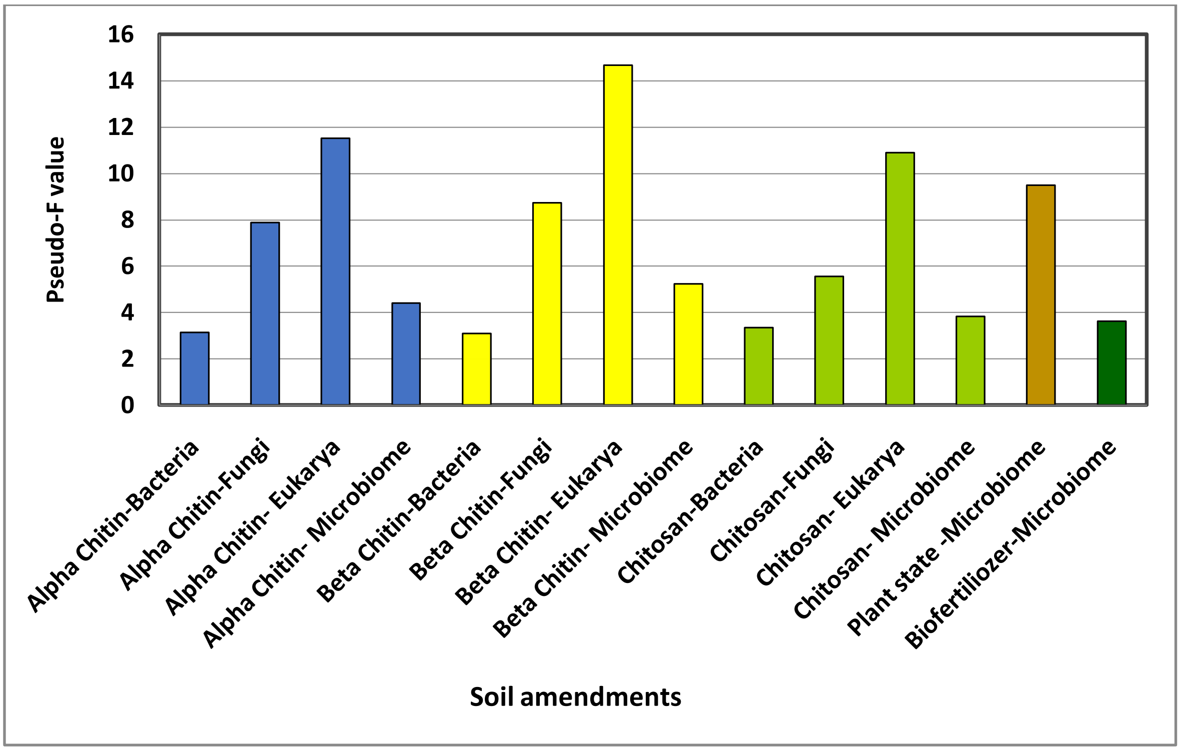


Fig 3.40: PERMANOVA output measuring the influence of different factors. Influences of different factors were calculated in rice rhizosphere microbiome using the pseudo-F value as a proxy.

Plant developmental stage plays a vital role in shaping the host microbiome, irrespective of the plant system. It was also observed in rice rhizosphere microbiome with different soil amendements. ANOSIM test was computed to determine the differences across factors, i.e. mono-, bi- and tri-partite combinations of soil amendments represented in Table 3.1 (treatment), 3.2 (treatment x plant state), 3.3 (treatment x dosage) and 3.4 (treatment x plant state x dosage), respectively.

Important abundant features were identified with different soil amendments to rice rhizosphere (bactera, fungi and eukarya) for alpha chitin (Fig 3.41), beta chitin (Fig 3.42), chitosan (Fig 3.43), all amandemnts (Fig 3.44) and plant developmental stages (Fig 3.45) by EdgeR algortihm with FDR adjusted at $p<0.05$ and expressed in RLE (Relative Log Expression).

Soil amendment with chitinous substrates showed a less significant dose-dependent response between alpha chitin high dosage and alpha chitin low dosage (R=0.03,P<0.253), alpha chitin high dosage and pre-treatment (R=0.593,P<0.024); alpha chitin high dosage and rice rhizosphere at vegetative stage (control) (R=0.593,P<0.024); alpha chitin high dosage and rice rhizosphere at flowering stage (control) (R=0.994,P<0.012); alpha chitin low dosage and pre treatment (R=0.272,P<0.06); alpha chitin low dosage and rice rhizosphere at vegetative stage (control) (R=0.259,P<0.143); alpha chitin low dosage and rice rhizosphere at flowering stage (control) (R=0.34,P<0.06). Similar results observed with beta chitin amendments, lowest found in beta chitin high dosage and beta chitin low dosage (R=-0.026,P<0.535); beta chitin high dosage and pre treatment (R=0.346,P<0.06); beta chitin high dosage and rice rhizosphere at vegetative stage (control) (R=0.272,P<0.107); beta chitin high dosage and rice rhizosphere at flowering stage (control) (R=0.574,P<0.024); beta chitin low dosage and pre treatment (R=0.512,P<0.024); beta chitin low dosage and rice rhizosphere at vegetative stage (control) (R=0.364,P<0.036); beta chitin low dosage and rice rhizosphere at flowering stage (control) (R=0.716,P<0.012). Chitosan dosage-related results were comparable to those of alpha and beta chitin, i.e., chitosan high dosage and chitosan low dosage (R=-0.002,P<0.416); chitosan high dosage and pre-treatment (R=0.556,P<0.024); chitosan high dosage and rice rhizosphere at vegetative stage (control) (R=0.512,P<0.024); chitosan high dosage and rice rhizosphere at flowering stage (control) (R=0.741,P<0.012); chitosan low dosage and pre treatment (R=0.451,P<0.024); chitosan low dosage and rice rhizosphere at vegetative stage (control) (R=0.358,P<0.048); chitosan low dosage and rice rhizosphere at flowering stage (control) (R=0.426,P<0.036); pre treatment, rice rhizosphere at vegetative stage (control) (R=0.556,P<0.1); pre treatment, rice rhizosphere at flowering stage (control) (R=1,P<0.1); rice rhizosphere at vegetative stage (control), rice rhizosphere at flowering stage (control) (R=1,P<0.1) (Table 3.3).

When three variables were combined, treatment (alpha, beta, and chitosan) x dosage (low and high) x developmental stage (vegetative and flowering), there were significant variations. Alpha chitin (high dosage at flowering stage) and alpha chitin (low dosage at flowering stage) (R=0.37, P<0.001); alpha chitin (high dosage at vegetative stage) and alpha chitin (high dosage at flowering stage) (R=1, P<0.001); alpha chitin (high dosage at vegetative stage) and alpha chitin (low dosage at vegetative stage) (R=1, P<0.001); beta chitin (high dosage at flowering stage), beta chitin (low dosage at flowering stage) (R=0.259, P<0.001); beta chitin (high dosage at vegetative stage) and beta chitin (high dosage at flowering stage) (R=1, P<0.001); beta chitin (high dosage at vegetative stage) and beta chitin (low dosage at vegetative stage) (R=1, P<0.001); chitosan (high dosage at flowering stage) and chitosan (low dosage at flowering stage) (R=1, P<0.001); chitosan (high dosage at vegetative stage) and chitosan (high dosage at flowering stage) (R=1, P<0.001); chitosan (high dosage at vegetative stage) and chitosan (low dosage at flowering stage) (R=1, P<0.001). Overall, ANOISM data imply that plant developmental stage-driven responses are greater than dose responses in all rice rhizosphere with chitin treatments (Table 3.4).

Groups	% of difference	P value
Alpha Chitin *vs.* Beta Chitin	12.1	0.053
Alpha Chitin *vs.* Chitosan	18.5	0.024
Alpha Chitin *vs.* Biofertilizer	15.9	0.031
Alpha Chitin *vs.* Pretreatment	39.4	0.04
Alpha Chitin *vs.* Vegetative control	28.3	0.086
Alpha Chitin *vs.* Flowering control	56.2	0.004
Beta Chitin *vs.* Chitosan	11.2	0.047
Beta Chitin *vs.* Biofertilizer	21.9	0.008
Beta Chitin *vs.* Pretreatment	42.9	0.026
Beta Chitin *vs.* Vegetative control	27.3	0.068
Beta Chitin *vs.* Flowering control	51.8	0.007
Chitosan *vs.* Biofertilizer	11.2	0.056
Chitosan *vs.* Pretreatment	49.7	0.002
Chitosan *vs.* Vegetative control	37.3	0.009
Chitosan *vs.* Flowering control	55.2	0.002
Biofertilizer *vs.* Pretreatment	45.2	0.013
Biofertilizer *vs.* Vegetative control	32.9	0.037
Biofertilizer *vs.* Flowering control	49.3	0.004
Pretreatment *vs.* Vegetative control	55.6	0.1
Pretreatment *vs.* Flowering control	100	0.1
Vegetative control *vs.* Flowering control	100	0.1
Vegetative *vs.* Flowering	74.6	0.001
Vegetative *vs.* Pretreatment	55.4	0.013
Flowering *vs.* Pretreatment	82.6	0.001

Table 3.1: ANOSIM differences between various factors of rice rhizosphere microbiome. Comparative differences were calculated using the Bray-Curtis Similarity distance matrix by ANOSIM for various soil amendments of rice rhizosphere.

Groups	% of difference	P value
Alpha Chitin - Vegetative *vs.* Alpha Chitin – Flowering	82.2	0.002
Alpha Chitin - Vegetative *vs.* Beta Chitin – Vegetative	67.6	0.002
Alpha Chitin - Vegetative *vs.* Beta Chitin – Flowering	89.3	0.002
Alpha Chitin - Vegetative *vs.* Chitosan – Vegetative	51.9	0.002
Alpha Chitin - Vegetative *vs.* Chitosan – Flowering	100	0.002
Alpha Chitin - Vegetative *vs.* Biofertilizer - Vegetative	59.1	0.002
Alpha Chitin - Vegetative *vs.* Biofertilizer - Flowering	100	0.002
Alpha Chitin - Vegetative *vs.* Pre treatment	85.8	0.012
Alpha Chitin - Vegetative *vs.* Rice - Vegetative Rhizosphere	96.9	0.012
Alpha Chitin - Vegetative *vs.* Rice - Flowering Rhizosphere	100	0.012
Alpha Chitin - Flowering *vs.* Beta Chitin - Vegetative	92.4	0.002
Alpha Chitin - Flowering *vs.* Beta Chitin - Flowering	24.3	0.019

Alpha Chitin - Flowering *vs.* Chitosan – Vegetative	87.8	0.002
Alpha Chitin - Flowering *vs.* Chitosan – Flowering	40.2	0.002
Alpha Chitin - Flowering *vs.* Biofertilizer - Vegetative	74.1	0.002
Alpha Chitin - Flowering *vs.* Biofertilizer - Flowering	46.3	0.002
Alpha Chitin - Flowering *vs.* Pre treatment	95.1	0.012
Alpha Chitin - Flowering *vs.* Rice - Vegetative Rhizosphere	95.7	0.012
Alpha Chitin - Flowering *vs.* Rice - Flowering Rhizosphere	91.4	0.012
Beta Chitin - Vegetative *vs.* Beta Chitin - Flowering	88.5	0.002
Beta Chitin - Vegetative *vs.* Chitosan – Vegetative	37.2	0.004
Beta Chitin - Vegetative *vs.* Chitosan – Flowering	100	0.002
Beta Chitin - Vegetative *vs.* Biofertilizer - Vegetative	57.8	0.002
Beta Chitin - Vegetative *vs.* Biofertilizer - Flowering	100	0.002
Beta Chitin - Vegetative *vs.* Pre treatment	87.7	0.012
Beta Chitin - Vegetative *vs.* Rice - Vegetative Rhizosphere	87	0.012
Beta Chitin - Vegetative *vs.* Rice - Flowering Rhizosphere	100	0.012
Beta Chitin - Flowering *vs.* Chitosan – Vegetative	87.4	0.002
Beta Chitin - Flowering *vs.* Chitosan – Flowering	38	0.004
Beta Chitin - Flowering *vs.* Biofertilizer - Vegetative	84.3	0.002
Beta Chitin - Flowering *vs.* Biofertilizer - Flowering	65.9	0.002
Beta Chitin - Flowering *vs.* Pre treatment	96.3	0.012
Beta Chitin - Flowering *vs.* Rice - Vegetative Rhizosphere	98.8	0.012
Beta Chitin - Flowering *vs.* Rice - Flowering Rhizosphere	90.7	0.012
Chitosan - Vegetative *vs.* Chitosan – Flowering	95.2	0.002
Chitosan - Vegetative *vs.* Biofertilizer - Vegetative	32.6	0.011
Chitosan - Vegetative *vs.* Biofertilizer – Flowering	97.2	0.002
Chitosan - Vegetative *vs.* Pre treatment	60.5	0.024
Chitosan - Vegetative *vs.* Rice - Vegetative Rhizosphere	58	0.012
Chitosan - Vegetative *vs.* Rice - Flowering Rhizosphere	91.4	0.012
Chitosan - Flowering *vs.* Biofertilizer - Vegetative	85.6	0.002
Chitosan - Flowering *vs.* Biofertilizer - Flowering	35.6	0.026
Chitosan - Flowering *vs.*Pre treatment	100	0.012
Chitosan - Flowering *vs.* Rice - Vegetative Rhizosphere	100	0.012
Chitosan - Flowering *vs.* Rice - Flowering Rhizosphere	100	0.012
Biofertilizer - Vegetative *vs.* Biofertilizer - Flowering	83.7	0.002
Biofertilizer - Vegetative *vs.* Pre treatment	57.4	0.024
Biofertilizer - Vegetative *vs.* Rice - Vegetative Rhizosphere	51.2	0.024
Biofertilizer - Vegetative *vs.* Rice - Flowering Rhizosphere	79.6	0.012
Biofertilizer - Flowering *vs.* Pre treatment	100	0.012
Biofertilizer - Flowering *vs.* Rice - Vegetative Rhizosphere	100	0.012
Biofertilizer - Flowering *vs.* Rice - Flowering Rhizosphere	100	0.012
Pre treatment *vs.* Rice - Vegetative Rhizosphere	55.6	0.1
Pre treatment *vs.* Rice - Flowering Rhizosphere	100	0.1
Rice - Vegetative Rhizosphere *vs.* Rice - Flowering Rhizosphere	100	0.1

Table 3.2: ANOSIM differences between various factors in bipartite-combinations (treatment X plant state) of rice rhizosphere microbiome. Comparative differences were calculated using the Bray-Curtis Similarity distance matrix by ANOSIM for various soil amendments of rice rhizosphere.

Groups	% Difference	P Value
Alpha Chitin (High) *vs.* Alpha Chitin (Low)	3	0.253
Alpha Chitin (High) *vs.* Beta Chitin (High)	19.8	0.087
Alpha Chitin (High) *vs.* Beta Chitin (Low)	9.6	0.128
Alpha Chitin (High) *vs.* Chitosan (High)	28.9	0.082
Alpha Chitin (High) *vs.* Chitosan (Low)	13.7	0.11
Alpha Chitin (High) *vs.* Biofertilizer (RD)	28.9	0.082
Alpha Chitin (High) *vs.* Biofertilizer (Half RD)	18.5	0.1
Alpha Chitin (High) *vs.* Pre treatment	59.3	0.024
Alpha Chitin (High) *vs.* Rice Rhizosphere (Vegetative control)	59.3	0.024
Alpha Chitin (High) *vs.* Rice Rhizosphere (Flowering control)	99.4	0.012
Alpha Chitin (Low) *vs.* Beta Chitin (High)	7.2	0.199
Alpha Chitin (Low) *vs.* Beta Chitin (Low)	20.9	0.084
Alpha Chitin (Low) *vs.* Chitosan (High)	29.1	0.05
Alpha Chitin (Low) *vs.* Chitosan (Low)	4.6	0.232
Alpha Chitin (Low) *vs.* Biofertilizer (RD)	18.1	0.078
Alpha Chitin (Low) *vs.* Biofertilizer (Half RD)	4.4	0.273
Alpha Chitin (Low) *vs.* Pre treatment	27.2	0.06
Alpha Chitin (Low) *vs.* Rice Rhizosphere (Vegetative control)	25.9	0.143
Alpha Chitin (Low) *vs.* Rice Rhizosphere (Flowering control)	34	0.06
Beta Chitin (High) *vs.* Beta Chitin (Low)	0	0.535
Beta Chitin (High) *vs.* Chitosan (High)	19.8	0.091
Beta Chitin (High) *vs.* Chitosan (Low)	8.3	0.216
Beta Chitin (High) *vs.* Biofertilizer (RD)	28.5	0.063
Beta Chitin (High) *vs.* Biofertilizer (Half RD)	15.7	0.11
Beta Chitin (High) *vs.* Pre treatment	34.6	0.06
Beta Chitin (High) *vs.* Rice Rhizosphere (Vegetative control)	27.2	0.107
Beta Chitin (High) *vs.* Rice Rhizosphere (Flowering control)	57.4	0.024
Beta Chitin (Low) *vs.* Chitosan (High)	11.5	0.117
Beta Chitin (Low) *vs.* Chitosan (Low)	2	0.333
Beta Chitin (Low) *vs.* Biofertilizer (RD)	22.8	0.078
Beta Chitin (Low) *vs.* Biofertilizer (Half RD)	16.7	0.087
Beta Chitin (Low) *vs.* Pretreatment	51.2	0.024
Beta Chitin (Low) *vs.* Rice Rhizosphere (Vegetative control)	36.4	0.036
Beta Chitin (Low) *vs.* Rice Rhizosphere (Flowering control)	71.6	0.012
Chitosan (High) *vs.* Chitosan (Low)	0	0.416
Chitosan (High) *vs.* Biofertilizer (RD)	24.3	0.078
Chitosan (High) *vs.* Biofertilizer (Half RD)	24.6	0.069
Chitosan (High) *vs.* Pretreatment	55.6	0.024
Chitosan (High) *vs.* Rice Rhizosphere (Vegetative control)	51.2	0.024
Chitosan (High) *vs.* Rice Rhizosphere (Flowering control)	74.1	0.012
Chitosan (Low) *vs.* Biofertilizer (RD)	13.9	0.104
Chitosan (Low) *vs.* Biofertilizer (Half RD)	1.9	0.344
Chitosan (Low) *vs.* Pretreatment	45.1	0.024
Chitosan (Low) *vs.* Rice Rhizosphere (Vegetative control)	35.8	0.048
Chitosan (Low) *vs.* Rice Rhizosphere (Flowering control)	42.6	0.036

Biofertilizer (RD) *vs.* Biofertilizer (Half RD)	9.3	0.134
Biofertilizer (RD) *vs.* Pretreatment	63	0.024
Biofertilizer (RD) *vs.* Rice Rhizosphere (Vegetative control)	54.9	0.024
Biofertilizer (RD) *vs.* Rice Rhizosphere (Flowering control)	89.5	0.012
Biofertilizer (Half RD) *vs.* Pretreatment	35.2	0.048
Biofertilizer (Half RD) *vs.* Rice Rhizosphere (Vegetative control)	30.9	0.048
Biofertilizer (Half RD) *vs.* Rice Rhizosphere (Flowering control)	45.1	0.036
Pretreatment *vs.* Rice Rhizosphere (Vegetative control)	55.6	0.1
Pretreatment *vs.* Rice Rhizosphere (Flowering control)	100	0.1
Rice Rhizosphere (Vegetative control) *vs.* Rice Rhizosphere (Flowering control)	100	0.1

Table 3.3: ANOSIM differences between various factors in bipartite-combinations (treatment X dosage) of rice rhizosphere microbiome. Comparative differences were calculated using the Bray-Curtis Similarity distance matrix by ANOSIM for various soil amendments of rice rhizosphere. RD- recommended dosage, Half RD – Half recommended dosage.

Groups	**% of difference**	**P value**
Alpha Chitin-High-Vegetative *vs.* Alpha Chitin-Low-Vegetative	100	0.1
Alpha Chitin-High-Vegetative *vs.* Alpha Chitin-High-Flowering	100	0.1
Alpha Chitin-High-Vegetative *vs.* Alpha Chitin-Low-Flowering	70.4	0.1
Alpha Chitin-High-Vegetative *vs.* Beta Chitin-High-Vegetative	100	0.1
Alpha Chitin-High-Vegetative *vs.* Beta Chitin-Low-Vegetative	100	0.1
Alpha Chitin-High-Vegetative *vs.* Beta Chitin-High-Flowering	96.3	0.1
Alpha Chitin-High-Vegetative *vs.* Beta Chitin-Low-Flowering	100	0.1
Alpha Chitin-High-Vegetative *vs.* Chitosan-High-Vegetative	96.3	0.1
Alpha Chitin-High-Vegetative *vs.* Chitosan-Low-Vegetative	55.6	0.1
Alpha Chitin-High-Vegetative *vs.* Chitosan-High-Flowering	100	0.1
Alpha Chitin-High-Vegetative *vs.* Chitosan-Low-Flowering	100	0.1
Alpha Chitin-High-Vegetative *vs.* Biofertilizer-RD-Vegetative	100	0.1
Alpha Chitin-High-Vegetative *vs.* Biofertilizer-Half RD-Vegetative	55.6	0.1
Alpha Chitin-High-Vegetative *vs.* Biofertilizer-RD-Flowering	100	0.1
Alpha Chitin-High-Vegetative *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Alpha Chitin-High-Vegetative *vs* .Pre treatment	55.6	0.1
Alpha Chitin-High-Vegetative *vs.* Vegetative control	100	0.1
Alpha Chitin-High-Vegetative *vs.* Flowering control	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Alpha Chitin-High-Flowering	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Alpha Chitin-Low-Flowering	85.2	0.1
Alpha Chitin-Low-Vegetative *vs.* Beta Chitin-High-Vegetative	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Beta Chitin-Low-Vegetative	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Beta Chitin-High-Flowering	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Beta Chitin-Low-Flowering	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Chitosan-High-Vegetative	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Chitosan-Low-Vegetative	44.4	0.1
Alpha Chitin-Low-Vegetative *vs.* Chitosan-High-Flowering	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Chitosan-Low-Flowering	100	0.1

Alpha Chitin-Low-Vegetative *vs.* Biofertilizer-RD-Vegetative	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Biofertilizer-Half RD-Vegetative	55.6	0.1
Alpha Chitin-Low-Vegetative *vs.* Biofertilizer-RD-Flowering	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Alpha Chitin-Low-Vegetative *vs.* Pre treatment	59.3	0.1
Alpha Chitin-Low-Vegetative *vs.* Vegetative control	92.6	0.1
Alpha Chitin-Low-Vegetative *vs.* Flowering control	100	0.1
Alpha Chitin-High-Flowering *vs.* Alpha Chitin-Low-Flowering	37	0.1
Alpha Chitin-High-Flowering *vs.* Beta Chitin-High-Vegetative	100	0.1
Alpha Chitin-High-Flowering *vs.* Beta Chitin-Low-Vegetative	100	0.1
Alpha Chitin-High-Flowering *vs.* Beta Chitin-High-Flowering	55.6	0.1
Alpha Chitin-High-Flowering *vs.* Beta Chitin-Low-Flowering	92.6	0.1
Alpha Chitin-High-Flowering *vs.* Chitosan-High-Vegetative	100	0.1
Alpha Chitin-High-Flowering *vs.* Chitosan-Low-Vegetative	100	0.1
Alpha Chitin-High-Flowering *vs.* Chitosan-High-Flowering	100	0.1
Alpha Chitin-High-Flowering *vs.* Chitosan-Low-Flowering	100	0.1
Alpha Chitin-High-Flowering *vs.* Biofertilizer-RD-Vegetative	100	0.1
Alpha Chitin-High-Flowering *vs.* Biofertilizer-Half RD-Vegetative	81.5	0.1
Alpha Chitin-High-Flowering *vs.* Biofertilizer-RD-Flowering	100	0.1
Alpha Chitin-High-Flowering *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Alpha Chitin-High-Flowering *vs.* Pre treatment	100	0.1
Alpha Chitin-High-Flowering *vs.* Vegetative control	100	0.1
Alpha Chitin-High-Flowering *vs.* Flowering control	100	0.1
Alpha Chitin-Low-Flowering *vs.* Beta Chitin-High-Vegetative	92.6	0.1
Alpha Chitin-Low-Flowering *vs.* Beta Chitin-Low-Vegetative	96.3	0.1
Alpha Chitin-Low-Flowering *vs.* Beta Chitin-High-Flowering	14.8	0.4
Alpha Chitin-Low-Flowering *vs.* Beta Chitin-Low-Flowering	33.3	0.1
Alpha Chitin-Low-Flowering *vs.* Chitosan-High-Vegetative	88.9	0.1
Alpha Chitin-Low-Flowering *vs.* Chitosan-Low-Vegetative	100	0.1
Alpha Chitin-Low-Flowering *vs.* Chitosan-High-Flowering	59.3	0.1
Alpha Chitin-Low-Flowering *vs.* Chitosan-Low-Flowering	44.4	0.1
Alpha Chitin-Low-Flowering *vs.* Biofertilizer-RD-Vegetative	81.5	0.1
Alpha Chitin-Low-Flowering *vs.* Biofertilizer-Half RD-Vegetative	92.6	0.1
Alpha Chitin-Low-Flowering *vs.* Biofertilizer-RD-Flowering	51.9	0.1
Alpha Chitin-Low-Flowering *vs.* Biofertilizer-Half RD-Flowering	48.1	0.1
Alpha Chitin-Low-Flowering *vs.* Pre treatment	100	0.1
Alpha Chitin-Low-Flowering *vs.* Vegetative control	100	0.1
Alpha Chitin-Low-Flowering *vs.* Flowering control	100	0.1
Beta Chitin-High-Vegetative *vs.* Beta Chitin-Low-Vegetative	100	0.1
Beta Chitin-High-Vegetative *vs.* Beta Chitin-High-Flowering	100	0.1
Beta Chitin-High-Vegetative *vs.* Beta Chitin-Low-Flowering	100	0.1
Beta Chitin-High-Vegetative *vs.* Chitosan-High-Vegetative	100	0.1
Beta Chitin-High-Vegetative *vs.* Chitosan-Low-Vegetative	66.7	0.1
Beta Chitin-High-Vegetative *vs.* Chitosan-High-Flowering	100	0.1
Beta Chitin-High-Vegetative *vs.* Chitosan-Low-Flowering	100	0.1
Beta Chitin-High-Vegetative *vs.* Biofertilizer-RD-Vegetative	100	0.1

Beta Chitin-High-Vegetative *vs.* Biofertilizer-Half RD-Vegetative	59.3	0.1
Beta Chitin-High-Vegetative *vs.* Biofertilizer-RD-Flowering	100	0.1
Beta Chitin-High-Vegetative *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Beta Chitin-High-Vegetative *vs.* Pre treatment	63	0.1
Beta Chitin-High-Vegetative *vs.* Vegetative control	100	0.1
Beta Chitin-High-Vegetative *vs.* Flowering control	100	0.1
Beta Chitin-Low-Vegetative *vs.* Beta Chitin-High-Flowering	96.3	0.1
Beta Chitin-Low-Vegetative *vs.* Beta Chitin-Low-Flowering	100	0.1
Beta Chitin-Low-Vegetative *vs.* Chitosan-High-Vegetative	100	0.1
Beta Chitin-Low-Vegetative *vs.* Chitosan-Low-Vegetative	40.7	0.1
Beta Chitin-Low-Vegetative *vs.* Chitosan-High-Flowering	100	0.1
Beta Chitin-Low-Vegetative *vs.* Chitosan-Low-Flowering	100	0.1
Beta Chitin-Low-Vegetative *vs.* Biofertilizer-RD-Vegetative	100	0.1
Beta Chitin-Low-Vegetative *vs.* Biofertilizer-Half RD-Vegetative	55.6	0.1
Beta Chitin-Low-Vegetative *vs.* Biofertilizer-RD-Flowering	100	0.1
Beta Chitin-Low-Vegetative *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Beta Chitin-Low-Vegetative *vs.* Pre treatment	70.4	0.1
Beta Chitin-Low-Vegetative *vs.* Vegetative control	100	0.1
Beta Chitin-Low-Vegetative *vs.* Flowering control	100	0.1
Beta Chitin-High-Flowering *vs.* Beta Chitin-Low-Flowering	25.9	0.2
Beta Chitin-High-Flowering *vs.* Chitosan-High-Vegetative	85.2	0.1
Beta Chitin-High-Flowering *vs.* Chitosan-Low-Vegetative	100	0.1
Beta Chitin-High-Flowering *vs.* Chitosan-High-Flowering	55.6	0.1
Beta Chitin-High-Flowering *vs.* Chitosan-Low-Flowering	55.6	0.1
Beta Chitin-High-Flowering *vs.* Biofertilizer-RD-Vegetative	96.3	0.1
Beta Chitin-High-Flowering *vs.* Biofertilizer-Half RD-Vegetative	96.3	0.1
Beta Chitin-High-Flowering *vs.* Biofertilizer-RD-Flowering	55.6	0.1
Beta Chitin-High-Flowering *vs.* Biofertilizer-Half RD-Flowering	85.2	0.1
Beta Chitin-High-Flowering *vs.* Pre treatment	100	0.1
Beta Chitin-High-Flowering *vs.* Vegetative control	100	0.1
Beta Chitin-High-Flowering *vs.* Flowering control	100	0.1
Beta Chitin-Low-Flowering *vs.* Chitosan-High-Vegetative	100	0.1
Beta Chitin-Low-Flowering *vs.* Chitosan-Low-Vegetative	100	0.1
Beta Chitin-Low-Flowering *vs.* Chitosan-High-Flowering	96.3	0.1
Beta Chitin-Low-Flowering *vs.* Chitosan-Low-Flowering	77.8	0.1
Beta Chitin-Low-Flowering *vs.* Biofertilizer-RD-Vegetative	100	0.1
Beta Chitin-Low-Flowering *vs.* Biofertilizer-Half RD-Vegetative	100	0.1
Beta Chitin-Low-Flowering *vs.* Biofertilizer-RD-Flowering	100	0.1
Beta Chitin-Low-Flowering *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Beta Chitin-Low-Flowering *vs.* Pre treatment	100	0.1
Beta Chitin-Low-Flowering *vs.* Vegetative control	100	0.1
Beta Chitin-Low-Flowering *vs.* Flowering control	100	0.1
Chitosan-High-Vegetative *vs.* Chitosan-Low-Vegetative	44.4	0.1
Chitosan-High-Vegetative *vs.* Chitosan-High-Flowering	100	0.1
Chitosan-High-Vegetative *vs.* Chitosan-Low-Flowering	100	0.1
Chitosan-High-Vegetative *vs.* Biofertilizer-RD-Vegetative	92.6	0.1

Chitosan-High-Vegetative *vs.* Biofertilizer-Half RD-Vegetative	55.6	0.1
Chitosan-High-Vegetative *vs.* Biofertilizer-RD-Flowering	100	0.1
Chitosan-High-Vegetative *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Chitosan-High-Vegetative *vs.* Pre treatment	55.6	0.1
Chitosan-High-Vegetative *vs.* Vegetative control	100	0.1
Chitosan-High-Vegetative *vs.* Flowering control	100	0.1
Chitosan-Low-Vegetative *vs.* Chitosan-High-Flowering	100	0.1
Chitosan-Low-Vegetative *vs.* Chitosan-Low-Flowering	100	0.1
Chitosan-Low-Vegetative *vs.* Biofertilizer-RD-Vegetative	59.3	0.1
Chitosan-Low-Vegetative *vs.* Biofertilizer-Half RD-Vegetative	7.4	0.5
Chitosan-Low-Vegetative *vs.* Biofertilizer-RD-Flowering	100	0.1
Chitosan-Low-Vegetative *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Chitosan-Low-Vegetative *vs.* Pre treatment	70.4	0.1
Chitosan-Low-Vegetative *vs.* Vegetative control	59.3	0.1
Chitosan-Low-Vegetative *vs.* Flowering control	100	0.1
Chitosan-High-Flowering *vs.* Chitosan-Low-Flowering	100	0.1
Chitosan-High-Flowering *vs.* Biofertilizer-RD-Vegetative	100	0.1
Chitosan-High-Flowering *vs.* Biofertilizer-Half RD-Vegetative	100	0.1
Chitosan-High-Flowering *vs.* Biofertilizer-RD-Flowering	100	0.1
Chitosan-High-Flowering *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Chitosan-High-Flowering *vs.* Pre treatment	100	0.1
Chitosan-High-Flowering *vs.* Vegetative control	100	0.1
Chitosan-High-Flowering *vs.* Flowering control	100	0.1
Chitosan-Low-Flowering *vs.* Biofertilizer-RD-Vegetative	100	0.1
Chitosan-Low-Flowering *vs.* Biofertilizer-Half RD-Vegetative	100	0.1
Chitosan-Low-Flowering *vs.* Biofertilizer-RD-Flowering	100	0.1
Chitosan-Low-Flowering *vs.* Biofertilizer-Half RD-Flowering	88.9	0.1
Chitosan-Low-Flowering *vs.* Pre treatment	100	0.1
Chitosan-Low-Flowering *vs.* Vegetative control	100	0.1
Chitosan-Low-Flowering *vs.* Flowering control	100	0.1
Biofertilizer-RD-Vegetative *vs.* Biofertilizer-Half RD-Vegetative	55.6	0.1
Biofertilizer-RD-Vegetative *vs.* Biofertilizer-RD-Flowering	100	0.1
Biofertilizer-RD-Vegetative *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Biofertilizer-RD-Vegetative *vs.* Pre treatment	63	0.1
Biofertilizer-RD-Vegetative *vs.* Vegetative control	100	0.1
Biofertilizer-RD-Vegetative *vs.* Flowering control	100	0.1
Biofertilizer-Half RD-Vegetative *vs.* Biofertilizer-RD-Flowering	77.8	0.1
Biofertilizer-Half RD-Vegetative *vs.* Biofertilizer-Half RD-Flowering	100	0.1
Biofertilizer-Half RD-Vegetative *vs.* Pre treatment	66.7	0.1
Biofertilizer-Half RD-Vegetative *vs.* Vegetative control	55.6	0.1
Biofertilizer-Half RD-Vegetative *vs.* Flowering control	100	0.1
Biofertilizer-RD-Flowering *vs.* Biofertilizer-Half RD-Flowering	96.3	0.1
Biofertilizer-RD-Flowering *vs.* Pre treatment	100	0.1
Biofertilizer-RD-Flowering *vs.* Vegetative control	100	0.1
Biofertilizer-RD-Flowering *vs.* Flowering control	100	0.1
Biofertilizer-Half RD-Flowering *vs.* Pre treatment	100	0.1

Biofertilizer-Half RD-Flowering *vs.* Vegetative control	100	0.1
Biofertilizer-Half RD-Flowering *vs.* Flowering control	100	0.1
Pre treatment *vs.* Vegetative control	55.6	0.1
Pre treatment *vs.* Flowering control	100	0.1
Vegetative control *vs.* Flowering control	100	0.1

Table 3.4: ANOSIM differences between various factors Tripartite-combinations (treatment X dosage X plant state) of rice rhizosphere microbiome. Comparative differences were calculated using the Bray-Curtis Similarity distance matrix by ANOSIM for various soil amendments of rice rhizosphere. RD-recommended dosage, Half RD – Half recommended dosage.

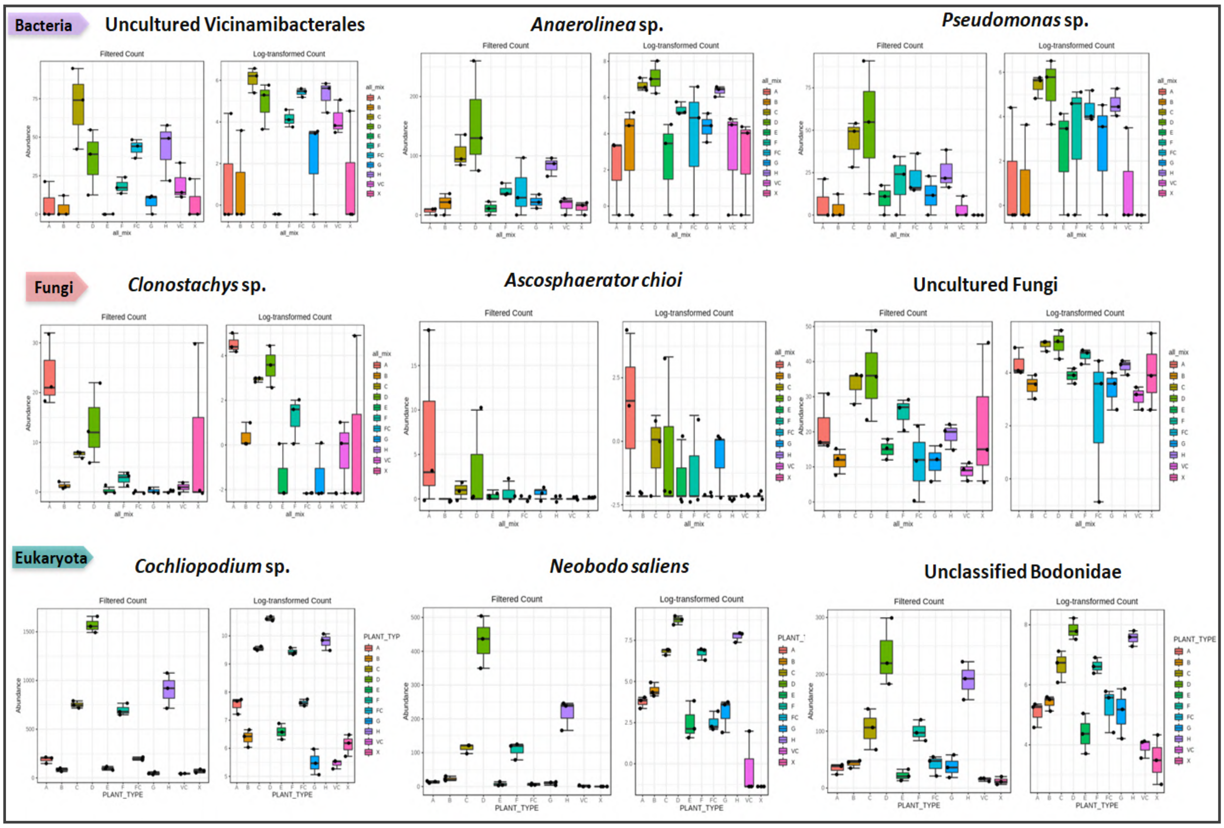


Fig 3.41: Important abundant features identified with alpha chitin amendement to rice rhizosphere. Alpha Chitin-High Dosage-Vegetative – A, Alpha Chitin-Low Dosage-Vegetative – B, Alpha Chitin-High Dosage-Flowering – C, Alpha Chitin-Low Dosage-Flowering – D, Bio fertilizer-RD-Vegetative – E, Bio fertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre treatment – X, Vegetative control – VC, Flowering control – FC

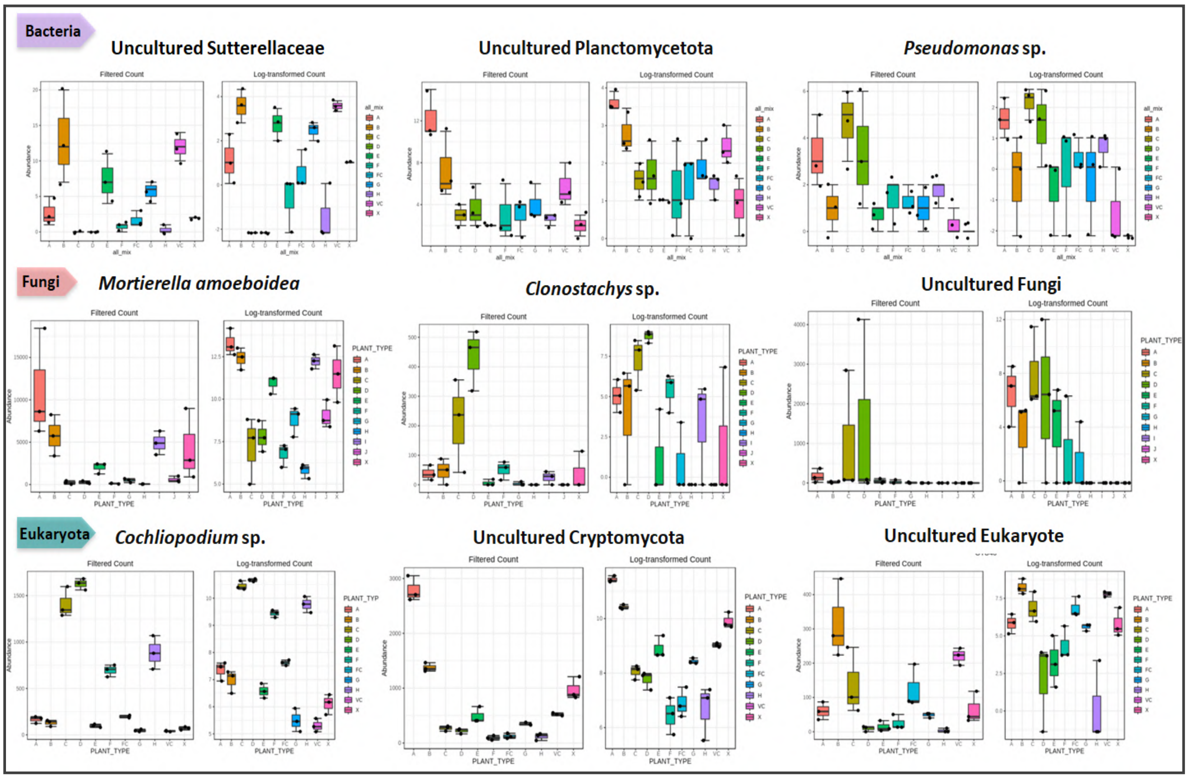


Fig 3.42: Important abundant features identified with beta chitin amendement to rice rhizosphere. Beta Chitin-High Dosage-Vegetative – A, Beta Chitin-Low Dosage-Vegetative – B, Beta Chitin-High Dosage-Flowering – C, Beta Chitin-Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre treatment – X, Vegetative control – VC, Flowering control – FC.

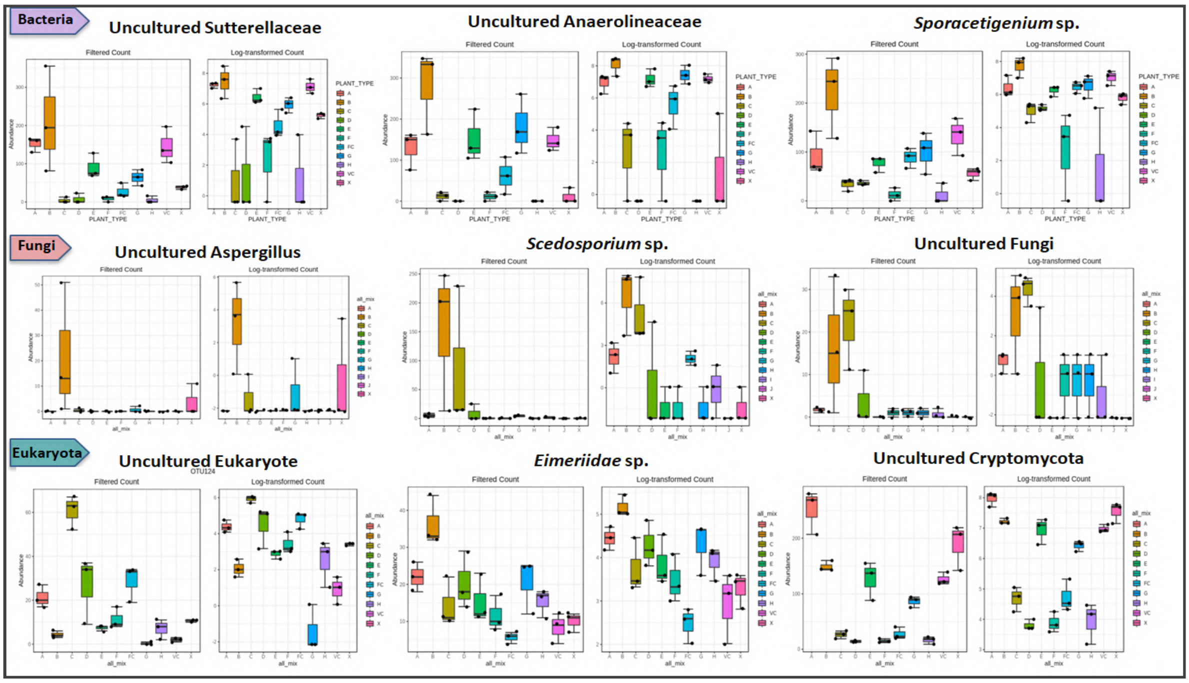


Fig 3.43: Important abundant features identified with chitosan amendement to rice rhizosphere. Chitosan -High Dosage-Vegetative – A, Chitosan - Low Dosage-Vegetative – B, Chitosan - High Dosage-Flowering – C, Chitosan - Low Dosage-Flowering – D, Biofertilizer-RD-Vegetative – E, Biofertilizer-Half RD-Vegetative – F, Biofertilizer-RD-Vegetative – G, Biofertilizer-Half RD-Flowering – H, Pre treatment – X, Vegetative control – VC, Flowering control – FC

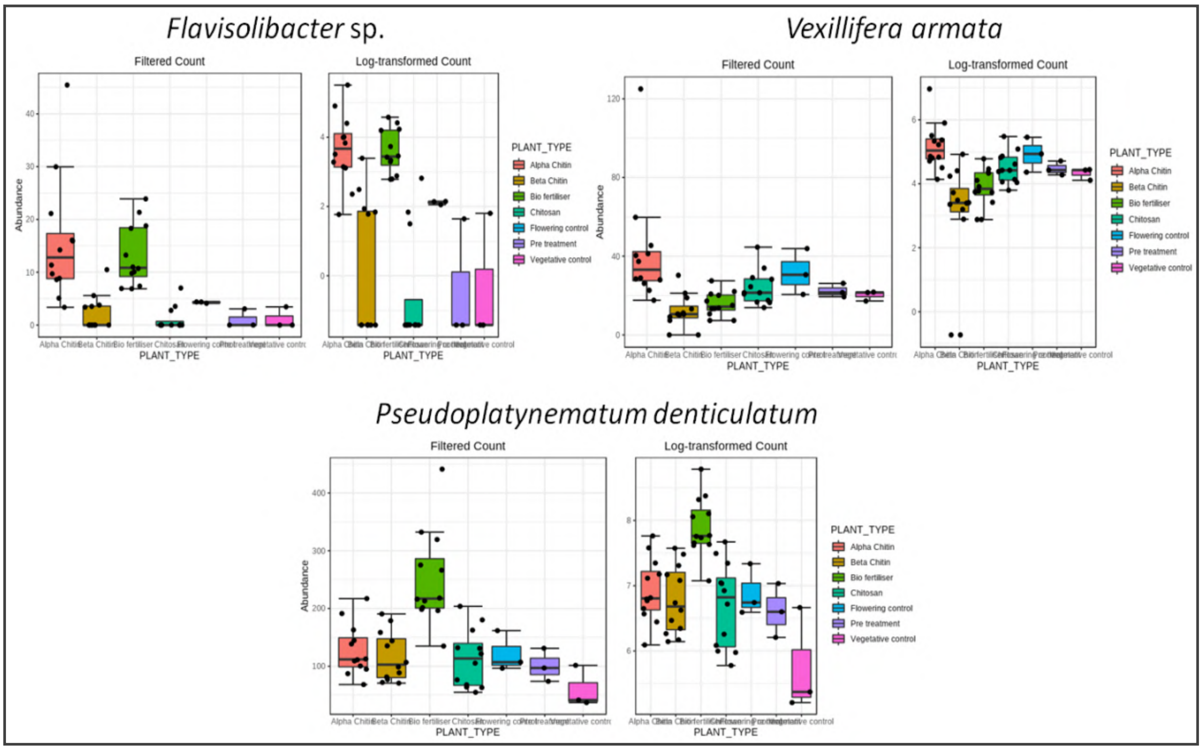


Fig 3.44: Important abundant features identified with diverse soil amendments to rice rhizosphere. EdgeR algortihm with FDR adjusted at $p < 0.05$ and expressed in RLE (Relative Log Expression) for chitosan, alpha and beta chitin in comparision with vegetative and flowering control, pretreatment.

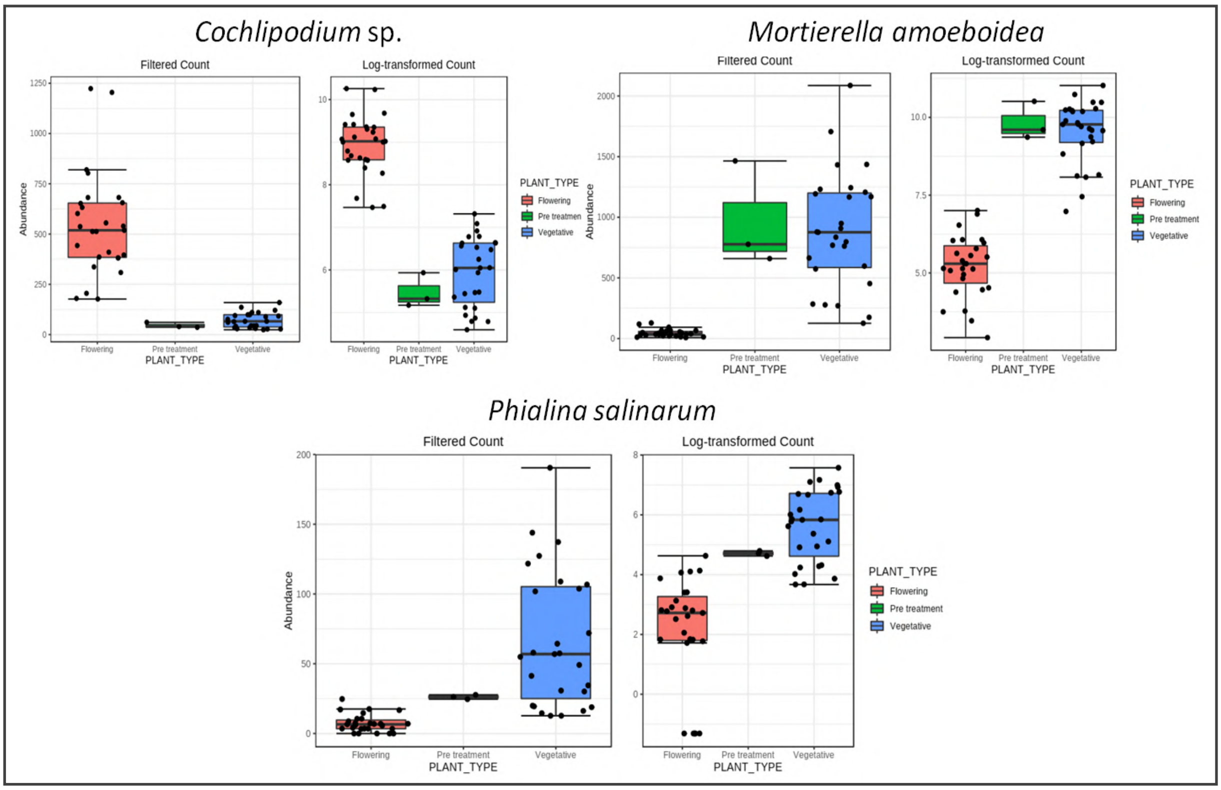


Fig 3.45: Important abundant features identified with different plant states in rice rhizosphere. EdgeR algortihm with FDR adjusted at $p < 0.05$ and expressed in RLE (Relative Log Expression) for vegetative and flowering stages in comparision with pretreatment.

3.1.14. Amendments with chitinous substrates increased rice yield

Soil amendment with chitinous substrates at different doses increased rice yield in terms of 100 seed weight, number of seeds per panicle and total seed weight per panicle, as represented in Fig 3.46. A one way ANOVA summary of the same represented in Table 3.5. Percentage improvement has been cacluated in comparision with control, number of seeds per panicle improved with various chitinous substartes along with other yield parameters and represented in Fig 3.46.

Rice yield has improved under application of various chitinous amendments and box plots with standard deviation were represented Fig 3.46. Yield characteristics such as 100 seed weight (g), number of seeds per panicle, and total seed weight (g) have been recorded.One way ANOVA calculated and represented in Table 3.5**,** each treatment is significant as F stat > P value, allowing us to reject the null hypothesis. Percentage improvemt has been calcluted in comparsion with untreated control for each yield trait and represented in Fig 3.7. It is observed that percentage improvement for 100 seed weight is alpha chitin - high dosage (13.56%), alpha chitin - low dosage (10.29%), Beta chitin - high dosage (5.56%), Beta chitin - low dosage (0.65%), Chitosan – high dosage (8.17%), Chitosan – low dosage (3.76%), Biofertilizer - RD (6.37%) and Biofertilizer - halfRD (2.61%). Seed weight per panicle improved by alpha chitin - high dosage (3.12%), alpha chitin - low dosage (0.93%), beta chitin - high dosage (2.07%), beta chitin - low dosage (1.88%), Chitosan - high dosage (3.60%), Chitosan - low dosage (1.87%), Biofertilizer - RD (1.89%), Biofertilizer - halfRD (0.09%). Total seedweight improved by alpha chitin - high dosage (20.43%), alpha chitin - low dosage (15.19%), beta chitin - high dosage (17.36%), beta chitin - low dosage (16.46%), chitosan - high dosage (23.51%), chitosan - low dosage (17.18%), biofertilizer - RD (15.37%) and biofertilizer – halfRD (4.70%).

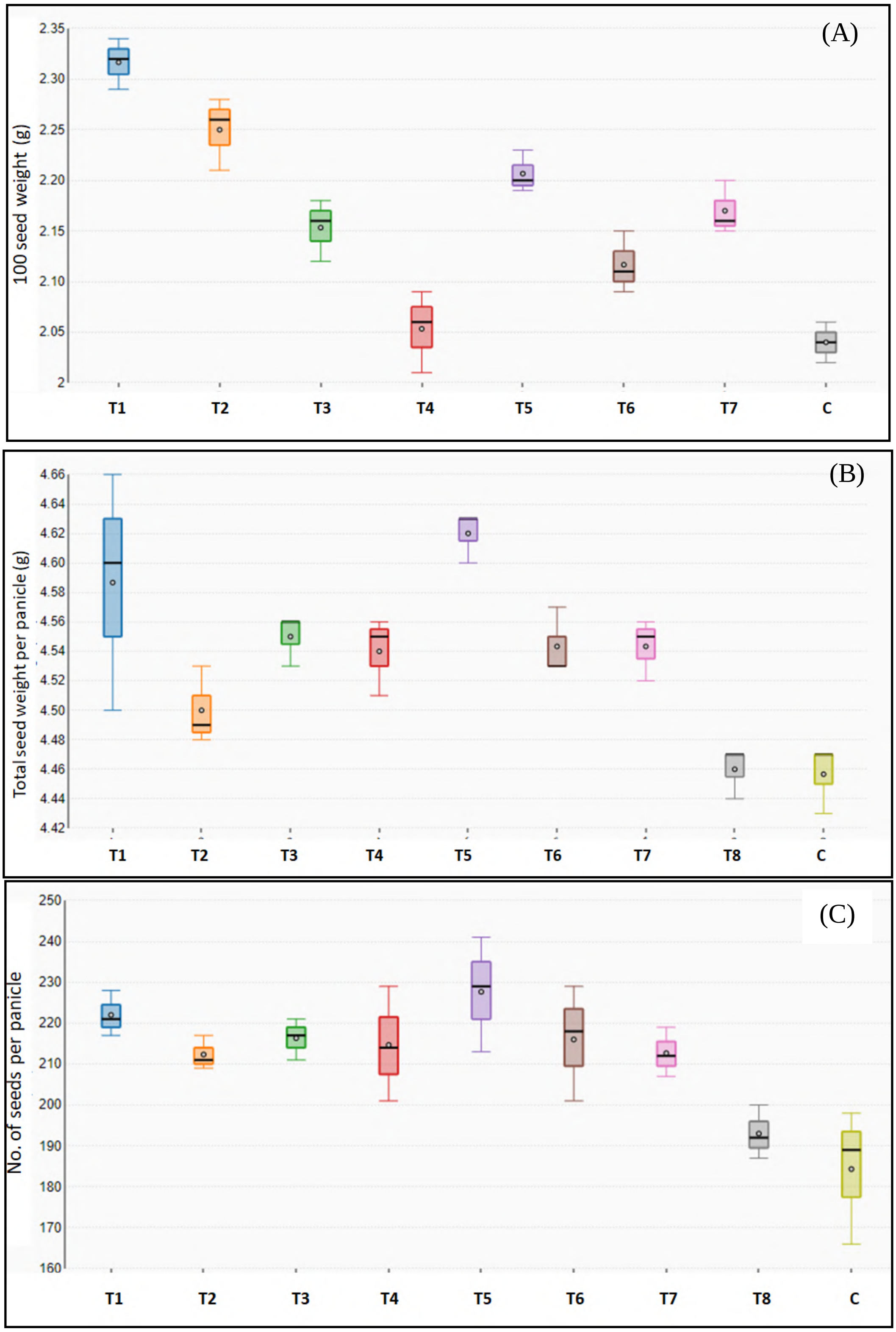


Fig 3.46: Rice yield under various chitinous substrate amendments. Yield under various chitinous substrates (A) 100 seed weight (g), (B) No. of seeds per panicle, (C) Total seed weight per panicle (g). T1- Alpha chitin (high dosage), T2- Alpha chitin (low dosage), T3- Beta chitin (High dosage), T4- Beta chitin (Low dosage), T5- Chitosan (High dosage), T6- Chitosan (Low dosage), T7-Biofertilzer (RD), T8- Bioferilizer (Half RD), control. High dosage (100 mg/Kg of soil), low dosage (50 mg/Kg of soil), RD- 5x10^{7} CFU (1g/kg of soil), Half RD –5X10^{7} CFU (0.5g/ kg of soil).

S.No	Source of variation	F -stat	P -value
1	Seeds per panicle	4.9907	0.002
2	100 seed weight (g)	26.838	0.001
3	Total seed weight per panicle (g)	7.5928	0.002

Table 3.5: Tabulated one way ANOVA values. P and F-Stat values with degrees of freedom measured between and among treated samples, for the harvested data samples.

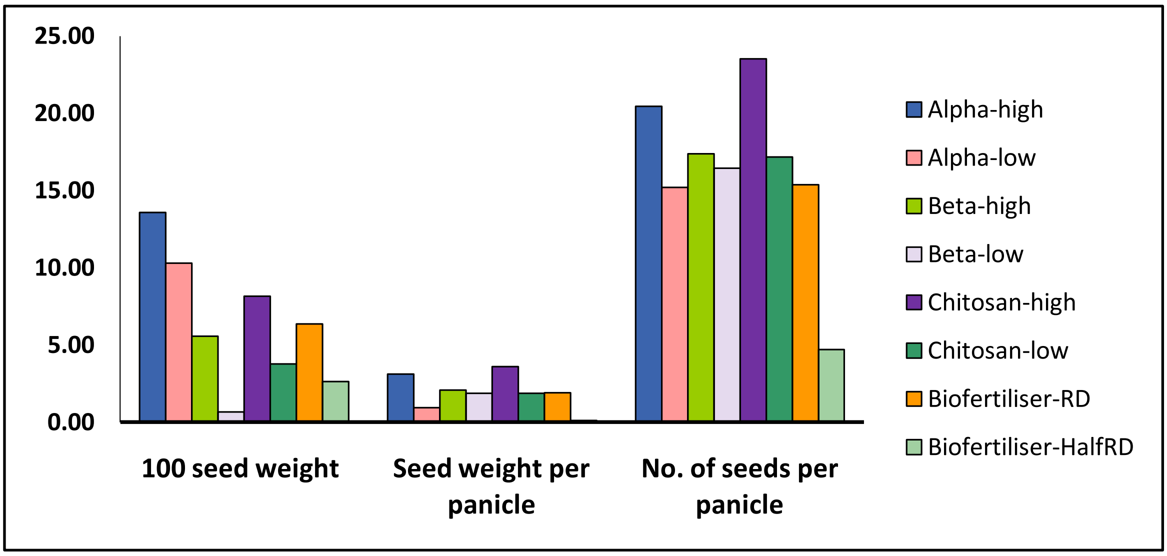


Fig 3.47: Percentage improvement of rice yield under chitinous substrates amendments. Percentage improvement in comparison with control calcultaed for 100 seed weight, seed weight per panicle and no. of seeds per panicle for different dosages of chitinous substrates.

3.2 Discussion

3.2.1. Rice rhizosphere microbiome is shaped by host, stages of growth and soil amendments

To understand the impact of soil amendment with chitinous substrates in shaping rice root microbiome, we have run PERMANOVA for soil amendments (alpha chitin, beta chitin and chitosan) and plant developmental stage (vegetative and flowering). It is observed that plant growth stage followed by soil amendments plays crucial role in rhizosphere microbiome. Chitinous substrate amendments, with high and low dosage, influenced the microbiome changes in rice rhizosphere. PCoA plot illustrates a sample location shift between all treatments, while the main boundary can be drawn noticeably between vegetative and flowering stages. ANOSIM differences between various factors were calculated and represented in Tables 3.1, 3.2 and 3.3. Major differences were observed during various plant stages i.e., vegetative and flowering stages, irrespective of soil amendments. In several crop species, the plant developmental stage is the primary determinant of community assembly, and significant variation was observed from vegetative to flowering stage (Chalasani et al. 2021; Lundberg et al. 2012; Voges et al. 2019; Xiong et al. 2021).

3.2.2. Bacteriome, mycobiome and eukaryome responded differently to chitinous substrate amendments to rice rhizosphere

The microbiome i.e., bacteriome, mycobiome and eukaryome responded differently to various soil amendments during different plant growth stages in rice rhizosphere (Fig 3.40). In the rice rhizosphere, the influence of soil amendments on bacteria was lowest, but the influence of Eukaryome was highest regardless of dosage and soil amendments. Organic amendment (4500 Kg of cow manure Ha^{-1}) increases barley (*Hordeum vulgare*) rhizosphere inter-kingdom associations especially bacteria and eukarya (Suleiman et al. 2019). Differential response was observed for bacteria and fungi to various organic amendments (biochar and organic N fertilizers) and fertilzers in chili pepper (*Capsicum annuum)* grown in Californian soil (Dangi et al. 2020).

Organic amendments (rice straw and cow manure) reduce salinity effects on rice microbiome in Bangladesh soils (Wichern et al. 2020). Soil amendments such as dry maize straw and regular nitrogen fertilization affect eukaryome communities more than other microorganisms in varied agricultural soils of china (Zhao et al. 2019). Organic amendements such as biochar prepared from bamboo, pig manure, cow manure and rice straw along with regular fertilizers improved microbial abundance, enzyme activity in maize rhizosphere (Ibrahim et al. 2020). In wheat–rice rotation, partial substitution of chemical fertilizer with organic fertilizer (pig manure) over seven years improves yields and restored soil bacterial diversity (Li et al., 2022).

3.2.3. Chitin amendments improved microbiome diversity in rice rhizosphere

Alpha chitin treatment had highest alpha diversity (Shannon diversity index) when compared to other treatments in rice rhizosphere (Fig 3.1A). Chitin amendments to the rice rhizosphere had a greater impact on fungi and eukaryotes than on bacteria. Organic amendments to soil improve species abundance, diversity and richness. These findings show that the rhizosphere has a higher richness than bulk soil, indicating a selection for certain microbes by plants that can help them in development and nutrient absorption. Crabshell chitin increased bacteria by 13-fold and fungus by 2.5-fold in *Trifolium repens* L. and *Lolium perenne* L. (perennial ryegrass) rhizosphere soils of New Zealand (Sarathchandra et al., 1996).

Chitin supplement in the potting soil boosted lettuce growth and reduced leaf pathogen survival followed by impoved alpha diversity in rhizosphere (Debode et al. 2016). Synthetic fertilizers increased crop yield but decreased soil fertility in *Eruca sativa* but organic additions enhance microbiome diversity and function (Cesarano et al., 2017). Chitin and hydrolysis products were used to enhance chitin degraders in 42-day soil incubation experiment and it was reported to be influenced by different fertilizations (Hui et al. 2020). Biochar and nitrogen fertilizer improve rice performance, reduce cadmium availability, and shape rice soil rhizosphere microorganisms (Zhang et al. 2022). By modifying the rhizosphere microbiome's community structure and metabolism, N-acetylglucosamine stimulates the growth of tomato (Sun et al., 2022). Nano-chitosan-treated maize seedlings have more Proteobacteria in their rhizospheres (Agri et al., 2022).

3.2.4. Core microbiome of rice rhizosphere dominated by Proteobacteria, Ascomycota and Euglenozoa

The phylum Proteobacteria, Ascomycota and Euglenozoa are core microbial taxa in rice rhizosphere (Fig 3.3A, 3.15A, 3.27A & 3.39A, 3.6A, 3.18A, 3.30A & 3.39A, 3.9A, 3.21A, 3.33A & 3.39A) with prevalence of 1-0.49%. Proteobacteria and ascomycota were most abundant phyla of rice rhizosphere treated with various organic amendments in Colombian soils (Jiménez et al., 2021). The phylum Proteobacteria responded to the chitin-amendment to soil (Cretoiu et al., 2014). Both members were predominant in rice rhizosphere (Guo et al., 2022), and associated with phosphate solubilisation in three wild cultivars grown under green house in Chinese soils (Sun et al., 2022). The wild rice cultivar *O. rufipogan* harbors *Mortierella* in their rhizosphere under extreme cold stress when grown in soils of south China (Xu et al., 2022). Most of the above abundant phyla are associated several plant species microbiomes reported from native and agriculture soils.

The bacterial genus *Pseudomonas* is a differentially abundant species that can be found in both alpha and beta chitin, but it is least abundant in rice rhizosphere soil that have been treated with chitosan (Fig 3.41 & 3.42). *Pseudomonas* is well-known plant growth promoting bacteria, antagonistic to fungal pathogens and it's highly abundant in both alpha and beta chitin during flowering stage of rice rhizosphere. *Pseudomonas* is a Gram-negative bacterium of class Gammaproteobacteria, belonging to the family Pseudomonadaceae. *Pseudomonas* associated with several plant species with diverse PGPR traits such as IAA production, phosphate, starch, zinc solubilisation, siderophore production, antifungal activity, and chitinase production and its part of many bacterial consortia applied for yield enhancement under field application (Saranraj et al., 2022; Rochimi et al., 2022; Das et al., 2022a). It is associated with rice grown under contrasting climates i.e. at Himachal Pradesh and Andaman & Nicobar islands (Sahu et al. 2022), as endophytes (Sessitsch et al. 2011), rhizosphere soil against rice fungal pathogens (Das et al., 2022b; Majumdar et al., 2022).

More prevalent fungal taxa *Mortierella* is highly abundant in rice rhizosphere with beta chitin amendment which is known as plant growth promoting fungus associated with several crop species and soil. The beneficial effects of *Mortierella* sp. and *M. elongata* in healthy rhizospheres and bulk soil improved plant growth in following cultivated plants *Pisum sativum* (Xu et al., 2012), mulberry fruit (Yu et al. 2016), Vanilla (Xiong et al., 2017), maize (Li et al., 2020) rice (Zhang, et al., 2022; Ding, et al., 2022).

One more prevelant, the fungal genus *Cordyceps* is abundant and a constituent of the paddy rhizosphere core microbiome when chitinous amendments were applied under field conditions. *Cordyceps* is a genus containing around 600 species of ascomycete fungi (sac fungi). Few *Cordyceps* species are parasitic on other fungus and often occur as entomopathogenic to arthropods, mainly insects most often called as endophytic entomopathogenic fungi (EEPF). *Cordyceps* sp. like *Cordyceps militaris* have at least 24 chitinases in their genomes (http: //www.cazy.org/e9472.html), thus it is not surprising that chitin addition to the soil may have promoted growth and resulted in it the most abundant fungal species in the paddy rhizosphere. There are many reports on its entomopathogenic trait, but only a few recent reports suggest that *Cordyceps* plays a role in promoting plant growth. For instance, *Cordyceps fumosorosea* isolates were observed to colonize *Solanum melongena* and promote plant growth. In addition, it also reduced the incidence of whiteflies (*Bemisia tabaci*) (Sun et al., 2020).

Tyrophagus putrescentiae, a common saprophagous soil mite`s species in soil, decomposes plant and vertebrate nests. It has been hypothesized that structural chitin from the cell walls of filamentous fungi, which can make up a sizable portion of the mite`s food, is broken up by chitin-digesting enzymes produced by symbiotic bacteria. Depending on the nutrition of the fungus, *T. putrescentiae* can have diverse communities of culturable chitinolytic bacteria, *Stenotrophomonas, Pseudomonas and Brevundimonas* (Smrž et al., 2016; Hubert et al., 2018). *T. putrescentiae* (saprophagous soil mites) observed to be part of core microbiome in rice (Fig 3.9B, 3.12B, 3.21B, 3.24B, 3.33B, 3.36B & 3.39B) (it is reported to form symbiotic relationship between chitinolytic bacteria) and digested fungi in mycophagous micro-arthropods.

3.2.5. Chitinous substrates improved rice yield

Chitionus substrates have been used as soil amendments in several crop species like lettuce (Tender et al. 2019), rocket plant (*Eruca vesicaria*) (Barouchas & Liopa, 2021), tomato (Sun et al., 2022). Organic amndements such as *Calothrix elenkinii* inoculation enhances rice biomass and growth. Selective interaction between *C. elenkinii* and the rhizosphere microbiota in tropical alluvial soils (Ranjan et al. 2016). Reports are availble on yield enhancemnt by organic amndemnts in rice with paddy straw (Otero-Jiménez et al., 2021), biochar and compost (Ghorbani et al., 2022), biochar from rice straw, cotton waste biochar (Mowrer et al., 2022), combined slag and biochar (Lin et al. 2022), olive mill waste (Peña et al. 2022), barley straw based biochar (Park et al., 2022). Use of fermented chitin waste as soil amendment for rice impoved the grain yeid to 4.7 folds, recomended as a plant growth stimulator for sustainable rice production (Kananont et al., 2016).

With this background, we attempted a field application of chitinous substrates (alpha chitin, beta chitin and chitosan) for rice crop and monitered the microbiome changes as discussed above. We further maintained the crop till maturity to check for the grain yield. It was observed that dosage postively correlated with all treatments in selected traits of rice yield. In conclusion the use of chitin substrates as amendments due to its low cost, the low concentration requirement, the large supply, and the environmental safety indicate their potential as useful supplments for agriculture to replace synthetic fertilizers/chemicals, over a period of time.

Chapter - IV

Application of Chitooligosaccharides on rice

Chapter IV: Application of chitooligosaccharides on rice

4.1. Introduction

Plants are constantly exposed to microbial pathogens including bacteria, fungi, and viruses. As a protection against the microbial pathogens, plants activate immune responses upon recognition of the evolutionarily conserved pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) (Das et al., 2015; Choi & Klessig, 2016). Plants detect the PAMPs with the help of membrane-anchored pattern recognition receptors (PRRs) and activate pattern-triggered immunity (PTI). PTI involves callose deposition, synthesis of antimicrobial compounds, activation of mitogen-activated protein kinase (MAPKs) cascade, generation of reactive oxygen species (ROS) etc., (Zipfel, 2014). Flagellin, lipopolysaccharides, peptidoglycans, glucans, and mannans represent some of the known MAMPs/PAMPs (Macho & Zipfel, 2014).

Chitin consists of β (1, 4)-linked N-acetyl-D-glucosamine (GlcNAc) units arranged in a linear form. It is a major structural component of fungal cell walls and insect exoskeletons, and also acts as a MAMP/ PAMP (Das et al. 2015; Li et al. 2016). Oligosaccharides generated from chitin are referred to as chitooligosaccharides (COS). The COS act as a broad-spectrum vaccine against several plant diseases making them potential candidates for crop protection (Yin, Zhao & Du, 2010). Treatment with COS was reported to induce defense reactions like generation of ROS, transient depolarization of membranes, extracellular alkalization and ion flow, production of phytoalexin in plants/cell suspension cultures (Kishimoto et al., 2010; Li et al., 2016), expression of several early responsive genes (Ramonell et al. 2005; Zhang et al. 2002), lignification (Kawasaki et al. 2006) etc., Activation of MAPK cascade, upon chitin elicitation, was reported in rice and *Arabidopsis* (Yamada et al., 2017; Wan & Stacey, 2004) .

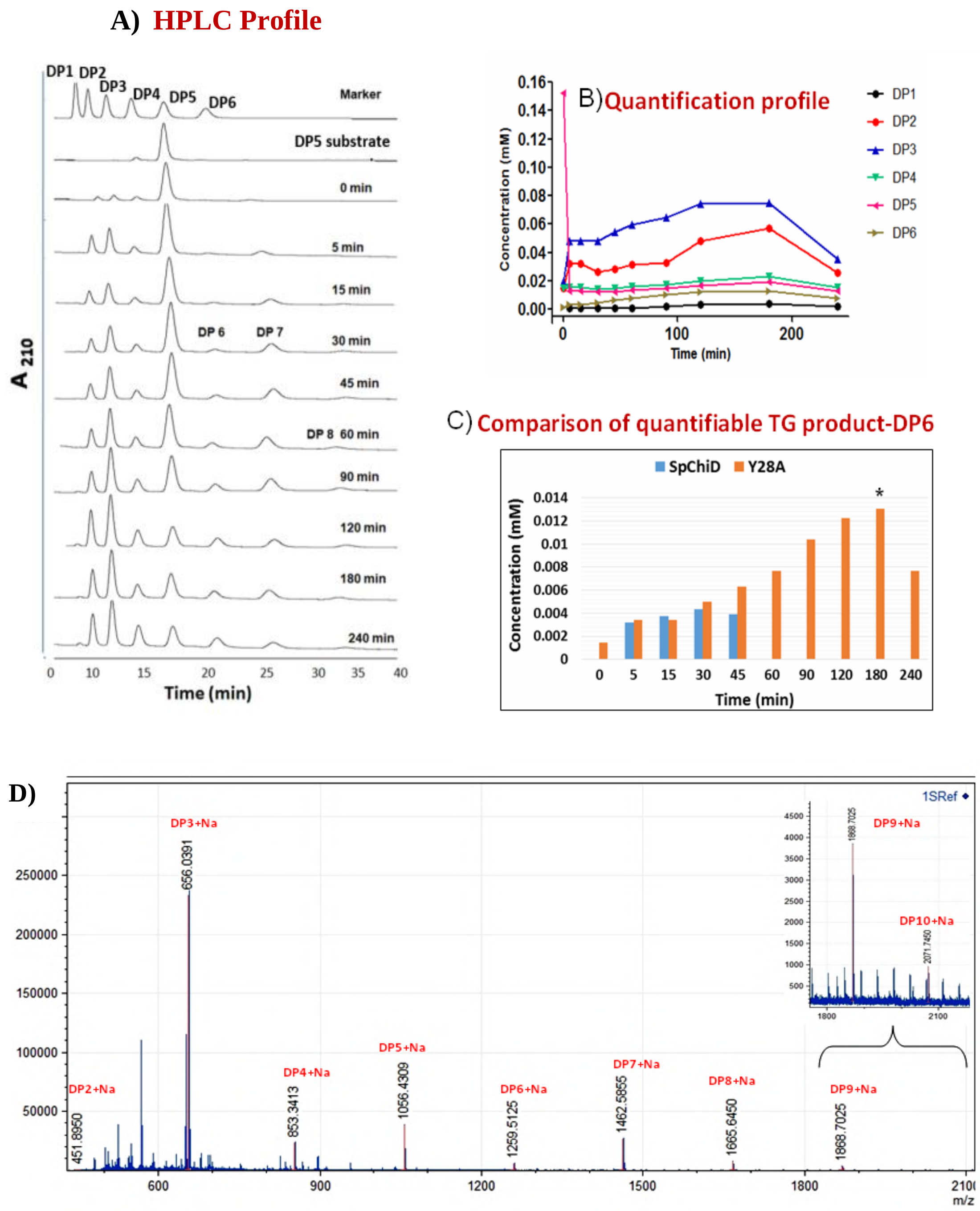
COS could be prepared from chitin by acid hydrolysis, alkaline hydrolysis, chemical synthesis, generated using microbial source or enzymatic hydrolysis. Chitinases (EC 3.2.1.14), which are glycosyl hydrolases (GHs), also perform trans-glycosylation (TG) using the sugar molecule as the acceptor resulting in the formation of a new glycosidic linkage (Zakariassen et al. 2011), are useful to generate long-chain COS. A few chitinases with TG activity (Purushotham & Podile, 2012) were reported to generate longer COS. A GH18 endo-chitinase from *Serratia proteamaculans* 568 (*Sp*ChiD) that exhibited hyper TG activity (Purushotham and Podile, 2012) was further improved by point mutations to increase the production of long-chain COS (Madhuprakash et al., 2015). Among them, Y28A (mutation in the substrate binding cleft of *Sp*ChiD) generated 21% of TG products from DP4 substrate (Madhuprakash et al. 2018) suggesting its suitability to generate long-chain COS. Hence, we have used *Sp*ChiD-Y28A to produce long-chain COS ranging from DP 5-7. Further, we have optimized the conditions (pH, substrate conc., reaction buffer and incubation time etc.,) to improve yield and purified DP5 (pentamer) DP6 (hexamer) and DP7 (heptamer) to homogeneity and prepared in bulk to test their effect on rice seedligs.

The purified COS were tested on rice seedlings for their ability to induce plant defence. The COS significantly activated the salicylic acid (SA) pathway and defence response genes. Taken together, the possible mechanism of defense activation could be through the induction of plant innate immunity *via* the MAPK6, which was followed by SA biosynthesis (phenylalanine ammonia lyase1 (*PAL1*) and isochorismate synthase 1(*ICS1*)) and SA signalling genes (enhanced disease susceptibility1 (*EDS1*) and phytoalexin deficient 4 *(PAD4*)). These signalling genes might trigger transcription factors such as Non-expressor of PR genes1 (*NPR1*) and WRKY45 which in turn induced the PR (pathogenesis related) and defense genes like *PR1a*, *PR4*, *PR10*, chitinase-1, peroxidase, and β -1,3-glucanase. Overall, the defense response in rice seedlings was primarily mediated by the SA-dependent pathway, with MAPK-mediated MAPK-mediated phosphorylation possibly playing a role. We have tested these purified COS (DP5-DP7) for the first time on rice under field conditions and assessed the growth and development including yield.

4.2. Results

4.2.1. Production optimization and purification of COS

To obtain COS>6, we chose a Y28A variant (Madhuprakash et al., 2014) of *Sp*ChiD that showed consistent production of COS>DP6 (Fig 4.1A,B) starting from 5th min itself. Comparison of the quantifiable TG product (DP6) showed improved yields and maximum at 180th min for mutant enzyme (Fig 4.1C). MALDI TOF-MS analysis of the concentrated sample detected COS up to DP 10 (Fig 4.1D). A white precipitate appeared in the bulk reaction (volumes of 150 ml), was eliminated and finally set up the reaction to obtain more quantity of TG products. Quantification of HPLC profiles from samples of different pH range of Tris-HCL buffer for TG product (DP6) revealed the highest yield obtained at 150 min sample of pH 7.0 (Fig 4.2A). Addition of NaCl at 50mM , BSA at 100ug/ml and substrate DP5 at 3mg/ml reaction mixture set up for 90 min duration at 37^{O}C yielded more quantifiable TG product DP6 (Fig 4.2B,C,D). To obtain higher chain length COS in more quantity, bulk reaction was set up (250 & 500 ml) using *Sp*ChiD Y28A mutant with optimized conditions Reaction was terminated by boiling the solution at 85^{O}C for 15 min. Comparison of HPLC profiles based on peak area analysis of the products obtained from the both above bulk reaction setup were quantified and represented in pie- charts (Fig 4.2E). Reactions set up (500ml) were prepared and purification of COS was carried out using semi-prep HPLC (Fig 4.3A). TLC analysis of DP6 and DP7 fractions showed the purity of the collected fractions. MALDI-ToF-MS analysis of DP7 purified fraction showed the exact mass of DP7 (Fig 4.3B, C). COS obtained after purification to homogenity is represented in Fig 4.3D.



A) HPLC Profile

B) Quantification profile

C) Comparison of quantifiable TG product-DP6

D)

Fig 4.1: Time course reaction of *Sp*ChiD-Y28A with DP5 substrate. A). *Sp*ChiD-Y28A was incubated with DP5 substrate in 50mM sodium acetate buffer, pH 5.6 at 40°C. Samples were collected at different time points and added with equal volume of 70% acetonitrile to stop the reaction and run on HPLC. B). Quantification profile showing different oligomers obtained from DP1-DP6 at different time points up to 4h. C). Graph depicting the quantifiable TG product [DP6] in comparison with *Sp*ChiD enzyme and the * mark in bar graph depicts the time point where concentration of DP6 is higher. D). MALDI-TOF-MS spectra are labelled according to their observed atomic mass and the degree of polymerization (DP) of the oligosaccharide. Most of the oligosaccharide species were observed as Na adducts of the oligosaccharide Na-salt. Inset shows the magnification for low peak area of m/z ratio.

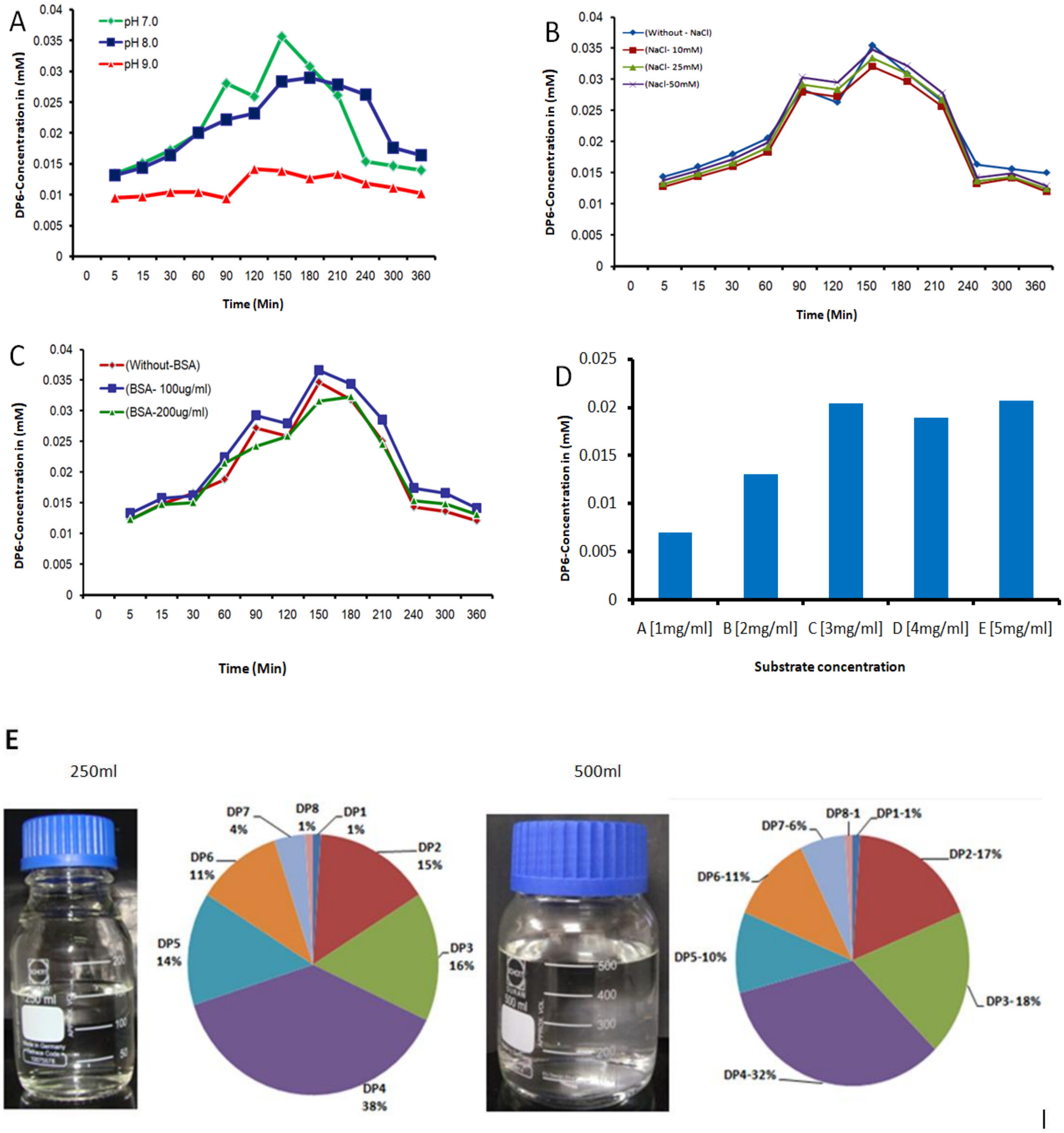
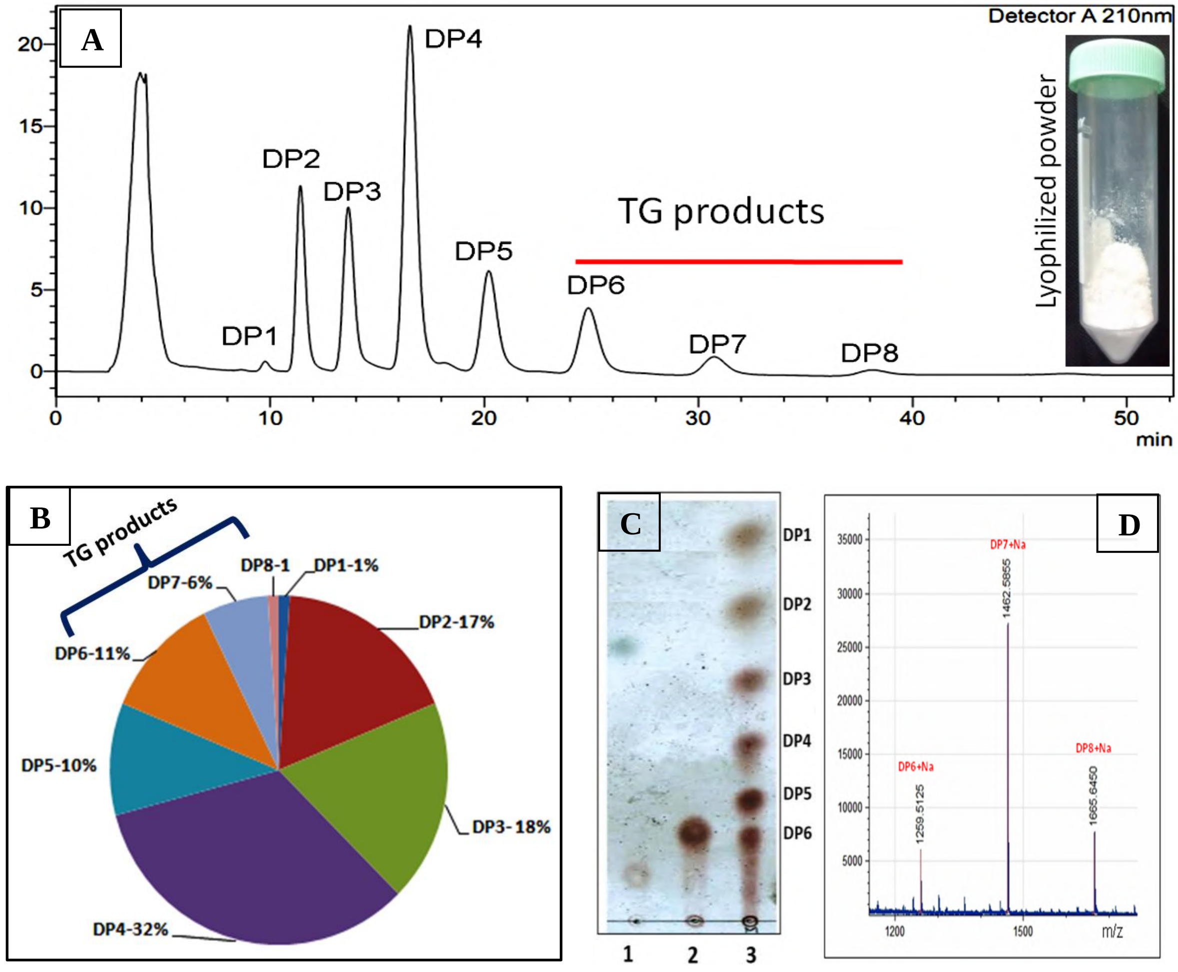


Fig 4.2: Reaction conditions optimization to obtain higher TG products. Quantifiable TG product DP6 obtained for following different optimizations for reaction having *Sp*ChiD-Y28A with DP5 substrate A). pH optimization using Tris–HCl buffer at different pH values of 7, 8, 9. B). Sodium chloride addition to the reaction set up at 10mM, 25mM and 50mM. C). Bovine Serum Albumin (BSA) addition at 100 and 200 µg/ml. D). Substrate (DP5) concentration ranging from 1 to 5mg/ml. E). Bulk reaction set up (250 & 500ml) for COS, Pie-chart representation of the concentration (in %) of different COS obtained based on the HPLC peak area.



E

TG products Conc. [DP6+DP7] (%)	Oligomer conc. (%)	Theoretical yield of oligomers [mg]	Yield obtained after 85 % purification [mg]	Recovery (%)
15	**DP6 [11]**	**165**	**54.45**	**55.0**
	DP7 [4]	**60**	**16.92**	**47.0**
	Total	**225**	**71.37**	**52.8**

Fig 4.3: Bulk preparation of COS. Reaction set up in bulk volume with the optimized reaction conditions A). HPLC profile of the reaction mixture showing a clear separation of COS including TG products DP6,DP7, DP8 (highlighted with red line) Inset picture shows lyophilized COS. B). MALDI_TOF-MS spectrum of purified COS DP7 also shows carryover of DP6, DP8 during separation process, all the masses were adducts of sodium. C). TLC figure showing the DP6 and DP7 obtained in pure form. D). Pie-charts showing the concentration (in %) of different COS obtained based on the HPLC quantification profiles of peak area. E). Yield calculations for purified TG products (DP6, DP7 oligomers).

4.2.2. Defense response in rice seedlings upon COS treatment

Induction of defense response by the purified COS with DP5, DP6 and DP7 was tested in rice seedlings at the 3rd leaf stage by foliar spray and root dip methods. Oxidative burst response of rice seedlings treated with pure COS (DP5, DP6 and DP7) and SA was evaluated by monitoring the time-dependent production of H_2O_2. In all the root-dip treatments, H_2O_2 formation peaked at 3h and dropped steadily until 72h (Fig 4.4A). SA increased H_2O_2 levels the most, followed by DP7, DP6, and DP5. The oxidative burst induced by COS foliar spray treatment was both quick (within 1h after elicitation) and prolonged compared to root dip treatment. Maximum H_2O_2 concentrations were detected at 3 hours with the DP5 and DP6 treatments, and at 6 hours with the DP7 and SA treatments. With the exception of the SA treatment, H_2O_2concentrations began to drop at 48 hours (Fig. 4.4B).

DP7 induced the maximum oxidative burst among the tested COS, followed by DP6 and DP5 in both foliar spray and root dip applications (Fig. 4.4A & B). The potential of COS to elicit PAL and POD in rice was studied. An increase in the PAL activity was observed up to 12h, after root treatment with DP6 and DP7 (Fig. 4.4C), and up to 24h with DP5 and SA. A strong increase in the PAL activity was observed with DP7 followed by DP5 and DP6 (Fig. 4.4D). SA application induced higher PAL activity compared to COS elicitations in both root dip and foliar treatments (Fig. 4.4C & D).

The time-response curve for the POD activity with root dip treatment revealed that the activity with DP5, DP6, and DP7 was highest at 24h, 12h, and 6h, respectively (Fig. 4.4E). In the case of foliar spray, maximum POD activity was observed at 24 h with DP5, DP7, and SA treatments and 48 h with DP6 treatment (Fig. 4.4F). The response to DP7 on the POD activity is on par with SA in the foliar application. The response of rice in terms of PAL and POD activities was active till 48 h in foliar spray application reflecting the efficacy of mode of application (Fig. 4.4D and F).

Notably, the induction of elicitation response was dependent on the size of the chitin oligomer i.e., the genes known to function in SA and JA signaling pathways in the seedlings after foliar application. In addition, expression of MAP kinase, WRKY transcription factors, defense responsive genes (chitinase-*I*, β -1,3-glucanase, peroxidase, and PAL), and PR gene was also studied. Our semi-qPCR results (data not shown) showed that most of the genes have higher PCR band intensity between 6 and 24h. Therefore, we have analyzed the expression of these genes at 6h, 12h, and 24h by real-time PCR. DP7 exhibited maximum elicitation effect (Table 4.1).

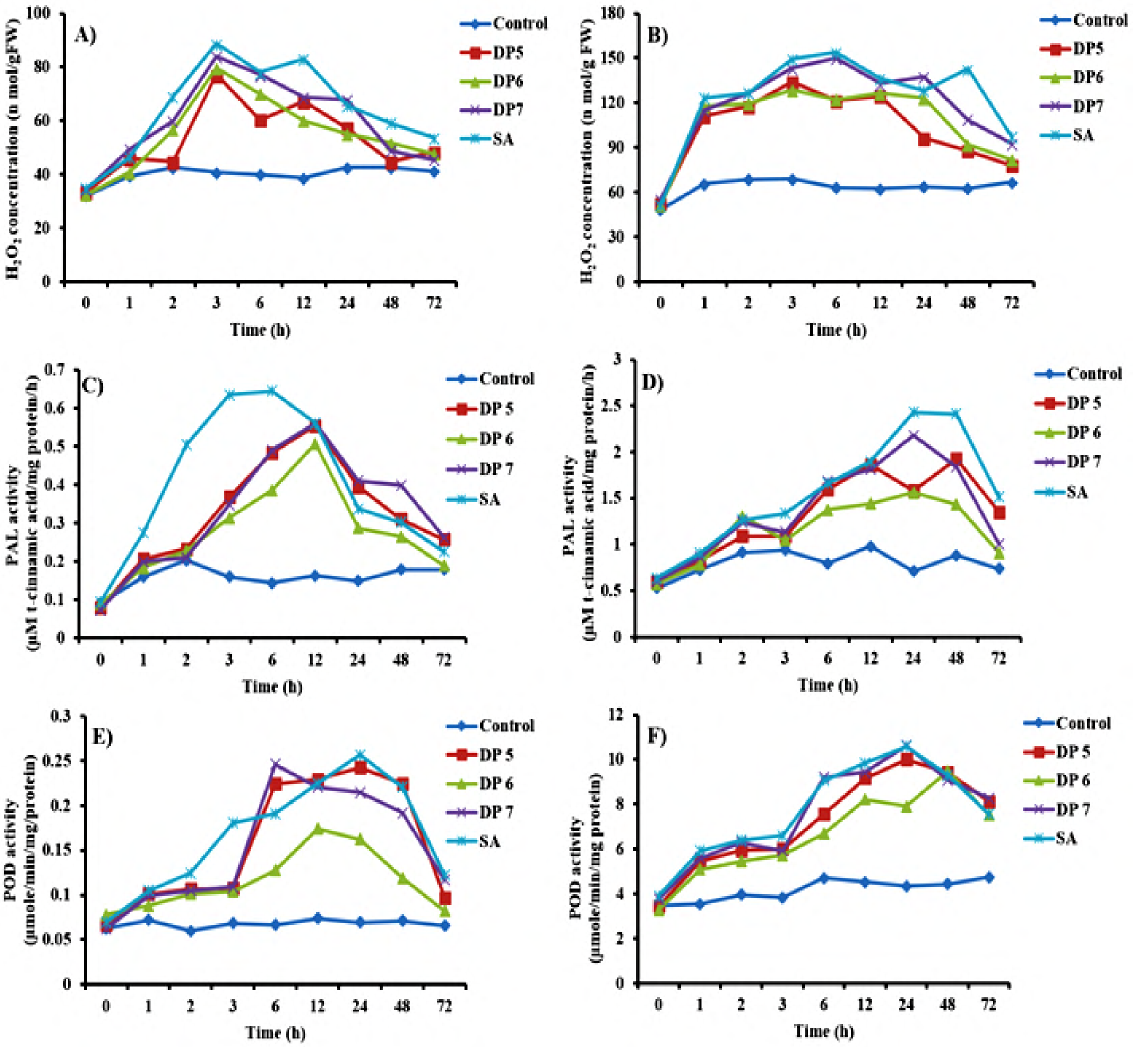
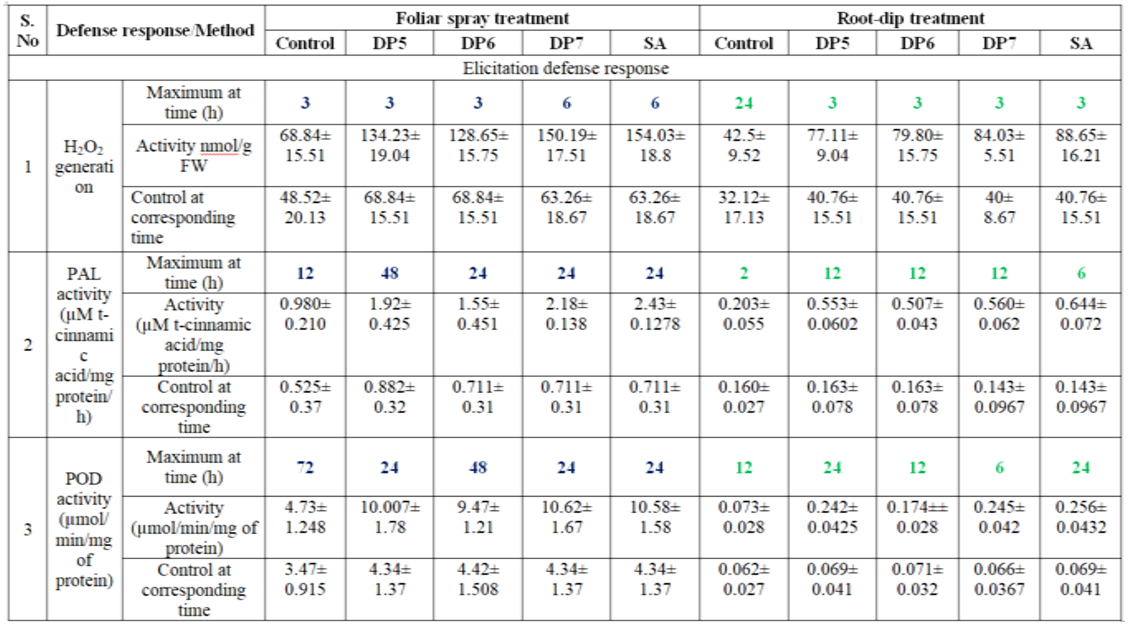


Fig 4.4: **Time response curves of H_2O_2 generation, PAL and POD elicitation by COS (DP5-7) upon root dip and foliar spray treatments of rice seedlings.** Time response curves of H_2O_2 generation (A & B), PAL (C & D) and POD (E & F) elicitation by COS and SA upon root dip (A, C & E) and foliar spray treatments (B, D & F) of rice seedlings. The data is mean (±SE) of two biological experiments with three technical replicates each. The error bars indicate mean (±SE) of three independent experiments.



S. No	Defense response/Method		Foliar spray treatment					Root-dip treatment				
			Control	DP5	DP6	DP7	SA	Control	DP5	DP6	DP7	SA
Elicitation defense response												
1	H_2O_2 generation	Maximum at time (h)	**3**	**3**	**3**	**6**	**6**	**24**	**3**	**3**	**3**	**3**
		Activity nmol/g FW	68.84±15.51	134.23±19.04	128.65±15.75	150.19±17.51	154.03±18.8	42.5±9.52	77.11±9.04	79.80±15.75	84.03±5.51	88.65±16.21
		Control at corresponding time	48.52±20.13	68.84±15.51	68.84±15.51	63.26±18.67	63.26±18.67	32.12±17.13	40.76±15.51	40.76±15.51	40±8.67	40.76±15.51
2	PAL activity (µM t-cinnamic acid/mg protein/h)	Maximum at time (h)	**12**	**48**	**24**	**24**	**24**	**2**	**12**	**12**	**12**	**6**
		Activity (µM t-cinnamic acid/mg protein/h)	0.980±0.210	1.92±0.425	1.55±0.451	2.18±0.138	2.43±0.1278	0.203±0.055	0.553±0.0602	0.507±0.043	0.560±0.062	0.644±0.072
		Control at corresponding time	0.525±0.37	0.882±0.32	0.711±0.31	0.711±0.31	0.711±0.31	0.160±0.027	0.163±0.078	0.163±0.078	0.143±0.0967	0.143±0.0967
3	POD activity (µmol/min/mg of protein)	Maximum at time (h)	**72**	**24**	**48**	**24**	**24**	**12**	**24**	**12**	**6**	**24**
		Activity (µmol/min/mg of protein)	4.73±1.248	10.007±1.78	9.47±1.21	10.62±1.67	10.58±1.58	0.073±0.028	0.242±0.0425	0.174±±0.028	0.245±0.042	0.256±0.0432
		Control at corresponding time	3.47±0.915	4.34±1.37	4.42±1.508	4.34±1.37	4.34±1.37	0.062±0.027	0.069±0.041	0.071±0.032	0.066±0.0367	0.069±0.041

Table 4.1: Summary of elicitation responses of rice treated with COS and SA. Table shows the maximum hydrogen peroxide (H_2O_2) accumulation and, phenylalanine ammonia lyase (PAL), and peroxidase (POD) activities at specific time points in rice seedlings upon root dip and foliar spray treatments with COS and SA. The data is mean (± SE) of three independent experiments with three technical replicates each.

4.2.3. COS induced differential expression of defense-related genes in SA and JA signaling pathways

To understand more details on the effect of COS, we examined the transcript levels of SA synthesis-related genes such as *PAL* and *ICS1*. The *PAL* gene was up-regulated significantly at 6h, 12h, and 24h after treatment with DP6, DP7 and SA, compared to their respective controls. However, expression of PAL was induced at all-time points, with different temporal behavior. It was highly induced at 6h, lower at 12h, and again high at 24h, after DP5 treatment (Fig. 4.5A). Treatments with COS (DP5-7) and SA induced the expression of the *ICS1* gene in rice seedlings compared to control. DP6 showed significant induction of the *ICS1* gene at 12h after elicitation, whereas, DP7 and SA treatments increased the transcript levels significantly at 12h and 24h (Fig. 4.5B). DP7 and SA significantly increased the *EDS1* transcripts at 12h and 24h after treatment, whereas the transcripts of *EDS1* in DP5 and DP6 treated seedlings increased marginally (Fig 4.5C). The *PAD4* transcripts accumulated significantly in the rice seedlings with DP7 (at 12h and 24h) and SA (at 24h) treatments (Fig. 4.5D), and no significant induction with DP5 and DP6. Rice seedlings, at 12h and 24h after DP7 treatment, exhibited significant induction of NPR1 transcription. Similarly, SA treatment accounted for a significant induction of NPR1 at 24h after treatment. There was no significant induction of the NPR1 gene in rice seedlings treated with DP5 and DP6 (Fig 4.5E).

Similarly, the expression pattern of the genes known to function in the JA-dependent signaling pathway were evaluated. In rice seedlings, treated with DP7, significant accumulation of *AOS2* transcripts was observed at 24h after treatment. Whereas, the basal expression of *AOS2* was weakly up-regulated in all other treatments (Fig 4.6A). Accordingly, the transcription of *JMT1* was marginal in rice seedlings in all treatments (Fig.4.6B). Concurrently, negative regulation or no change in transcription of *JMYB* was detected in rice treated with COS and SA (Fig. 4.6C).

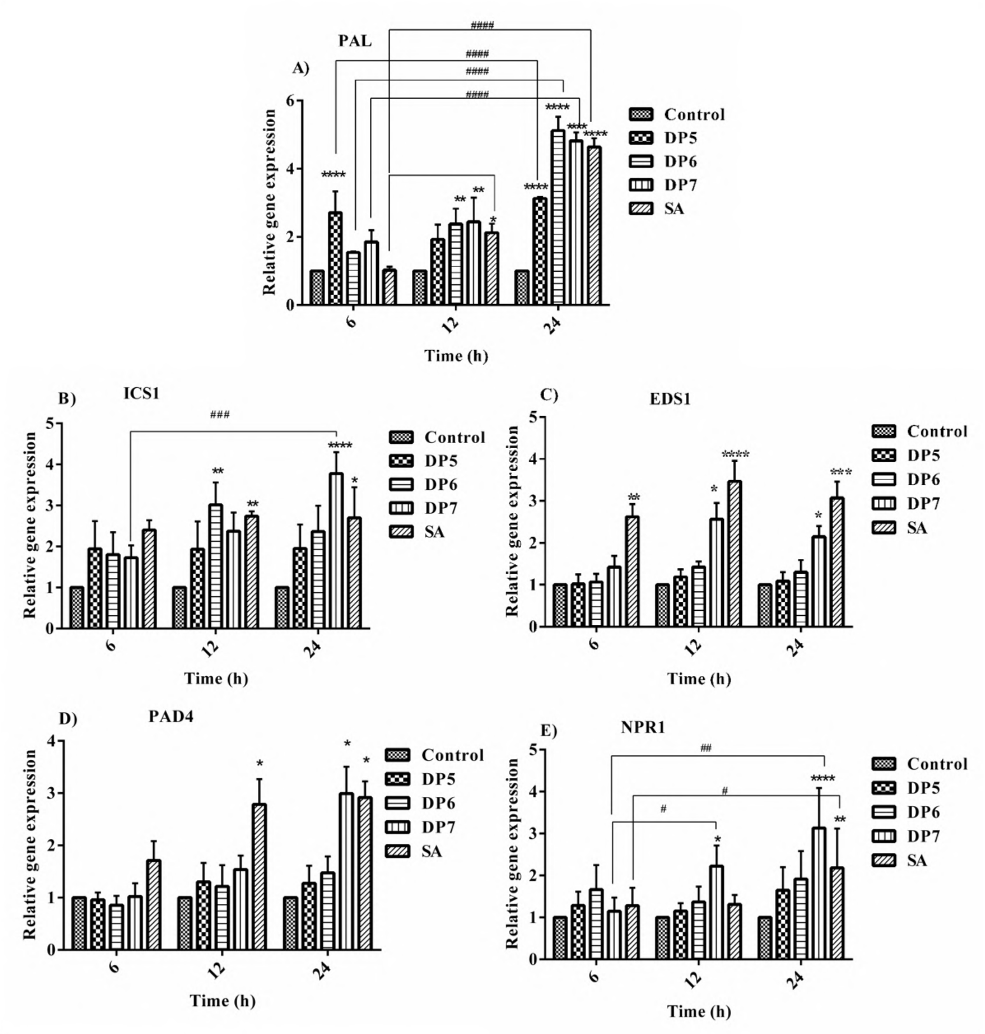


Fig 4.5: Expression of salicylic acid signaling pathway genes after COS and SA treatment. Salicyclic acid signaling pathway genes; Phenylalanine ammonia lyase-*PAL* (A), Iso-chorismate synthase1- *ICS1* (B), Enhanced disease susceptibility 1-*EDS1* (C), Phytoalexin deficient 4- *PAD4* (D), and Non-expressor of PR genes1-*NPR1* (E), were monitored for gene expression after COS and SA treatments. Error bars indicate mean (±SE) of three technical replicates. The asterisk symbol denotes significant difference of treated rice seedlings with respective controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). The hashtag symbol denotes significant differences between respective treatments among the time points (#$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$; ####$P < 0.0001$).

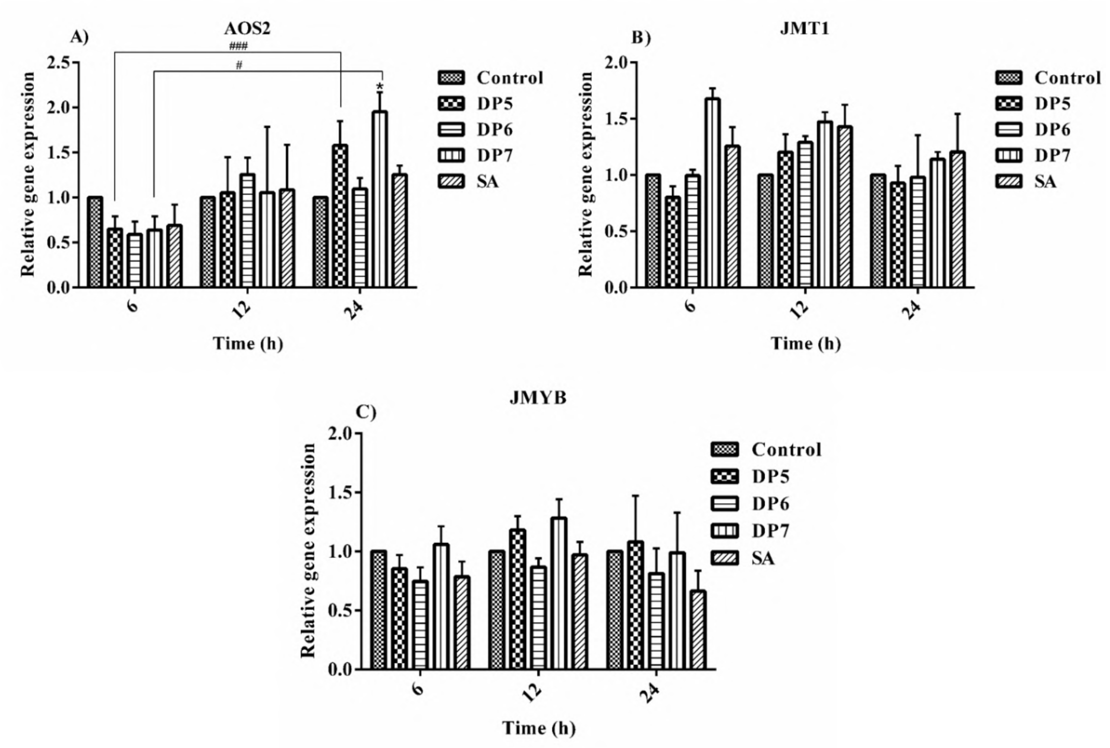


Fig 4.6: Expression of jasmonic acid signaling pathway genes after COS and SA treatment. Jasmonic acid signaling pathway genes; Allene oxidase synthase - *AOS2* (A), Jasmonic acid carboxyl methyltransferase- *JMT1* (B), and JA-inducible Myb transcription factor *JMYB* (C) were monitored for gene expression after COS and SA treatments. Error bars indicate mean (±SE) of three technical replicates. The asterisk symbol denotes significant difference of treated rice seedlings with respective controls (**P* <0.05; ***P* <0.01; ****P* <0.001; *****P*<0.0001). The hashtag symbol denotes significant differences between respective treatments among the time points (#*P* <0.05; ##*P* <0.01; ###*P* <0.001; ####*P* <0.0001). The significance was calculated using Tukey's post hoc multiple comparison test (mean ± SE, n=3). The experiment was repeated twice with similar results.

4.2.4. Differential response of MAPK and WRKY transcription factor genes upon COS elicitation in rice seedlings

MAPK5a transcripts were not induced significantly with COS and SA treatments at all-time points (Fig. 4.7A). Whereas, MAPK6a transcripts accumulated significantly with DP7 (at 24h) and SA (at 12h and 24h) treatments. SA-treated seedlings, at 24h, showed a significant transcription of MAPK6a in comparison to 6h and 12h (Fig. 4.7B). There were no significant differences in the temporal expression of *WRKY13* with COS and SA treatments at 6, 12, and 24h (Fig. 4.7C). WRKY45 gene expression was up-regulated significantly at 12h and 24h after treatment with DP7 compared to their respective controls. Whereas, expression of *WRKY45* was induced in SA treated seedlings at all-time points but showed a significant induction at 12h (Fig. 4.7D).

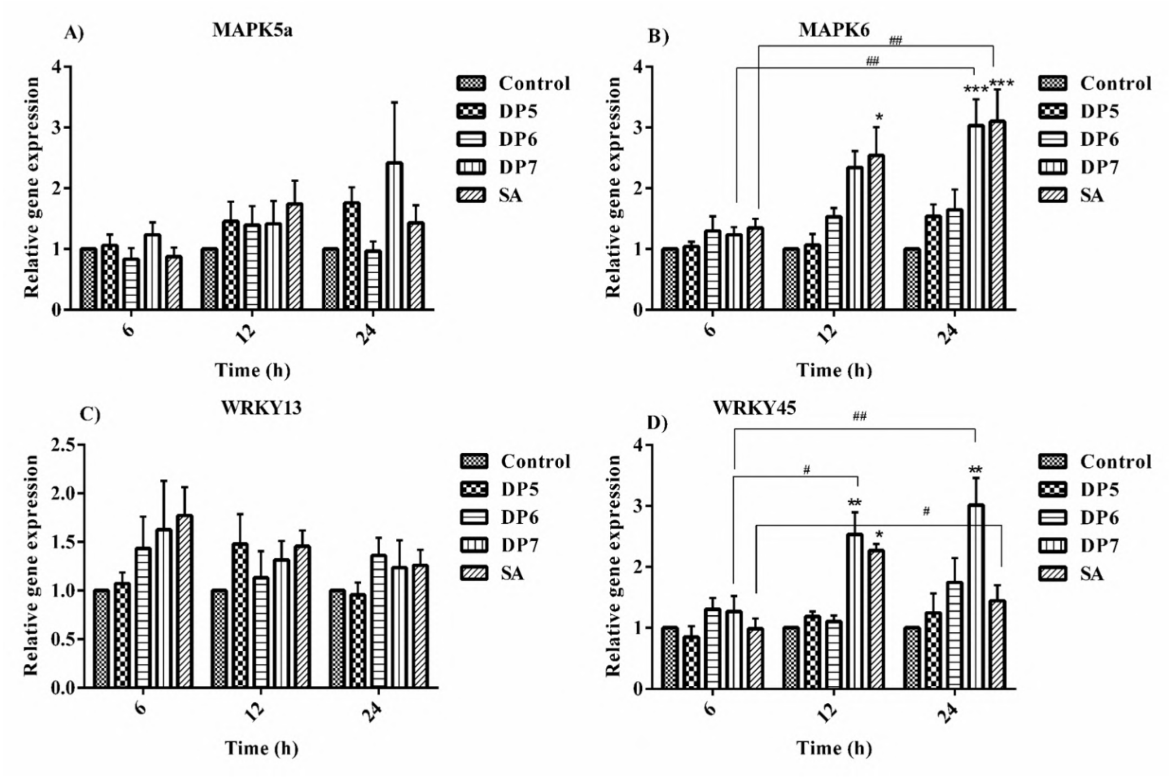


Fig 4.7: Expression of mitogen-activated protein kinase genes. *MAPK5a* (A) and *MAPK6* (B) and WRKY transcription factors: *WRKY13* (C) and *WRKY45* (D) in rice seedlings treated with COS and SA. Error bars indicate mean (± SE) of three technical replicates. The asterisk symbol denotes significant difference of treated rice seedlings with respective controls (**P*< 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001). The hashtag symbol denotes significant differences between respective treatments among the time points (#*P*< 0.05; ##*P* < 0.01; ###*P* < 0.001; ####*P* < 0.0001). The significance was calculated using Tukey's post hoc multiple comparison test.

4.2.5. General defense response to COS elicitation in rice seedlings

To elucidate the molecular mechanisms underpinning the general defense response of rice seedlings upon COS treatment, expression of PR protein genes (*PR1a*, *PR1b*, *PR4*, and *PR10*), chitinase1, β-1,3-glucanase, and peroxidase genes was evaluated. At 12h and 24h after DP7 treatment, and at 24h after SA treatment, there was a significant expression of *PR1a* in rice seedlings. DP5 and DP6 treatments account for the insignificant induction of *PR1a* (Fig 4.8A). There were no significant differences (P <0.05) in the temporal expression of *PR1b* at 6, 12, and 24h with COS and SA treatments (Fig. 4.8B). The *PR4* is not up-regulated in rice seedlings treated with COS and SA, except with DP7 at 24h after treatment (Fig 4.8C). The PR10 was up-regulated significantly at 12h and 24h with DP7 treatment, and at 24h with DP6 and SA treatments compared to their respective controls (Fig. 4.8D). There was a systemic and temporal expression of chitinase1 and β -1,3-glucanase genes in rice seedlings treated with COS and SA.

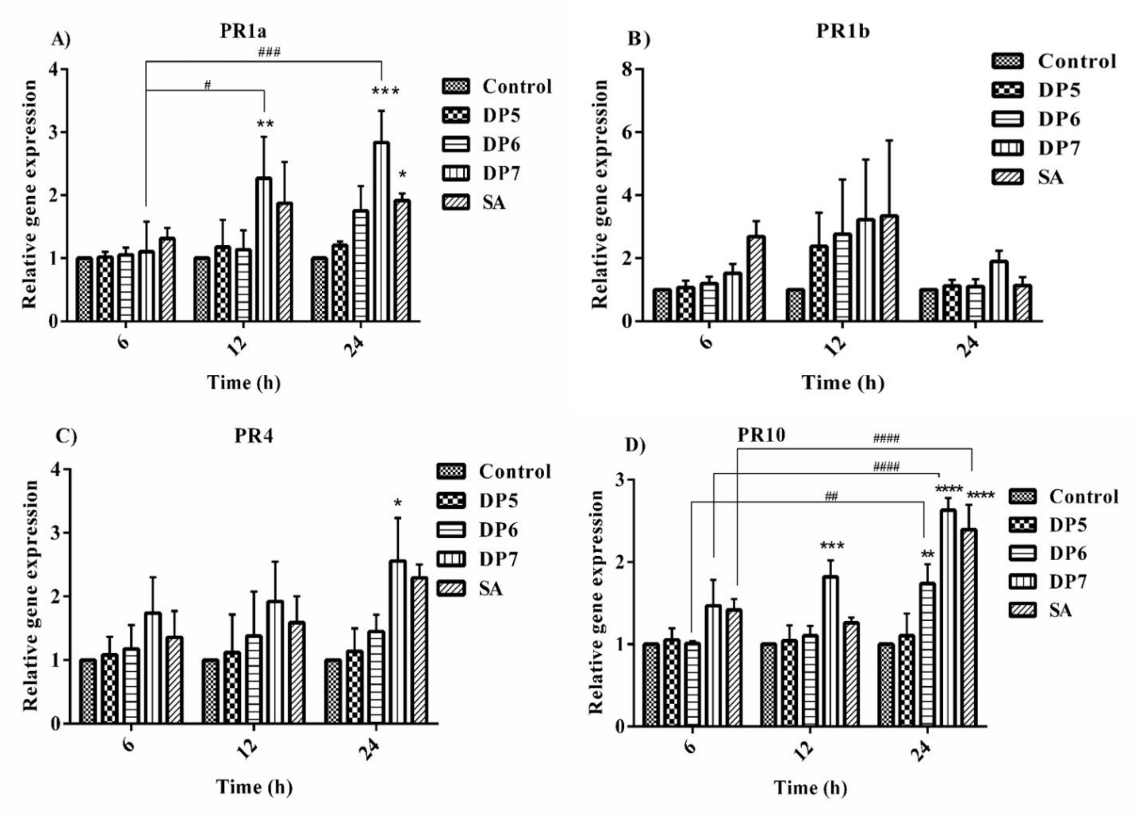


Fig 4.8: Expression of pathogenesis related genesafter COS and SA treatments. Genes related to pathogenesis *PR1a* (A), *PR1b* (B), *PR4* (C), and *PR10* (D) were monitored after COS and SA treatments. Error bars indicate mean (± SE) of three technical replicates. The asterisk symbol denotes significant difference of treated rice seedlings with respective controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). The hash tag symbol denotes significant differences between respective treatments among the time points (#$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$; ####$P < 0.0001$). The significance was calculated using Tukey's post hoc multiple comparison test.

The chitinase-*1* transcripts increased significantly at 12h and 24h with DP7 and SA treatments. DP5 treatment also enhanced the chitinase transcripts gradually and peaked at 24h considerably. At 12h and 24h, there was a significant induction of the chitinase1 gene, as compared to 6h (Fig. 4.9A). Similarly, the increase of β -1,3-glucanase transcripts was significant at 12h and 24h in DP7 and SA treatments. Whereas, the transcript levels of the β -1,3-glucanase gene in DP5 and DP6 treated plants were marginal (Fig. 4.9B). Both DP6 and DP7 induced the *POD* gene expression at all-time points with respective controls. However, in comparison to 6h and 24h treatments, expression was significant at 12h after treatment (DP6 = P < 0.0001 and DP7 = P < 0.0001). No significant induction of the *POD* gene was observed at different time points except for the SA treatment (Fig. 4.9C).

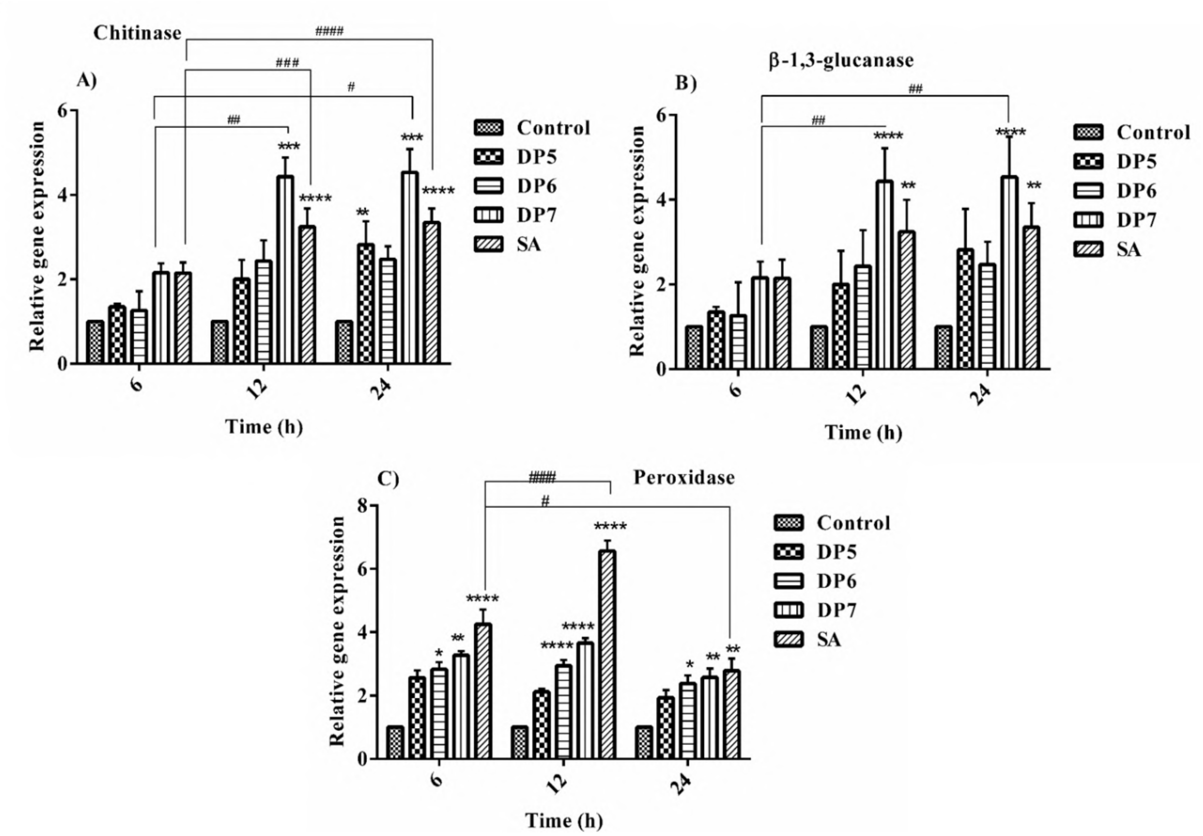


Fig 4.9: Induction of *chitinase*, β-1,3-*glucanase* and *peroxidase* genes after COS and SA treatments. Gene expression of *chitinase* (A), β-1,3-*glucanase* (B), and *peroxidase* (C) genes were monitored after COS and SA treatments. Error bars indicate mean (± SE) of three technical replicates. The asterisk symbol denotes significant difference of treated rice seedlings with respective controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). The hash tag symbol denotes significant differences between respective treatments among the time points (#$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$; ####$P < 0.0001$). The significance was calculated using Tukey's post hoc multiple comparison test.

4.2.6. Evaluation of purified COS on rice by foliar application at field condition

To understand the effect of purified COS on rice in terms of improvement of growth and yield, experiment was set up with pot cultivated rice (Zordar variety, NSL Pvt Ltd) under field conditions and treated with purified COS during both vegetative and flowering stage of the plant development (Fig 4.10). Crop was maintained till maturity and plants were harvested when about 95% of the grain had matured. Seeds from panicles were carefully collected for each treatment separately in brown paper bag and kept for open air drying till 12-15% (approx) of moisture content is reached.

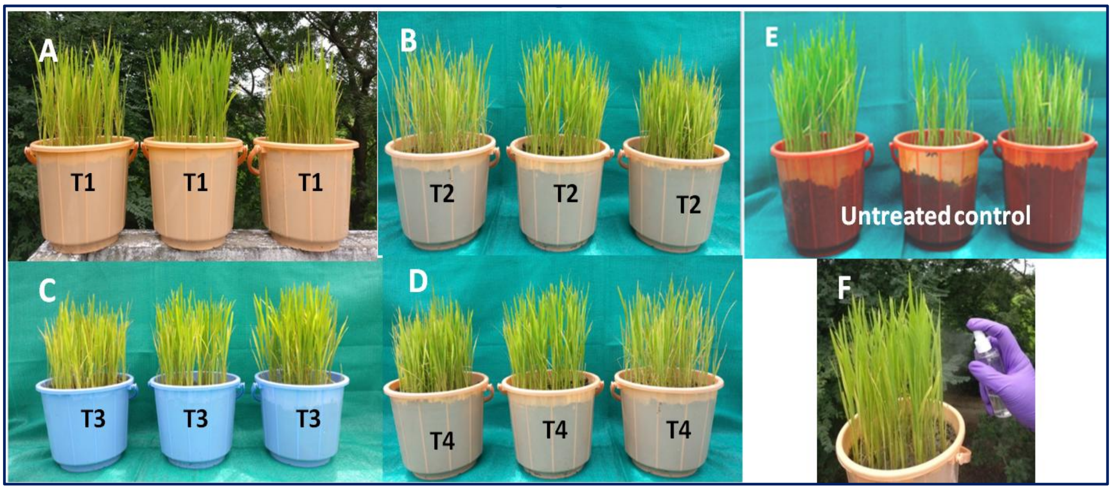
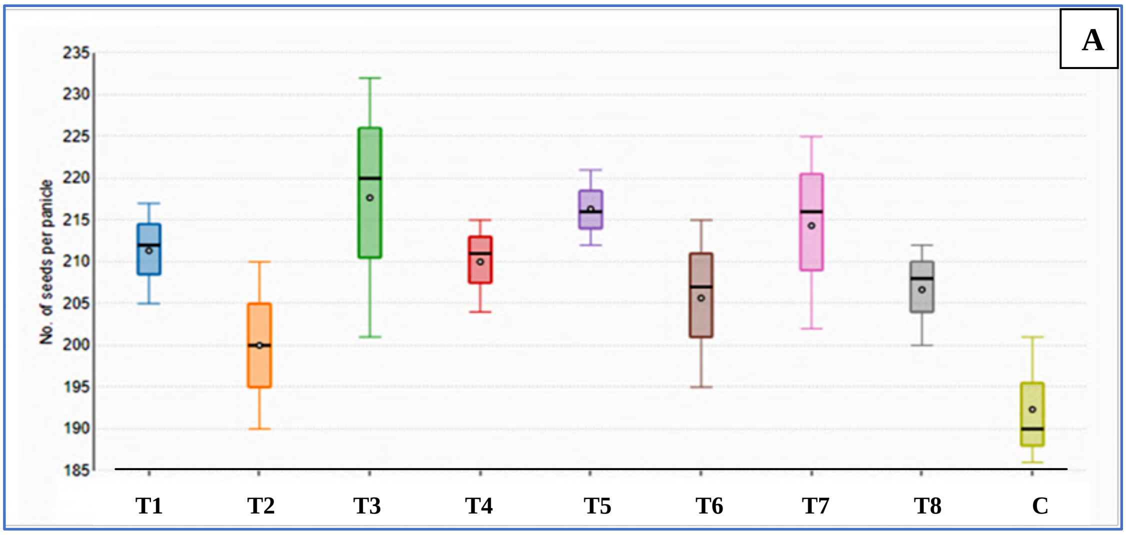


Fig 4.10: Foliar treatment of purified COS on rice. Pictorial representation of various COS treatments to rice cultivated in pots under field conditions, and foliar treatment of A; DP5-T1, B; DP6-T2, C; DP7-T3, and D; Salicylic acid-T4; untreated control. COS were sprayed during the vegetative and flowering stages of crop for each treatment (F), while water alone was sprayed on control plants.

Yield parameters were tabulated and shown in box plots for each treatment in terms of the number of seeds per panicle, total seed weight, and 100 seed weight (Fig 4.11). Statistical analysis performed using (PRIMER v7 software, Quest Research Limited, NZ) One-way ANOVA to evaluate the significant differences among the means (Table 4.2). Further, Grain yield was also analysed for percentage of improvement with respect to COS treatments in comparison with untreated control (Fig 4.12).



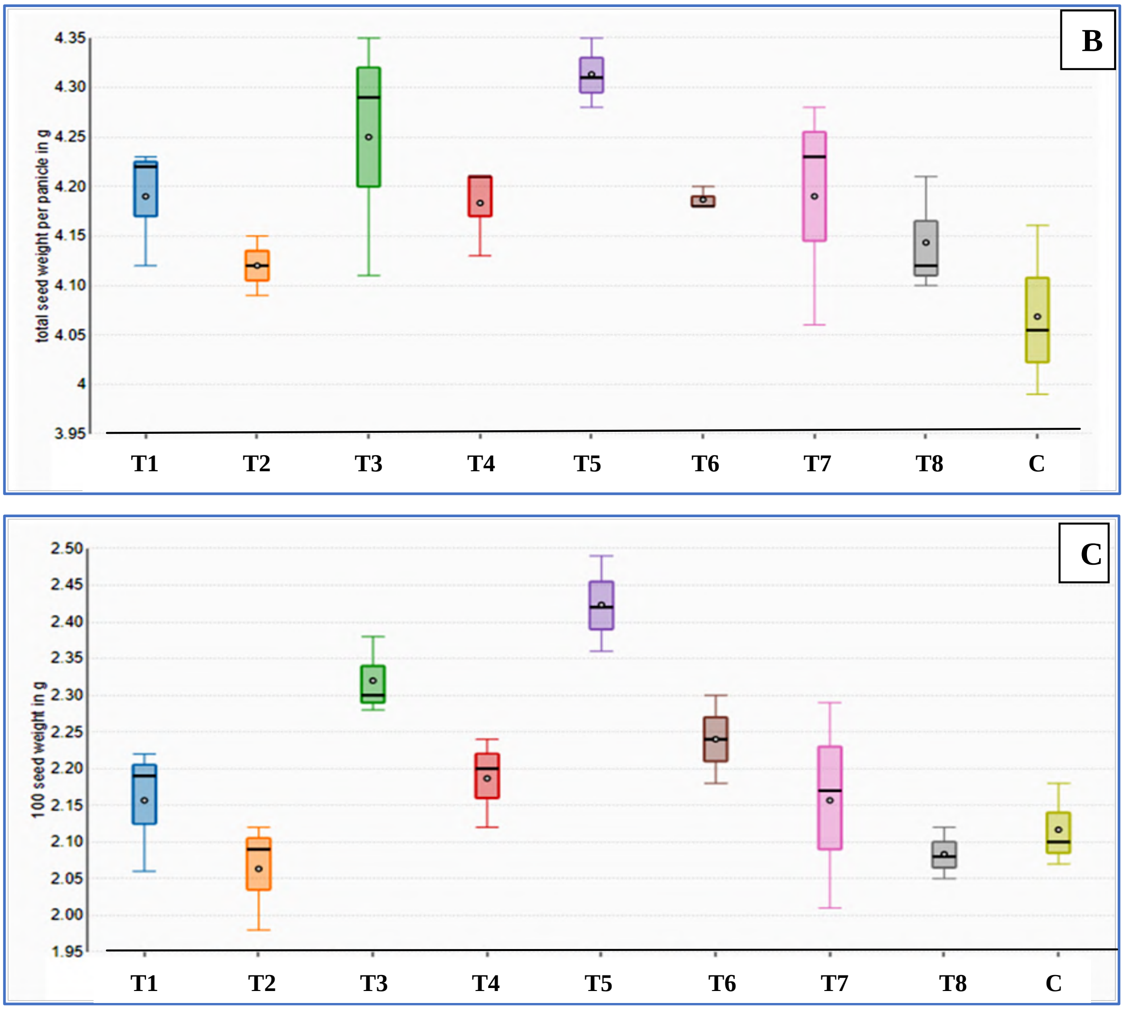


Fig. 4.11: Rice grain yield after COS treatments. Box plot depicting the yield parameters obtained from the harvested rice crop treated with following purified COS, DP5 (T1, T2), DP6 (T3, T4), DP7 (T5, T6), Salicyclic acid (T7, T8) along with untreated control (C). (a) Seeds per panicle obtained, (b) 100 Seed weight calculated, (c) Total seed weight per panicle.

S.No	Source of variation	P- value	F- stat
1	Seeds per panicle	0.0625	2.355
2	100 seed weight	0.003	7.161
3	Total seed weight per panicle	0.314	2.839

Table 4.2: One way ANOVA values (P and F-Stat values with degrees of freedom measured between and among treated samples) for harvested rice data samples

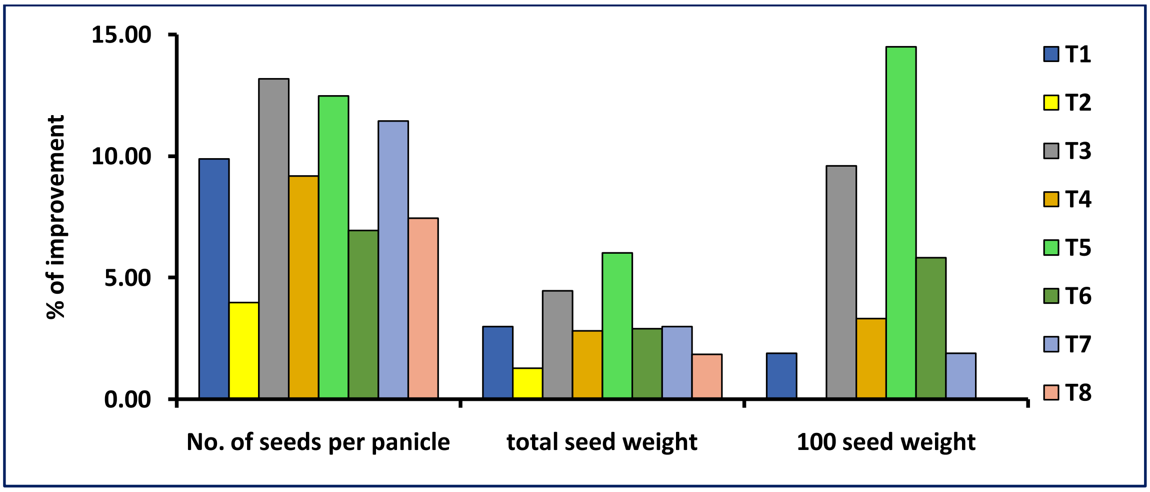


Fig 4.12: Percentage improvement of rice yield for COS treatments. Improved yield in terms of Seeds per panicle, total seed weight and 100 seed weight are compared with untreated control for different COS treatments.

Application of purified COS (DP5, DP6 and DP7) improved yield of rice, represented in box plots with standard deviation (Fig 4.11). It is observed that in all the treatments, plants were healthy in comparison with the control and also observed that the flowering stage reached little early (7-10 days) for the treated plants in comparison with untreated control. Yield parameters such as 100 seed weight (g), number of seeds per panicle, and total seed weight (g) have been recorded. One way ANOVA (Table 4.2) was performed to statistically analyse the harvested data and each treatment was found to be significant as F-stat value was higher than its P-value, which allowed us to reject the null hypothesis. The percentage improvement in grain yield has been calculated in comparison to the control for each yield trait (Fig 4.12). It is noted that DP7 (T5) (14.49%) treatment demonstrate the greatest percentage improvement for 100 seed weight, followed by DP6 (T3) (9.61%).

Similarly, treatment with DP7 (T5) (6.93%) exhibited the greatest response for total seed weight, followed by DP6 (T3) (4.46%). In terms of seeds per panicle, DP6 (T3) (13.17%), followed by DP7 (T5) (12.48%), and salicylic acid treatment SA (T7) (11.44%) were effective. In conclusion, various treatments employing the purified COS, the response with heptamer (DP7) was substantial, followed by hexamer (DP6) in terms of grain yield (per panicle) improvement as well as weight of the seed in particular. In conclusion the COS could be used as natural bio-stimulants of plant growth and development.

4.3 Discussion

4.3.1. Preparation of higher DP COS

Selection of TG enzyme for production of COS >6 is crucial for developing a bioprocess. Y28A variant of *Sp*ChiD that showed consistent production of TG product DP6, even at extended at 180 min. To obtain longer chain COS in higher quantity, bulk reaction set-up (150ml) using *Sp*ChiD-Y28A mutant upto 180min resulted in a white precipitate. Modified reaction conditions and subsequent analysis of MALDI-TOF for the clear liquid and the precipitate showed similar profile of COS up to DP10.

To reduce the formation of precipitate, we tested the influence of Tris-HCl buffer at different pH range (7.0, 8.0 and 9.0) on the reaction set up. Quantification of HPLC profiles for TG product (DP6) revealed the highest yield obtained at 150 min sample of pH 7.0 reaction set up in comparison with reactions set up at pH-8.0 and pH 9.0. Appearance of white precipitate started from 120th min for pH 7.0 and 150th min for pH 8.0 and not observed in samples with pH 9.0. Further, comparison of TG product (DP6) formation in pH 7.0 (Tris-HCl) reactions set up at substrate concentration 3mg/ml, 50mM NaCl and 100µg/ml BSA for 90min and 120min of incubation, revealed the highest yield obtained at 3mg/ml for 90 min without formation of white precipitate.

All further reactions were carried out with above optimized conditions. HPLC analysis of the products obtained from the both (250 & 500 ml) bulk reaction setup were quantified. The concentration`s of DP6 and DP7 obtained were 11% and 4%, respectively, indicating that the total TG product concentration of ~15% in 250ml reaction. Where as in 500ml reaction, concentration`s of DP6 and DP7 obtained were 11% and 6% with a total TG product concentration ~17% (Fig 26B) indicating only a marginal increase of 2%.

Purification of COS by semi-preparative HPLC yielded ~15% of TG product concentration`s with DP6 and DP7 at 11% and 4%, respectively. Repeated injections of the reaction mixtures were run in semi-preparative HPLC and the collected fractions were pooled before lyophylization. We could recover up to 50% of yield for DP6 and DP7 after purification. TLC and MALDI-TOF-MS, showed pure DP6 and DP7 but MALDI-ToF-MS analysis of DP7 purified fraction revealed a minor contamination of DP6 and DP8 COS.

4.3.1. COS induced elicitation response in rice seedlings

Most of the previous studies on chitin elicitation responses in plants were based on suspension cultures and COS prepared chemically from chitin (Felix & Boller, 1993; Kaku et al., 1997; Day et al., 2001; Okada et al., 2002, 2007; Gallão et al., 2007). The present study involves the evaluation of the application of COS (prepared by enzymatic method) by root dip and foliar spray application in rice seedlings. The increase of H_2O_2 levels (accumulation of ROS) is the initial key indicator during the plant defense responses to pathogen attack and it acts as the signaling molecule in the induction of systemic acquired resistance (SAR) (Dodds & Rathjen, 2010).

In the present study, the H_2O_2 generation was observed in rice seedlings with all treatments and it depends on the degree of polymerization of chitin. Similarly, the root application and pre-treatment of DP7 induced the H_2O_2 generation in *Medicago tranculata* and rice seedlings, respectively (Desaki et al. 2012; Nars et al. 2013). Egusa et al., (2015) reported that COS and chitin nanofiber induced the ROS production in *Arabidopsis*. The present study along with previous reports further supports that the plants induce H_2O_2 generation upon COS treatment.

Both modes of COS treatments increased PAL and POD enzyme activities, which are known to be involved in plant defense responses (Gallão et al. 2007; Jung et al. 2008). Both PAL and POD enzymes displayed their maximum activities between 12h to 48h in foliar treatment. Levels of PAL and POD activities were increased with chitin chain length, i.e., the larger the COS chain length (DP7) the greater the level of enzyme activity. Chitin oligomers induced POD and PAL activities in wheat and soybean leaves (Jung et al. 2008; Vander et al. 1998). Burkhanova et al., (2007) observed the enhanced peroxidase enzyme activity in resistant and susceptible verities of wheat seedlings to root rot upon the treatment of low-MW COS (5-10 kDa). It was reported that chitin-oligomers increased peroxidase and L-phenylalanine ammonia lyase activities in suspension-cultured cells of *Citrus aurantium* (Gallão et al., 2007).

Arabidopsis seedlings treated with microbially- digested lobster shell extract also enhanced PAL and chitinase enzyme activities (Ilangumaran et al. 2017). Compared to DP5 and DP6, which were active at the concentration tested, the DP7 was more active, enhancing the ROS generation, and POD and PAL activities. These results indicate that the exogenous application of COS elicited the defense response. Based on the present results, the foliar application was more effective than the root application, as the elicitation response was quick and persistent for a long time. This could be probably due to the difference in adherence and penetration properties of COS (by simplistic and apoplastic routes) in targeted cells of different organs i.e., leaves and roots which results in rapid diffusible signal (Pereira et al., 2019; Tanabe et al., 2006). Therefore, foliar mode of treatment was chosen to assess the elicitation response at the molecular level.

4.3.2. COS induced differential regulation of SA and JA-signaling pathways

In plants, SA can be synthesized *via* iso-chorismate pathway by *ICS1* and phenylpropanoid pathway by *PAL*. *EDS1* and *PAD4* function in a positive feedback loop to regulate SA synthesis upstream of *ICS1* (Seyfferth & Tsuda, 2014). In the present study, COS treatment, especially DP7 show the gradual significant induction of *ICS1* expression until 24h after elicitation. Consistent with these results, DP7 increased the *EDS1* transcript levels in rice at 12h after elicitation. Expression levels were higher in the DP7 treatment than DP5 and DP6 treatments. Whereas, DP7 and SA, induced the significant expression of *PAD4* expression levels at 24h after the treatment.

The *PAL* gene was strongly elevated in treatments with DP6 and DP7. Besides, the *NPR1* gene, which was suggested to act in transducing the SA signal to downstream and PR gene activation in rice (Shimono et al. 2007), was significantly expressed in COS and SA treatments. Similarly, Zhang et al., (2002) suggested that *EDS1, PAD4,* and *CPR1* act upstream of *NPR1* in the SA signal transduction pathway upon chitin treatment in *Arabidopsis* seedlings. Ilangumaran et al., (2017) also observed the strong up-regulation of the *ICS1* gene in *Arabidopsis* treated chitin extract at 24h after treatment. Similarly, induced expression of the *PAL* gene in *Medicago tranculata* seedlings was reported following the COS treatments (Nars et al. 2013). The present data suggest that the phenylpropanoid pathway is the major route for SA biosynthesis, though the contribution of the iso-chorismate pathway is evident in rice seedlings with COS treatment (especially DP7) during the plant SAR response. Therefore, these results suggest that the COS treatment could elicit the SAR response through SA-dependent pathway.

The expression patterns of biosynthesis and signaling genes of JA, another marker involved in plant defense responses (Glazebrook, 2005) was also evaluated. The expression of the *AOS2* gene, which encodes alleneoxide synthase 2, a key enzyme in JA biosynthesis was significantly higher only in DP7 treatment at 24h. Whereas, the transcript levels of *JMT1* (converts JA to volatile methyl jasmonate) and *JMYB* (JA-response) genes were weakly/or not induced in rice treated with COS. It was observed that chitosan oligosaccharide induced the resistance to *tobacco mosaic virus* (TMV) in *Arabidopsis* through SA-mediated signalling (Jia et al. 2016). These results indicate that the activation of SA synthesis and the SA signaling pathway over the JA pathway upon COS treatment in rice seedlings.

4.3.3. COS treatment regulates the expression of signaling and defense-related genes

In plants, MAPK cascades are evolutionarily conserved signalling modules that govern the action of numerous transcription factors such as WRKY and enzymes such as protein kinases, eventually regulating the induction of PR proteins in host responses to a variety of biotic stressors. (Dodds & Rathjen, 2010; Yamada et al., 2017). In this study, DP7 and SA induced the expression of *MAPK6* at 12h and 24h after treatment. However, the expression of *MAPK5a* was significant only in DP7 treatment at 24h. It was reported that *OsMPK6* was activated by several MAMPs (Kurusu et al. 2005; Lieberherr et al. 2005). Kishi-Kaboshi et al., (2010) found that chitin perception by the *OsCEBiP/OsCERK1* complex activates the conserved MAPK cascade, *OsMKK4/5-OsMPK3/MPK6*. This cascade triggers the synthesis of antimicrobial compounds, induction of immune responses such as defense-related gene expression, and cell death in rice.

The WRKY family of transcription factors is known to play a role in regulating the transcription of defense genes through W-box in their promoters. Several WRKY transcription factors play role in disease resistance (Shimono et al., 2007; Shimono et al., 2012) and a number of WRKY genes were identified in the rice genome (Ramamoorthy et al. 2008). WRKY transcription factors also have a role in the modulation of SA and JA-responsive gene expression and disease resistance (Shimono et al., 2012). Our results demonstrated that *WRKY45* transcript levels were significantly induced upon DP7 treatment in rice seedlings. In contrast, the *WRKY13* gene was weakly induced upon COS treatment. In rice plants, knock-down of *WRKY45* compromised the benzo-thiadiazole induced defense against rice blast, whereas, the over-expression of *WRKY45* conferred resistance (Shimono et al. 2007). Qiu et al., (2007) also showed that the over-expression of *OsWRKY13* enhanced the rice resistance to bacterial blight and fungal blast by regulating the defense-related genes in SA and JA dependent signaling. Similarly, chitin elicitor-responsive photon emission potentiation occurred through SA signalling *via OsWRKY45* in rice cell suspension culture (Iyozumi, Nukui & Kato, 2016). Our results were further supported by Ueno et al., (2017), where it was reported that *OsMPK6* activates the *WRKY45* by phosphorylation, which induces the disease resistance mediated by SA signaling in rice.

To capture more details of the defense response in eliciting the SAR, the expression of PR genes was also studied. In rice seedlings, DP6, as well as DP7, stimulated the expression of *PR1a* and *PR10* genes. However, the *PR4* gene was induced considerably only in DP7 treated seedlings at 24h after treatment. By contrast, expression of the *PR1b* gene was not considerably induced by COS treatments, suggesting the enhanced expression of *WRKY45* may activate the *PR1a* and *PR10* genes in SA-dependent pathway. Results presented here are consistent with the findings of Tanabe et al., (2006) who showed that DP7 induced SAR was accompanied by the activation of *PR1* and *PR10* genes in rice. Moreover, our results show that DP7 and SA induced strong expression of *POD*, *chitinase*, and *1,3*-β-*glucanase* genes. Previously, it was reported that the transcripts of *POD*, *chitinase*, and *1,3*-β-*glucanase* accumulate to higher levels in chitin-treated plants and/or suspension cultures (Kaku et al., 1997; Nars et al., 2013). These results indicate that the COS especially, DP7 induce the expression of defense-related proteins for initiation of PTI. Based on the results, it can be attributed that, DP higher than five is detected by chitin elicitor receptor kinase (*OsCERK1*) with high specificity (Kaku et al., 2006), which forms a complex with chitin elicitor binding protein (*OsCEBiP*) thereby downstream chitin signalling (Hayafune et al., 2014; Shimizu et al., 2010) through SA signaling pathway over JA pathway.

4.3.4. Improvement of grain yield with application of COS in rice

COS are being used as important functional materials in a variety of applications. The benefits of utilization of COS in agriculture closely mimic the purpose for which both the bio-fertilizers and bio-pesticides are used, as there is induction of defense against pathogens besides enhanced plant growth. Ramírez et al. (2010) reviewed the applications of chitin and its derivatives as biopolymers with potential agricultural applications and predicted that these biopolymers will be utilised more broadly, particularly to replace chemical pesticides and as growth regulators in future. Chitin and its derivatives are a prospective alternative due to their biological activity and easy availability. Multiple studies demonstrate the mechanisms of action and efficacy of such active principles in agriculture (Sharp, 2013), but are limited to a laboratory scale or controlled environments/green houses and field experiments were not reported.

Several methods of utilising chitin and their derivatives for plant growth stimulation have been attempted, including foliar spraying, seed coating, and soil supplementation. Use of oligo-chitosan as a broad-spectrum vaccine against plant diseases (Yin et al., 2010) highlights the need to produce specific COS for crop protection and production. El-Serafy, (2020) reported the use of ologo-chitosan in *Cordyline* seedlings for improved plant growth and root development. Similarly COS mixture increased plant growth and mineral accumulation in *Phaseolus vulgaris*, although chrolphyll content remained similar (Chatelain et al., 2014). Similarly, many studies reported the application of oligo-chitosan / COS mix in groundnut (Dung & Thang 2014), soybean (Dzung & Thang 2002), sugarcane, rice (El-Sawy et al. 2010), and wheat (Ma et al. 2012) towards improvement in plant growth and yield but no work is reported on specific application of purified COS at field level.

Cabrera and Cutsem (2005) used non-purified commercial enzymes to produce defined COS mixtures enriched in oligomers with DP >6. COS of DP <6 can be converted to DP>6 by an efficient transglycosylation (TG) (Usui et al., 1990; Zakariassen et al., 2011; Purushotham & Podile, 2012). We have employed a mutant of TG enzyme *Sp*ChiD Y28A (Madhuprakash et al., 2015) and prepared higher DP COS. Further, we have tested these COS (DP5, DP6, DP7) on rice in terms of plant growth and grain yield. Total seed weight in rice treated with purified COS DP7 and DP6 improved ~7% and ~5% respectively along with improvement in seeds per panicle and 100 seed weight. In conclusion, we report that these COS could potentially be used as natural bio-stimulants of plant growth and development.

Chapter - V

Major findings

Chapter 5: Major Findings

5. Major findings reported in the thesis

- Plant developmental stage shapes rice rhizosphere microbiome, regardless of soil amendments.
- The highest difference, 100% ($P \leq 0.005$), was observed between vegetative and flowering stages. Highest alpha diversity is observed during flowering stage (ANOSIM)
- Bacteriome, mycobiome and eukaryome responded differently to chitinous substrate amendments to rice rhizosphere.
- Alpha diversity of bacteria, fungi, and eukaryota, are affected by chitinous substrate amendments. The alpha diversity index is highest for bacteria, then for eukaryota, and lowest for fungi.
- In the rice rhizosphere, the influence of soil amendments on bacteria was lowest, but the influence of eukaryome was highest regardless of dosage and soil amendments (based on Pseudo F value at $P \leq 0.001$ obtained during one way ANOVA)
- Core rhizosphere microbiome of rice comprises of Uncultivated Eukaryote, Uncultivated Fungi, Uncultivated Cryptomycota, *Tyrophagus putrescentiae,* Uncultivated Micro Eukaryote, *Mortierella*, *Cochliopodium* and *Cordyceps* respectively
- Rhizosphere microbiome of rice is shaped by host i.e., plant growth stages and different soil amendments with various dosages.
- The bacterial genus *Pseudomonas* sp. is highly abundant in both alpha and beta chitin treatments to rice rhizosphere and low with respect to chitosan treatments. Abundance of a known PGPR organism like *Pseudomonas* is positively correlated with chitinous treatments.
- The fungal genus *Cordyceps* is part of core microbiome during various chitinous amendments. *Cordyceps* is reported as endophytic entomopathogenic fungus possessing more than 24 chitinase genes in its genome.
- *Tyrophagus putrescentiae* (saprophagous soil mite`s) observed to be part of core microbiome (it is reported to form symbiotic relationship between chitinolytic bacteria and digested fungi in mycophagous micro-arthropods).

- Rare fungi *Mortierella* is highly abundant in rice rhizosphere under chitinous substrates and it is reported be PGPR for several crop species.
- The fungal genus *Clonostachys* is highly abundant during both alpha and beta chitin treatments to rice. It is reported as a endophytic fungus with plant growth promotion.
- *Scedosporium* sp. (fungi) is highly abundant in chitosan treatments and not prevelant in other treatments.
- *Ascosphaera torchioi* is abundant in alpha chitin treatment and widely reported as pathogens to insects in nature.
- Differential abundance of *Clonostachys* (genus of fungi) is observed, i.e., in the vegetative stage after alpha chitin treatment, it is highly abundant during the flowering stage of beta chitin treatment.
- The eukaryotic genus *Eimeriidae* is abundant during chitosan treatment at vegetative stage.
- *Cochliopodium* sp. belongs to eukaryotes is highly abundant in alpha and beta chitin treatments especially during plant flowering stage.
- *Neobodo sailens* are bacterivores protists and abundant in alpha chitin treatment especially during flowering stage of rice
- The bacterial genus *Flavisoilbacter* sp. is highly abundant during vegetative growth stage of rice rhizosphere microbiome
- Chitinous substrate amendments were effective in improving seed weight and total number of seeds, alpha chitin was effective followed by chitosan.
- Conditions were optimized for bulk production of COS using *Sp*ChiDY28A mutant and purified COS was prepared in required quantities for further application.
- Enzymatically generated COS- DP5, DP6 and DP7 elicited plant defense response in rice seedlings by root dip and foliar spray, the latter being more effective.
- Foliar treatment with COS elicited rapid and longer oxidative burst response in rice in comparison with root dip method.
- COS with DP7 exhibited higher induction of defense-related genes than DP6 and DP5.
- Foliar treatment with COS elicited a rapid and longer oxidative burst response in rice.
- COS triggered Salicylic Acid signaling pathway genes for defense in rice over Jasmonic Acid pathway.
- Pathogenesis related proteins - *PR1a*, *PR10*, *chitinase1*, *peroxidase* and β *-1,3-glucanase* genes were induced by DP7.

- COS-induced activation of PAL and ICS1 and defense-related genes, imply SA-mediated SAR activation, possibly related to *MAPK6* and *WRKY45* expression.
- Induction of *MAPK6a*, *WRKY 13* and *WRKY45* genes was enhanced with DP7 treatment.
- Field evaluation was carried out by spraying purified COS (DP5-DP7) on rice during vegetative and booster dose at flowering stage.
- Harvest index parameters like total seed weight, 100 seed weight and seeds per panicle were improved.
- The total quantity of seeds harvested from each panicle across all treatments indicated that DP6 treatment was the most effective.
- DP7 treatment resulted in the highest total seed weight when all of the treatments were evaluated, followed by the DP6 treatment in rice.

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Annexure – I

Bash Code used for data analysis

```
#mbSet<-Init.#mbSetObj()
#mbSet<-SetModuleType(#mbSet, "mdp")
#mbSet<-ReadSampleTable(#mbSet, "Alpha chitin meta.csv");
#mbSet<-Read16STaxaTable(#mbSet, "sorted taxa bact of top 100.csv");
#mbSet<-Read16SAbundData(#mbSet, "sorted otu taxa bact 100 for alpha chitin.csv","text","SILVA","T");
#mbSet<-SanityCheckData(#mbSet, "text");
#mbSet<-SanityCheckSampleData(#mbSet);
#mbSet<-SetmetaAttributes(#mbSet, 0)
#mbSet<-PlotLibSizeView(#mbSet, "norm_libsizes_0","png");
#mbSet<-CreatePhyloseqObj(#mbSet, "text","SILVA","F")
#mbSet<-ApplyAbundanceFilter(#mbSet, "prevalence", 1, 0.1);
#mbSet<-ApplyVarianceFilter(#mbSet, "sd", 0.0);
#mbSet<-PerformNormalization(#mbSet, "none", "CSS", "none");
#mbSet<-PlotTaxaAundanceBar(#mbSet, "taxa_alpha_0","Phylum","PLANT_TYPE", "null", "barraw",10, "set3","sum",10, "bottom", "F", "png");
#mbSet<-PlotTaxaAbundanceBarSamGrp(#mbSet, "taxa_alpha_1","Phylum","Treatment", "none", "barnorm",10,"set3","sum", 10, "bottom", "F", "png");
#mbSet<-PlotTaxaAbundanceBarSamGrp(#mbSet, "taxa_alpha_2","Phylum","Treatment", "none", "barnorm",10,"cont28","sum", 10, "bottom", "F", "png");
#mbSet<-PlotTaxaAbundanceBarSamGrp(#mbSet, "taxa_alpha_3","Genus","Treatment", "none", "barnorm",10,"cont28","sum", 10, "bottom", "F", "png");
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#mbSet<-PCoA3D.Anal(#mbSet, "PCoA","bray","OTU","expfac","Treatment","OTU1","Chao1","beta_diver3d_0.json")
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#mbSet<-PerformCategoryComp(#mbSet, "Genus", "adonis","bray","Treatment");
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#mbSet<-PlotTaxaAbundanceBarSamGrp(#mbSet, "taxa_alpha_3","Genus","Treatment", "none", "barnorm",10,"cont28","sum", 10, "bottom", "F", "png");
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#mbSet<-PlotTaxaAbundanceBarSamGrp(#mbSet, "taxa_alpha_2","Phylum","Treatment", "none", "barnorm",10,"cont28","sum", 10, "bottom", "F", "png");
#mbSet<-PlotTaxaAbundanceBarSamGrp(#mbSet, "taxa_alpha_3","Genus","Treatment", "none", "barnorm",10,"cont28","sum", 10, "bottom", "F", "png");
#mbSet<-PerformBetaDiversity(#mbSet, "beta_diver_0","PCoA","bray","expfac","Treatment","none","OTU","OTU1","Chao1", "yes", "png", 72, "default");
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#mbSet<-CoreMicrobeAnalysis(#mbSet, "core_micro_2",0.1,0.01,"Genus","viridis","overview", "all_samples", "Treatment", "Paddy", "png");
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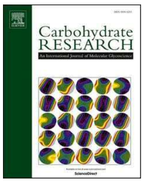

Elicitation of defense response by transglycosylated chitooligosaccharides in rice seedlings

Bellamkonda Ramakrishna [1], P.V.S.R.N. Sarma [1], Sravani Ankati, Bhoopal Bhuvanachandra, Appa Rao Podile *

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Gachibowli, Hyderabad, 500046, Telangana, India

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Transglycosylation
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Elicitor activity
Defense mechanism

ABSTRACT

Long-chain chitooligosaccharides (COS) with degree of polymerization (DP) more than 4 are known to have potential biological activities. A hyper-transglycosylating mutant of an endo-chitinase from *Serratia proteamaculans* (*Sp*ChiD-Y28A) was used to synthesize COS with DP6 and DP7 using COS DP5 as substrate. Purified COS with DP5-7 were tested to elicit the defense response in rice seedlings. Among the COS used, DP7 strongly induced oxidative burst response as well as peroxidase, and phenylalanine ammonia lyase activites. A few selected marker genes in salicylic acid (SA)- and jasmonic acid-dependent pathways were evaluated by real-time PCR. The expression levels of pathogenesis-related (PR) genes *PR1a* and *PR10* and defense response genes (chitinase1, peroxidase and *-1,3-glucanase*) were up regulated upon COS treatment in rice seedlings. The DP7 induced *Phenylalanine ammonia lyase* and *Isochorismate synthase* 1 genes, with concomitant increase of *Mitogen-activated protein kinase* 6 and *WRKY45* transcription factor genes indicated the possible role of phosphorylation in the transmission of a signal to induce SA-mediated defense response in rice.

1. Introduction

Plants are constantly exposed to microbial pathogens including bacteria, fungi, and viruses. As a protection against the microbial pathogens, plants activate immune responses upon recognition of the evolutionarily conserved pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) [1,2]. Plants detect the PAMPs with the help of membrane-anchored pattern recognition receptors (PRRs) and activate pattern-triggered immunity (PTI). PTI involves callose deposition, synthesis of antimicrobial compounds, activation of mitogen-activated protein kinase (MAPKs) cascade, generation of reactive oxygen species (ROS) etc., [3,4]. Flagellin, lipopolysaccharides, peptidoglycans, glucans, and mannans represent some of the known MAMPs/PAMPs [4].

Chitin consists of (1, 4)-linked *N*-acetyl-D-glucosamine (GlcNAc) units arranged in a linear form. It is a major structural component of fungal cell walls and insect exoskeletons, and also acts as a MAMP/PAMP [1,5]. Oligosaccharides generated from chitin are referred to as chitooligosaccharides (COS). The COS act as a broad-spectrum vaccine against several plant diseases making them potential candidates for crop protection [6]. Treatment with COS was reported to induce defense reactions like generation of ROS, transient depolarization of membranes, extracellular alkalization and ion flux, phytoalexin production in plants/cell suspension cultures [5,7,8], expression of several early responsive genes [9,10], lignification [11] etc., Activation of MAPK cascade, upon chitin elicitation, was reported in *Arabidopsis* and rice [12,13].

The COS could be prepared from chitin by acid hydrolysis, alkaline hydrolysis, chemical synthesis, and oligosaccharide mixture generated using microbial source or by enzymatic hydrolysis. Chitinases (EC 3.2.1.14), which are glycosyl hydrolases (GHs), also perform transglycosylation (TG) using the sugar molecule as the acceptor resulting in the formation of a new glycosidic linkage [14], are useful to generate long-chain COS. A few chitinases with TG activity [15 17] were reported to generate longer COS. A GH18 endo-chitinase from *Serratia proteamaculans* 568 (*Sp*ChiD) that exhibited hyper TG activity [17] was further improved by point mutations to increase the production of long-chain COS [18]. Among them, Y28A (mutation in the substrate binding cleft of *Sp*ChiD) generated 21% of TG products from DP4 substrate [19] suggesting its suitability to generate long-chain COS. Hence, we made an attempt to produce long-chain COS ranging from DP5-7

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[1] These authors contributed equally to this work.

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RESEARCH ARTICLE

Poor Competitiveness of *Bradyrhizobium* in Pigeon Pea Root Colonization in Indian Soils

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Danteswari Chalasani, Anirban Basu, and Sarma V. S. R. N. Pullabhotla contributed equally to this work. Author order was determined as female author first followed by males alphabetically and in order of increasing seniority.

ABSTRACT Pigeon pea, a legume crop native to India, is the primary source of protein for more than a billion people in developing countries. The plant can form symbioses with N_2-fixing bacteria; however, reports of poor crop nodulation in agricultural soils abound. We report here a study of the bacterial community associated with pigeon pea, with a special focus on the symbiont population in different soils and vegetative and non-vegetative plant growth. Location with respect to the plant roots was determined to be the main factor controlling the bacterial community, followed by developmental stage and soil type. Plant genotype plays only a minor role. Pigeon pea roots have a reduced microbial diversity compared to the surrounding soil and select for *Proteobacteria*, especially for *Rhizobium* spp., during vegetative growth. While *Bradyrhizobium*, a native symbiont of pigeon pea, can be found associating with roots, its presence is dependent on plant variety and soil conditions. A combination of 16S rRNA gene amplicon survey, strain isolation, and co-inoculation with nodule-forming *Bradyrhizobium* spp. and non-N_2-fixing *Rhizobium* spp. demonstrated that the latter is a much more successful colonizer of pigeon pea roots. Poor nodulation of pigeon pea in Indian soils may be caused by a poor *Bradyrhizobium* competitiveness against non-nodulating root colonizers such as *Rhizobium*. Hence, inoculant strain selection of symbionts for pigeon pea should be based not only on their nitrogen fixation potential but, more importantly, on their competitiveness in agricultural soils.

IMPORTANCE Plant symbiosis with N_2-fixing bacteria is a key to sustainable, low-input agriculture. While there are ongoing projects aiming to increase the yield of cereals using plant genetics and host-microbiota interaction engineering, the biggest potential lies in legume plants. Pigeon pea is a basic food source for a billion low-income people in India. Improving its interactions with N_2-fixing rhizobia could dramatically reduce food poverty in India. Despite the Indian origin of this plant, pigeon pea nodulates only poorly in native soils. While there have been multiple attempts to select the best N_2-fixing symbionts, there are no reliable strains available for geographically widespread use. In this article, using 16S rRNA gene amplicon, culturomics, and plant co-inoculation assays, we show that the native pigeon pea symbionts such as *Bradyrhizobium* spp. are able to nodulate their host, despite being poor competitors for colonizing roots. Hence, in this system, the establishment of effective symbiosis seems decoupled from microbial competition on plant roots. Thus, the effort of finding suitable symbionts should focus not only on their N_2-fixing potential but also on their ability to colonize. Increasing pigeon pea yield is a low-hanging fruit to reduce world hunger and degradation of the environment through the overuse of synthetic fertilizers.

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REVIEW ARTICLE

Biotechnological approaches to develop bacterial chitinases as a bioshield against fungal diseases of plants

[illegible] PVSRN Sarma[3] [illegible]

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Abstract
Fungal diseases of plants continue to contribute to heavy crop losses in spite of the best control efforts of plant pathologists. Breeding for disease-resistant varieties and the application of synthetic chemical fungicides are the most widely accepted approaches in plant disease management. An alternative approach to avoid the undesired effects of chemical control could be biological control using antifungal bacteria that exhibit a direct action against fungal pathogens. Several biocontrol agents, with specific fungal targets, have been registered and released in the commercial market with different fungal pathogens as targets. However, these have not yet achieved their full commercial potential due to the inherent limitations in the use of living organisms, such as relatively short shelf life of the products and inconsistent performance in the field. Different mechanisms of action have been identified in microbial biocontrol of fungal plant diseases including competition for space or nutrients, production of antifungal metabolites, and secretion of hydrolytic enzymes such as chitinases and glucanases. This review focuses on the bacterial chitinases that hydrolyze the chitinous fungal cell wall, which is the most important targeted structural component of fungal pathogens. The application of the hydrolytic enzyme preparations, devoid of live bacteria, could be more efficacious in fungal control strategies. This approach, however, is still in its infancy, due to prohibitive production costs. Here, we critically examine available sources of bacterial chitinases and the approaches to improve enzymatic properties using biotechnological tools. We project that the combination of microbial and recombinant DNA technologies will yield more effective environment-friendly products of bacterial chitinases to control fungal diseases of crops.

Keywords: *Biocontrol; chitinolytic bacteria; chitinases; fungal pathogens; chitin*

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