MICROBIOME-BASED RHIZOSPHERE ENGINEERING FOR MITIGATION OF SALINITY STRESS IN *VIGNA RADIATA*

SHUBHAM DUBEY

DEPARTMENT OF BIOCHEMICAL ENGINEERING AND BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY DELHI

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by

SHUBHAM DUBEY

Department of Biochemical Engineering and Biotechnology

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Dedicated

To my Grandparents, who inspired this and will not read it.....

CERTIFICATE

This is to certify that the thesis entitled **"Microbiome-based rhizosphere engineering for mitigation of salinity stress in *Vigna radiata*"** being submitted by **Mr. Shubham Dubey** is worthy of consideration for the award of the degree of Doctor of Philosophy. The thesis has been prepared under my supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology Delhi, and is a record of the original bonafide research work. The results presented in this thesis have not been submitted in part or full to any other universities or institutes for the award of any other degree or diploma.

Prof. Shilpi Sharma

Professor,
Department of Biochemical Engineering and Biotechnology,
Indian Institute of Technology Delhi
New Delhi-110016
INDIA

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“वर्णानामर्थसंघानां रसानां छन्दसामपि।
मंगलानां च कर्त्तारौ वन्दे वाणीविनायकौ॥1॥

First Shloka from Sri Ramcharitmanas

By the **Grace of God**, I have successfully completed my **Ph.D. thesis work**. As I stand at the crossroads, on the verge of thesis submission, I am fondly ready to take a walk down memory lane, remembering with gratitude, all the individuals who have supported me throughout this journey and beyond.

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Shubham Dubey

ABSTRACT

The indiscriminate use of chemical fertilizers and pesticides in agriculture, and rising global temperatures (rising sea levels, fluctuations in weather cycles and increase in primary salinization), lead to salt accumulation in the soil, thereby negatively impacting plant physiology and crop yield worldwide. This has significantly affected the global economies, as agriculture is essential for ensuring food security and reducing poverty in many parts of the world. To address this grave issue, researchers have turned to eco-friendly approaches of utilizing beneficial microbes residing in the rhizosphere. However, using single strains or consortia of different strains has often proven ineffective due to issues concerning their ability to colonize roots while facing competition from the native soil microbiome. To combat this challenge, rhizosphere engineering has been employed as a sustainable strategy. It can be categorized into plant-based, metaorganism-based, and microbiome-based methods, wherein the transformation of the non-optimized metaorganism takes place, resulting in an optimized metaorganism that exhibits enhanced fitness. The microbiome-based method can be attained by employing top-down or bottom-up approaches.

In present study, **an indirect plant-assisted microbiome-selection strategy was adopted over several passaging cycles to acclimatize the indigenous microbiome such that it benefitted the growth of *Vigna radiata* plants under salt-stress conditions.** The plant fitness was assessed over successive cycles in terms of plant growth and physiological parameters. Results showed an increased tolerance of plants to salinity stress, as evidenced by enhancement in plant growth attributes and decreased stress marker levels/indicators such as proline, malondialdehyde (MDA), and membrane stability index (MSI). Amplicon sequencing, employing the 16S rRNA and ITS as genetic markers, revealed significant changes in the microbial community inhabiting the rhizosphere of *Vigna radiata* under salinity stress across the passages. A significant increase

in the relative abundance of dominant bacterial phyla such as *Proteobacteria*, *Actinobacteria*, and *Firmicutes*, coupled with a concomitant decrease in the abundance of the fungal phylum *Ascomycota*, was observed in the acclimatized microbiome during successive passages. Moreover, a decline in the abundance of *Mortierellomycota* and *Chytridiomycota* was explicitly noted during the 5^{th} and 13^{th} passages, respectively.

Once the salt stress-adapted rhizospheric microbiome was obtained, the next objective was to understand the mechanism of action in salinity stress mitigation in *Vigna radiata*. Bacterial strains were isolated from stress-acclimatized microbiome using group-specific media to generate a culture bank. As determined by split plate assay, specific bacterial isolates were found to produce volatiles, possibly contributing to salt stress mitigation in Arabidopsis. The best-performing plant growth-promoting bacterial strains were selected to analyze their volatilomes by gas chromatography–mass spectrometry (GC-MS). Key metabolites, like 7 methyl-hexadecane, 2-(2-butoxyethoxy) ethanol, sulfur-containing volatiles, etc. were identified after a detailed investigation of volatilomes of stress-acclimatized bacterial strains. These volatiles may promote plant development and stress tolerance. Hence the impact of selected volatiles was then examined on *Vigna radiata* under controlled conditions. In the presence of salinity stress, the volatiles were able to mitigate the effects of stress, promote plant growth and improve their fitness.

To overcome the technical limitations associated with the storage of such an acclimatized microbiome, synthetic microbial communities (SynComs) were designed from the culture bank generated from the acclimatized microbiome. SynComs were generated to perform *at par* with the stress-acclimatized microbiome, while mimicking its functionality in mitigating salinity stress in plants grown in arable land. The salinity stress mitigation potential of these SynComs was assessed

first under control, and subsequently in field conditions, resulting in significant enhancement in the plant growth and stress amelioration under salinity stress condition.

The efficacy of the multi-passaging technique in rhizosphere engineering and the utilization of SynComs has been demonstrated through an extensive investigation conducted in laboratory and field settings. The study presents opportunities for implementing such a unique approach of combining top-down and bottom-up strategies with other crops and under diverse stress regimes. This approach promises an essential and favourable step toward ushering sustainable agricultural practices worldwide.

सार/सारांश

कृषि में रासायनिक उर्वरकों और कीटनाशकों के अत्यधिक उपयोग और बढ़ते वैश्विक तापमान के कारण मृदा में लवण संचयन हो रहा है, जो दुनिया भर में पादप संरचना और फसल की उपज को नकारात्मक रूप से प्रभावित कर रहा है। इसने वैश्विक अर्थव्यवस्थाओं को काफी प्रभावित किया है, क्योंकि दुनिया के कई हिस्सों में गरीबी को कम करने के लिए और खाद्य सुरक्षा सुनिश्चित करने के लिए कृषि आवश्यक है। इस गंभीर मुद्दे को हल करने के लिए शोधकर्ताओं ने मूल परिवेश, राइजोस्फीयर (rhizosphere) में रहने वाले लाभकारी जीवाणुओं के उपयोग के लिए पर्यावरण के अनुकूल तरीकों की ओर रुख किया है। हालांकि, जीवाणुओं के एकल उपयोग या संयुक्त अनुप्रयोग करना अक्सर देशी मिट्टी माइक्रोबायोम से प्रतिस्पर्धा का सामना करते हुए जड़ों को उपनिवेशित करने की उनकी क्षमता से संबंधित मुद्दों के कारण अप्रभावी साबित हुआ है। इस चुनौती का मुकाबला करने के लिए, राइजोस्फीयर इंजीनियरिंग (rhizosphere engineering) को एक स्थायी रणनीति के रूप में अपनाया गया है। इसे पादप-आधारित, मेटाऑर्गेनिज्म (metaorganism)-आधारित और माइक्रोबायोम (microbiome)-आधारित विधियों में वर्गीकृत किया जा सकता है, जिसमें गैर-अनुकूलित मेटाऑर्गेनिज्म का परिवर्तन होता है, जिसके परिणामस्वरूप एक अनुकूलित मेटाऑर्गेनिज्म में परिवर्तित हो जाता है, जो बढ़ी हुई उपयोगिता प्रदर्शित करता

है। माइक्रोबायोम-आधारित विधि को टॉप-डाउन (top-down) या बॉटम-अप (bottom-up) दृष्टिकोणों को अपना करके प्राप्त किया जा सकता है।

वर्तमान अध्ययन में, निवासी माइक्रोबायोम को अनुकूलित करने के लिए बहु पारगमन चक्रों (multi-passaging cycle) पर आधारित एक अप्रत्यक्ष पादप-सहायता प्राप्त माइक्रोबायोम-चयन रणनीति अपनाई गई थी ताकि लवण-तनाव की स्थिति में *विग्ना रेडिएटा* (*Vigna radiata*) पौधों के विकास को लाभ हो। पौधे की फिटनेस का मूल्यांकन पौधे के विकास और शारीरिक मापदंडों के संदर्भ में क्रमिक चक्रों में किया गया था। परिणामों ने लवणता तनाव के लिए पौधों की बढ़ती सहनशीलता दिखाई, जैसा कि पौधे के विकास गुणों में वृद्धि और प्रोलिन (proline), मालोंडिअलडिहाइड (Malondialdehye, एमडीए), और मेम्ब्रेन स्टैबिली इंडेक्स (Membrane stability index, एमएसआई) जैसे तनाव मार्कर स्तर / संकेतकों में कमी से स्पष्ट है। 16 एस आरआरएनए (16S rRNA) और आईटीएस (ITS) को आनुवंशिक मार्करों के रूप में नियोजित करने वाले एम्प्लिकॉन (amplicon) अनुक्रमण ने पारगमन चक्रों में लवणता तनाव के तहत विग्ना रेडिएटा के राइजोस्फीयर में रहने वाले माइक्रोबियल समुदाय में महत्वपूर्ण परिवर्तनों को उजागर किया। प्रोटीओबैक्टीरिया (*Proteobacteria*), एक्टिनोबैक्टीरिया (*Actinobacteria*) और फर्मिक्यूट्स (*Firmicutes*) जैसे प्रमुख बैक्टीरियल फ़ाइला (phyla) की सापेक्ष बहुतायत में एक महत्वपूर्ण वृद्धि, फंगल फाइलम एस्कोमाइकोटा की बहुतायत में सहवर्ती कमी के साथ मिलकर, बहु पारगमन

चक्रों के दौरान अनुकूलित माइक्रोबायोम में देखी गई थी। इसके अलावा, क्रमशः 5 वें और 13 वें चक्र के दौरान मोर्टिएरेलोमीकोटा (*Mortierellomycota*) और चिट्रिडियोमाइकोटा (*Chytridiomycota*) की बहुतायत में गिरावट स्पष्ट रूप से नोट की गई थी। गैस क्रोमैटोग्राफी-मास स्पेक्ट्रोमेट्री (Gas Chromatography-Mass Spectrometry, जीसी-एमएस) द्वारा उनके वोलाटिलोम (volatilome) का विश्लेषण करने के लिए सबसे अच्छा प्रदर्शन करने वाले पौधे के विकास को बढ़ावा देने वाले जीवाणु उपभेदों का चयन किया गया था। प्रमुख मेटाबोलाइट्स (metabolites), जैसे 7 मिथाइल-हेक्साडेकेन (7 methyl-hexadecane), 2-(2-ब्यूटॉक्सीथोक्सी) इथेनॉल (2-(2-butoxyethoxy) ethanol), सल्फर युक्त वाष्पशील, आदि। तनाव-अनुकूलित जीवाणु उपभेदों के ज्वालामुखी की विस्तृत जांच के बाद उनकी पहचान की गई थी। ये वाष्पशील पौधे के विकास और तनाव सहिष्णुता को बढ़ावा दे सकते हैं। इसलिए चयनित वाष्पशील पदार्थों के प्रभाव की जांच तब नियंत्रित परिस्थितियों में विग्ना रेडिएटा पर की गई थी। लवणता तनाव की उपस्थिति में, वाष्पशील तनाव के प्रभाव को कम करने, पौधे के विकास को बढ़ावा देने और उनकी फिटनेस (fitness) में सुधार करने में सक्षम थे।

एक बार नमक तनाव-अनुकूलित राइजोस्फेरिक माइक्रोबायोम प्राप्त हो जाने के बाद, अगला उद्देश्य विग्ना रेडिएटा में लवणता तनाव शमन में कार्रवाई के तंत्र को समझना था। कल्चर बैंक (culture bank) उत्पन्न करने के लिए समूह-विशिष्ट मीडिया का उपयोग करके तनाव-अनुकूलित

माइक्रोबायोम से बैक्टीरियल (bacterial) उपभेदों को अलग किया गया था। जैसा कि स्प्लिट प्लेट (split-plate) परख द्वारा निर्धारित किया गया था, विशिष्ट बैक्टीरियल आइसोलेट्स (bacterial isolates) वाष्पशील पदार्थों का उत्पादन करते पाए गए थे, ये वाष्पशील पदार्थ संभवतः एराबिडोप्सिस (Arabidopsis) में नमक तनाव शमन में योगदान देते थे।

इस तरह के एक अनुकूलित माइक्रोबायोम के भंडारण से जुड़ी तकनीकी सीमाओं को दूर करने के लिए, सिंथेटिक माइक्रोबियल समुदायों (सिनकॉम्स, SynComs) को अनुकूलित माइक्रोबायोम से उत्पन्न माइक्रोबियल कल्चर बैंक (microbial culture bank) से डिजाइन किया गया था। कृषि योग्य भूमि में उगाए गए पौधों में लवणता तनाव को कम करने में इसकी कार्यक्षमता की अनुकृति करते हुए, तनाव-अनुकूलित माइक्रोबायोम के बराबर प्रदर्शन करने के लिए सिनकॉम्स को बनाया गया था। इन सिनकॉम्स की लवणता तनाव शमन क्षमता का मूल्यांकन पहले नियंत्रित परिस्थितियों में किया गया, और बाद में वास्तविक कृषि क्षेत्र की स्थितियों में परीक्षण किया गया।

राइजोस्फीयर इंजीनियरिंग (rhizosphere engineering) में बहु पारगमन चक्र तकनीक की प्रभावकारिता और सिनकॉम (SynCom) के उपयोग को प्रयोगशाला और वास्तविक कृषि क्षेत्र में आयोजित एक व्यापक जांच के माध्यम से प्रदर्शित किया गया है। वर्तमान अध्ययन अन्य फसलों तथा विविध तनाव व्यवस्थाओं के उपचार हेतु टॉप-डाउन (top-down) और बॉटम-अप

(bottom-up) रणनीतियों के संयोजन का एक अनूठा दृष्टिकोण लागू करने के अवसर प्रस्तुत करता है। यह दृष्टिकोण दुनिया भर में टिकाऊ कृषि प्रथाओं को शुरू करने की दिशा में एक आवश्यक और अनुकूल कदम का वादा करता है।

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Formula/Abbreviation	Media, Molecules and Chemicals
$(NH_4)_2SO_4$	Ammonium sulphate
ACC	1-aminocyclopropane-1-carboxylate
ARE	Artificial root exudate
$CaCO_3$	Calcium carbonate
CAS	Chrome-azurol S
CMS	Carboxymethylcellulose
CTAB	Cetyl trimethyl ammonium bromide
EDTA	Ethylene diamine tetra-acetic Acid
EPS	Exopolysaccharide
$FeCl_3$	Ferric chloride
$FeSO_4$	Ferrous sulphate
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
$HClO_3$	Chloric acid
$HClO_4$	Perchloric acid
HCN	Hydrogen cyanide
HNO_3	Nitric acid
IAA	Indole acetic acid
KCL	Potassium chloride
KH_2PO_4	Dihydrogen potassium phosphate
LB	Luria–Bertani
MDA	Malondialdehyde
$MgCl_2$	Magnesium dichloride
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
MS	Murashige and Skoog medium
Na_2MoO_4	Sodium molybdate
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NBRIP	National Botanical Research Institute's phosphate

Terminology used in the study

AMF	Arbuscular mycorrhizal fungus
ASV	Amplicon sequence variants
CFU	Colony forming unit
CPA	Cryoprotective agents
DAS	Days after sowing
DNA	Deoxyribonucleic acid
DW	Dry weight

EC	Electrical conductivity
FAO	Food and Agriculture Organization
FW	Fresh weight
GC-MS	Gas Chromatography-Mass Spectrometry
HMME	Host mediated microbiome engineering
IARI	Indian Agricultural Research Institute
ICAR	Indian Council of Agricultural Research
ITS	Internal transcribed spacer
LC/MS	Liquid Chromatography-Mass Spectrometry
LDA	Linear discriminant analysis
MCT	Microcentrifuge tube
MSI	Membrane stability index
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NIST	National Institute of Standards and Technology
OUT	Operational taxonomic unit
PCA	Principal component analysis
PCD	Programmed cell death
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PERMANOVA	Permutational multivariate analysis of variance
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative polymerase chain reaction
RO	Reverse osmosis
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SRA	Sequence read archive
SynComs	Synthetic microbial communities
TOC	Total organic carbon
USDA	United States Department of Agriculture
VOCs	Volatile organic compounds

Elements

N	Nitrogen
P	Phosphorus
K	Potassium
C	Carbon
Na	Sodium
Cl	Chlorine

Units

%	Percent
Cm	Centimetre
dS/m	DeciSiemens per metre
g	Grams
Kg	Kilogram
°C	Degrees Celsius
Cl	Chlorine
L	Litre
M	Metre
M	Molar
mL	Millilitre
nm	Nanometre
min	Minutes
rpm	Revolutions per minute
S	Seconds
v/v	Volume by volume
RT	Retention time
µL	Microlitre
µM	Micromolar
q/h	Quintals per Hectare