EFFICACY AND RISK ASSESSMENT OF BIOINOCULANTS ON *CAJANUS CAJAN*

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by

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Submitted

in the fulfillment of the requirement of the degree of Doctor of Philosophy

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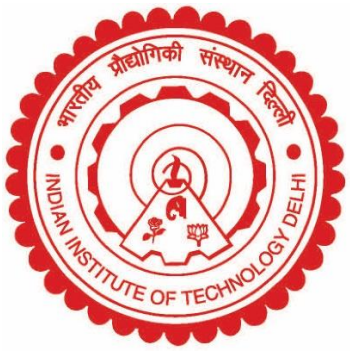

INDIAN INSTITUTE OF TECHNOLOGY DELHI

AUGUST 2019

Dedicated

To

My Parents

CERTIFICATE

This is to certify that the thesis entitled "**Efficacy and risk assessment of bioinoculants on *Cajanus cajan***" being submitted by **Ms Richa Sharma** is worthy of consideration for the award of the degree of Doctor of Philosophy. The thesis has been prepared under our 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.

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Richa Sharma

ABSTRACT

Due to rapid urbanization and industrialization, deleterious effects have been observed on ecosystem, which includes impact on water, soil, air and biodiversity. To address this issue, more attention has been laid on sustainable agriculture. Employment of bioinoculants is one of the several methods used as a substitute of chemical fertilizers for sustainable agriculture, because of their eco-friendly nature. Although the direct positive effect of bioinoculants on plant growth has been well-established, study of role of their indirect effects, contributing to the observed plant growth promotion, is still at its infancy. These agricultural amendments when introduced in soil, in numbers much greater than their usual numbers, result in transient perturbations in equilibrium of resident microflora. These introduced bioinoculants interact with resident microbial community to exert cumulative effect on plants. Such indirect effects on resident microflora, which can be both beneficial and deleterious, are known as 'non-target effects'.

The present study aimed to fill prime research gaps in the area, viz. efficacy and risk assessment of bioinoculants, apart from their persistence in the rhizosphere of *Cajanus cajan*. Efficacy assessment comprised of strategically designing a bacterial consortium for plant growth enhancement *Cajanus cajan*. Risk assessment focussed on analysis of non-target effects of these bioinoculants on resident bacterial community structure and function. A polyphasic approach was adopted by employing both cultivation-dependent and cultivation-independent methods to study the interplay of the consortium (*Azotobacter chroococcum, Bacillus megaterium* and *Pseudomonas fluorescens*) on both resident and active bacterial community structure and function (microbes participating in various steps of nitrogen cycle), alongwith their impact on plant growth attributes. Culturable fraction of microflora was enumerated on different selective media, while the total bacterial community was targeted by community-level physiological profiling (CLPP), denaturing gradient gel electrophoresis (DGGE), quantitative

polymerase chain reaction (qPCR) and next-generation sequencing (NGS). 16S rRNA marker was used for DGGE and NGS, while for qPCR both 16S rRNA and molecular markers involved in nitrogen cycle (*nif*H, *amo*A, *nir*K and *nar*G) were used. These effects were also compared with the impact exerted by chemical fertilizers at recommended dose. The amount of chemical fertilizers that could be replaced by these bioinoculants, without compromising on grain yield, was also assessed. Finally, their impact was seen on the survivability of human pathogen *Listeria monocytogenes*. The bioactive compounds responsible for the inhibition of *L.monocytogenes* were identified.

From the present piece of work it was concluded that bioinoculants competed well with the chemical fertilizers in terms of plant growth parameters and grain yield at field level. Upon tracking of bioinoculants they were observed to persist in soil only till flowering stage, though their effects were evident till harvest stage of the plant. The positive non-target impact of the consortium was visible on beneficial rhizospheric bacterial population, viz. nitrogen fixers, nitrifiers, phosphate solubilizers etc., rendering them "safe" for field application at larger scale. The consortium negatively impacted the survivability of human pathogen *L.monocytogenes*. Their negative effect on the survivability of human pathogen broadens the horizon of the application of these agricultural amendments not only for the benefits on plant health but also on humans.

सार

तेजी से शहरीकरण और औद्योगिकीकरण के कारण, पारिस्थितिकी तंत्र पर हानिकारक प्रभाव देखा गया है, जिसमें पानी, मिट्टी, वायु और जैव विविधता पर प्रभाव शामिल है। इस मुद्दे को हल करने के लिए, स्थायी कृषि पर अधिक ध्यान दिया गया है। जैव-कृषि का रोजगार स्थायी कृषि के लिए रासायनिक उर्वरकों के विकल्प के रूप में कई तरीकों में से एक है, क्योंकि उनके पर्यावरण के अनुकूल प्रकृति है। यद्यपि पौधे की वृद्धि पर जैव-रसायनों के प्रत्यक्ष सकारात्मक प्रभाव को अच्छी तरह से स्थापित किया गया है, उनके अप्रत्यक्ष प्रभावों की भूमिका का अध्ययन, मनाया संयंत्र विकास संवर्धन में योगदान, अभी भी अपनी प्रारंभिक अवस्था में है। मिट्टी में पेश किए जाने वाले ये कृषि संशोधन, उनकी सामान्य संख्या से बहुत अधिक संख्या में, निवासी माइक्रोफ्लोरा के संतुलन में क्षणिक गड़बड़ी के परिणामस्वरूप होते हैं। ये पौधों पर संचयी प्रभाव डालने के लिए निवासी माइक्रोबियल समुदाय के साथ जैव-जैव-रासायनिक संपर्क करते हैं। निवासी माइक्रोफ्लोरा पर इस तरह के अप्रत्यक्ष प्रभाव, जो लाभकारी और निंदनीय दोनों हो सकते हैं, 'गैर-लक्षित प्रभाव' के रूप में जाने जाते हैं। वर्तमान अध्ययन का उद्देश्य क्षेत्र में प्रमुख अनुसंधान अंतराल को भरना है, अर्थात, बायोइनोकुलेंट्स की प्रभावकारिता और जोखिम का आकलन, कजानस कजान के प्रकंद में उनकी दृढ़ता के अलावा, प्रभावकारिता मूल्यांकन में संयंत्र विकास वृद्धि कजानस कजान के लिए रणनीतिक रूप से एक जीवाणु कंसोर्टियम डिजाइन करना शामिल था। जोखिम मूल्यांकन निवासी जीवाणु समुदाय संरचना और कार्य पर इन बायोइनोकुलेंट्स के गैर-लक्ष्य प्रभावों के विश्लेषण पर ध्यान केंद्रित किया। दोनों निवासी और सक्रिय जीवाणु समुदाय संरचना और कार्य (रोगाणुओं के विभिन्न चरणों में भाग लेने वाले) पर कंसोर्टियम (एज़ोटोबैक्टर क्रोकुकोम, बैसिलस मेगाटेरियम और स्यूडोमोनास फ्लोरेसेंस) के परस्पर क्रिया का अध्ययन करने के लिए खेती-निर्भर और खेती-स्वतंत्र दोनों तरीकों को नियोजित करके एक पॉलीपसिक दृष्टिकोण अपनाया गया था। नाइट्रोजन चक्र), पौधों की वृद्धि विशेषताओं पर उनके प्रभाव के साथ। माइक्रोफ्लोरा के कृष्य अंश को अलग-अलग चयनात्मक मीडिया पर एन्यूमरेट किया गया था, जबकि कुल बैक्टीरियल समुदाय को समुदाय-स्तरीय शारीरिक रूपरेखा (सीएलपीपी) द्वारा लक्षित किया गया था, जो कि ढाल जेल वैद्युतकणसंचलन (डीजीजीई), मात्रात्मक

पोलीमरेज़ चेन रिएक्शन (क्यूपीसीआर) और अगली पीढ़ी के अनुक्रमण (एनजीएस) को दर्शाता है।)। 16S rRNA मार्कर का उपयोग डीजीजीई और एनजीएस के लिए किया गया था, जबकि क्यूपीसीआर के लिए 16S rRNA और नाइट्रोजन चक्र (*nif*H, *amo*A, *nir*K और *nar*G) में शामिल आणविक मार्करों का उपयोग किया गया था। अनुशंसित खुराक पर रासायनिक उर्वरकों द्वारा लगाए गए प्रभाव के साथ इन प्रभावों की तुलना भी की गई थी। अनाज की पैदावार पर कोई समझौता किए बिना इन जैव सूचनाओं द्वारा प्रतिस्थापित किए जा सकने वाले रासायनिक उर्वरकों की मात्रा का भी आकलन किया गया। अंत में, उनका प्रभाव मानव रोगज़नक़ लिस्टेरिया मोनोसाइटोजेन्स की उत्तरजीविता पर देखा गया। लिस्टेरिया मोनोसाइटोजेन्स के निषेध के लिए जिम्मेदार जैव सक्रिय यौगिकों की पहचान की गई थी।

काम के वर्तमान टुकड़े से यह निष्कर्ष निकाला गया कि जैव विकासकों ने रासायनिक उर्वरकों के साथ खेत के विकास के मानकों और क्षेत्र स्तर पर अनाज की उपज के मामले में अच्छी तरह से प्रतिस्पर्धा की। बायोइनोकुलेंट्स पर नज़र रखने पर, उन्हें केवल फूलों के चरण तक मिट्टी में बने रहने के लिए मनाया गया, हालांकि उनके प्रभाव पौधे की फसल की अवस्था तक स्पष्ट थे। कंसोर्टियम का सकारात्मक गैर-लक्षित प्रभाव लाभप्रद राइज़ोस्फेरिक बैक्टीरिया की आबादी, अर्थात पर दिखाई दे रहा था। नाइट्रोजन फिक्सर, नाइट्रिफ़ियर्स, फॉस्फेट सॉल्युबलाइज़र आदि, उन्हें बड़े स्तर पर फील्ड अनुप्रयोग के लिए "सुरक्षित" प्रदान करते हैं। कंसोर्टियम ने मानव रोगज़नक़ लिस्टेरिया मोनोसाइटोजेन्स की उत्तरजीविता को नकारात्मक रूप से प्रभावित किया। मानव रोगज़नक़ की उत्तरजीविता पर उनका नकारात्मक प्रभाव न केवल स्वास्थ्य पर बल्कि मानव पर लाभ के लिए इन कृषि संशोधनों के आवेदन के क्षितिज को व्यापक बनाता है।

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ABBREVIATIONS

Molecules

N_2	Dinitrogen gas
NH_3	Ammonia
NH_4^+	Ammonium
NO_2^-	Nitrite
NO_3^-	Nitrate

Functional genes catalyzing processes in the microbial nitrogen cycle

*amo*A	Gene encoding the α-subunit of the ammonia monooxygenase
*nar*G	Gene encoding the membrane-bound nitrate reductase
*nif*H	Gene encoding the nitrogenase reductase
*nir*K	Gene encoding the copper-containing nitrite reductase
*nir*S	Gene encoding the cytochrome *cd*1 nitrite reductase
Bacterial *amoA*	Gene encoding subunit A of ammonia monooxygenase in bacteria

Elements

N	Nitrogen
P	Phosphorus
K	Potassium
Fe	Iron
Mn	Manganese
Zn	Zinc
Cu	Copper

Prokaryotes involved in the nitrogen turnover processes

AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria

Important terminology

ANOVA	Analysis of variance
ARDRA	Amplified ribosomal DNA restriction analysis

ARISA	Automated Ribosomal Intergenic Spacer Analysis
bp	Base pair
cDNA	Complementary DNA
cfu	Colony forming unit
CLPP	Community-level Physiological Profiling
CRD	Completely randomized design
Ct	Cycle threshold
DAS	Days after sowing
DEPC	Diethyl pyrocarbonate
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
EC	Electrical conductivity
EDTA	Ethylene diamine tetra acetic acid
g	Gram
h	Hour
ha	Hectare
HCN	Hydrogen cyanide
IAA	Indole acetic acid
kg	Kilogram
LB	Luria Bertani
^{o}C	Degree centigrade
min	Minutes
MTCC	Microbial type culture collection
NTC	No template control
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGP	Plant growth promoting
PGPR	Plant growth promoting rhizobacteria
PyNAST	Python Nearest Alignment Space Termination
QIIME	Quantitative insight into microbial ecology
qPCR	Real-time quantitative PCR
r	Correlation coefficient
RAPD	Random Amplified Polymorphic DNA
RBD	Randomized block design
RDF	Recommended dose of fertilizer
RNA	Ribonucleic acid

rpm	Rotations per minute
RT-PCR	Reverse transcription PCR
s	Second
SSCP	Single strand conformation polymorphism
TGY	Tryptone glucose yeast extract
TLC	Thin layer chromatography
T-RFLP	Terminal restriction fragment length polymorphism
TSA	Tryptone soy agar
UPGMA	Unweighted pair group method with arithmetic mean
UV	Ultravoilet
Viz.	Videlicet
VSEARCH	Vectorized search
α	Alpha
β	Beta
γ	Gamma
δ	Delta
μ	Micro