ENHANCEMENT OF GROWTH ATTRIBUTES OF *CAJANUS CAJAN* BY THE SYNERGISTIC ACTION OF BIOINOCULANTS

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Enhancement of growth attributes of *Cajanus cajan* by the synergistic action of bioinoculants

by

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ALWAYS GRATEFUL TO
THE SUPREME SOUL
AND MY PARENTS

Certificate

This is to certify that the thesis entitled" **Enhancement of growth attributes of *Cajanus cajan* by the synergistic action of bioinoculants**" being submitted by **Priyanka** to the Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, for the award of the degree of **Doctor of Philosophy** is the record of the bona-fide research work carried out by her under my supervision. In my opinion, the thesis has reached the standards fulfilling the requirements of the regulations relating to the degree. The results contained in this thesis have not been submitted either in part or in full to any other university or institute for the award of any degree or diploma.

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Abstract

The swift pace of urbanization and industrialization has subjected the ecosystem to harmful consequences encompassing the degradation of water, soil, air, and biodiversity. To address this issue, a greater emphasis has been placed on sustainable agriculture. The application of bioinoculants is one of the various methods employed for sustainable agriculture, owing to their environment-friendly characteristics. However, despite the numerous advantages associated with the use of plant growth promoting rhizobacteria (PGPR) as bioinoculants, there are limitations like reduced efficacy and persistence under field conditions. This is due to limited knowledge of the interactions of these microorganisms as members of the consortium, and with the native microflora, especially under environmental conditions.

The present study focused on the characterization of three bacterial strains (*Azotobacter chroococcum*, *Priestia megaterium,* and *Pseudomonas* sp. SK3) for their plant growth promoting (PGP) properties, individually and as multiple inoculants (dual and triple). This study compared their efficacy on fitness of *Cajanus cajan* (pigeonpea) under controlled and natural conditions. After establishing the synergistic impact of these bacterial strains on plant growth, the mechanism of synergism was studied by assessing the metabolome of the bacterial strains (individually and in different combinations) using High Resolution Liquid Chromatograph Mass Spectrometer, and quantitative estimation of PGP attributes of bioinoculants determined.

Spontaneous mutants of the three bacterial strains comprising the consortium were generated through antibiotic-based screening. Root colonization by the bacterial strains was assessed during plant growth in Hoagland medium. To enhance the shelf life of bioinoculants, solid and liquid bioformulations were developed after carrying out optimization studies for different additives, using Response Surface Methodology. Based on the better performance of liquid bioformulation, a pot experiment was conducted to study the impact of the formulation

on plant growth, soil nutrient status, the resident bacterial community diversity, and markers of the phosphorus cycle (*phoD* and *pqqC*).

The present work concluded that the strategic development of a consortium with three bioinoculant strains led to the synergistic enhancement of growth attributes in *C. cajan*. The mechanism of synergism could be delineated by identification of metabolites such as 6,6-dimethoxy-2,5,5-trimethyl-2-hexene, N6-hydroxy-L-lysine, N-methyltryptamine, 2,2-dimethyl-3,4-bis(4-methoxyphenyl)-2H-1-benzopyran-7-ol acetate, L-furosine and antipyrine, which were uniquely produced in triple inoculant culture, and exhibited successful competence in the rhizosphere. An efficient method for simultaneous monitoring of bacterial bioinoculant strains was devised to track their persistence during plant growth. The developed bioformulation exhibited a shelf life of six months and was found to be more stable at lower temperatures. The consortium also exhibited significant impacts on soil health by positively enhancing the soil nutrient status and bacterial diversity.

The study established a triple-membered bacterial consortium for enhancing growth and yield attributes of an economically important crop, *Cajanus cajan*. It reflected upon the plausible mechanism of synergism between bioinoculant strains used as a consortium. Further, to take the consortium into the application, stable bioformulations were developed. Finally, the positive impact of the application of the bioformulation was assessed in a holistic manner on plant and soil health. It, therefore, puts forth the systematic and sequential process of development of a robust and efficient bioformulation for ushering in agricultural sustainability.

सार

शहरीकरण और औद्योगीकरण की तेज गति से पारिस्थितिकी तंत्र (ecosystem) को हानिकारक परिणामों का सामना करना पड़ा है, जिसमें पानी, मिट्टी, हवा और जैव विविधता का क्षरण शामिल है। इस मुद्दे के समाधान के लिए टिकाऊ कृषि (sustainable agriculture) पर अधिक जोर दिया गया है। बायोइनोकुलेंट्स (bioinoculants) का अनुप्रयोग उनकी पर्यावरण अनुकूल विशेषताओं के कारण टिकाऊ कृषि के लिए नियोजित विभिन्न तरीकों में से एक है। हालाँकि, बायोइनोकुलेंट्स (bioinoculants) के रूप में पौधों की वृद्धि को बढ़ावा देने वाले राइजोबैक्टीरिया (rhizobacteria) (पीजीपीआर, PGPR) के उपयोग से जुड़े कई फायदों के बावजूद, क्षेत्र की स्थितियों के तहत कम प्रभावकारिता और अटलता जैसी सीमाएं हैं। इसका कारण यह है की वास्तबिक समय की पर्यावरणीय परिस्थिति में संघ के सूक्ष्मजीवियों आपसी और निवासी जीवाणु के साथ उनके इंटरेक्शन के बारे में जानकारी सीमित है

वर्तमान अध्ययन में पौधों के विकास को बढ़ावा देने वाले (पीजीपी, PGP) गुणों के लिए तीन जीवाणु उपभेदों एज़ोटोबैक्टर क्रोकोकम (*Azotobacter chroococcum*) , प्रीस्टिया मेगाटेरियम (*Priestia megaterium*) और स्यूडोमोनास एसपी एसके 3) (*Pseudomonas* sp. SK3) के लक्षण वर्णन पर ध्यान केंद्रित किया गया है, व्यक्तिगत रूप से और विभिन्न संयोजनों (दो- सदस्यीय और त्रि-सदस्यीय) के रूप में। इस अध्ययन में नियंत्रित और प्राकृतिक परिस्थितियों में कैजानस कैजन (*Cajanus cajan*) की फिटनेस पर उनकी प्रभावकारिता की तुलना की गई। पौधों की वृद्धि पर इन जीवाणु उपभेदों के सहक्रियात्मक प्रभाव को स्थापित करने के बाद, उच्च रिज़ॉल्यूशन तरल क्रोमैटोग्राफ मास स्पेक्ट्रोमीटर (HRLCMS) का उपयोग करके जीवाणु उपभेदों (व्यक्तिगत रूप से और विभिन्न संयोजनों में) के चयापचय (metabolome) का आकलन करके सहक्रिया (synergism) के तंत्र का अध्ययन किया गया, और बायोइनोकुलेंट्स (bioinoculants) के पीजीपी (PGP) गुणों को मात्रात्मक रूप से निर्धारित किया गया।

कंसोर्टियम (consortium) में शामिल तीन जीवाणु उपभेदों के सहज उत्परिवर्ती एंटीबायोटिक (antibiotic)-आधारित स्क्रीनिंग (screening) के माध्यम से उत्पन्न हुए थे। होगलैंड (Hoagland) माध्यम में पौधों की वृद्धि के

दौरान जीवाणु उपभेदों द्वारा जड़ उपनिवेशण का मूल्यांकन किया गया था। बायोइनोकुलेंट्स (bioinoculants) के शेल्फ जीवन (shelf life) को बढ़ाने के लिए, रिस्पांस सरफेस मेथडोलॉजी (response surface methodology) का उपयोग करके विभिन्न योगज (additives) के लिए अनुकूलन अध्ययन करने के बाद ठोस और तरल बायोफॉर्म्यूलेशन (bioformulation) विकसित किए गए थे। तरल बायोफॉर्म्यूलेशन (bioformulation) के बेहतर प्रदर्शन के आधार पर, पौधों की वृद्धि, मिट्टी के पोषक तत्व की स्थिति, निवासी जीवाणु समुदाय विविधता और फॉस्फोरस चक्र (phosphorus cycle) (*phoD* और *pqqC*) के मार्करों पर फॉर्मूलेशन (formulation) के प्रभाव का अध्ययन करने के लिए एक पॉट (pot) प्रयोग किया गया था।

वर्तमान कार्य ने निष्कर्ष निकाला कि तीन बायोइनोकुलेंट (bioinoculant) उपभेदों के साथ एक संघ के रणनीतिक विकास से सी. कैजन (*C. cajan*) में विकास विशेषताओं में सहक्रियात्मक वृद्धि हुई। तालमेल के तंत्र को 6,6-डाइमेथॉक्सी-2,5,5-ट्राइमेथाइल-2-हेक्सिन (6,6-dimethoxy-2,5,5-trimethyl-2-hexene), एन 6-हाइड्रॉक्सी-एल-लाइसिन (N6-hydroxy-L-lysine), एन-मिथाइलट्रिप्टामाइन (N-methyltryptamine), 2,2-डाइमिथाइल-3,4-बीआईएस(4-मेथॉक्सीफेनिल)-2एच-1-बेंजोपाइरन-7-ओएल एसीटेट (2,2-dimethyl-3,4-bis(4-methoxyphenyl)-2H-1-benzopyran-7-ol acetate), एल-फ़्यूरोसिन (L-furosine), और एंटीपाइरिन (antipyrine) जैसे मेटाबोलाइट्स (metabolites) की पहचान करके चित्रित किया जा सकता है, जो त्रि-सदस्यीय संयोजन में विशिष्ट रूप से उत्पादित किए गए थे और राइजोस्फीयर (rhizosphere) में सफल क्षमता प्रदर्शित की थी। पौधों की वृद्धि के दौरान उनकी अटलता (persistence) को पता करने के लिए जीवाणु बायोइनोकुलेंट उपभेदों की एक साथ निगरानी के लिए एक कुशल विधि तैयार की गई थी। विकसित बायोफॉर्म्यूलेशन (bioformulation) ने छह महीने का शेल्फ (shelf) जीवन प्रदर्शित किया, और कम तापमान पर अधिक स्थिर पाया गया। संघ ने मिट्टी की पोषक स्थिति और जीवाणु विविधता को सकारात्मक रूप से बढ़ाकर मिट्टी के स्वास्थ्य पर महत्वपूर्ण प्रभाव भी प्रदर्शित किया है।

अध्ययन ने आर्थिक रूप से महत्वपूर्ण फसल कैजानस कैजन (*Cajanus cajan*) की वृद्धि और उपज विशेषताओं को बढ़ाने के लिए एक त्रि-सदस्यीय जीवाणु संघ की स्थापना की। यह एक कंसोर्टियम (consortium) के रूप में उपयोग किए जाने वाले बायोइनोकुलेंट (bioinoculant) उपभेदों के बीच तालमेल के प्रशंसनीय तंत्र पर प्रतिबिंबित

करता है। इसके अलावा, कंसोर्टियम (consortium) को अनुप्रयोग में शामिल करने के लिए, स्थायी बायोफ़ॉर्म्यूलेशन (bioformulation) विकसित किए गए। अंत में, पौधे और मिट्टी के स्वास्थ्य पर बायोफॉर्म्यूलेशन (bioformulation) के अनुप्रयोग के सकारात्मक प्रभाव का समग्र तरीके से मूल्यांकन किया गया। इसलिए, यह कृषि स्थिरता की शुरुआत के लिए एक मजबूत और कुशल बायोफॉर्म्यूलेशन (bioformulation) के विकास की व्यवस्थित और अनुक्रमिक प्रक्रिया को सामने रखता है।

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ABBREVIATIONS

Abbreviations/Formula	Chemicals, Molecules and Media
$(NH_4)SO_4$	Ammonium sulphate
ACC	1-aminocyclopropane-1-carboxyclic acid
AHL	N-acyl-homoserine lactone
$Ca_3(PO_4)_2$	Calcium phosphate
$CaCO_3$	Calcium carbonate
CTAB	Cetyl trimethyl ammonium bromide
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EPS	Exopolysaccharides
$FeCl_3$	Ferric chloride
HCN	Hydrogen cyanide
IAA	Indole acetic acid
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
LB	Luria Bertani
MDA	Malondialdehyde
$MgCl_2.6H_2O$	Magnesium dichloride hexahydrate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
NA	Nutrient agar
NaCl	Sodium chloride
NaClO	Sodium hypochlorite
Na_2CO_3	Sodium carbonate
NB	Nutrient broth
NBRIP	National Botanical Research Institute's phosphate
PBS	Phosphate buffer saline
SOD	Superoxide dismutase
TBA	Tributyrin agar

Important terminology

ANOVA	Analysis of variance
APCI	Atmospheric Pressure Chemical Ionization
BNF	Biological nitrogen fixation
CAS	Chrome azurol sulfonate
CCD	Central Composite Design
CI	Chloroform: isoamyl alcohol
CMC	Carboxymethylcellulose
DAS	Days after sowing
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
Dual AJS ESI	Dual Agilent Jet Stream Electrospray Ionization
EC	Electrical conductivity
ESI	Electrospray Ionization
FISH	Fluorescent *in situ* hybridization
GDP	Gross domestic product
HRLCMS	High resolution Liquid Chromatograph mass spectrometer
IARI	Indian Agricultural Research Institute
ICAR	Indian Council of Agricultural Research

IPS	Inorganic phosphorus solubilization
ITS	Internal transcribed spacer
MCT	Microcentrifuge tubes
MS	Mass spectrometry
MSSHPA	Multiple-strain soil health-promoting agents
NMR	Nuclear magnetic resonance
OD	Optical density
OFAT	One factor at a time
OPM	Organic phosphorus mineralization
PAL	Phenylalanine ammonia-lyase
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGPM	Plant growth promoting microorganisms
PGPR	Plant growth promoting rhizobacteria
PO	Plant peroxidase
phoD	Gene encoding alkaline phosphatase
pqqC	Gene encoding quinoprotein glucose dehydrogenase
PSB	Phosphate solubilizing bacteria
PVP	Polyvinyl pyrrolidone
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
RFLP	Restriction fragment length polymorphism
RO	Reverse osmosis
rRNA	Ribosomal ribonucleic acid
RSM	Response Surface Methodology
SA	Sodium alginate
SCAR	Sequence-characterized amplified region
SEM	Scanning Electron Microscopy
SHPA	Soil health-promoting agent
TRFLP	Terminal restriction fragment length polymorphism
α	Alpha
β	Beta

Element

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

Units

%	Percent
°C	Degree Celsius
µg	Micrograms
µL	Microliter
amu	Atomic mass unit
CFU	Colony forming units
cm	Centimeter

g	Grams
h	Hour
Kg	Kilogram
L	Liter
min	Minutes
mL	Milliliter
mM	Millimolar
nm	Nanometer
rpm	Revolution per minute
v/v	Volume by volume