POLYPHASIC APPROACH FOR ASSAYING THE ROLES OF BIOCONTROL AGENTS AGAINST *FUSARIUM* WILT IN *SOLANUM LYCOPERSICUM*

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by

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Dedicated

To

The Almighty

&

My family

CERTIFICATE

This is to certify that the thesis entitled **"Polyphasic approach for assaying the roles of biocontrol agents against *Fusarium* wilt in *Solanum lycopersicum*"** submitted by **Ms. Monika Jangir** has been prepared under our guidance with the rules and regulations of Indian Institute of Technology Delhi India. The results presented in this thesis have not been submitted for any degree or diploma in any other institute or university.

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Monika Jangir

ABSTRACT

Globally tomato (*Solanum lycopersicum*), belonging to the family Solanaceae, is the second important vegetable crop after potato. *Fusarium* wilt is one of the most devastating soil-borne diseases affecting tomato crop and is responsible for major economical losses in its yield. To obtain an environment friendly alternative for wilt disease control in tomato, utilizing the potential of biocontrol agents and oil seed cakes to mitigate *Fusarium* wilt control has been explored in the present study. In an attempt to develop a method of biological control against *Fusarium oxysporum* f. sp. *lycopersici*, efficacy of *Bacillus subtilis* and *Trichoderma harzianum* was evaluated in wilt control. In addition, present work comprised an extensive study to understand the biocontrol mechanisms of both antagonistic microorganisms and their non-target effects on soil bacterial community.

Dual culture assay revealed that *B. subtilis* (MTCC 2274) and *T. harzianum* (MTCC 3928) showed highest inhibition against fungal pathogenic mycelia. Additionally, indigenous fungal (3T) and bacterial (B44) strains were isolated from rhizospheric soil of tomato exhibiting antagonistic efficacy against pathogen. Further, characterization of isolated and procured strains was done for their antifungal and plant growth promoting properties. After ribotyping, it was found that 3T showed similarity with *Aspergillus flavus* and B44 with *Bacillus* sp. As the fungal isolate was identified as a plant pathogen it was not considered further for *in planta* assay whereas the sequence of bacterial isolate (B44) was submitted to NCBI database (accession number: MG779639). Disease control efficacy of all strains was tested in *in planta* assay; it was observed that dual inoculation of *B. subtilis* and *T. harzianum*

exhibited higher efficacy with disease reduction of 56% as compared to chemical treatment (48.7%). The dual inoculation evidently enhanced the plant growth parameters. Furthermore, non-target effect of biocontrol agents on soil bacterial microbiome was assessed using denaturing gradient get electrophoresis (DGGE). DGGE fingerprints revealed positive shifts in bacterial community of rhizospheric soil upon treatment with dual inoculation of *B. subtilis* and *T. harzianum* as compared to untreated and uninoculated control. Also, several ecologically important species were identified in the rhizospheric soil treated with dual inoculation.

In the present study, an extensive study was carried out for the identification of antifungal metabolites produced by antagonistic strains. It was obsered that both biocontrol agents were capable of producing hydrolytic enzymes, volatile and non-volatile bioactive compounds. For *B. subtilis* activity of β-1,3-glucanase was found maximum (12.69 U ml^{-1} min^{-1}) on 7^{th} day whereas for *T. harzianum* it was highest (21.47 U ml^{-1} $min^{-1)}$ at 5^{th} day of incubation. Protease production was maximal at 7^{th} day of incubation (929 and 846 U ml^{-1} min^{-1}) for *B. subtilis* and *T. harzianum*, respectively. For *B. subtilis*, highest value of chitinase activity (33.69 U ml^{-1} min^{-1}) was observed after 2 days of incubation. However, in case of *T. harzianum* highest activity (154.23 U ml^{-1} min^{-1}) was observed at day 5. In *in vitro* volatile assay, an inhibition of 61.7% and 47.2% against fungal pathogen was observed with *B. subtilis* and *T. harzianum* whereas culture filtrates (40%) exhibited 58.51 and 44.66% inhibition against pathogen, respectively. The volatile compounds were extracted with solid phase microextraction (SPME) and identified with gas chromatography-mass spectrophotometry (GC-MS). However, antifungal bioactive fractions of non-volatile compound were screened using direct bioautography and further UPLC-MS identified morpholine, 4-tridecanoyl and N~2~,N~4~-Bis[3-(piperidin-1-yl)propyl]-1,3,5-

triazine-2,4,6-triamine as one of the major compounds for *T. harzianum* and *B. subtilis*, respectively.

Different formulations viz., talc formulation, alginate beads and water dispersible granules (WDG) were developed for biocontrol agents. In *in planta* study, it was notable that WDG was the most efficient in disease reduction followed by talc formulation and alginate beads. Also, utilization of oil seed cakes (OSCs) was taken into consideration for disease control. Among all OSCs, mustard cake showed highest inhibition (36.8%) of fungal mycelial growth with 10% aqueous extract. Then, mustard cake was tested as a growth substrate for biocontrol agents, and evaluated its efficacy in *in planta* assay. In pot study, mustard cake formulation showed 48% disease reduction after 6 months of growth, with increased fruit yield, fresh weight, shoot length and root length over untreated control.

The findings of the present study identified a sustainable and environment friendly alternative for control of *Fusarium* wilt. The application of biocontrol agent with organic amendment could aid in mitigating disease and enhancement of plant health. Dual inoculation of biocontrol agents, potential source of bioactive volatile and non-volatile metabolites, exhibited promising effect on disease suppression and plant growth promotion. In addition, the treatment showed non-target effect by exerting an enhancement on the abundance of bacteria in rhizosphere of host plant over untreated control as compared to single inoculation. In addition, dual inoculation exhibited the presence of some species of ecological importance and reduced the deteriorating effect of fungal pathogen on bacterial community.

सार

विश्व स्तर पर टमाटर (सोलेनम लाइकोपर्सिकम), सोलनैसे परिवार से संबंधित, आलू के बाद दूसरी महत्वपूर्ण सब्जी फसल है। टमाटर की फसल को प्रभावित करने वाली सबसे अधिक विनाशकारी मृदा जनित बीमारियों में से एक है विल्ट और इसकी पैदावार में बड़े आर्थिक नुकसान के लिए जिम्मेदार है। टमाटर में इस बिमारी के नियंत्रण के लिए पर्यावरण के अनुकुल विकल्प प्राप्त करने के लिए, वर्तमान अध्ययन में बायोकन्ट्रोल एजेंटों और तेल बीज केक की क्षमता का उपयोग किया गया है। ऑक्सीस्पोरम एफ०एसपी० लाईकोपर्सिकी के खिलाफ जैविक नियंत्रण की एक विधि विकसित करने के प्रयास में, बेसिलस सबटिलिस और ट्राइकोडर्मा हर्जियानम का मूल्याकन विल्ट कंट्रोल में किया गया है। इसके अलावा, वर्तमान काम में दोनों विरोधी सूक्ष्मजीवों के जैव तंत्र और मिट्टी जीवाणू समुदाय पर उनके गैर–लक्ष्य प्रभावों को समझने के लिए एक व्यापक अध्ययन शामिल है।

डूयल कलचर परख से पता चला कि बी० सबटिलिस (एम०टी०सी०सी० 2274) एवं टी० हर्जियानम (एम०टी०सी०सी० 3928) ने फंगल रोगजनक मायसेलिया के खिलाफ उच्चतम अवरोध दिखाया। इसके अतिरिक्त, इनडीजिनस फंगल (3T) और बैक्टीरियल (B44) उपभेदों को टमाटर की राइजोस्फेरिक मिट्टी से अलग किया गया था जो रोगजनक के खिलाफ विरोधी प्रभाव दिखा रहा था। इसके अलावा, पृथक और खरीदे गए उपभेदों के लक्षणों का वर्णन उनके एंटिफंगल और पौधो के विकास को बढ़ावा देने वाले गुणों के लिए किया गया था। राइबोटाइपिंग के बाद, यह पाया गया कि 3T ने एस्परजिलस फलेवस और B44 ने बेसिलस के साथ समानता दिखाई। चूकि फंगल उपभेद (3T) को पौधो के लिए रोगजनक के रूप में पहचाना गया है, इसलिए इसे अध्ययन में आगे नही लिया गया, जबकि बैक्टीरियल (B44) आइसोलेट को अनुक्रम NCBI डेटाबेस (परिग्रहण संख्याः MG 779639) में जमा किया गया है।

पॉट अध्ययन में सभी उपभेंदों के रोग नियंत्रण प्रभावकारित का परीक्षण किया गया, यह देखा गया कि बी० सबटिलिस एवं टी० हर्जियानम के डुयल

इनोक्यूलेशन ने रासायनिक उपचार (48.7%) की तुलना में 56% बीमारी की रोक के साथ उच्च प्रभावकारित का प्रदर्शन किया। डुयल इनोक्यूलेशन ने पौधे के विकास के मापदंडो को स्पष्ट रूप से बढ़ाया। इसके अलावा, मृदा जीवाणु माइक्रोबायोम पर बायेकन्ट्रोल एजेंटों के गैर – लक्षित प्रभाव का मूल्यांकन डी.जी.जी.ई. का उपयोग करके किया गया था।

डी.जी.जी.ई. फिंगर प्रिटं ने बी0 सबटिलिस और टी0 हर्जियानम के दोहरे इनोक्यूलेशन के साथ उपचार पर राइजोस्फेरिक मिट्टी के जीवाणु समुदाय में सकारात्मक बदलावों का पता लगाया। इसके अलावा, कोई पारिस्थितिक रूप से महत्वपूर्ण प्रजातियों की पहचान दोहरे इनोक्यूलेशन की मिट्टी में की गई थी।

वर्तमान अध्ययन में, प्रतिपक्षी जीवाणु द्वारा उत्पादित एंटिफगल मेटाबोलाइट्स की पहचान के लिए एक व्यापक अध्ययन किया गया था। यह पाया गया था कि दोनो बायोकन्ट्रोल एजेंट हाइड्रोलिटिक एंजाइमों, वाष्पशील और गैर–वाष्पशील अनाकर यौगिको का उत्पादन करेन में सक्षम थें। बी. सबटिलिस के लिए बीटा–1, 3–गलूकेनेस की गतिविधि 7 वें दिन अधिकतम ($12.64\ Uml^{-1}\ min^{-1}$) पाई गई जबकि टी. हर्जियानम के लिए 5 वें दिन ($21.74\ Uml^{-1}\ min^{-1}$) उच्चतम थी। बी. सबटिलिस और टी. हर्जियानम के लिए प्रोटीज का उत्पादन अधिकतम 926 एवं 846 $Uml^{-1}\ min^{-1}$ था।

फाईटिनेस उत्पादन बी0 सबटिलिस के लिए, इंक्योबेशन के 2 दिनों बाद देखा गया था ($33.69\ Uml^{-1}\ min^{-1}$)। हालांकि, टी0 हर्जियानम के लिए उच्चतम गतिविधि 5 दिन के बाद देखी गई ($154.23\ Uml^{-1}\ min^{-1}$)। इन विट्रो वाष्शील तकनीक में, बी0 सबटिलिस और टी0 हर्जियानम के साथ 61.7% 47.2% निषेध देखा गया, जबकि कल्चर फिलट्रेटस (40%) ने 58.51 और 44.6% रोगजनक के खिलाफ क्रमश निषेध दिखाया। वाष्पशील यौगिकों को एस.पी.एम.ई. के साथ निकाला गया और गौर क्रोमैट्रोग्राफी–मास स्पेक्ट्रोफोटोमेट्री (जी सी–एमएस) के साथ पहचाना

गया। हालांकि गैर–वाष्पशील यौगिक के ऐंटिफंगल अंशो को डाईरेक्ट बायोआटोग्राफी और आगे यू.पी.एल.सी.–एम.एस. द्वारा जांचा गया।

बायोकंट्रोल एजेंटो के लिए अलग–अलग फॉर्मुलेशन जैसे टॉल्क फॉर्मुलेशन, एल्जिनेट बीड्स और वाटर डिस्पर्सिवल गैन्यूल्स बनाए गए थे। पॉट अध्ययन में, यह उल्लेखनीय था, कि वाटर डिस्पर्सिबल गैन्यूल्स रोग के रोकथाम में सबसे कुशल था, इसके बाद टॉल्क फॉर्मुलेशन और एल्जिनेट बीड्स थे। इसके अलावा रोग नियंत्रण के लिए ऑयल सीड केक (ओ.एस.सी.) के उपयोग को ध्यान में रखा गया। सभी ओएससी के बीच, सरसों के केक में उच्चतम निषेध (36.8%) देखा गया। फिर, सरसों के केक को बायोकन्ट्रोल एजेटो के लिए एक विकास सब्सट्रेट के रूप में परीक्षण किया गया था, और पॉट अध्ययन में इसकी प्रभावकारिता का मूल्यांकन किया। पॉट अध्ययन में, सरसों के केक उपचार ने 48% रोग में कमी दिखाई, जिसमें फल की पैदावार, शूट की लंबाई और वजन अनुपचारित नियंत्रण में ज्यादा था।

वर्तमान अध्ययन के निष्कर्षो ने फ्यूजेरियम विल्ट के नियंत्रण के लिए एक स्थायी और प्र्यावरण के अनुकूल विकल्प की पहचान की। जैविक संशोधन के साथ बायोकंट्रोल एजेंट के आवेदन से रोग को कम करने ओर पौधो के स्वास्थ्य को बढ़ाने में मदद मिल सकती है। बायोकंट्रोल एजेंटो के दोहरे इनोक्यूलेशन, बायोएक्टिव वाष्पशील और गैर– वाष्पशील सेटाबोलाइट्स के संभावित स्रोत, रोग दमन और पौधे के विकास को बढ़ावा देने वाले प्रभाव का प्रदर्शन करते है। इसके अलावा, एकल इनोक्यूलेशन की तुलना में दोहरे इनोक्यूलेशन में राइजोस्फेरिक जीवाणुओं की प्रचुरता को बढ़ाकर गैर–लक्ष्य प्रभाव दिखाया। इसके अलावा दोहरे इनोक्यूलेशन में पारिस्थतिक महत्व की कुछ प्रजातियों की उपस्थित भी पाई गई और बैक्टीरिया समुदाय पर फगंल रोगजनक के बिगड़ते प्रभाव को भी कम किया।

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ABBREVIATIONS

°C	Degree centigrade
°E	Degree east
°N	Degree north
6-PP	6-pentyl-2H-pyran-2-one
ACC	1-aminocyclopropane-1-carboxylate
AI	Active ingredient
ANNOVA	Analysis Of Variance
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CCD	Central composite design
CFU	Colony forming unit
$CHCl_3$	Chloroform
CMC	Carboxymethylcellulose
DAI	Days after incubation
DAS	Days after sowing
df	Degree of freedom
DGGE	Denaturing gradient gel electrophoresis
DMRT	Duncan's new multiple range test
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
DSI	Disease severity index
EC	Electrical conductivity
Fig.	Figure
g	Gram
GC	Guanine-cytosine
GC	Gas chromatography
GP	Germination percentage
h	Hour
H	Shannon-index
HCN	Hydrogen cyanide
HPLC	High performance liquid chromatography
IAA	Indole acetic acid
ITCC	Indian Type Culture Collection
l	Litre
LB	Luria-Bertani broth
M	Molar
m/z	Mass/charge

mg	Milligram
min	Minutes
ml	Milliletre
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
MS	Mass spectrophotometry
MTCC	Microbial Type Culture Collection
MTT	3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
N	Normal
NA	Nutrient agar
NAG	N-acetyl glucosamine
NCBI	National Center for Biotechnology Information
nm	Nanometre
OCSs	Oil seed cakes
OD	Optical density
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PE	Petroleum ether
PGP	Plant growth promoting
PGPR	Plant growth promoting rhizobacteria
pmole	Picomoles
PO_4	Phosphate
ppm	Parts per million
psi	Pound-force per square inch
qpcr	Quantitative-polymerase chain reaction
Rf	Retention factor
rpm	Revolutions per minute
RSM	Response surface methodology
s	Seconds
S	Richness
SD	Standard deviation
SEM	Scanning electron microscope
SPME	Solid phase microextraction
SSF	Solid state fermentation
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TE	Tris- ethylenediaminetetraacetic acid
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TSA	Tryptic soy sgar
U ml^{-1} min^{-1}	Units of activity per ml per minute

UPGMA	Unweighted pair group method with arithmetic mean
UPLC	Ultra performance liquid chromatography
USD	United states dollars
UV-VIS	Ultraviolet–visible
v/v	Volume/volume
VOCs	Volatile organic compounds
w/v	Weight/volume
WDG	Water dispersible granules
Zn	Zinc
µl	Microlitre
µS/cm	Microsiemens per centimetre