

**DEVELOPMENT OF BOTANICAL
FORMULATIONS FOR MANAGING FUNGAL
PHYTOPATHOGENS IN *CAPSICUM ANNUUM* L.**

HIMANSHU ARORA



**CENTRE FOR RURAL DEVELOPMENT AND TECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY DELHI**

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CAPSICUM ANNUUM L.**

by

HIMANSHU ARORA

Centre for Rural Development and Technology

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*“Dedicated to all those
who believed in me.”*

CERTIFICATE

This is to certify that the thesis entitled "**Development of botanical formulations for managing fungal phytopathogens in *Capsicum annum* L.**", being submitted by **Mr. Himanshu Arora** to the **Indian Institute of Technology Delhi** for the award of "**Doctor of Philosophy**" is a record of bonafide research work carried out by him. He has worked under our guidance and supervision and has fulfilled the requirements for the submission of this thesis. To the best of our knowledge, the results contained in this thesis have not been submitted in part or full to any other university or institute for the award of any degree or diploma.



Dr. Satyawati Sharma
Professor

Centre for Rural
Development and
Technology

Indian Institute of
Technology Delhi

New Delhi – 110016



Dr. Abhishek Sharma
Assistant Professor

Amity Food and Agriculture
Foundation

Amity University, Noida

Uttar Pradesh- 201313

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Himanshu Arora

ABSTRACT

Capsicum annuum L. is a significant horticulture crop known for its pungent varieties and use as a spice. The pungent character in the plant, known as capsaicinoid, has been discovered to have various health benefits. However, its production has been affected due to various biotic factors, including fungal diseases such as anthracnose, vascular wilt, and damping-off caused by *Colletotrichum capsici*, *Fusarium aphanidermatum*, and *Pythium aphanidermatum*, respectively. In contrast to chemical fungicides, which often come with drawbacks such as environmental concerns and the potential for resistance development, various plant extracts and essential oils are gaining significant recognition as plant protectants due to their inherent antifungal effectiveness and environmentally friendly attributes. Contemplating these findings, the present research entitled "*Development of botanical formulations for managing fungal phytopathogens in Capsicum annuum L.*" primarily focuses on developing multi-targeted fungicidal nanoformulations employing plant extracts and essential oils. This comprehensive study encompasses various stages, starting with the assessment of numerous plant extracts and essential oils against the targeted phytopathogens. Subsequently, it involves the characterization of the bioactive components within the selected botanicals, an *in-silico* evaluation of these identified compounds against the phytopathogens, exploration of synergistic antifungal interactions among these botanicals, formulation development, and culmination in a thorough *in-planta* assessment of the developed formulation.

The study commenced by evaluating the *in-vitro* antifungal activity of several plant extracts and essential oils against the targeted phytopathogens. Within this investigation, licorice (*Glycyrrhiza glabra*) cold water extract (LAE), and thyme (*Thymus vulgaris*) essential oil (TO) emerged as remarkably potent botanicals against the *C. annuum* pathogens. LAE at 200 mg ml⁻¹ demonstrated the maximum antifungal activity of 89.9% against *P. aphanidermatum*, whereas TO at 0.25 mg ml⁻¹ showed 100% inhibition of *C. capsici*. Enhanced cellular

component leakage observed in response to TO and LAE treatments provided clear evidence of damage to the fungal cell wall and plasma membrane. This phenomenon can be attributed to the TO lipophilicity and triterpenoid saponins of LAE. Furthermore, TO and LAE treatments also led to a decrease in ergosterol biosynthesis.

Metabolite profiling using gas chromatography-mass spectrometry and high resolution-liquid chromatography-mass spectrophotometry analysis showed the presence of several bioactive compounds. The co-application of TO and LAE, at notably lower concentrations of 100 mg ml⁻¹ LAE and 0.125 mg ml⁻¹ TO, demonstrated a remarkable synergistic effect in controlling fungal pathogens. The combined application also resulted in more significant cellular component leakage and a more substantial reduction in ergosterol biosynthesis compared to TO or LAE applied alone. Additionally, the research extended its focus to the identification of the key bioactive molecules within the thyme oil and licorice water extract that act to inhibit the Cox1 enzymes of the phytopathogens, employing the *in-silico* approach. From a wide range of bioactive molecules screened, the molecular docking indicated trans-carveol, carvacrol, kaempferol 3-rhamnoside 7-xyloside, kaempferitrin, and astragalol 7-rhamnoside as the potential inhibitors for Cox1 of *C. capsici*, β -Caryophyllene, Caryophyllene acetate, hispaglabridin A, kaempferol 3-rhamnoside 7-xyloside and licorice glycoside A for Cox1 of *F. oxysporum* and (+)-Longifolen, Caryophyllene acetate, Hispaglabridin A, Neoliquiritin 2''-apioside and Licorice-saponin A3 for Cox1 of *P. aphanidermatum*. Molecular dynamic simulations confirmed the stability of docked complexes when evaluated through multiple descriptors. Additionally, MM/PBSA analysis supported the findings, indicating the spontaneous binding of the enzymes to the screened ligands.

Furthermore, the study involves developing oil-in-water nanoemulsions using thyme oil (TO) alone and in combination with licorice aqueous extract (LAE) and comparing their efficacies against the targeted phytopathogens. The optimized nanoemulsions developed with TO alone

(TFO4) and with LAE (TFO4-10LAE) with droplet size (Z-average diameter) of 24.33 ± 0.86 nm and 217.0 ± 5.9 nm, respectively, exhibited promising results pertaining to their stability and shelf-life. This stability was substantiated through rigorous two-year shelf-life testing. The life cycle assessment showed that the production of TFO4-10LAE nanoemulsion has a lesser negative impact on the environment than that of carbendazim pesticides. TFO4-10LAE, characterized by the preservation of the bioactive molecules present in LAE and TO (as confirmed through GC-MS and FTIR analyses), demonstrated a synergistic and superior antifungal activity when compared to TFO4, which contains only TO. During the *in-planta* assay against chilli anthracnose, nanoemulsion was applied as seed, foliar and seed-foliar treatment. TFO4-10LAE applied as a seed-foliar application significantly reduced the disease severity index (DSI) to 20.37% over control (62.96%), along with the improved activity of induced resistance enzymes and plant growth parameters. Nanoemulsion was applied as a seed, seedling, and seed-seedling treatment during the *in-planta* experiment against Fusarium wilt in chilli. TFO4-10LAE applied as a seed-seedling application against Fusarium wilt significantly reduced the disease severity index (DSI) to 25.33% over control (68.92%), with enhanced induced resistance enzyme activity and plant growth metrics. TFO4-10LAE applied as a seed application against the pythium damping-off exhibited a high reduction in disease incidences, showing pre- and post-emergence damping-off incidence of 11.9 and 5.9%, respectively, as opposed to 43.7 and 12.6% in control.

Drawing from the achieved results, this work will pave the path for future research and development of fungicide alternatives that are not only safer and more sustainable but also surpass the effectiveness of traditional chemical options.

सारांश

मिर्च एक महत्वपूर्ण बागवानी फसल है जो अपनी तीखी किस्मों और मसाले के रूप में उपयोग के लिए जानी जाती है। पौधे में मौजूद तीखापन, जिसे कैप्सिसिनॉइड के नाम से जाना जाता है, के विभिन्न स्वास्थ्य लाभ पाए गए हैं। हालाँकि, इसका उत्पादन विभिन्न जैविक कारकों के कारण प्रभावित हुआ है, जिनमें *कोलेटोट्राइकम कैप्सिसी*, *पाइथियम एफैनिडर्मेटम* और *फ्यूसेरियम ऑक्सीस्पोरम* के कारण होने वाले फंगल रोग क्रमशः एन्थेक्नोज, फ्यूसेरियम विल्ट, और डैम्पिंग-ऑफ शामिल हैं। विभिन्न पौधों के अर्क और आवश्यक तेलों को उनकी प्रभावकारिता और पर्यावरण-अनुकूल प्रकृति के कारण पौधों के संरक्षक के रूप में व्यापक ध्यान मिल रहा है।

इस साक्ष्य पर विचार करते हुए, "कैप्सिकम एन्युम एल. में फंगल फाइटोपैथोजेन के प्रबंधन के लिए वनस्पति फॉर्मूलेशन का विकास" शीर्षक वाला वर्तमान शोध मुख्य रूप से पौधे के अर्क और आवश्यक तेल का उपयोग करके एक बहु-लक्षित वनस्पति फॉर्मूलेशन विकसित करने पर केंद्रित है। अनुसंधान में फाइटोपैथोजेन के खिलाफ कई पौधों के अर्क और आवश्यक तेलों की जाँच, चयनित वानस्पतिक के जैव सक्रिय घटकों का वर्णन, फाइटोपैथोजेन के खिलाफ पहचाने गए वनस्पति का इन-सिलिको मूल्यांकन, वानस्पतिक की सहक्रियात्मक एंटीफंगल क्षमता, फॉर्मूलेशन विकास और विकसित फॉर्मूलेशन का पौधों पर प्रभाव की जाँच शामिल हैं।

अध्ययन की शुरुआत फाइटोपैथोजेन के खिलाफ कई पौधों के अर्क और आवश्यक तेलों की इन-विट्रो एंटीफंगल गतिविधि के मूल्यांकन के साथ हुई। इस अध्ययन में, मुलेठी (*ग्लाइसीराइजा ग्लबरा*) के ठंडे पानी का अर्क (एलएई), और अजवायन (*थाइमस वल्गारिस*) का आवश्यक तेल (टीओ) मिर्च के रोगजनकों के खिलाफ अत्यधिक प्रभावी पाए गए। 200 मि. ग्रा./ एम.एल. की मात्रा पर एलएई ने *पी. एफैनिडर्मेटम* के खिलाफ 89.9% की अधिकतम एंटीफंगल गतिविधि का प्रदर्शन किया, जबकि 0.25 मि. ग्रा./ एम.एल. पर टीओ ने *सी. कैप्सिसी* का 100% निषेध दिखाया। इस अध्ययन से टीओ और एलएई उपचार के कारण कवक कोशिका दीवार और झिल्ली को नुकसान का पता चला, जिसका श्रेय टीओ लिपोफिलिसिटी और एलएई के ट्राइटरपेनॉइड सैपोनिन को दिया जा सकता है। टीओ और एलएई उपचारों के कारण एर्गोस्टेरोल जैवसंश्लेषण में भी कमी आई।

गैस क्रोमैटोग्राफी-मास स्पेक्ट्रोमेट्री और उच्च रिज़ॉल्यूशन-तरल क्रोमैटोग्राफी-मास स्पेक्ट्रोफोटोमेट्री विश्लेषण का उपयोग करके मेटाबोलाइट प्रोफाइलिंग की गयी जिसमें कई जैव सक्रिय यौगिकों की उपस्थिति देखी गयी। जब इन पौधे संरक्षकों को संयोजन में उपयोग किया गया, तो इनकी बहुत कम खुराक (100 मि. ग्रा./ एम.एल. एलएई और 0.125 मि. ग्रा./ एम.एल. टीओ) ने फंगल रोगजनकों को नियंत्रित करने में एक सहक्रियात्मक प्रभाव प्रदर्शित किया। अकेले टीओ या एलएई उपचार की तुलना में, टीओ और एलएई के संयुक्त अनुप्रयोग के परिणामस्वरूप कोशिकीय रिसाव में वृद्धि हुई और एर्गोस्टेरोल जैवसंश्लेषण में अधिक कमी आई। इसके अलावा, इस अध्ययन में अजवायन और मुलेठी के प्रमुख जैव सक्रिय यौगिकों की पहचान करने पर ध्यान केंद्रित किया गया, जो इन-सिलिको दृष्टिकोण को नियोजित करते हुए फाइटोपैथोजेन के कॉक्स1 एंजाइम की गतिविधि को रोकते हैं। जांचे गए जैव सक्रिय यौगिकों की एक विस्तृत श्रृंखला से, आणविक डॉकिंग ने *सी. कैप्सिसी* के कॉक्स1

के लिए संभावित अवरोधकों के रूप में ट्रांस-कार्वेओल, कार्वाक्रोल, काएम्फेरोल 3-रम्नोसाइड 7-ज़ाइलोसाइड, काएम्फेरिट्रिन और एस्ट्रैगैलिन 7-रैम्नोसाइड का संकेत दिया। एफ. ऑक्सीस्पोरम के कॉक्स1 के लिए β -कैरियोफिलीन, कैरियोफिलीन एसीटेट, हिस्पाग्लाइडिन ए, काएम्फेरोल 3-रम्नोसाइड 7-ज़ाइलोसाइड और लिकोरिस ग्लाइकोसाइड ए और पी. एफैनिडर्मेटम के कॉक्स1 के लिए (+)-लॉन्गिफोलेन, कैरियोफिलीन एसीटेट, हिस्पाग्लाइडिन ए, नियोलिक्विरिटिन 2"-एपियोसाइड और लिकोरिस-सैपोनिन ए3 । कई विवरणों के माध्यम से मूल्यांकन करने पर आणविक गतिशील सिमुलेशन ने डॉक किए गए संकुल की स्थिरता की पुष्टि की। इसके अतिरिक्त, एमएम/पीबीएसए विश्लेषण ने निष्कर्षों का समर्थन किया, जो छांटे गए लिगेण्ड्स के साथ एंजाइमों के सहज बंधन का संकेत देता है।

इसके अलावा, अध्ययन में अकेले अजवायन तेल (टीओ) का उपयोग करके और लिकोरिस जलीय अर्क (एलएई) के संयोजन में ऑयल-इन-वॉटर नैनोइमल्शन विकसित करना और फाइटोपैथोजेन के खिलाफ उनकी प्रभावशीलता की तुलना करना शामिल है। क्रमशः 24.33 ± 0.86 एन.एम. और 217.0 ± 5.9 एन.एम. के ड्रॉपलेट आकार (जेड-औसत व्यास) के साथ अकेले टीओ (टीएफओ4) और एलएई (टीएफओ4-10एलएई) के साथ विकसित अनुकूलित नैनोइमल्शन ने स्थिरता और जीवनावधि से संबंधित आशाजनक परिणाम प्रदर्शित किए। जैसा कि उनके दो साल के जीवनावधि परीक्षण से पता चला है, नैनोइमल्शन स्थिर पाए गए। जीवन चक्र मूल्यांकन से पता चला कि टीएफओ4-10एलएई नैनोइमल्शन के उत्पादन का रासायनिक कीटनाशकों की तुलना में पर्यावरण पर कम नकारात्मक प्रभाव पड़ता है। टीएफओ4-10एलएई, जिसमें एलएई और टीओ के जैव सक्रिय योगिक बरकरार हैं (जी सी-एम एस और अफ टी-आई आर विश्लेषण), टीएफओ4 की तुलना में सहक्रियात्मक और बेहतर एंटीफंगल गतिविधि प्रदर्शित करता है। मिर्च एन्थ्रेकनोज के खिलाफ *इन-प्लान्टा* परख के दौरान, नैनोइमल्शन को बीज, पर्पीय और बीज-पर्पीय उपचार के रूप में उपयोग किया गया था। बीज-पर्पीय छिड़काव अनुप्रयोग के रूप में उपयोग किए गए टीएफओ4-10एलएई ने रोग की गंभीरता सूचकांक को नियंत्रण (62.96%) से 20.37% तक कम कर दिया, साथ ही प्रेरित प्रतिरोध एंजाइमों और पौधों के विकास मापदंडों में सुधार किया। मिर्च में फ्यूजेरियम विल्ट के खिलाफ *इन-प्लान्टा* प्रयोग के दौरान बीज, अंकुर और बीज-अंकुर उपचार के रूप में नैनोइमल्शन का उपयोग किया गया था। टीएफओ4-10एलएई को फ्यूजेरियम विल्ट के खिलाफ बीज-अंकुर अनुप्रयोग के रूप में उपयोग करने से रोग की गंभीरता सूचकांक काफी हद तक नियंत्रण (68.92%) से 25.33% कम हो गई, जिससे प्रेरित प्रतिरोध एंजाइम गतिविधि और पौधों की वृद्धि मापों में वृद्धि हुई। टीएफओ4-10एलएई को पाइथियम डैम्पिंग-ऑफ के खिलाफ बीज अनुप्रयोग के रूप में उपयोग करने से रोग की घटनाओं में काफी कमी देखी गई, जिसमें क्रमशः 11.9 और 5.9% की पूर्व और बाद की डैम्पिंग-ऑफ घटनाएं देखी गईं, जबकि नियंत्रण में ये 43.7 और 12.6% थीं।

यह कार्य भविष्य में ऐसे कवकनाशक विकल्पों के अनुसंधान और विकास का मार्ग प्रशस्त करेगा जो रासायनिक विकल्पों की तुलना में अधिक सुरक्षित, टिकाऊ और अधिक प्रभावी हैं।

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ABBREVIATIONS

°C	Degree centigrade
µg	Microgram
µL	Microlitre
µM	Micro molar
ANOVA	Analysis of variance
Cm	Centimetre
Cox1	Cytochrome c oxidase subunit 1
DMRT	Duncan's new multiple range test
DMSO	Dimethylsulfoxide
DSI	Disease severity index
DW	Dry weight
FAO	Food and Agriculture Organization
Fig.	Figure
FT-IR	Fourier transform infrared spectroscopy
Fw	Fresh weight
GAE	Gallic acid equivalents
GC-MS	Gas chromatography mass spectrophometer
h or hr	Hour
HPLC	High performance Liquid Chromatography
HR-LCMS	High-resolution liquid chromatograph mass spectroscopy
ICAR	Indian Council of Agricultural Research
ISR	Induced systemic resistance
ITCC	Indian type culture collection
Kg	Kilogram
L	Liters
LAE	Licorice cold aqueous extract
LCA	Life-cycle assessment
MD	Molecular Dynamics
Mg	Milligram
MIC	Minimum inhibitory concentration
Min	Minutes
Mm	Millimetre
Nm	Nanometers
PAL	Phenylalanine ammonia-lyase
PBS	Phosphate buffer saline
PDA	Potato dextrose agar
PDP	Plant derived protectant
PO	Peroxidase
PPO	Polyphenol oxidase
QE	Quercetin

RMSD	Root-mean-square deviation
RPM	Revolutions per minute
s or sec	seconds
SD	Standard deviation
SEM	Scanning Electron Microscopy
TFC	Total flavonoid content
TFO-10LAE	Thyme oil-Licorice cold water extract nanoemulsion
TFO4	Thyme oil nanoemulsion
TO	Thyme oil
TPC	Total phenolic content