Comprehensive Studies and Management of Charcoal Rot of Cowpea caused by *Macrophomina phaseolina* (Tassi) Goid

लोबिया के चारकोल जड़ सड़न रोग के रोगजनक *मैक्रोफोमिना फेजियोलिना* (तासी) गोइड का विस्तृत अध्ययन एवं प्रबंधन

HANSA CHOUDHARY
(23-02-02-10-08)

Thesis

Master of Science in Agriculture
(Plant Pathology)

2025

Department of Plant Pathology
Rajasthan Agricultural Research Institute,
Durgapura, Jaipur 302 018
Sri Karan Narendra Agriculture University, Jobner (Raj.)

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हंसा चौधरी
(23-02-02-10-08)

शोध प्रबंध

कृषि विज्ञान में स्नातकोत्तर
(पादप रोग विज्ञान)

2025

पादप रोग विज्ञान विभाग
राजस्थान कृषि अनुसंधान संस्थान,
दुर्गापुरा, जयपुर–302 018
श्री कर्ण नरेन्द्र कृषि विश्वविद्यालय, जोबनेर (राज.)

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Thesis

Rajasthan Agricultural Research Institute,
Durgapura, Jaipur 302018, Rajasthan

In partial fulfillment of the requirement for

the degree of

Master of Science

in the

Faculty of Agriculture

(Plant Pathology)

By

HANSA CHOUDHARY
(23-02-02-10-08)

2025

Sri Karan Narendra Agriculture University, Jobner
Rajasthan Agricultural Research Institute, Durgapura- Jaipur

CERTIFICATE- I

Date…….

On the basis of office record, it is certified that **Miss. Hansa Choudhary** has successfully completed the comprehensive examination held on 13.06.2025 as required under the regulation of **M.Sc.** degree.

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Head
Department of Plant Pathology
RARI, Durgapura

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Rajasthan Agricultural Research Institute, Durgapura- Jaipur

CERTIFICATE – II

Date…….

This is to certify that the thesis entitled "**Comprehensive Studies and Management of Charcoal Rot of Cowpea caused by *Macrophomina phaseolina* (Tassi) Goid**" submitted for the degree of **Master of Science** in Agriculture in the subject of Plant Pathology embodies bonafide research work carried out by **Miss. Hansa Choudhary** under my guidance and supervision and that no part of this thesis has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged. The draft of the thesis was also approved by the advisory committee on 10.07.2025.

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Rajasthan Agricultural Research Institute, Durgapura- Jaipur

CERTIFICATE – III

Date:……

This is to certify that the thesis entitled entitled **"Comprehensive Studies and Management of Charcoal Rot of Cowpea caused by *Macrophomina phaseolina* (Tassi) Goid"** submitted by **Miss. Hansa Choudhary** to the Sri Karan Narendra Agriculture University, Jobner in partial fulfillment of the requirements for the degree of **Master of Science** in the subject of Plant Pathology after recommendation by the external examiner was defended by the candidate before the following members of the examination committee. The performance of the candidate in the oral examination conducted on her thesis has been found satisfactory; we therefore, recommended that the thesis be approved.

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(Arvind M)
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(Ved Prakash Yadav)
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CERTIFICATE – IV

Date: ……

This is to certify that **Miss. Hansa Choudhary** of the Department of Plant Pathology, Rajasthan Agricultural Research Institute, Durgapura has made all corrections / modifications in the thesis entitled **"Comprehensive Studies and Management of Charcoal Rot of Cowpea caused by *Macrophomina phaseolina* (Tassi) Goid"** which were suggested by the external examiner and the advisory committee in the oral examination held on …….. The final copies of the thesis duly bound and corrected were submitted on……. and are enclosed herewith for approval.

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At this inexplicable moment, words are not in lexicon to express my sincere sense of gratitude, but with full honor and ecstasy of delight, I express my heartfelt thanks to my major guide ***Dr. Nitin Chawla****, Asstt. Professor, Rajasthan Agricultural Research Institute, Durgapura, (SKN Agriculture University, Jobner). I owe a special debt of gratitude to him, for his valuable guidance, constant encouragement, keep interest, constructive criticism and suggestion throughout the course of his investigation. I am highly indebted to him for his generous advice, cooperation in preparing the manuscript of this dissertation.*

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I am again ecstatic to express my inherent sense of gratitude to ***Dr. R.K. Bagri****, Head of Department, Professor,* ***Dr. Bhavya Mishra****, Asstt. Professor,* ***Dr. P.S. Shekhawat****, Professor,* ***Dr. R.S. Sharma,*** *Asstt. Professor,* ***Dr. M.A. Khan****, Asstt. Professor,* ***Dr. Anita Jat,*** *Asstt. Professor, other members of Department of Plant Pathology, Rajasthan Agricultural Research Institute, Durgapura, (SKN Agriculture University, Jobner) whose co-operation made this investigation smooth and easy.*

I pleasure to express my heartiest gratitude towards the help rendered by my senior Dr. Pinki, Dr. Sunita, Dr. Rajpal and Sanju, Manish, dearest classmates Sonu, Ranu, Mona, Subhash and with whom I went hand in hand during study and research work. I would like to thanks to my dearest friends Anju, Megha, Monu, Neelam, Siddharth, Mohit, Jagveer and lovely juniors whose co-operation and help in various ways brought this task to completion.

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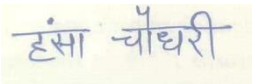
*Last but not the least, a million of thanks to "****The Almighty God****" who made me to complete this task and made every job a success for me.*

Place : Durgapura

Date:



(Hansa Choudhary)

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ABBREVIATION

%	:	Percentage
/	:	Per
@	:	at the rate
°C	:	Degree Celsius
µg	:	Microgram
µl	:	Micro liter
µm	:	Micrometer
Bp	:	Base pair
CD	:	Critical difference
cm	:	Centimetre
Conc.	:	Concentration
CTAB	:	Cetyl trimethyl ammonium bromide
DNA	:	Deoxy ribonucleic acid
Dntp	:	Deoxyribonucleotide triphosphate
e.g.	:	for example
EDTA	:	Ethylenediamine tetra acetic acid
et al.	:	et alia (and associate)
EtBr	:	Ethidium bromide
etc.	:	et cetera
Fig.	:	Figure
FS	:	Flowable Suspension
g	:	Gram
Ha/ha	:	Hectare
hpi	:	hours post inoculation
hrs	:	Hour
i.e.	:	(id est.) that is
Kg	:	Kilogram
L	:	Litre
m	:	Meter
MEGA X	:	Molecular evolutionary genetic analysis
mg	:	Milligram
ml	:	Milliliter
mm	:	Millimeter
NCBI	:	National Center for Biotechnology Information
nm	:	Nanometer
No.	:	Number
PCR	:	Polymerase chain reaction
Psi	:	Pounds per square inch
pv	:	Pathovar
RPM	:	Revolutions per minute
S. No.	:	Serial number
S.Em	:	Standard Error of Mean
Spp.	:	Species
T. No.	:	Treatment number
viz.	:	Namely
WP	:	Wettable powder

Comprehensive Studies and Management of Charcoal Rot of Cowpea caused by *Macrophomina phaseolina* (Tassi) Goid

Hansa Choudhary* (Researcher)

Dr. Nitin Chawla** (Major Advisor)

ABSTRACT

Cowpea [*Vigna unguiculata* (L.) Walp.], an annual legume, is also commonly referred as southern pea. Diseased roots of cowpea showing typical symptoms of root rot were collected from Rajasthan Agricultural Research Institute, farm, All India Network Project trials on cowpea during summer 2024. Pathogen was isolated from infected roots of cowpea and purified by hyphal tip method. Pathogenicity test of *Macrophomina phaseolina* was confirmed by soil inoculation method in the pots. On the basis of mycelial, sclerotial characters and internal transcribed spacer (ITS) sequence of rDNA amplified using the primers ITS1/ITS4, the pathogen was identified and confirmed as *M. phaseolina*, with NCBI Gen Bank Accession number PQ399952.

Thirty-five entries/varieties were screened against *M. phaseolina* under artificial conditions. None of the entries were found immune or resistant while, 4 entries namely, CPD 307, CPD 347, CPD 332, and CPD 324 were found to be moderately resistant and the disease incidence in susceptible check (RC 19) was 74.67%. During biochemical studies on moderately resistant and susceptible entries, an increase in phenolic content at 96 hours post inoculation (hpi) in moderately resistant entries and decrease in content after 72 hpi was observed in susceptible entries. Proline concentration did not show any significant difference; however, total antioxidant enzyme activity exhibited a sharp increase at 48 hpi in moderately resistant entries, while the increase in susceptible entries was comparatively less pronounced.

In the field study, among eight treatments, seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS was found to be the most effective which showing the lowest disease incidence at 20.27% and maximum grain yield of 6.88 q/ha. While, both *Trichoderma harzianum* and *Trichoderma asperellum* showed more than 50% disease reduction.

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** M.Sc. (Ag.) Thesis submitted to Sri Karan Narendra Agriculture University, Jobner for partial fulfillment of the requirement of degree under the supervision of **Dr. Nitin Chawla**, Asstt. Professor, Rajasthan Agricultural Research Institute, Durgapura Jaipur (Rajasthan) 302 018

लोबिया के चारकोल जड़ सड़न रोग के रोगजनक *मैक्रोफोमिना फेजियोलिना* (तासी) गोइड का विस्तृत अध्ययन एवं प्रबंधन

हंसा चौधरी*
(शोधार्थी)

डॉ. नितिन चावला**
(मुख्य सलाहकार)

अनुक्षेपण

लोबिया [*विग्ना अनगुइकुलाटा* (एल.) वाल्प.] एक वार्षिक दलहनी फसल है, जिसे सामान्यतः दक्षिणी मटर के नाम से जाना जाता है। ग्रीष्म ऋतु 2024 के दौरान, राजस्थान कृषि अनुसंधान संस्थान, फार्म में अखिल भारतीय नेटवर्क परियोजना के अंतर्गत संचालित परीक्षणों से जड़ सड़न रोग के विशिष्ट लक्षणों वाले लोबिया पौधों की संक्रमित जड़ें एकत्रित की गईं।

लोबिया की संक्रमित जड़ों से रोगजनक को पृथक किया गया जिसको हाइफल टिप विधि द्वारा शुद्धिकृत किया गया। *मैक्रोफोमिना फेजियोलिना* की रोगकारकता की पुष्टि पॉट में मृदा–संक्रमण विधि द्वारा की गई। माइसीलियल, स्क्लेरोटियल और प्राइमर आई टी एस 1/आई टी एस 4 का उपयोग करके प्रतिवर्धित आरडीएनए के आंतरिक प्रतिलेखित के अनुक्रम के आधार पर रोगजनक की पहचान व पुष्टी *एम. फेजियोलिना* के रूप में की गई, जिसका एन. सी. बी. आई. जीनबैंक एक्सेशन नंबर PQ399952 है।

कृत्रिम स्थितियों में कुल 35 प्रविष्टियों/प्रजातियों की रोग प्रतिरोधकता की जांच की गई। कोई भी प्रविष्टि पूर्णतः रोगमुक्त या प्रतिरोधी नहीं पाई गई, जबकि चार प्रविष्टियाँ सीपीडी 307, सीपीडी 347, सीपीडी 332 एवं सीपीडी 324 को मध्यम प्रतिरोधक के रूप में पाई गई और संवेदनशील जांच (आरसी 19) में रोग आपतन 74.67 प्रतिशत पाई गई।

मध्यम प्रतिरोधी और संवेदनशीलता प्रविष्टियों पर जैव रासायनिक अध्ययनों के दौरान, जिसमें मध्यम प्रतिरोधी प्रविष्टियों में संरोपण 96 घंटे बाद (एचपीआई) फिनॉलिक घटक में वृद्धि की मात्रा में उल्लेखनीय वृद्धि देखी गई एवं संवेदनशील प्रविष्टियों में 72 एचपीआई के बाद मात्रा में कमी देखी गई।

प्रोलिन की मात्रा में कोई भी सार्थक अंतर नहीं पाया गया, जबकि कुल एंटीऑक्सिडेंट एंजाइम गतिविधि में मध्यम रूप से प्रतिरोधी प्रविष्टियों में 48 एचपीआई के उपरांत तीव्र वृद्धि दर्ज की गई, जबकि संवेदनशील प्रविष्टियों में यह वृद्धि अपेक्षाकृत कम रही।

क्षेत्रीय परीक्षण के दौरान, कुल आठ उपचारों में से एज़ॉक्सीस्ट्रोबिन 2.5 प्रतिशत + थियोफेनेट मेथाइल 11.25 प्रतिशत एफ.एस. + थायामेथॉक्साम 25 प्रतिशत एफ.एस. द्वारा किया गया बीजोपचार सबसे अधिक प्रभावी पाया गया। जिसमें *ट्राइकोडर्मा हरजिएनम* और *ट्राइकोडर्मा एस्पेरेलम* उपचार के उपचारित भूखण्ड में 50 प्रतिशत से अधिक रोग में कमी देखी गई।

---

* कृषि स्नातकोत्तर छात्रा, पादप रोग विज्ञान विभाग, राजस्थान कृषि अनुसंधान संस्थान, दुर्गापुरा, जयपुर–302 018

** कृषि में स्नातकोत्तर उपाधि प्राप्ति की आंशिक आवश्यकता की पूर्ति के लिये वर्तमान शोधकार्य **डॉ. नितिन चावला,** सहायक आचार्य, राजस्थान कृषि अनुसंधान संस्थान, दुर्गापुरा, (श्री कर्ण नरेन्द्र कृषि विश्वविद्यालय, जोबनेर) जयपुर राजस्थान के निर्देशन में पूर्ण किया गया।

Chapter - 1

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp] (2n=22), commonly referred to as "Lobia", is an annual legume that ranks among the earliest food sources for humans. Widely cultivated in tropical and subtropical regions, it is a short-duration pulse crop such as fodder, hay or silage. Cowpea is believed to have originated in Africa, as wild varieties are found exclusively in Africa and Madagascar (Steele 1976). It belongs to the Fabaceae family.

Cowpea is often referred to as "vegetable meat" because its grains are rich in protein (23.4%) and possess high biological value on a dry weight basis. It is also known as the "hungry season crop" since it is typically harvested earlier than cereal crops, providing food during periods of scarcity (Gomez 2004).

Cowpea is also popularly known by other names such as southern pea, black-eyed pea, barbatti, chawali, asparagus bean and crowder pea (Srinivas et al. 2016). The seeds are edible in their fresh form along with the pods and the green beans are commonly used as a vegetable. Dry seeds of cowpea are used to prepare many snacks and main food dishes. It is highly adaptable to different farming systems and is cultivated as a cover crop, mixed crop, catch crop, intercrop with millets or as a green manure crop in several Indian regions such as Punjab, Delhi and Haryana. Additionally, cowpea forms a dense ground cover which helps protect the soil from erosion and reduces water loss from the field (Gupta and Saxena 2015).

In Rajasthan, cowpea holds significant value because of its short growing period, high yield potential, rapid growth and rich protein content. Commonly used as an alternative crop in dryland agriculture, cowpea is occasionally cultivated as green manure to enhance soil health. Its capacity to replenish soil nutrients makes it an essential part of crop rotation systems, particularly for improving the fertility of the soil for subsequent cereal crops. The natural decomposition of cowpea root residues within the soil contributes organic matter and essential nutrients. Various studies have demonstrated

that cowpea cultivation enhances soil nitrogen content by 40-80 kg N per hectare. This increase is due to a symbiotic relationship with nitrogen-fixing bacteria from the *Bradyrhizobium* genus, which reside in root nodules and convert atmospheric nitrogen into a usable form.

Cowpea is a vital contributor of both macro and micronutrients in the human diet. Its tender leaves, beans and pods are rich in vitamins and minerals, making cowpea suitable for both human food and animal feed (Nielsen et al. 1997). It is also recognized as one of the earliest cultivated legumes used for protein by both humans and livestock (Steele 1972). Cowpea is rich in carbohydrates (60.3%) and contain a small amount of fat (1.8%) (Gupta et al. 2019). It also provides significant levels of essential minerals such as calcium (76 mg/100 g) and iron (57 mg/100 g) along with vitamins including thiamine (0.92 mg/100 g), riboflavin (0.18 mg/100 g) and nicotinic acid (1.9 mg/100 g) (Chatterjee and Bhattacharya, 1986). It is abundant in lysine. Although its total protein is relatively low in methionine and cysteine compared to animal-based proteins, it contains higher levels of essential amino acids than most cereals (Gonclaves et al. 2016).

In India, cowpea is cultivated in 1.50 million hectares with a production of 0.50 million tonnes of grain (Anonymous 2022). It is mainly grown in Gujrat, West Bengal, Tamil Nadu, Andhra Pradesh, Kerala and Odisha (Detroja et al. 2020). In Rajasthan, it is cultivated in 0.5 lakh hectares with a production of 0.23 lakh tonnes (Punia et al. 2025). It mainly grows in Jaipur, Tonk, Dausa, Jhunjhunu, Sikar, Ajmer, Bhilwara, Chittor and Banswara.

Cowpea is susceptible to a variety of diseases. These include charcoal rot and dry root rot caused by *Macrophomina phaseolina*, anthracnose caused by *Colletotrichum lindemuthianum*, powdery mildew caused by *Erysiphe polygoni* and cercospora leaf spot caused by *Cercospora canescens*. Other common diseases affecting cowpea are fusarium wilt caused by *Fusarium solani*, bacterial blight due to *Xanthomonas axonopodis* pv. *vignicola*, rust caused by *Uromyces vignae* and yellow mosaic disease caused by the *Cowpea yellow mosaic virus*.

Among these diseases, charcoal rot is most prevalent in Rajasthan owing to dry climates and low soil moisture. Charcoal rot caused by *M. phaseolina*, is considered one of the most destructive diseases affecting cowpea. It causes severe crop losses, particularly during the *Kharif* season. In Rajasthan, the infection typically begins at the seedling stage, just below the cotyledon node. The initial symptom of the disease is leaf yellowing which progresses to leaf drying within 2 to 3 days. In many cases, the affected plant dies within a week of the onset of symptoms (Pickel et al. 2020). Upon close inspection of the stem, dark-colored lesions are seen on the bark near the soil surface. The taproot and lower portion of the stem often appear light brown. Tiny dark specks may develop beneath the outer layer and within the inner stem and root tissues, giving them a charcoal-dusted look. Additionally, reddish-brown discoloration can be observed in the pith and vascular tissues of the root and stem. In severe stages of infection, sclerotial structures become visible across the damaged areas (Singh et al. 1998; Dhingra & Sinclair 1978)

The pathogen *Macrophomina phaseolina* is classified under the Kingdom-Fungi, Phylum-Ascomycota, Class-Dothideomycetes, Order-Botryosphaeriales, Family-Botryosphaeriaceae, Genus-Macrophomina and Species-phaseolina.

M. phaseolina is a seed- and soil-borne fungal pathogen known to infect around 500 plant species across more than 100 families. Charcoal rot in cowpea characterized by the formation of microsclerotia or pycnidia in the roots and stems of infected plants, allowing the fungus to persist in the soil and plant debris for extended periods (Romero Luna et al. 2017). As a facultative parasite, it can also survive saprophytically on dead organic matter. The fungus invades host tissues both between and within cells, spreads rapidly and affects large portions of plant tissue, ultimately leading to plant death in a short period of time. The fungus spreads from plant to plant through irrigation water, implements and cultural operation. (Rangaswami and Mahadevan 2008).

The pathogen exhibits significant variability with different isolates showing differences in microsclerotia size and the presence or absence of pycnidia. Initially, pycnidia develop within the host tissue and later emerge as they mature. These structures are dark to greyish in color, turning black over time and are typically globose or slightly flattened with a membranous. Pycnidia produce simple, rod-shaped conidiophores. The conidia are single-celled, hyaline and oval. Microsclerotia are formed by clusters of hyphal cells bound together by melanin, with each microsclerotium consisting of 50 to 200 individual cells (Huda-Shakirah et al. 2019).

Charcoal rot lead to significant yield losses in cowpea, ranging from 5% to 40% (Mohanpriya et al. 2017). Under favourable conditions, specifically when temperatures exceed 30°C and soil moisture drops below 60% crop losses reach up to 100% (Marquez et al. 2021). The disease typically remains undetected during the cooler, early stages of the growing season. However, as temperatures rise and the plant approaches flowering, fruit development and weight gain, symptoms begin to emerge and worse especially when the crop experiences heat or drought stress (Pickel et al. 2020; Romero Luna et al. 2017).

Owing to the unavailability of resistance genotype in the germpool and lack of information on biochemical basis of resistance this research was undertaken with the following objectives:

1. Morphological and molecular characterization of pathogen associated with charcoal rot of cowpea
2. Screening of varieties and biochemical evaluation of susceptible and resistant cultivars under artificial inoculation conditions
3. Management of charcoal rot of cowpea under field condition using novel strategies

Chapter - 2

REVIEW OF LITERATURE

This chapter provides a concise overview of existing literature related to the morphological and molecular characterization of charcoal rot disease, biochemical differences between healthy and infected plants, screening of entries and disease management using fungicides and bioagents. Additionally, relevant studies on dry root rot affecting other legume crops such as chickpea, black gram and pigeon pea are also included where extensive information is available.

The literature has been summarized under the following key sections:

2.1 Morphological and molecular characterization

2.2 Screening of varieties and biochemical evaluation of susceptible and resistant cultivars

2.3 Management of charcoal rot of cowpea

2.1 Morphological and molecular characterization of pathogen associated with charcoal rot of cowpea

2.1.1 Morphological characterization

Hinguera (1991) conducted studies on three different methods of inoculation to establish pathogenicity of *Macrohomina phaseolina* in cowpea *viz*., toothpick inoculation, inoculation using dry sclerotia and infested rice seeds-based inoculation and concluded that inoculum multiplied on rice seeds was most effective in disease development in cowpea seedlings.

Lodha (1998) studied the effect of sources of inoculum on population dynamics of *M. phaseolina* and disease intensity in cluster bean and suggested significant correlation between higher plant mortality and increased soil inoculum under moisture stress conditions. He also reported that *M. phaseolina* and *Fusarium solani* produce charcoal rot and root rot of jojoba by inoculating the soil as well as by incorporation of

inoculum of *M. phaseolina* through seed-cum-soil and drenching 15 days after sowing in cluster bean.

Amusa et al. (2007) isolated *M. phaseolina* from six different leguminous species including cowpea and studied induced systemic response. In this study, isolate 93-295 from winged bean leaf tissue was the most virulent, inducing necrotic lesions of 4.8 mm in diameter on cowpea stem tissues while the smallest necrotic lesion size of 3.2 mm was induced on the same cowpea by isolate 93-52-1.

El–Araby et al. (2009) observed symptoms such as shrunken, unfilled pods and brown wilted attached to dead petioles and stems along with the stem cortex due to *M. phaseolina* causing charcoal rot in soybean. The fungus was soil dweller and spreaded from plant to plant through irrigation water, flood, implements and cultural operations.

Csondes (2012) reported that morphological analysis of microsclerotia characters (size, shape and production of microsclerotia) revealed substantial differences in diameter between microsclerotia originating from the same isolation in different media such as maize-flour agar, followed by Sabouraud-glucose, maltextract, potato-dextrose, Czapek-Dox and watery agar media. The diameter range was wide between 39 and 308 µm. The shape of microsclerotia varied from spherical to oval.

Garg and Kumar (2012) studied the morphological and cultural characteristics of *M. phaseolina* of *Euphorbia lathyrism* L. The fungal mycelium was hyaline and light to dark brown, profusely branched and constricted at septa. Sclerotia were oval to spherical, dark brown to black and measured 62.5 µm to 100 µm diameter in culture.

Javaid *et al*. (2012) used cowpea plants showing typical features of charcoal rot disease caused by *M. phaseolina* fungus. Disease infected region of stem was cut into small pieces and surface sterilized with 0.1 *per cent* mercuric chloride ($HgCl_2$) then stem pieces were placed on autoclaved nutrients medium. The plates were incubated for 6 to 7 days at 28°C

temperature. Colony morphology, pycnidia, conidia, microsclerotia and hyphal branching were observed under microscope.

Rhizoctonia bataticola was isolated from seed, root and shoot of pulses where seed and root isolates of black gram and soybean produced moderate sclerotia while the shoot isolates of soybean produced less sclerotia. Among the isolates, the diameter of sclerotia ranged from 43.6 µm to 89.9 µm. The sclerotia of black gram root rot isolate recorded a maximum diameter of 89.9 µm and followed by red gram root isolate (86.85 µm) (Sundravadana et al. 2012).

Kumar et al. (2013) tested eight isolates of *M. phaseolina* for their pathogenicity by using susceptible varieties *viz.,* RMO-225 of mothbean, RMG-62 of mungbean, Varsha-Uphar of okra and M-83 of clusterbean in pot culture. The disease first appeared in okra followed by clusterbean, mothbean and mungbean in sterilized as well as unsterilized soil.

Mahdizadeh et al. (2011) confirmed that fifty-two *M. phaseolina* isolates were recovered from 24 host plant species. The feathery colony phenotype was the most common (63.7%) on the chlorate selective medium and represented the chlorate sensitive phenotype of the Iranian *M. phaseolina* population.

Manjunath (2014) noticed variation among the twenty isolates of *M. phaseolina* from chickpea with respect to microsclerotia diameter which varied from 160.73 µm (KAMP-4) and at par with MHMP-12, KAMP-1, APMP-6, APMP-7, APMP-8, TNMP-10 and significantly superior over the isolate TNMP-11 which is having a diameter of 36.98 µm.

Tandel et al. (2015) successfully conducted pathogenicity of leaf blight in green gram due to *M. phaseolina*. In this experiment various techniques were used namely pin aprick, injury caused by a toothbrush and by a carborandum powder. All techniques significantly showed typical symptoms of leaf blight in mungbean.

Gade et al. (2018) observed significant variation among the 40 isolates of *Rhizoctonia bataticola* in soybean regarding the size of their sclerotia.

Maximum sclerotial size was observed in case of isolate Rb-33 (120.11 µm) while the isolate Rb-40 produced the smallest size sclerotia (42.03 µm). The average sclerotial size of isolates ranged from 42.03-120.11 µm. The sclerotial size of 16 isolates were above 80 µm and were classified as large sized while 16 isolates with sclerotia size less than 60 µm were rated as small sized. The remaining eight isolates ranged between 60 and 80 µm sclerotia size and were categorized as medium sized.

Satpathi and Gohel (2018) collected five isolates of stem and root rot of sesame from different paths of Gujarat and tested on different culture media *viz*., PDA, oatmeal agar and host leaf extract agar. In the study, PDA was found best for sclerotial formation and mycelial growth. Out of five isolates, Mp 2 and Mp 3 exhibited the highest radial growth (90.00 mm) and average sclerotial size ranged from 62.93-129.36 µm x 46.93-11.28 µm.

A comprehensive survey of sunflower was conducted in Sindh and 32 isolates of *M. phaseolina* were collected from Badin, Dadu, Hyderabad, Khairpur, Mirpurkhas, Sanghar, Shaheed Benazirabad, Sukkur, Tando and Thatta. The variation in colony colour (gray and blackish gray to grayish black), pattern (dense, feathery and restricted) and sclerotia size were reported (Wagan et al. 2018).

Vengadesh et al. (2019) observed during survey conducted in different regions of Tamil Nadu revealed the endemic nature of the root rot disease of cowpea with the maximum disease incidence (18.43%) recorded in Kaveripattinam of Krishnagiri region. Also, the isolates of *M. phaseolina* exhibited cultural and pathogenic variability. Among these isolate, MP5 exhibited faster mycelial growth, maximum sclerotial production and sclerotial size and recorded the maximum incidence of root rot disease under pot culture conditions.

Gaikwad et al. (2020) studied cultural and genetic diversity of *R. bataticola* causing in chickpea. The isolated Rb-6 exhibited maximum mycelial growth (90.00 mm) followed by Rb-9 (89.50 mm) and Rb-2 (88.50 mm) indicating a great cultural variability amongst *R. bataticola* isolates.

Basbagci and Dolar (2022) examined 19 chickpea isolates of *R. bataticola* obtained from different locations of Turkey in 2016-17 and reported that the sclerotial size varied between 65.6 and 127.4 µm. The colony colour of the pathogen was grayish black and the colony texture was velvety and fluffy.

2.1.2 Molecular characterization

Babu et al. (2007) observed that species-specific primers MpKFI and MpKRI, internal transcribed spacers (ITS) and oligonucleotide probes MpKFI (19 MER) can be used to rapidly detect and identify *M. phaseolina* by polymerase chain reaction (PCR) and hybridization.

Aghakhani and Dube (2009) determined genetic diversity among six isolates of *R. bataticola* in chickpea representing five different ITS-RFLP groups with respect to their nucleotide sequences of ITS I, 5.8 rDNA and ITS II regions. The ITS of Karnataka isolate (Rb1) was largest (670 bp), whereas it was smallest (499 bp) in Haryana (Rb21) isolated. The phylogeny tree constructed from the nucleotide sequence showed similarity of these six isolates along with 27 other ITS sequences of fungi. The isolates Rb16 (Jharkhand), Rb21 (Haryana) and Rb26 (Madhya Pradesh) clustered in one group and showed 96 to 100 per cent nucleotide similarity with the sequences of *M. phaseolina* (*R. bataticola*) available in the GenBank.

Mahdizadeh et al. (2011) confirmed that fifty-two *M. phaseolina* isolates were recovered from 24 host plant species from the 14 Iranian provinces using species-specific primers MpKFI and MpKRI.

Mahmoud et al. (2012) studied molecular characterization of the pathogenic plant fungus *R. solani* isolated from Egypt based on protein and PCR-RAPD profiles. Twenty-one isolates of *R. solani* were categorized into three anastomosis groups consisting of AG-4-HG-I (eight isolates), AG-2-2 (nine isolates) and AG-5 (four isolates). The Pathogenic capacities were also tested on cotton cultivar Giza 86. The pre-emergence damping off varied in response to the different isolates.

Manjunath (2014) studied ITS region of rDNA amplified using ITS1 and ITS4 primers for sequencing of the 18S rDNA region. Among the 20 isolates of *M. phaseolina* evaluated, the highest similarity (100%) was observed between most of the isolates while the lowest similarity (94%) was observed between the isolates KAMP-2 and PUMP-14.

Sun et al. (2015) studied the causal agent of adzuki bean disease. Four fungal isolates were obtained and identified as *M. phaseolina* based on morphological and molecular characteristics along with species-specific primer detection. The resulting sequences showed 99% identity with more than 60 strains of *M. phaseolina* from diverse hosts.

Khan et al. (2017) tested virulence of three strains of *M. phaseolina,* namely 1156, 1160 and PCMC/F1 on sunflower and chickpea. The strains increased the hydrogen per oxide content by 1.4- to 1.6-fold in root as well as shoot of chickpea and sunflower. The *M. phaseolina* strains also produced hydrolytic enzymes such as lipase, amylase and protease with solubilization zones of 5-43 mm, 5-45 mm and 12-35 mm, respectively. The *M. phaseolina* strains were identified by 18S rRNA.

The molecular characterization of twenty isolates of *M. phaseolina* with species specific primers MpKFI (5'- CCGCCAGAGGACTATCAAAC-3') and MpKRI (5' CGTCCGAAGCGAGGTGTATT-3') yielded single amplified product of 350 bp in all the isolates tested and the PCR amplified 18S rDNA region using ITS primers produced a band size of about 600 bp (Jyothi et al. 2019).

Pandey et al. (2020) isolated *M. phaseolina* from urdbean, vegetable soybean and mungbean using ITS region of 18S rRNA and 43 mungbean genotypes were screened against *M. phaseolina.* Out of these three genotypes, IPM99-125 showed consistently higher plant survival rate followed by EC693368 and EC693369 as compared to susceptible checks (VC3960-88, KPS1).

Santos et al. (2020) used four nuclear gene primers actin (ACT), β-tubulin (βT), calmodulin (CAL) and translation elongation factor 1-α (TEF1- α) for the identification of *M. phaseolina*, *M. pseudophaseolina* and *M.*

euphorbiicola in different crops. PCR-based assays were conducted to verify the specificity with isolates of the three species of Macrophomina and 42 species of other genera. Three primer sets to amplify of regions CAL (MpCalF/MpCalR, MsCalF/MsCalR and MeCalF/MeCalR) and three primer sets to amplify of regions TEF-1α (MpTefF/MpTefR, MsTefF/MsTefR and MeTefF/MeTefR) were designed for *M*. *phaseolina*, *M. pseudophaseolina* and *M. euphorbiicola*, respectively.

Sodji et al. (2024) identified and characterized *M. phaseolina* isolates using both molecular and morphological approaches and determined the virulence of the isolates. Eleven *M. phaseolina* isolates from cowpea growing areas in northern Ghana (Manga, Nyankpala, Yendi, Damongo, Tumu, Wa, Akomadan and Ejura) were morphologically and molecularly characterized. DNA of the *M. phaseolina* isolates were amplified using species-specific PCR primers MpKF1 and MpKR1 and sequencing of the *M. phaseolina* specific amplicons was obtained. All isolates were confirmed as *M. phaseolina* based on the amplicon size obtained and the sequencing homology. Isolate Mp-3Mc was the most virulent while isolate Mp-6Ec was the least virulent, resulting in 76.7% and 26.7% seed rot and damping off, respectively.

2.2 Screening of varieties and biochemical evaluation of susceptible and resistant cultivars under artificial inoculation conditions

2.2.1 Screening of cowpea germplasm against charcoal rot disease

Gangwar et al. (2002) tested thirty-five chickpea cultivars for resistance to dry root rot caused by *R. bataticola* (*M. phaseolina*) in field conditions. Disease intensity was recorded 30 days after disease symptoms. Ten cultivars (IICC-2644, 10384, 10630, 112244, 11332, ICCL 81002, 81010, ICC 12263, 12441 and ICCV) were found resistant whereas, five cultivars (GCP 9504, phule G-96020, 96105, 96313 and GL 91059) were found moderately resistance to dry root rot.

Khan and Shuaib (2007) screened twenty-nine mungbean genotypes against charcoal rot disease caused by *M. phaseolina*. Two genotypes NCM

252-10 and 40536 were highly resistant, whereas five genotypes *viz.,* 40504, NCM 257-5, 40457, NCM 251-4 and 6368-64-72 were resistant and six were moderately resistant.

Sixty-one Indian bean (*Phaseolus vulgaris*) lines collected from different sources were screened for resistance to dry root rot disease (*M. phaseolina*) under natural field conditions of Tirupati, Andhra Pradesh, India, during *kharif*, 2005 observed. Among sixty-one genotypes/varieties, only 2 genotypes/varieties were found to be completely resistant (PDR 14 and Pant Bean 2), 1 moderately resistant against dry root rot disease (Prasanthi 2007).

Iqbal et al. (2010) screened 100 blackgram germplasm accessions for resistance against charcoal rot under artificial conditions in greenhouse as well as in the field. At the seedling stage, 5 genotypes appeared to be highly resistant, 11 resistant and 30 tolerant in green house whereas rest of the 54 genotypes showed susceptible to highly susceptible response. At reproductive stage, twelve genotypes were found to be highly resistant, 17 resistant and 25 tolerant, whereas 16 genotypes appeared to be susceptible and 30 highly susceptible under field conditions. Three genotypes *viz*., 013468, 013663 and 013468 showed resistance both at seedling and reproductive stages.

Choudhary et al. (2011) screened twenty-five mungbean genotypes to identify sources of resistance to dry root rot caused by *M. phaseolina*. They observed three genotypes namely MSJ-118, KM-4-44 and KM-4-59 as resistant. These resistant genotypes had significantly greater root and shoot length, root and shoot weight than those of the susceptible check RMG-62.

Muchero et al. (2011) evaluated 14 cowpea genotypes against *M. phaseolina* under moderate water stress conditions. The genotypes IT98K-499-39, Suvita 2, IT93K-503-1 and Mouride were found to be highly resistant with mortality below 10%.

Oladimeji et al. (2012) screened five cultivars of cowpea against *M. phaseolina* using two methods of inoculation *viz*., pouring of spore/mycelium suspension in the soil and wrapping of inoculums meal around wounded lower

stem of the seedling. Cowpea cultivar ITO4K-217-5 was found to be resistant to the pathogen in both inoculation methods.

Khan et al. (2013) reported management of chickpea dry root rot through resistance germplasm lines in Jammu & Kashmir. Sixty germplasm lines of chickpea were screened for their resistance against dry root rot disease in pot. Only nine lines namely KGD-1189, KGD-1201, KGD-1209, KGD-1215, KGD-1217, KGD-1220, KGD-1221, KGD-1248 and KGD-1289 were found to be resistant.

Kumar et al. (2013) tested eight isolates of *M. phaseolina* for their pathogenicity by using susceptible varieties *viz*., RMO-225 of mothbean, RMG-62 of mungbean, Varsha-Uphar of okra and M-83 of clusterbean in pot culture. The disease first appeared in okra followed by clusterbean, mothbean and mungbean in sterilized as well as unsterilizes soil.

Tanzeel-u-Rehman (2015) screened seven varieties/lines (SA dandy, Elite, Rawan-2003, White star, CP1, P-518, P-2127) against seedling blight disease of cowpea caused by *M. phaseolina*. Among seven cultivars, White star and Elite showed resistant reactions and SA dandy, CP1 and Rawan-2003 showed tolerance against the disease.

Ou'edraogo et al. (2021) screened eighty cultivars of cowpea against *M. phaseolina*. In this study, five genotypes B05-5a, B27-07a, CB27, SP369 A Profil-39B and SP88 Profil-13A were free from disease, four genotypes, *viz*., Komsare, Kaya local, 58-57 and Gaoua local-2 showed low severity (S < 10%) and 11 other genotypes including KV x 295-2-124-51, Pa local-2, Boalga local, TVU 14-676, Pouytenga-3, Apagbaala, NE91 profil-4, IT82D-849, B301, TV286b profil-12 and IT98K-317-2 showed moderate disease severity indexes (S < 20%) to both isolates of *M. phaseolina*.

Anupriya and Chawla (2022) screened fifty-two different mungbean genotype/varieties lines against *M. phaseolina* under artificially seed and soil inoculated conditions in the field. Among fifty-two genotypes/varieties, only 2 genotypes/varieties were found to be completely resistant (Pusa 0871 and SML 1839), 1 moderately resistant against dry root rot disease.

De araujo et al. (2022) evaluated the response of 100 cowpea lines to two isolates of *M. phaseolina* using toothpick method. Out of the evaluated accessories, 15% of the lines were resistant to isolate 59 and 11 % of the lines were resistant to isolate CMM 2106.

Elmerich et al. (2022) examined *M. phaseolina* associated with charcoal rot in black gram and screened 41 black gram genotypes for charcoal rot resistance in South Asia and identified two genotypes, CO-5 and IPU 07-3, with charcoal rot resistance (disease scores: ≤3) and 18 genotypes with moderate resistance (disease scores >3 to ≤5).

Lamini et al. (2022) screened cowpea lines using molecular technology to identify potential sources of resistance to the Macrophomina root rot disease (MRRD). A sick pot evaluation method comprinsing 49 cowpea lines indicated that 10 lines were resistant to MRRD. In this study, a selection of eight resistant lines (Suvita 2, Abagbaala, IT99K573-1-1, IT93K-503-1-1, Hewale, AV2 3224, Nhyira and T2T4) and a susceptible check (Songotra) were evaluated against 10 isolates of *M. phaseolina* using a sick pot method. Results showed that the highly resistant lines based on the 1–9 reaction scale were Suvita 2, Apagbaala, 1793K-503-1 and IT99K573-1-1, with disease severity scores of 1.0, 1.8, 2.2 and 2.6, respectively. Hewale, AV2 3224, Nhyira and T2T4 were found to be resistant lines against MRRD.

Sasode et al. (2023) screened nineteen cowpea genotypes against *M. phaseolina*. In this study, none of the entries were found free from dry root rot. Eleven entries *viz.,* GC-3, TPTC-29, RC 10, KBC-9, Pant Lobia-3, Pant Lobia-4, MC 17-2 (KBC-12), CPD-313, PGCP-69, GC-1602, PGCP-70 were found moderate resistant.

2.2.2 Studies on biochemical alteration in cowpea post inoculation with *M. phaseolina*

Ramanathan et al. (2001) isolated an elicitor from *M. phaseolina*, in greengram. Suspension-cultured cells of greengram were established which responded to the fungal elicitor. When greengram leaves were inoculated with *M. phaseolina* two new peroxidases appeared. Similarly, two new peroxidases

could be detected in suspension-cultured greengram cells when treated with the fungal elicitor. These peroxidases were purified by column chromatography and their molecular masses were 27 and 38 kDa. The new peroxidases detected in both leaves and cultured cells appear to be similar with the same molecular weights.

Mehta (2004) revealed that the protein content and peroxidase activity increased in healthy and diseased leaves of three green gram genotypes *viz*., K 851, RMG 268 and MUM 2 having separate different levels of resistance. K 851 was highly susceptible, RMG 268 was moderately susceptible, and MUM 2 was moderately resistant in this study.

Sharma et al. (2011) revealed that maximum accumulation of phenolic acids in *M. phaseolina* infected guar plants than in uninoculated plants after 120 hours of infection in all the genotypes. The maximum accumulation of phenolic acids was 23 per cent higher than the control.

Srinivas (2016) observed that plants grown in *R. bataticola* inoculated soil had more phenol content (2.75 mg/100 mg of fresh weight) than plants grown in uninoculated soil (2.17 mg/100 mg of fresh weight) in chickpea crop. The highly susceptible genotypes showed lower concentration of total phenol than others.

Shoaib et al. (2018) an experiment conducted in potted soil to screen out soybean genotypes against *M. phaseolina*. The genotypes AJMERI, PSC-60, RAWAL-I, NARC-II and NARC-I were artificially inoculated with the pathogen kept in a completely randomized designed in triplicate for 45 days. Based on disease severity, genotypes were categorized into four groups *i.e*. resistant (AJMERI), moderately resistant (PSC-60 and RAWAL-I), susceptible (NARC-II) and highly susceptible (NARC-I). The infection of pathogen led to many morphological and biochemical changes including reduction in growth attributes, upregulation in total phenolics and activities of the antioxidant enzymes (peroxidase, polyphenol oxidase and catalase) in susceptible groups but not in resistant groups. Therefore, the growth inhibition index was significantly increased in susceptible genotypes.

Shruti et al. (2018) studied the biochemical basis of resistance of three (two susceptible RMO-40, CZM-3 and one resistant FMM-96) varieties of mothbean inoculated against *M. phaseolina*. The polyphenol, flavonoid, PAL, Total protein content, proline content and oxidative enzymes such as peroxidase, catalase and superoxide dismutase and hydrogen peroxide were recorded at different time intervals after pathogen inoculation. The increase in production of these biochemical parameters was higher in the inoculated plants compared to control plants and their response in the resistant cultivar was faster and higher than in the susceptible cultivars. Resistant variety FMM-96 exhibited maximum increase in polyphenol, flavonoid, PAL, Total protein content, proline content, peroxidase, catalase and superoxide dismutase and hydrogen peroxide.

Chavan et al. (2019) recorded that highest protein content in the soybean genotype AMS MB 5-19 (43.22 g/100 g) and the lowest level was recorded in TAMS-38 (31.31 g/100 g) genotype. The highest proline was found in AMS MB 5-19 (24.42 g/100 g) and lowest amount of proline was found in AMS 475 (21.42 g/100 g).

Kumar et al. (2019) studies the effect of total sugar, protein and phenolic content in mungbean root rot caused by *M. phaseolina*. The total sugar, reducing sugar, non-reducing sugar and soluble protein were higher in healthy roots as compared to diseased roots in all tested varieties *i.e.,* SML-668, MH-2-15 and IPM-02-03. The maximum reduction in these parameters was found in SML-668 followed by MH-2-15 while total phenol content was higher in diseased roots as compared to healthy tissues of all the tested varieties. The maximum increase in total phenol was observed in diseased roots of SML-668 followed by MH-2-15.

Monaim et al. (2019) studied the activation of peroxidase and phenolic contents in plants inoculated with *M. phaseolina*. Total phenols and peroxidase increased in cowpea plants inoculated with *M. phaseolina*. The highest accumulation of phenols and peroxidase was recorded 6 and 8 days after the application, respectively.

2.3 Management of charcoal rot of cowpea under field condition using novel strategies

Shahina et al. (2001) reported that seed treatment of carbendazim 0.2% significantly reduced incidence of root rot caused by *M. phaseolina* in cowpea by 58.9%.

Prajapati et al. (2002) tested the efficacy of 11 fungicides and 2 biological control agents against *M. phaseolina* in chickpea and found that carbendazim, carbendazim + thiram, carboxin and Topsin-M completely inhibited the growth of *M. phaseolina*.

Kumar and Jain (2004) found that seeds treated with Carbendazim, Tebuconazole, Thiophanate-methyl and Thiram were effective against blight and root rot diseases caused by *M. phaseolina* in clusterbean.

Rathore (2006) tested seed treatment and foliar spray of fungicides *viz*., carbendazim, topsin M-70, captan, thiram, mancozeb, copper oxychloride against *M. phaseolina* and leaf spots in greengram and observed that seed treatment with carbendazim (@ 2 g/kg seeds) was most effective as it recorded the minimum disease incidence of root rot (14%), thiophanate methyl WP 2 g/kg seed treatment was at par with carbendazim. Untreated plots recorded maximum disease incidence (35%).

Lokesha and Benagi (2007) studied biological management of *M. phaseolina* causing dry root rot of pigeon pea and reported that maximum percent inhibition by *Trichoderma virens* (78.22%), followed by *Pseudomonas fluorescens* (76.66%), *Bacillus subtilis* (74.11%).

Konde et al. (2008) reported that the seed treatment of carbendazim + thiram (1+2 g/kg) was most effective in reducing root / collar rot of soybean caused by *M. phaseolina* in field and pot studies. This was followed by thiram (3 g/kg), carbendazim (1 g/kg) with soil application of *T. viride* + *T. harzianum* (1+1 kg/ha).

Jaiman and Jain (2010) tested efficacy of six different fungicides *viz*., carbendazim, tebuconazole, thiophanate methyl, captan, mancozeb and

thiram under field condition against charcoal rot in cluster bean. The highest mycelial growth inhibition was found in carbendazim 50% WP followed by thiophanate methyl 70% WP.

Manjunatha et al. (2011) observed that the application of biocontrol agents individually either through soil application or seed treatment showed good germination with reduced mortality due to dry root rot of chickpea as compared to treatments. Among bioagents, *T. viride* (Tv-R) isolate showed maximum inhibition of mycelial growth 78.50% followed by *T. viride* (Tv-32) with inhibition of 74.92%.

Deepthi et al. (2014) evaluated different fungicides under *in vivo* and *in vitro* conditions against *M. phaseolina* in which carboxin + thiram and penflufen gave 100% inhibition at 500 ppm while tricyclazole gave 100% inhibition at 1000 ppm. The seed treatment with vitavax power gave highest seed germination percentage and reduced seedling mortality and lower yield losses. The vitavax power as seed treatment along with one foliar application of carbendazim was found most effective for enhancing increasing seed germination and reducing pre, post emergence mortality and lowering losses in yield of sesame.

Kaithikeyan et al. (2015) found that among the *Trichoderma spp*. tested against dry root rot of black gram, *Trichoderma viride* exhibited strong inhibition of the growth (77.77%) against *M. phaseolina*. Seed treatment of *T. viride* recorded the minimum root rot incidences (21.4%) followed by *T. harzianum* (26.6%).

Gupta et al. (2018) observed that seed treatment with *T. viride* (5g/kg seed and soil application of *T. viride* @ 2.5 kg/ha was found effective and economical for the management of Macrophomina root /stem rot of sesame.

Khan et al. (2019) studied charcoal rot complex caused by *M. phaseolina* in mungbean by *Trichoderma* spp. under field conditions. Effectiveness of *Trichoderma harzianum*, *T. hamatum*, *T. viride*, *T. polysporum* and *T. asperallum* against charcoal rot *M. phaseolina* was evaluated *in vitro* and *in vivo* and reports that farmyard manure colonized by *T. harzianum*, *T.*

hamatum and *T. viride* was applied in the soil at 250 kg/ha (50 g/microplot) under field condition soil application of *T. viride* or *T. harzianum* effectively controlled the root-rot disease complex (5–15%) and improved the grain yield of mungbean (14–19%) in diseased plots.

Thombre and Kohire (2018) evaluated different fungicides, bioagents and phytoextracts in pot and field conditions against Macrophomina blight of mungbean. Among the foliar sprays under pot condition, carbendazim 12 WP + mancozeb 63 WP (@ 0.2%) recorded significantly higher incidence and intensity 82.35 and 85.18%, respectively, followed by @ 0.1% carbendazim (75.00 and 78.61%). The foliar sprays of carbendazim 12 WP + mancozeb 63 WP (@ 0.2%) recorded significantly least mean Macrophomina blight incidence (14.63 and 17.29%) and intensity (11.87 and 13.82%) and significantly highest mean disease reduction (67.07 and 69.17%), with significantly highest seed yield (539.63 and 535.60 kg/ha) over unsprayed control (yield 358.31 and 351.58 kg/ha) during 2011-12 and 2012-13 crop seasons, respectively.

Iqbal and Mukhtar (2020) evaluated nine fungicides *in vitro* and *in vivo* conditions effectiveness against *M. phaseolina*. All the fungicides caused significant inhibition of the fungus over control in green gram and blackgram. Maximum individual inhibition of growth of the fungus was recorded with benomyl (83.89%) followed by carbendazim (79.11%).

Santosh et al. (2023) studied the efficacy of different fungicides in fenugreek by *in vivo* and *in vitro* experiment against charcoal rot. Among all the tested fungicides, tebuconazole 50% + trifloxystrobin 25% WG was found most effective in controlling the mycelium growth of pathogen. In field condition, it gave maximum disease control (85.72%) with highest grain yield (19.83 q/ha) when applied as seed treatment with tebuconazole 50% + trifloxystrobin 25% WG @ 1.5 g/kg seed + soil application of *T. harzianum* @ 10 kg/ha + SD with tebuconazole 50% + trifloxystrobin 25% WG.

Malagi *et al*. (2024) reported that tebuconazole 50% + trifloxystrobin 25% WG as seed treatment @ 1.5 g/kg along with *T. harzianum* @ 10 kg/ha as soil application gave maximum (83.76%) disease control in chickpea under field conditions.

Chapter - 3

MATERIALS AND METHODS

The current research titled “Comprehensive Studies and Management of Charcoal Rot of Cowpea Caused by *Macrophomina phaseolina* (Tassi) Goid.” was conducted at the RARI Farm and the Department of Plant Pathology, Rajasthan Agricultural Research Institute, Jaipur, during the *Kharif* season of 2024-25. This chapter outlines the materials utilized and the methodologies followed throughout the investigation.

3.1 Morphological and molecular characterization of pathogen associated with charcoal rot of cowpea

3.1.1 Collection of diseased samples

Diseased plant samples were collected from the RARI farm, AINP trials on cowpea during summer 2024 and subsequently transported to the Department of Plant Pathology laboratory, RARI, Jaipur for detailed investigation and further research.

3.1.2 Isolation and purification of pathogen

Prior to the isolation and subsequent laboratory experiments, all glasswares were thoroughly cleaned with a potassium dichromate ($K_2Cr_2O_7$) and sulfuric acid (H_2SO_4) followed by rinsing with sterile distilled water and then sterilized in a hot air oven at 180°C for a duration of two hours. The culture medium Potato Dextrose Agar (PDA) was sterilized by autoclaving at 15 psi pressure for 15 minutes.

Pathogen isolation was carried out using infected roots of cowpea plants exhibiting characteristic disease symptoms by hyphal tip method. The infected root pieces were surface sterilized using 0.1% sodium hypochlorite solution for one minute followed by sequential rinsing with sterile distilled water for one minute to eliminate any residual chemicals. The sterilized pieces were then placed on PDA in sterile Petri plates which were incubated at temperature (27°C). Following 48 hours of incubation, the advancing hyphal tips of the growing mycelium were observed under a light microscope and

marked on the base of the Petri plates. Hyphal tips from the colony margins were excised using a sterilized 2 mm cork borer and transferred to fresh Petri plates containing PDA. All purified cultures were stored on PDA slants at 4 ± 1°C for further experimental use.

3.1.3 Morphological characterization

The fungal isolates were identified as *M. phaseolina* based on the morphological features of the mycelium and sclerotia. To study the morphological traits of fungal pathogen isolated from decayed roots, seven-day-old cultures cultivated on PDA and incubated at 27 ± 2°C were utilized. Aseptic preparation of slides was carried out in a laminar air flow chamber and the samples were observed under a compound microscope. Morphological characteristics such as hyphal branching, septation as well as the structure, shape and length of sclerotia were examined under 10X and 40X magnifications.

3.1.4 Molecular characterization

3.1.4.1 DNA extraction of pathogen

Genomic DNA was isolated from mycelial mat, showing typical disease symptoms, collected from RARI Farm, Durgapura. The extraction was carried out using a modified CTAB (Cetyl Trimethyl Ammonium Bromide) method as described by Amer et al. (2011). The detailed protocol is as follows:

The mycelial mat grown on Potato Dextrose Broth (PDB) was grinded into a fine powder using a mortar and pestle in the presence of CTAB buffer. The homogenized tissue was then transferred into properly labeled 2.0 ml centrifuge tubes.

1. The tubes were placed in a water bath at 65°C for 40 minutes, with gentle inversion every 10 minutes to ensure uniform mixing during incubation.
2. Post incubation, 800 µl of a phenol: chloroform: isoamyl alcohol solution (25:24:1) was added to each tube and the contents were gently inverted to form a stable emulsion.

3. The tubes were then positioned on a rocker shaker and agitated for 15 to 20 minutes to enhance phase separation.
4. Following mixing, the samples were centrifuged at 10,000 rpm for 10 minutes using an Eppendorf 5820 centrifuge (Eppendorf, Germany).
5. The clear supernatant was gently transferred using a micropipette into a freshly labeled 1.5 ml microcentrifuge tube.
6. To this, 600-700 µl of pre-chilled isopropanol was added and mixed thoroughly. The mixture was then stored at –20°C for approximately two hours to facilitate DNA precipitation.
7. After incubation, the samples were centrifuged at 10,000 rpm for 10 minutes. The supernatant was carefully removed, retaining the DNA pellet.
8. The pellet was washed with 500 µl of 70% ethanol and again centrifuged at 13,000 rpm for 3 minutes. The ethanol was cautiously decanted to avoid disturbing the pellet.
9. The DNA pellet was left to air dry at room temperature and was subsequently dissolved in 100 µl of Tris-EDTA (TE) buffer for further use.

3.1.4.2 Quantitative and Qualitative measurement of nucleic acid

The optical surfaces of the spectrophotometer (Thermo Scientific NanoDrop™ 1000) were cleaned by applying 2 µl of distilled water to the measurement surface. The lever arm was then closed and gently tapped several times to ensure the upper surface was also rinsed. Both optical surfaces were subsequently wiped dry using clean tissue paper.

- The NanoDrop software version 3.8.1 was clicked to open and the “Nucleic Acid” measurement mode was selected.
- The spectrophotometer was initialized by placing 2 µl of distilled water onto the lower optical surface and selecting the “Initialize” function within the software.

- A blank reading was taken using 2 µl of TE buffer to calibrate the instrument.
- For DNA quantification, the upper optical surface was cleaned using a lint-free tissue, 2 µl of DNA sample was applied and the “Measure” button was selected. DNA purity was assessed using the 260/280 absorbance ratio, with pure DNA typically showing values between 1.80 and 1.90.

3.1.4.3 Polymerase Chain Reaction

The DNA extracted using the CTAB method was utilized as a template for performing PCR amplification reactions. The primers were ITS1 and ITS 4 (White et al. 1990). The amplification profile was as follows, initial denaturation of 95°C for 4 min, 35 cycles each of denaturation- 94°C (50 sec), annealing 58°C (45 sec), elongation 72 °C- 90 sec, followed by final elongation of 72 °C for 10 min and hold at 4°C. The component of PCR reaction mixture was added as per Table 3.1. The expected amplicon size 600-900 bp.

Table 3.1: PCR Reaction mixture

Component	**Conc Used**	**Volume**
Emerald Amp GT PCR Master Mix (2X Premix) (Takara, Japan)	1X	12.5µl
Forward Primer (100 pmol/ µl)	20 pmol/ µl	2.0 µl
Reverse Primer (100 pmol/ µl)	20 pmol/ µl	2.0 µl
DNA Sample		2.0 µl
Nuclease Free Water		6.5 µl
Total		20 µl

3.1.4.4 Analysis of PCR amplified products

Buffers and chemicals used

TAE buffer (Tris-Acetic Acid-EDTA), pH 8.3 (50X Stock)

Tris Base	242 g
Glacial Acetic Acid	57.1 ml
EDTA, pH 8.0	37.2 g
Distilled Water	1000 ml

Preparation of TAE

A solution was prepared by dissolving 242.0 g of Tris base and 37.2 g of EDTA in 1000 ml of distilled water followed by the addition of 57.1 ml of glacial acetic acid. The mixture was stirred thoroughly until all components were completely dissolved. The pH was adjusted to 8.3 and the solution was sterilized through autoclaving and stored at room temperature for further use.

Ethidium Bromide (10 mg/ml)

An amount of 0.5 mg of ethidium bromide was dissolved in 1 ml of distilled water and stored in a dark-colored bottle at 4°C to protect it from light.

Agarose gel

Agarose gel was prepared by dissolving 1.0 g of agarose powder (Himedia, India) in 100 ml of 1X TAE buffer. The mixture was heated in a microwave until it fully melted. Once dissolved, the solution was cooled under running tap water until it reached a lukewarm temperature. At this stage, 3 µl of ethidium bromide (10 mg/ml) was added. The molten gel was then poured into a casting tray and allowed to solidify before use.

Protocol for Agarose gel preparation

- The gel casting apparatus was thoroughly cleaned, and the frame was set on a level surface to ensure even gel formation. A comb was carefully inserted approximately 2 mm above the base, aligned parallel to the open edge of the casting tray.
- The slightly cooled agarose solution containing ethidium bromide (3 µl per 100 ml) was gently poured into the mold and left undisturbed until the gel solidified.
- Once the gel had set, it was transferred to the electrophoresis chamber with the wells oriented toward the cathode. The gel tank was then filled with 1X TAE buffer until the gel surface was fully submerged.
- The combs were gently removed, and the PCR-amplified samples were loaded into the wells of the submerged agarose gel using a suitable micropipette.

- 3 µl of 100 bp DNA ladder (Himedia, India) was also loaded to serve as a reference.
- The gel electrophoresis unit was connected to a power supply and the run was performed at 100 mA and 200 volts for one hour.
- Following electrophoresis, the DNA bands were visualized and recorded using UV transilluminator (Himedia, India). The amplified products were then sent to Barcode Biosciences, Bangalore for sequencing. The resulting genome sequences were obtained and utilized for further analysis.

3.1.4.5 Analysis of sequence

The obtained partial forward and reverse nucleotide sequences were assembled into a single contig using Bioedit V 7.2 software. The assembled contig was then used as a query sequence for homology search in the GenBank database. This was done using the BLASTn (Basic Local Alignment Search Tool – nucleotide) program, with the query uploaded in FASTA format. Under the ‘Choose Search Set’ section, the nucleotide collection (nr/nt) database was selected for analysis. The 'mega blast' option, optimized for detecting highly similar sequences was chosen for the BLAST search and the query nucleotide sequence was submitted using the default algorithm settings. Sequences with a query coverage of 98–99%, an E-value of 0.0 or lower and a maximum identity exceeding 95% were selected. These high-confidence matches were downloaded in FASTA format and subsequently utilized for phylogenetic analysis and comparative studies.

Multiple sequence alignment (MSA) was carried out using the ClustalW algorithm (Thompson et al. 1994) integrated within the Molecular Evolutionary Genetics Analysis software (MEGA version 12) (Kumar et al. 2024). The FASTA file containing the query sequence along with the most closely related sequences was imported into MEGA V 12.0. From the main interface, the Alignment Explorer was accessed and the ‘Align’ option was selected. The alignment was initiated by clicking the ClustalW icon (denoted by a capital 'W') and the process was run using default parameters. The resulting aligned

sequences were displayed in the Alignment Explorer window and saved for subsequent phylogenetic analysis. For constructing the phylogenetic tree, the neighbour joint tree method was employed. The final tree was generated and exported in JPG format for documentation and interpretation. Finally, sequence was submitted to NCBI (https://www.ncbi.nlm.nih.gov) and accession number was retrieved.

3.1.5 Pathogenicity test

To establish the pathogenicity, the isolated and purified *M. phaseolina* was propagated on sterilized sorghum grains. The cowpea germplasm against *M. phaseolina* was tested using soil inoculation methods. First, soaked sorghum in a bucket (20 L) overnight. Next morning, extract the extra water from the sorghum and fill 200-250 g in Polypropylene (PP) bag. After that put a rubber pipe in it and pack it with an aluminium sheet and rubber band. Then, autoclaved at 15 psi pressure for 15 minutes and when it cools down, inoculate it with mycelium bit. After optimum growth (16 days), soil was mixed with inoculated sorghum and applied in the pots before sowing.

Soil used in the experiments was collected from the field and sterilized by autoclaving at 1.05 kg/cm² pressure for 30 minutes over three successive days. The fungal inoculum, cultured on sorghum grains was incorporated into pots (24 cm diameter). Prior to filling, the pots were disinfected using a 2% formalin solution to ensure aseptic conditions. The pathogen inoculum was mixed into the soil. Pots without fungal inoculum served as untreated controls. Disease symptoms were monitored after sowing. Plants exhibiting charcoal rot symptoms were collected and the pathogen was re-isolated and compared with the original culture to confirm the Koch’s postulates.

3.2 Screening of varieties and biochemical evaluation of susceptible and resistant cultivars under artificial inoculation conditions

3.2.1 Inoculation of pathogen in field

The pathogen was mass multiplied on sterilized sorghum using methodology followed in section 3.1.5. After optimum growth (16 days), soil was mixed with inoculated sorghum and applied in the furrows of each entry before sowing.

3.2.2 Screening of cowpea germplasm against charcoal rot disease

The total of thirty-five cowpea entries lines sourced from RARI, Durgapura were assessed for their response to charcoal rot under natural field conditions during the *Kharif* season of 2024-25. The seeds were rinsed thoroughly with sterile water before sowing. For each entry/variety, seeds were sown per plot with three replications as per Tabe 3.2. Observations on disease incidence recorded 30 days after sowing (DAS) and continued up to 75 days. Based on the recorded data, PDI was calculated as per Wheeler (1969) and the entries were categorized for their reaction to the disease as per Table 3.3.

Conduction of performance trial	:	*Kharif* season 2024-25
Date of sowing	:	19 July 2024
Number of entries	:	35
Design	:	Randomized Block Design (RBD)
Replication	:	3
Spacing	:	45 cm x 15 cm

Table 3.2: List of different entries screened against charcoal rot of cowpea

S. No.	Entries/Varieties	S. No.	Entries/Varieties
1	CPD 334	19	CPD 229
2	CPD 324	20	CPD 269
3	CPD 305	21	CPD 330
4	CPD 302	22	CPD 317
5	CPD 265	23	CPD 343
6	CPD 333	24	CPD 288
7	CPD 332	25	CPD 119
8	CPD 336	26	CPD 273
9	CPD 335	27	RC 19R
10	CPD 347	28	CPD 260
11	CPD 307	29	CPD 261
12	CPD 249	30	CPD 286
13	CPD 276	31	CPD 290
14	CPD 315	32	CPD 279
15	CPD 348	33	CPD 268
16	RC 19BR	34	CPD 345
17	CPD 254	35	CPD 320
18	RC 19		

Table 3.3: Disease scale used for categorization of cowpea entries/varieties based on PDI against *M. phaseolina* (Anonymous 2024-25)

S. No.	PDI	Reaction	Designation
1	0	Free	F
2	0.01-10.0%	Resistant (R)	R
3	10.1-20.0%	Moderately Resistant (MR)	MR
4	20.1-30.0%	Moderately Susceptible (MS)	MS
5	30.1-50.0%	Susceptible (S)	S
6	Above 50.01%	Highly Susceptible (HS)	HS

3.2.3 Studies on biochemical alteration in cowpea post inoculation with *M. phaseolina*

For biochemical analyses, cowpea plants were grown in pots and leaf tissues from 30-days-old seedlings were utilized for different biochemical assays.

Plant material and growth condition

Biochemical analyses were carried out using four cowpea entries that ranged from highly susceptible to moderately resistant, showing varying degrees of response to *M. phaseolina* infection. The seeds were sown in pots filled with steam-sterilized soil and kept in a controlled growth chamber with a 14-hour light and 10-hour dark cycle, maintained at a temperature of 28±2°C and 60% relative humidity. The levels of phenol, proline and total antioxidant enzyme activity were assessed at 0, 24, 48, 72 and 96 hours after inoculation with 30-days-old cowpea plants with pathogen

Preparation of spore suspension and pathogen inoculation

The fungal strain isolated and characterized during this study (NCBI accession no. PQ399952) was activated on PDB under proper aseptic conditions in the laminar flow. Five mm mycelial Bits were added in the flasks and were incubated in BOD (27˚C) for 7 days. The cowpea leaves were slightly injured with sandpaper to facilitate the entry of test pathogen on 30 days after sowing (DAS). Then, the mycelium mat along with surrounding liquid media was swabbed on injured leaves with cotton. Meanwhile, liquid media was also added to the soil. After inoculation, leaf samples were collected from 30-days old, inoculated plants at 0, 24, 48, 47 and 96 hpi. The control plants of each entry were maintained and leaf samples were collected at same duration for cross comparison (Shruti et al. 2018). For sample collection, first the leaf portion was removed with the help of sterilized scissors and sheath portion was carefully collected in aluminium foil and later the aluminium foil was wrapped and dipped liquid nitrogen to stop the enzyme activity. The samples were brought to lab, sorted and stored in -80 °C freezer. Labelling was done for the proper identification of samples during biochemical analysis.

3.2.3.1 Estimation of Phenol

Total phenol content was determined following the procedure outlined by Thimmaiah (1999).

A one-gram sample of cowpea leaves were ground using a mortar and pestle in 10 ml of 80% ethanol. The resulting mixture was centrifuged at 10,000 rpm for 20 minutes. The supernatant was collected after filtration while the residue was subjected to a second extraction using five times the volume of 80% ethanol. The combined supernatants were cooled and evaporated to dryness in a water bath. The remaining residue was then dissolved in 5 ml of distilled water. From this solution, 0.2 ml was taken into a test tube, and the volume was adjusted to 3 ml with distilled water. Subsequently, 0.5 ml of Folin-Ciocalteu reagent was added. After waiting for three minutes, 2 ml of 20% sodium carbonate solution was added, and the mixture was thoroughly mixed. The test tubes were then heated in boiling water for one minute. After cooling, absorbance was measured at 650 nm using a reagent blank for comparison. A standard curve was generated using various concentrations of catechol and the phenolic content was calculated and expressed as mg GAE/g of fresh tissue.

3.2.3.2 Estimation of Proline

The estimation of proline content was carried out following the procedure outlined by Bates et al. (1973).

Fresh leaf samples weighing 100 mg were measured using an electric balance and ground in a mortar with 5 ml of 3% aqueous sulfosalicylic acid. The homogenate was centrifuged at 5000 rpm for 5 minutes and the aqueous layer was collected. The volume was then adjusted to 10 ml using additional sulfosalicylic acid. For proline estimation, 2 ml of the extract was transferred into a test tube. Simultaneously, a standard proline solution was prepared by dissolving 10 mg of proline in 100 ml of 3% sulfosalicylic acid and test tubes were prepared with varying volumes (0.2, 0.4, 0.6, 0.8 and 1.0 ml) of this solution. A test tube containing 2 ml of sulfosalicylic acid alone was used as a blank.

Each test tube was supplemented with 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent (prepared by dissolving 1.25 g of ninhydrin in 20 ml of 6N ortho-phosphoric acid), then incubated in a boiling water bath at 100°C for 10 minutes. After incubation, the tubes were immediately transferred to an ice bucket to halt the reaction. Following this, 4 ml of toluene was added to each tube and the mixture was vortexed thoroughly for 10 seconds. The optical density was measured at 520 nm using a UV-Visible spectrophotometer with a blank reagent as the reference. The amount of free proline in 100 mg of stem tissue was determined using a standard curve. The absorbance of the blank was subtracted from that of the samples. Proline concentration was calculated using the standard curve developed with L-Proline and was expressed as µmol/g fresh weight.

3.2.3.3 Estimation of Total Antioxidant Enzymes Activity

The total antioxidant enzyme activity was determined following the procedure outlined by Maheswari et al. (2024).

One gram of finely powdered leaf material was soaked overnight in 15 ml of 70% acetone. The mixture was then centrifuged at 3000 rpm for 10 minutes and the resulting supernatant was collected. In a test tube, 1 ml each cupric chloride, ammonium acetate buffer and neocuproine were added. Then 100 µl of the supernatant was introduced and the total volume was adjusted to 4.1 ml using distilled water. The tubes were kept in the dark for 30 minutes after which absorbance was measured at 450 nm against a reagent blank. The antioxidant activity was then calculated accordingly.

$$\mu mol\ TE/g = (A_f/E_{TR})\ (V_F/V_S)\ r\ (V_{initial}/m)$$

$V_{initial}$ = Initial volume, M = Weight of sample, R = Dilution factor, V_f = Final volume, V_S = Volume of aliquot, A_f = Absorbance, $E_{TR} = 1.67*10^4$ 1-1/mol-1/cm

3.3 Management of charcoal rot of cowpea under field condition using novel strategies

A field experiment was laid out using an RBD with three replications in plots measuring 4 m x 2 m at the RARI Farm, RARI College, during the *Kharif* season of 2024. Cowpea seeds (variety RC-101) were treated with seed dressers and bioagent a fungicide for 30 minutes and then air-dried in the shade (Table 3.4). Seeds without fungicide treatment, grown under the same conditions served as control. At sowing, inoculum cultured on sorghum grains @ 20 g was applied in each row and 100 g in a plot as per section 3.1.5. Standard agronomic practices were followed for crop cultivation. Sowing was carried out in the third week of July.

Experimental details:

(a) Location	:	Research farm, Rajasthan Agricultural Research Institute, Durgapura
(b) Crop and variety	:	Cowpea, RC 101
(c) Year and season	:	*Kharif*, 2024
(d) Experimental design	:	Randomized Block Design
(e) Plot size	:	4 m x 2 m
(f) Spacing	:	45 cm x 15 cm
(g) Replication	:	3
(h) Treatment	:	8

Table 3.4: Details of fungicides using *in vivo* condition against *M. phaseolina*:

Treatment No.	Treatment Details	Dose
T1	Seed treatment with Carboxin 37.5% + Thiram 37.5% WP	3.0 g/kg seed
T2	Seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% + Thiamethoxam 25% FS	10.0 ml/kg seed
T3	Seed treatment with Thiophanate Methyl 45% + Pyraclostrobin 5% FS	2.0 ml/kg seed
T4	Seed treatment with Penflufen 154 + Trifloxystrobin 154 FS (13.28% w/w + 13.28% w/w)	1.0 ml/kg seed
T5	Seed treatment with Fluxapyroxad 33.3% w/v	1.0 ml/kg seed
T6	Seed treatment with *Trichoderma* strain (RARI) mixed with jaggery (PQ409413)	10.0 g/kg seed
T7	Seed treatment with Karan Narendra Trichoderma (KNT) mixed with jaggery	10.0 g/kg seed
T8	Control	

Observations recorded

1. Per cent disease incidence
2. Grain yield
3. B:C ratio

Calculation

$$\text{Per cent Disease Incidence (\%)} = \frac{\text{Number of diseased plants}}{\text{Total no. of plants}} \times 100$$

$$\text{Disease control (\%)} = \frac{\text{Disease incidence in inoculated control (\%)} - \text{Disease incidence in treatment (\%)}}{\text{Disease incidence in inoculated control (\%)}} \times 100$$

$$\text{Increase in yield over control (\%)} = \frac{\text{Yield of plants in treatment (\%)} - \text{Yield of plants in inoculated control (\%)}}{\text{Yield of plants in inoculated control}} \times 100$$

Statistical analysis was done using OPSTAT (Sheoran et al. 2020).

Chapter - 4

EXPERIMENTAL RESULTS

The experiments were carried out as per the planned methodology, using suitable statistical designs and standardized procedures. Observations were recorded at scheduled intervals, adhering to the appropriate rating scale.

4.1 Morphological and molecular characterization of pathogen associated with charcoal rot of cowpea

4.1.1 Collection, Isolation and Purification of pathogen

The infected cowpea root samples obtained from AINP on Arid legume were aseptically transferred onto PDA medium. Following fungal isolation, the inoculated PDA plates were incubated leading to profuse fungal growth within seven days. Initially, the culture exhibited white mycelial growth which gradually turned brownish black as it matured. After 10-15 days post-inoculation, the fungus produced hard, black sclerotia, primarily at the colony margins. The culture was subsequently purified using the hyphal tip method.

Identification of the pathogen was based on the morphological characteristics of its mycelium and sclerotia. The mycelium was hyaline and septate, while the sclerotia appeared brown to black. On PDA medium, numerous black sclerotia developed within 5 to 6 days. A pathogenicity test confirmed the identity of the isolate as *Macrophomina phaseolina*. Based on these morphological characteristics, the fungal isolate was identified as *M. phaseolina* (pycnidial stage of *Rhizoctonia bataticola*).

4.1.2 Pathogenicity

Pathogenicity of *M. phaseolina* was tested under pot conditions by soil inoculation technique. These pots were kept in cage house and when required were regularly watered and maintained carefully. Initial symptoms include yellowing of the leaves, which is soon followed by drooping within 2–3 days and complete plant withering shortly thereafter. Typically, the entire plant collapses within a week of symptom appearance. A closer examination reveals dark lesions on the stem near the soil surface. When such plants are

uprooted, the basal stem and main root exhibit dry rot symptoms, with tissues becoming brittle and easily breakable (Plate 1). These symptoms were not observed in un-inoculated pots.

4.1.3 Morphological characterization

1. Mycelium

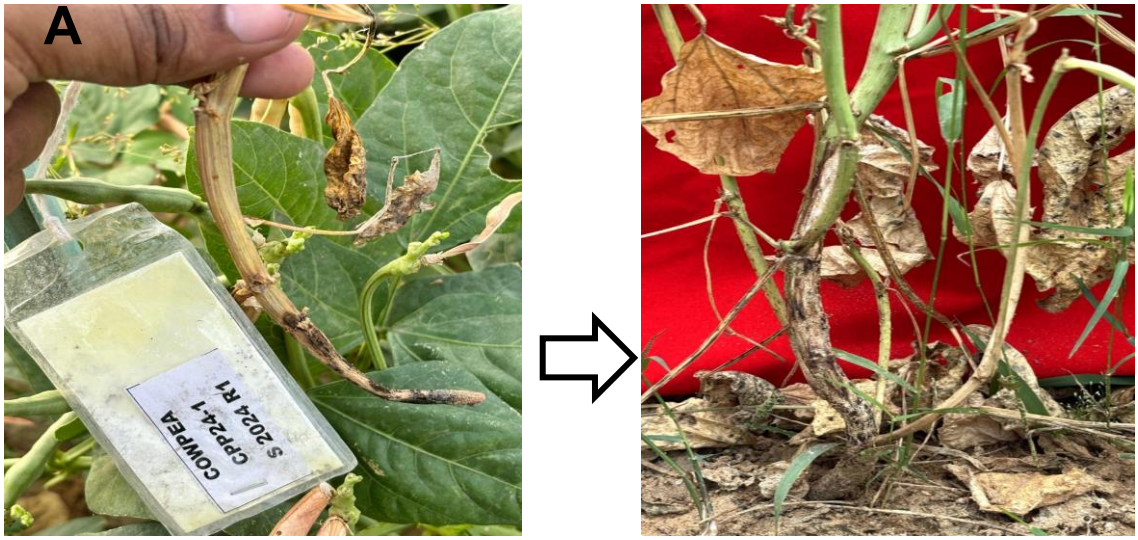
M. phaseolina was cultured on PDA medium and incubated at 27°C. Mycelial growth initiated within 24 hours of incubation. The mycelium initially appeared white, gradually turning grey within 48 hours due to the development of sclerotia. The hyphae were densely septate, thick-walled and blackish in color. Lateral hyphal branches emerged at right angles at 90° from the main hyphae (Plate 2).

2. Sclerotia

The sclerotia initially appeared brownish blackish in color and progressively turned completely black upon maturation. They exhibited variable shapes, ranging from irregular to spherical forms and 10-20 µm in length (Plate 2).

4.1.4 Molecular characterization

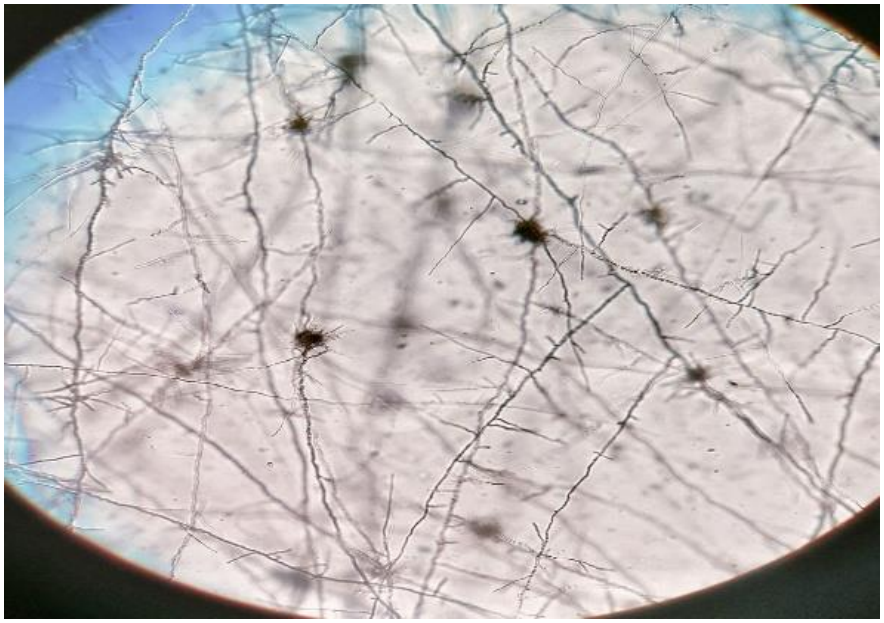
To confirm the presence of *M. phaseolina*, PCR was performed using universal primers. The samples yielded an expected size of amplicon (600-900 bp) with ITS1, 4 (Fig. 4.1). Phylogenetic analysis was performed on the partial genome sequence of *M. phaseolina* isolate M1 internal transcribed spacer 1 and full genome of 5.8S ribosomal RNA gene and internal transcribed spacer 2 (Fig. 4.2). Top ten hits of Blastn suite of NCBI of each sequence were downloaded and used for analysis (Table 4.1). The Blastn results revealed that the sequence of M1 shared highest similarity (99.14 per cent) with *M. phaseolina* isolate Mp31 from maize in Gurdaspur, Punjab, followed by *Macrophomina* sp. isolate Mavin 01 from sesamum in Chidambaram, Tamil Nadu (98.98 per cent) and *M. phaseolina* isolate Mp35 from chickpea in Ludhiana, Punjab (98.49 per cent). The sequence was submitted to NCBI and accession number of PQ399952.



Source of Infection **Result**

Plate B

1: Pathogenecity test of charcoal rot of cowpea against *M. phaseolina* (A) in field conditions and (B) in pot conditions



Morphological characters

Pure culture

Plate 2: Pure culture and morphological characteristic of *M. phaseolina*

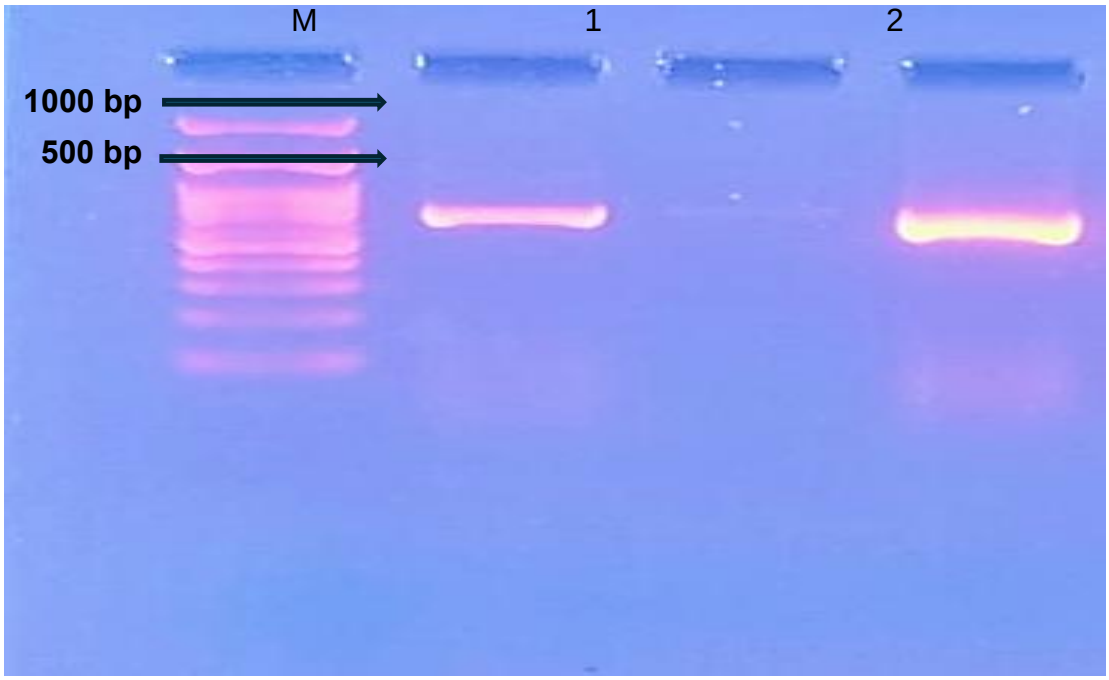


Fig. 4.1: PCR amplification of *Macrophomina phaseolina* using Fungus specific universal ITS 1 and ITS 4 primers (Lane M: 100 bp Ladder (Himedia), Lane 1-Sample M1, Lane 2- Negative control, Lane 3-Positive control)

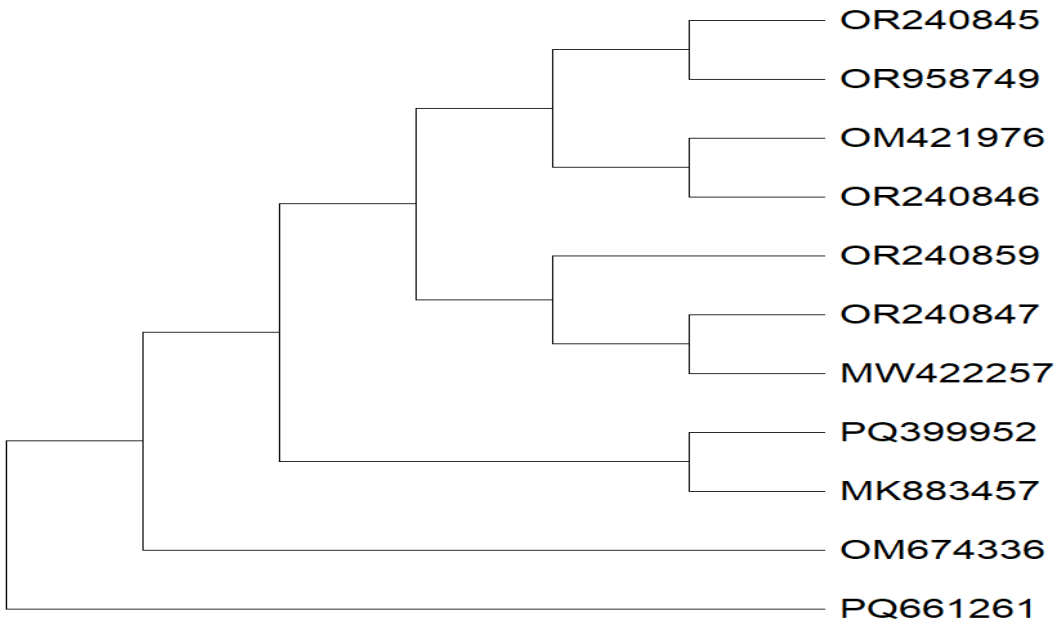


Fig. 4.2: Phylogenetic analysis using MEGA V 12.0

The sequence under study shared a close common ancestory with MK883457 forming a clade which is distantly related to *M. pseudophaseolina* isolate M1 (MW422257) isolated from lentil, *M. phaseolina* isolate Mp10 (OR240847) isolated from sesamum, *M. phaseolina* isolate Mp31 (OR240859) isolated from maize, *M. phaseolina* isolate Mp26 (OR240846) isolated from soybean, *M. pseudophaseolina* isolate CRb6 (OM421976) isolated from groundnut, *M. phaseolina* isolate Mp-II (OR958749) and *M. phseolina* isolate Mp35 (OR240845) isolated from chickpea. Among the sequences tested, PQ399952 was least related to *M. phaseolina* isolate Mp13 (OM674336) from chickpea. The result was also confirmed with the position of the outgroup (PQ661261), which was not found to be related to any of the sequence.

Table 4.1: Sequences of fungus retrieved from NCBI phylogenetic analysis

Accession No.	**Host**	**Name of the Fungus**	**% nucleotide identity**
OR240859	Maize	*Macrophomina phaseolina* isolate Mp31, Gurdaspur, Punjab	99.14
MK883457	Sesamum	*Macrophomina* sp. isolate Mavin 01, Chidambaram, Tamil Nadu	98.98
OR240845	Chickpea	*Macrophomina phaseolina* isolate Mp35, Ludhiana, Punjab	98.49
OR240846	Soybean	*Macrophomina phaseolina* isolate Mp26, Ludhiana, Punjab	98.00
OR958749	-	*Macrophomina phaseolina* isolate MP- II (S), CAZRI, Jodhpur	98.00
OM674336	Chickpea	*Macrophomina phaseolina* isolate MP13_Guntur2, Guntur, AP	97.84
OR240847	Sesamum	*Macrophomina phaseolina* isolate Mp10, Ropar, Punjab	97.70
OM421976	Groundnut	*Macrophomina pseudophaseolina* isolate CRb6, Tirupati, AP	97.55
MW422257	Lentil	*Macrophomina pseudophaseolina* isolate M1, Relizane, Algeria	97.39
PQ661261	Insect	*Metarhizium anisopliae isolate MZ-AR*	79.81

4.2 Screening of varieties and biochemical evaluation of susceptible and resistant cultivars under artificial inoculation conditions

4.2.1 Screening of cowpea germplasm against charcoal rot disease

A total of thirty-five cowpea entries including susceptible check RC 19, were evaluated under field conditions through artificial inoculation conditions at the Rajasthan Agricultural Research Institute, Durgapura. Disease incidence was recorded, and the entries were categorized accordingly. Among these entries, the lowest per cent disease incidence was recorded in CPD 307 (13.33%) and highest disease incidence in CPD 249 (94.67%) (Table 4.2). Observations recorded during *Kharif*-2024 indicated that none of the thirty-five cowpea entries exhibited complete free and resistance to charcoal rot disease. Among them, four entries CPD 307, CPD 347, CPD 332 and CPD 324 were classified as moderately resistant which showed disease incidence 13.33%, 14.67%, 16.00% and 16.00%, respectively. Whereas six moderately susceptible CPD 269, CPD 343, CPD 119, CPD 345, CPD 333 and CPD 336. Twelve entries CPD 334, CPD 302, CPD 265, CPD 335, CPD 276, CPD 348, CPD 288, CPD 273, CPD 261, CPD 286, CPD 279 and CPD 268 were found susceptible whereas, thirteen highly susceptible CPD 305, CPD 249, CPD 315, RC 19BR, CPD 254, RC 19, CPD 229, CPD 330, CPD 317, RC 19R, CPD 260, CPD 290 and CPD 320 (Table 4.3 and Fig. 4.3).

Table 4.2: Disease reaction of cowpea entries/varieties against charcoal rot pathogen

S. No.	Entries/Varieties	PDI	Reaction
1	CPD 334	32.00 (34.33)*	S
2	CPD 324	16.00 (23.46)	MR
3	CPD 305	66.67 (54.93)	HS
4	CPD 302	40.00 (38.84)	S
5	CPD 265	34.67 (35.91)	S

6	CPD 333	22.67 (28.04)	MS
7	CPD 332	16.00 (23.11)	MR
8	CPD 336	29.33 (32.73)	MS
9	CPD 335	41.33 (39.95)	S
10	CPD 347	14.67 (22.18)	MR
11	CPD 307	13.33 (20.37)	MR
12	CPD 249	94.67 (76.80)	HS
13	CPD 276	37.33 (37.44)	S
14	CPD 315	57.33 (49.59)	HS
15	CPD 348	48.00 (43.98)	S
16	RC 19BR	73.33 (59.18)	HS
17	CPD 254	53.33 (46.90)	HS
18	RC 19	74.67 (60.18)	HS
19	CPD 229	81.33 (70.76)	HS
20	CPD 269	24.00 (29.15)	MS
21	CPD 330	53.33 (47.27)	HS
22	CPD 317	85.33 (68.49)	HS
23	CPD 343	28.00 (31.30)	MS
24	CPD 288	41.33 (39.87)	S
25	CPD 119	25.33 (30.19)	MS
26	CPD 273	43.33 (42.23)	S
27	RC 19R	70.67 (57.40)	HS
28	CPD 260	54.67 (47.67)	HS
29	CPD 261	38.67 (38.40)	S

30	CPD 286	37.33 (37.61)	S
31	CPD 290	65.33 (53.92)	HS
32	CPD 279	34.67 (36.00)	S
33	CPD 268	37.33 (37.56)	S
34	CPD 345	28.00 (31.62)	MS
35	CPD 320	74.67 (60.73)	HS
	C.D. at 5%	13.25	
	SE.m (±)	4.68	

*Angular transformation value

Values are average of three replications

Table 4.3: Classification of cowpea entries/varieties based on PDI against *M. phaseolina*

Disease reaction	PDI	Entries/Varieties	Total
Free	0	No	0
Resistant	0.01-10.0	No	0
Moderately resistant	10.1-20.0	CPD 307, CPD 347, CPD 332, CPD 324	4
Moderately susceptible	20.1-30.1	CPD 269, CPD 343, CPD 119, CPD 345, CPD 333, CPD 336	6
Susceptible	30.1-50.0	CPD 334, CPD 302, CPD 265, CPD 335, CPD 276, CPD 348, CPD 288, CPD 273, CPD 261, CPD 286, CPD 279, CPD 268	12
Highly susceptible	Above 50.1	CPD 305, CPD 249, CPD 315, RC 19BR, CPD 254, RC 19, CPD 229, CPD 330, CPD 317, RC 19R, CPD 260, CPD 290, CPD 320	13

4.2.2 Studies on biochemical alteration in cowpea post inoculation with *M. phaseolina*

Plants have developed an arsenal of defense mechanisms to protect themselves against pathogen attacks. In this study, biochemical parameters

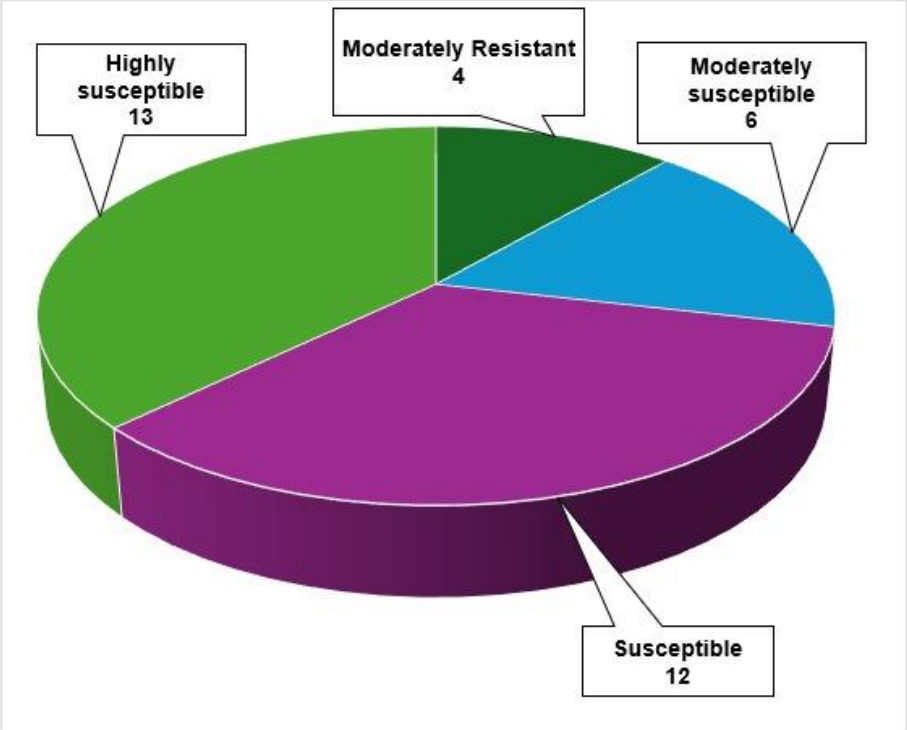


Fig. 4.3: Distribution of cowpea entries in different disease reaction categories

such as total phenol, proline and total antioxidant enzyme activity levels were measured in both diseased and healthy plant samples at 0, 24, 48, 72 and 96 hpi HS (CPD 249 and RC 19) and MR entries (CPD 307 and CPD 347).

The results indicated noticeable differences in the biochemical profiles of healthy and diseased leaves among four cowpea entries - CPD 307 and CPD 347 (MR), RC 19 and CPD 249 (HS) showing a spectrum of responses from resistance to susceptibility to *M. phaseolina*.

4.2.2.1 Phenol

Total phenol content was assessed in both healthy and diseased leaves of resistant and susceptible cowpea cultivars at 0, 24, 48, 72 and 96 hpi. The results revealed that the diseased leaves of the MR entry CPD 307 recorded the highest phenol level at 7.09 mg GAE/g at 96 hours (hrs), which decrease significantly to 1.29 mg GAE/g in its healthy leaves. This was followed by another MR entry CPD 347 with 6.57 mg GAE/g in diseased leaves, which decreased to 1.17 mg GAE/g in healthy leaves after pathogen infection at 96 hrs (Fig. 4.4).

The HS entry CPD 249 exhibited the lowest phenol content of 1.85 mg GAE/g in diseased leaves, which decreased to 0.96 mg GAE/g in healthy leaves. Similarly, the HS entry RC 19 showed the phenol content of 1.93 mg GAE/g in diseased leaves, which decreased to 1.02 mg GAE/g after pathogen infection at 96 hrs (Table 4.4). Overall, total phenol content was increased after 24 hpi in all the inoculated entries, whereas a sharp increase after 72 hpi was found in resistant entries (CPD 307, CPD 347) and declined after 72 hpi in susceptible entries (CPD 249, RC 19) of cowpea.

Table 4.4: Changes in phenol content in *M. phaseolina* inoculated and un-inoculated entries of cowpea

	Phenol (mg GAE/g)				
Entries	Hours after inoculation				
	0 hpi*	24 hpi	48 hpi	72 hpi	96 hpi
CPD 307 (MR)	1.84	2.45	3.37	3.91	7.09
CPD 347 (MR)	1.77	2.33	3.09	3.54	6.57
CPD 249 (HS)	1.53	1.85	2.65	3.05	1.85
RC 19 (Susceptible check)	1.69	1.93	2.89	3.19	1.93
CPD 307 Control	0.96	1.04	1.11	1.17	1.29
CPD 347 Control	0.80	0.88	0.96	1.05	1.17
CPD 249 Control	0.56	0.61	0.68	0.72	0.96
RC 19 Control	0.64	0.72	0.86	0.91	1.02

*hpi- hours after post inoculation

4.2.2.2 Proline

Proline content was assessed in both healthy and diseased leaves of resistant and susceptible cowpea cultivars at 0, 24, 48, 72 and 96 hpi. The results revealed that the diseased leaves of the MR entry CPD 307 recorded the highest proline level at 12.33 µmol/g at 48 hrs, which decreased significantly to 4.65 µmol/g in their healthy leaves. This was followed by another MR entry CPD 347 with 11.38 µmol/g in diseased leaves, which decreased to 4.24 µmol/g in healthy leaves after pathogen inoculation at 48 hrs (Fig. 4.5).

The HS entry CPD 249 exhibited the lowest proline content of 8.52 µmol/g in diseased leaves, which decreased 3.41 µmol/g in healthy leaves. Similarly, the HS entry RC 19 showed the proline content of 9.48 µmol/g in diseased leaves, which decreased 3.87 µmol/g after pathogen infection at 48 hrs (Table 4.5). Overall, proline content increased rapidly up to 48 hpi, followed by a gradual decline across all the studied entries.

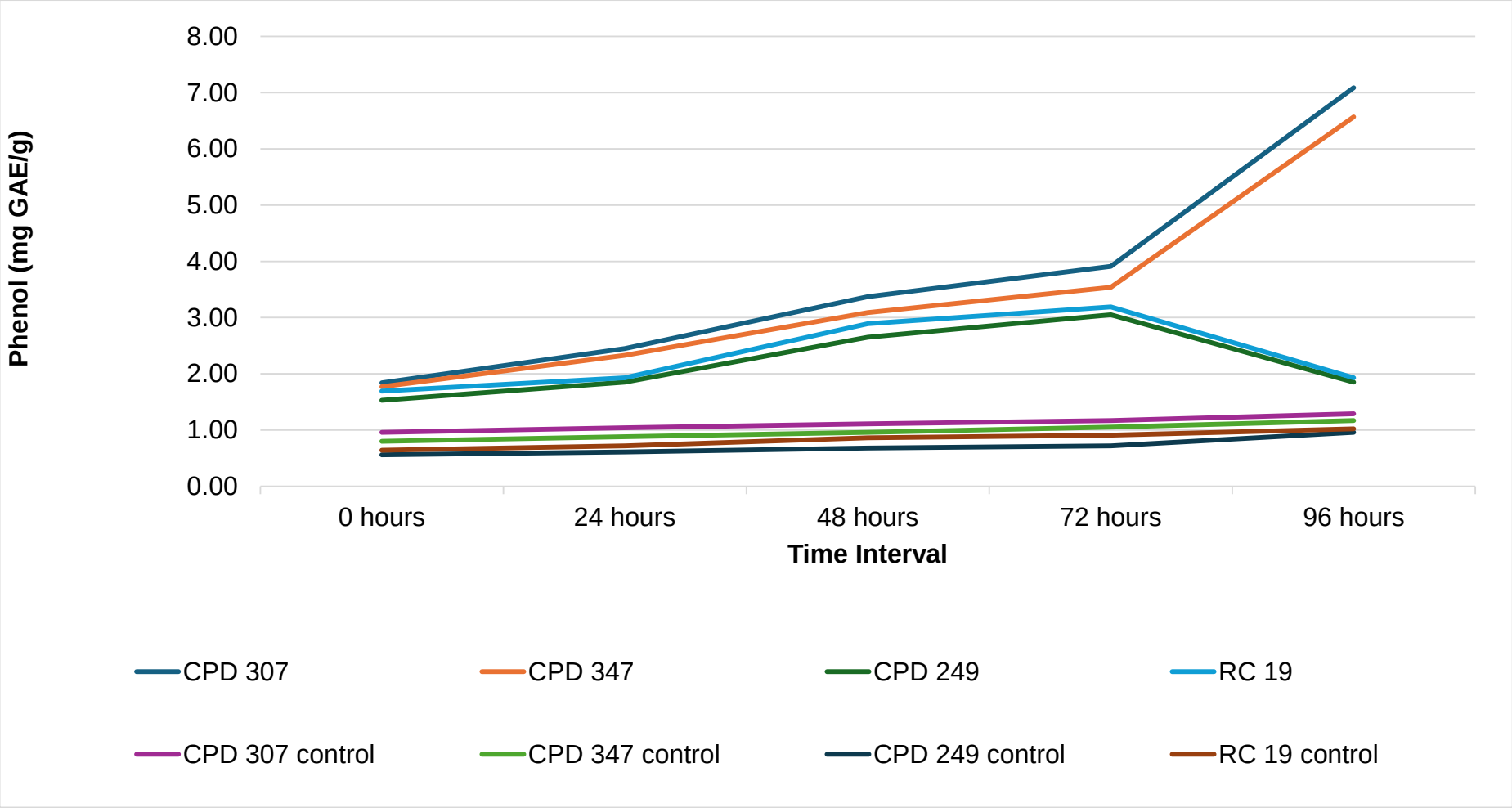


Fig. 4.4: Changes in phenol content in *M. phaseolina* inoculated and un-inoculated entries of cowpea

Table 4.5: Changes in proline content in *M. phaseolina* inoculated and un-inoculated entries of cowpea

Entries	Proline (µmol/g)				
	Hours after inoculation				
	0 hpi*	24 hpi	48 hpi	72 hpi	96 hpi
CPD 307 (MR)	6.62	10.43	12.33	11.86	11.38
CPD 347 (MR)	6.14	9.48	11.38	10.90	10.43
CPD 249 (HS)	5.19	7.57	8.52	8.52	8.05
RC 19 (Susceptible check)	5.67	8.05	9.48	9.00	8.52
CPD 307 Control	4.24	4.59	4.65	4.73	4.78
CPD 347 Control	4.12	4.19	4.24	4.25	4.29
CPD 249 Control	3.29	3.36	3.41	3.41	3.44
RC 19 Control	3.76	3.78	3.87	3.93	4.04

4.2.2.3 Total Antioxidant Enzymes Activity

Total antioxidant enzymes activity was assessed in both healthy and diseased leaves of resistant and susceptible cowpea cultivars at 0, 24, 48, 72 and 96 hpi. The results revealed that the diseased leaves of the MR entry CPD 307 recorded the highest total antioxidant level at 3.28 mmol TE/g at 48 hrs, which decreased significantly to 0.65 mmol TE/g in its healthy leaves. This was followed by another MR entry CPD 347 with 2.98 mmol TE/g in diseased leaves, which decreased 0.59 mmol TE/g in diseased roots after pathogen inoculation (Fig. 4.6).

The highly susceptible entry CPD 249 exhibited the lowest antioxidant enzyme activity of 1.91 mmol TE/g in diseased leaves, which decreased to 0.46 mmol TE/g in healthy leaves. Similarly, the highly susceptible entry RC 19 showed the antioxidant enzyme activity of 2.43 mmol TE/g in diseased leaves, which decreased 0.52 mmol TE/g after pathogen infection at 48 hrs (Table 4.6). Thereafter, a decline was observed across all entries, with a notably sharper decline in the susceptible entries CPD 249 and RC 19.

Table 4.6: Changes in total antioxidant enzymes activity content in *M. phaseolina* inoculated and un-inoculated entries of cowpea

Entries	Total Antioxidant Enzymes activity (mmol TE/g)				
	Hours after inoculation				
	0 hpi*	24 hpi	48 hpi	72 hpi	96 hpi
CPD 307 (MR)	1.66	1.84	3.28	2.87	2.71
CPD 347 (MR)	1.51	1.71	2.98	2.65	2.49
CPD 249 (HS)	1.18	1.36	1.91	1.31	1.18
RC 19 (Susceptible check)	1.25	1.44	2.43	1.62	1.44
CPD 307 Control	0.61	0.64	0.65	0.66	0.67
CPD 347 Control	0.55	0.57	0.59	0.60	0.62
CPD 249 Control	0.45	0.45	0.46	0.47	0.49
RC 19 Control	0.49	0.51	0.52	0.54	0.57

4.3 Management of charcoal rot of cowpea under field condition using novel strategies

4.3.1 Effect of different fungicides and bioagents against charcoal rot incidence under field conditions

During the *Kharif* season of 2024, eight treatments were evaluated for their efficacy against charcoal rot in cowpea under artificially inoculated field conditions. Prior to sowing, all treatments were seed treated at their respective recommended doses as per companies technical data sheet. At harvest, observations were recorded in terms of PDI and grain yield.

The results indicated that among the eight fungicides tested against *M. phaseolina* under field conditions, PDI of T_2 (Seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS) treatment was found to be the most effective, showing the lowest disease incidence at 20.27%, followed by T_3 (Seed treatment with Thiophanate Methyl 45% + Pyraclostrobin 5% FS) with 24.80%. Compared to this T_8 (Control) showed PDI of 86.40% (Table 4.7).

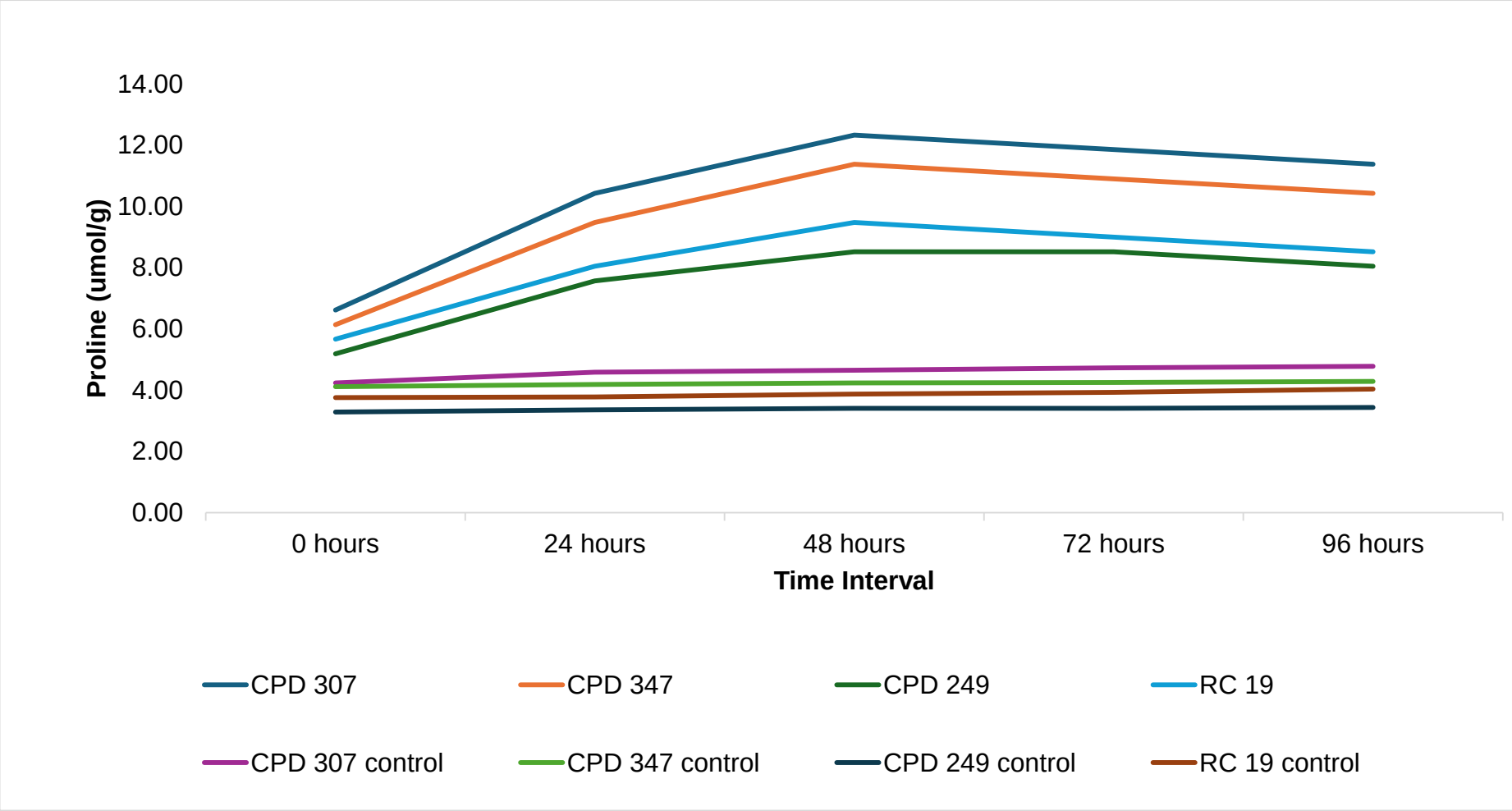


Fig. 4.5: Changes in proline content in *M. phaseolina* inoculated and un-inoculated entries of cowpea

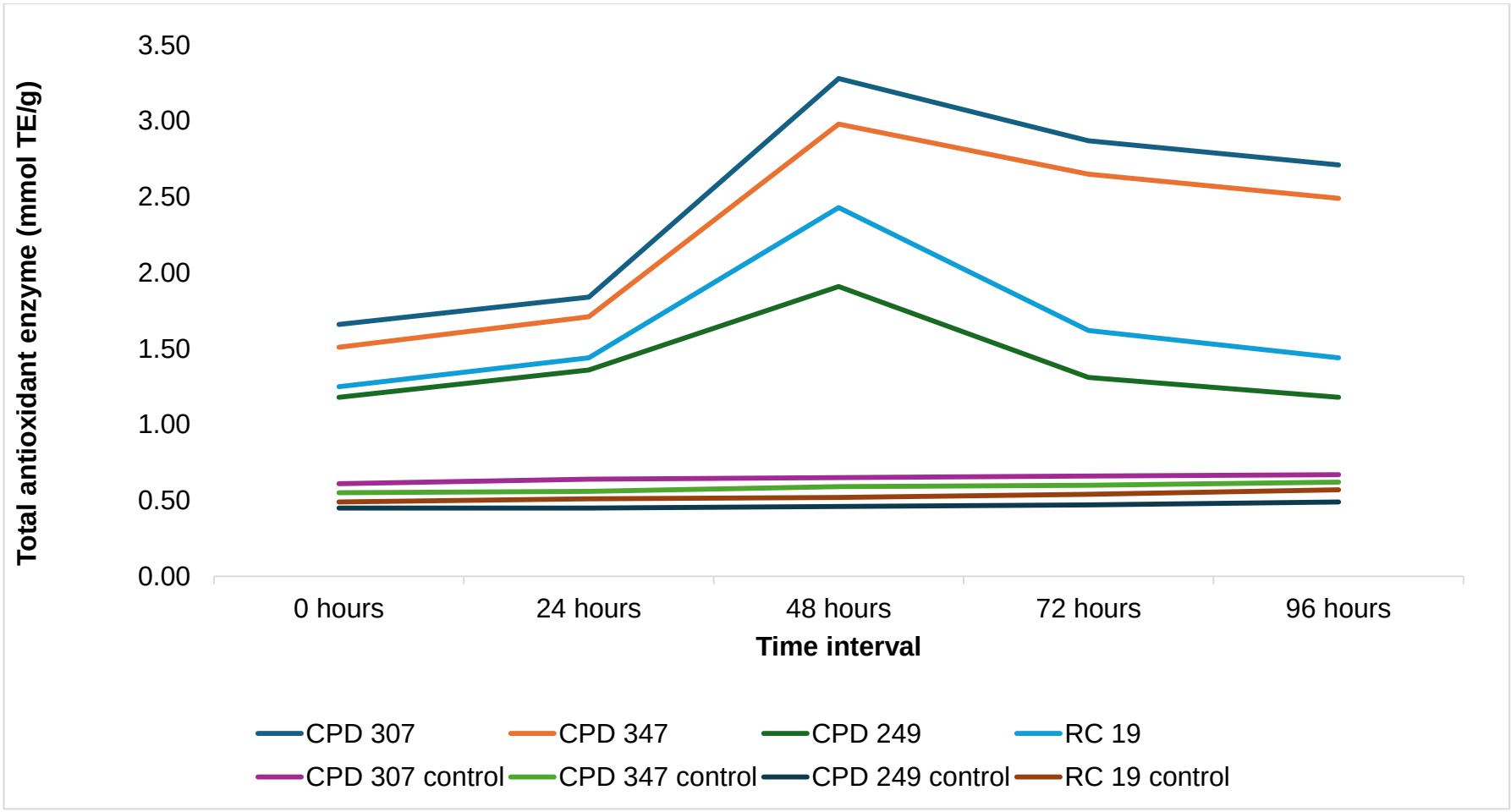


Fig 4.6: Changes in total antioxidant enzymes activity content in *M. phaseolina* inoculated and un-inoculated entries of cowpea

Table 4.7: Management of charcoal rot of cowpea through fungicides and bioagents under artificial inoculation conditions

Treatment No.	Treatment Details	Disease incidence	Disease Reduction (%)
T1	Seed treatment with Carboxin 37.5% + Thiram 37.5% WP	35.47 (36.51)*	58.95
T2	Seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS	20.27 (26.73)	76.54
T3	Seed treatment with Thiophanate Methyl 45% + Pyraclostrobin 5% FS	24.80 (29.83)	71.29
T4	Seed treatment with Penflufen 154 + Trifloxystrobin 154 FS (13.28% w/w + 13.28% w/w)	37.33 (37.64)	56.79
T5	Seed treatment with Fluxapyroxad 33.3% w/v	43.20 (41.06)	50.00
T6	Seed treatment with Trichoderma strain mixed with jaggery	41.07 (39.83)	52.46
T7	Seed treatment with Karan Narendra Trichoderma (KNT) mixed with jaggery	40.27 (39.37)	53.39
T8	Control	86.40 (68.87)	0.00
	C.D. at 5%	5.21	
	SE.m ±	1.70	

*Angular transformation value

In terms of disease reduction, the highest percentage was observed in T_2 (Seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS) with 76.54% reduction, followed by T_3 (Seed treatment with Thiophanate Methyl 45% + Pyraclostrobin 5% FS) with

76.90%. The lowest disease reduction was recorded in T_5 (Seed treatment with Fluxapyroxad 33.3% FV) showing only 50.00% reduction. Both bioagents showed more than 50% disease reduction (Table 4.7 and Fig. 4.7). The treatments were found to be significantly different in terms of PDI.

4.3.1 Effect of different fungicides and bioagents on grain yield of cowpea under artificial inoculation conditions

Grain yield per plot was measured for each treatment in the field experiment. The results indicated that the highest yield (6.88 q/ha) was recorded under treatment T_2 (Seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS), followed by T_3 (Seed treatment with Thiophanate Methyl 45% + Pyraclostrobin 5% FS) treatment (6.14 q/ha). The lowest yield (3.34 q/ha) was observed in treatment T_8 (Control). All the treatments were significantly different from each other and found superior over control.

The maximum percentage increase in grain yield over the control was observed in T_2 (Seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS) treatment (105.98%), followed by T_3 (Seed treatment with Thiophanate Methyl 45% + Pyraclostrobin 5% FS) treatment (83.83%). The minimum grain yield q/ha increase over control was also recorded (32.63%) in T_5 (Seed treatment with Fluxapyroxad 33.3% FV) treatment, as presented in Table 4.8 and Fig. 4.8. All treatments had a positive impact on the grain yield of cowpea.

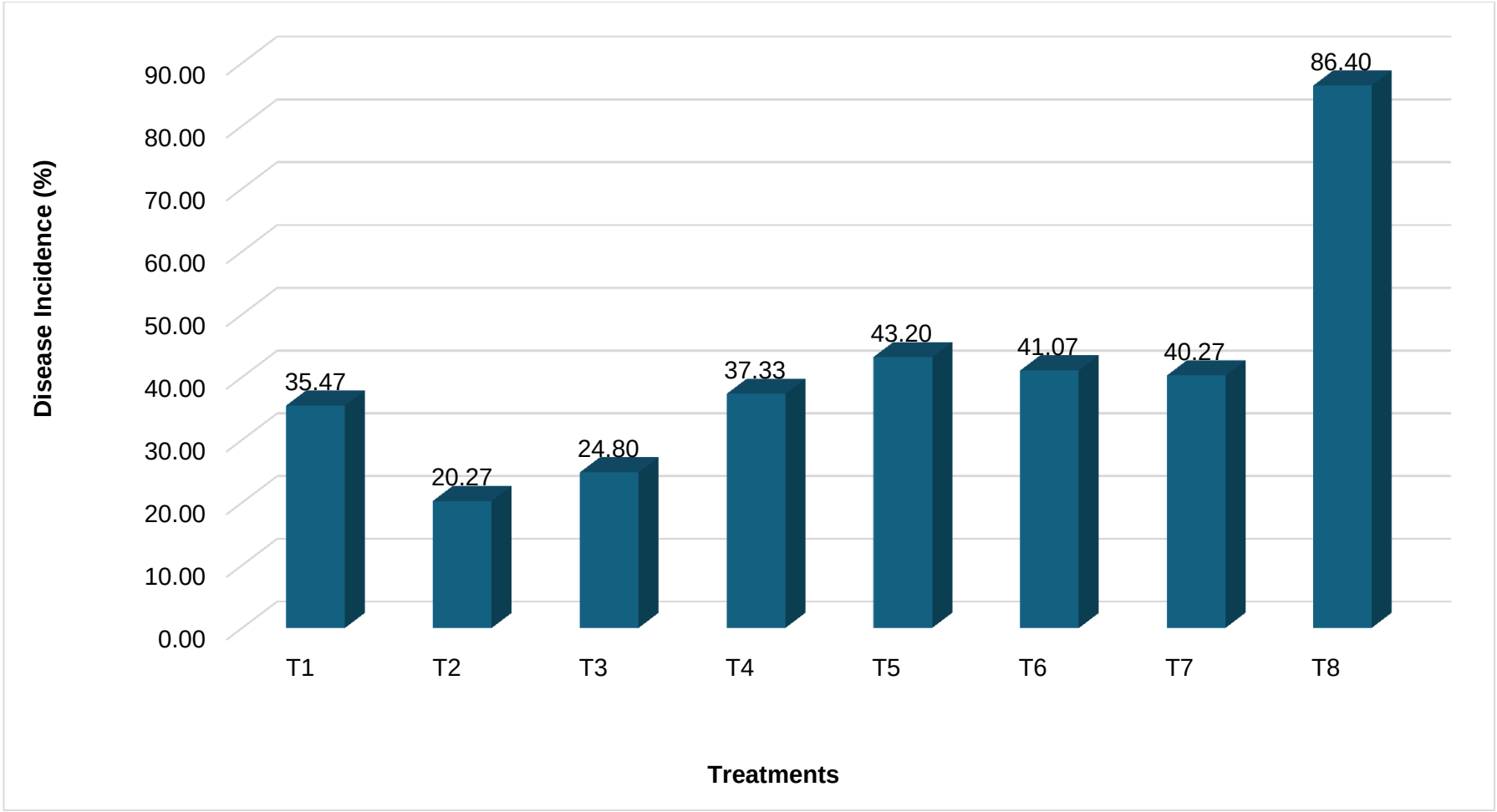


Fig. 4.7: Management of charcoal rot of cowpea through fungicides and bioagents under sick plot condition

Table 4.8: Effect of different fungicides and bioagents on grain yield of cowpea under artificial inoculation conditions

Treatment No.	Treatment Details	Grain yield (q/ha)	Increased over control (%)
T1	Seed treatment with Carboxin 37.5% + Thiram 37.5% WP	5.56	66.46
T2	Seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS	6.88	105.98
T3	Seed treatment with Thiophanate Methyl 45% + Pyraclostrobin 5% FS	6.14	83.83
T4	Seed treatment with Penflufen 154 + Trifloxystrobin 154 FS (13.28% w/w + 13.28% w/w)	5.24	56.88
T5	Seed treatment with Fluxapyroxad 33.3% w/v	4.43	32.63
T6	Seed treatment with Trichoderma strain (RARI) mixed with jaggery	4.64	38.92
T7	Seed treatment with Karan Narendra Trichoderma (KNT) mixed with jaggery	4.82	44.31
T8	Control	3.34	0.00
	C.D. at 5%	1.84	
	SE.m ±	0.54	

Table 4.9: Effect of different fungicides and bioagents on benefit cost ratio of cowpea under artificial inoculation conditions

Treatments	Cost of treatment (Rs)	Cost of cultivation (Rs)	Total cost of cultivation (Rs)	Mean yield (q/ha)	Gross return (Rs)	Net return (Rs)	B:C ratio
T1 - Seed treatment with Carboxin 37.5% + Thiram 37.5% WP	165.5	10000	10165.5	5.56	33360	23195.5	2.28
T2 - Sees treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS	1310	10000	11310	6.88	41280	29970	2.64
T3 - Seed treatment with Thiophanate Methyl 45% + Pyraclostrobin 5% FS	287.5	10000	10287.5	6.14	36840	26552.5	2.58
T4 - Seed treatment with Penflufen 154 + Trifloxystrobin 154 FS (13.28% w/w + 13.28% w/w)	292.5	10000	10292.5	5.24	28440	18147.5	1.76
T5 - Seed treatment with Fluxapyroxad 33.3% w/v	210	10000	10210	4.43	20580	9939	1.01
T6 - Seed treatment with Trichoderma strain mixed with jaggery	215	10000	10215	4.64	21780	11565	1.13
T7 - Seed treatment with Karan Narendra Trichoderma (KNT) mixed with jaggery	215	10000	10215	4.82	22140	11925	1.16
T8 - Control	0	10000	10000	3.34	20040	8900	1.00

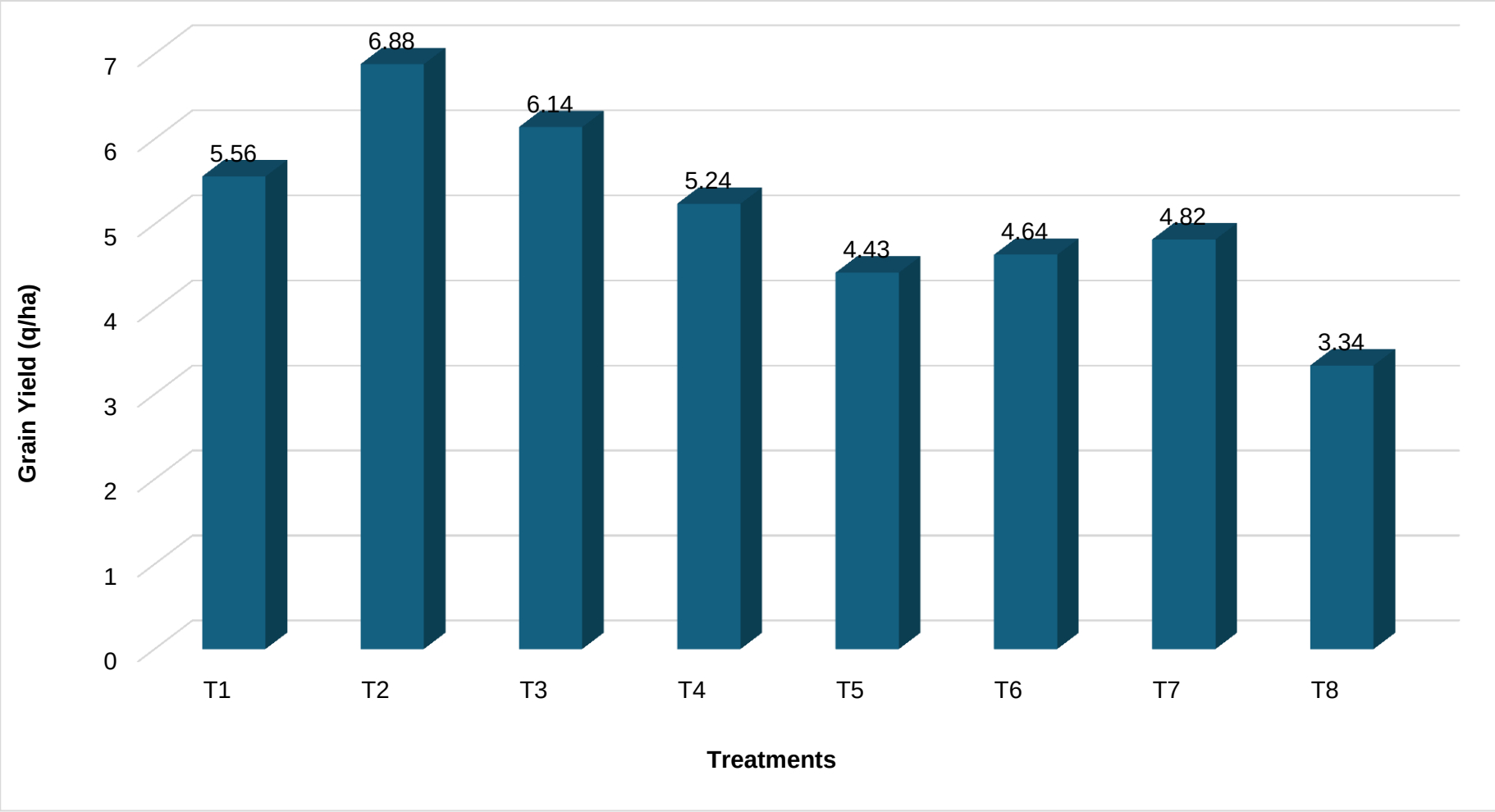


Fig 4.8: Effect of different fungicides and bioagents on grain yield of cowpea under sick plot condition

Plate 3: An overall view of disease screening experiment in the field

Plate 4: An overall view of biochemical experiment in pot

Chapter - 5

DISCUSSION

Cowpea (*Vigna unguiculata* (L.) Walp.) is an annual legume, commonly known as southern pea, black-eyed pea or crowder pea. Its seeds can be eaten fresh along with the pods, while the green pods are often consumed as a vegetable. Additionally, cowpea's dense canopy plays a crucial role in conserving soil moisture and preventing erosion. Because of these multifaceted uses, cowpea holds significant importance in diverse agricultural systems, especially in Rajasthan (Gupta and Saxena 2015).

Cowpea is susceptible to various diseases, out of these which charcoal rot, caused by *Macrophomina phaseolina* (Tassi) Goid., is considered one of the most devastating. This disease is responsible for substantial yield losses, particularly during the *kharif* season. In Rajasthan, the infection typically begins at the seedling stage, just below the cotyledon node. The first symptom of disease is yellowing of the leaves which droop in next 2-3 days and withers off. Other symptoms include drying and discoloration of tap root, longitudinal crack of the stem, blackish growth when split open, stunting, wilting and death of the plant.

5.1 Morphological and molecular characterization of pathogen associated with charcoal rot of cowpea

In the current investigation, the diseased samples were collected from the RARI Farm during summer 2024 at Rajasthan Agricultural Research Institute, Durgapura.

The fungal pathogen was isolated from infected root samples collected from the RARI farm using PDA medium under aseptic laboratory conditions. To obtain a pure culture, hyphal tip technique was employed. Following isolation, the PDA plates were incubated in a BOD incubator under controlled environmental conditions and later stored in a refrigerator for further research. The pathogen exhibited uniformly dense colony growth on PDA. The pathogen was identified based on the mycelium branching and sclerotia characteristics. Javaid et al. (2012) previously reported comparable results in the isolation and identification of *M. phaseolina*.

A pathogenicity test was conducted under pot conditions to verify the disease-causing potential of the isolated fungus. Artificial inoculation was performed using soil inoculation method. Mass multiplication was done in sterilized sorghum.

The conformity of this study is the earlier findings of Hinguera (1991) conducted studies on three different methods to establish pathogenicity of *M. phaseolina* in cowpea *viz*., toothpick inoculation, inoculation using dry sclerotia of *M. phaseolina* and inoculation using rice seeds colonized by *M. phaseolina* and concluded that soil-borne inoculum multiplied on rice seeds was more effective in disease development in cowpea seedlings. In a similar study, symptoms noticed on population dynamics of *M. phaseolina* and disease intensity in cluster bean and suggested significant correlation between higher plant mortality and increased soil inoculum under moisture stress conditions (Lodha 1998). He also reported that *M. phaseolina* and *Fusarium solani* produce charcoal rot of jojoba by inoculating the soil as well as by incorporation of inoculum of *M. phaseolina* through seed-cum-soil and drenching 15 days after sowing in cluster bean. Oladimeji et al. (2012) screened five cowpea cultivars for resistance against *M. phaseolina* using two inoculation methods: pouring of spore/mycelial suspension into the soil and wrapping inoculum meal around the wounded lower stem of seedlings.

The mycelia appeared white, gradually darkening to a brownish-black as the culture matured, accompanied by the formation of sclerotia after 10–15 days along the colony's periphery. The sclerotia of *M. phaseolina* isolates were black to dark brown in colour, round to irregular in shape, and were between 10-20 µm in length, according to microscopic examinations. The mycelium was hyaline, septate in nature and profusely rise at right angle of 90°. In case of Tandel et al. (2015), the same fungus was found to produce initially white mycelial growth on PDA later changing to brown to black in center due to the formation of numerous small black sclerotia. The mycelium was hyaline to brown, branched, septate and 1.78 to 6.58 µm in width. The sclerotia formed in culture were black, hard and 67.92 to 195.18 µm in

diameter. The pycnidial stage was not produced in culture. Similar to this, pycnidial stage was not found in current study.

The molecular identification of the fungal isolate was performed using the ITS-DNA PCR method. The isolate produced the expected amplicons ranging from 600 to 900 bp using ITS1 and ITS4 primers after successful amplification through PCR. In a similar study conducted by Aghakhani and Dube (2009), primer sequence based on ITS I, 5.8 rDNA and ITS II regions were used to determine genetic diversity among six isolates of *Rhizoctonia bataticola*. The result indicated that ITS of Karnataka isolate (Rb1) was largest (670 bp), whereas it was smallest (499 bp) in Haryana (Rb21) isolate.

5.2 Screening of varieties and biochemical evaluation of susceptible and resistant cultivars under artificial inoculation conditions

5.2.1 Screening of cowpea germplasm against charcoal rot disease

Among different entries/varieties, no entries were found immune and resistant, 4 were found MR CPD 324, CPD 332, CPD 347 and CPD 307, whereas 6 MS, 12 S and 13 HS.

Compared to the studies, Muchero et al. (2011) evaluated 14 cowpea genotypes against *M. phaseolina* under moderate water stress conditions. The genotypes IT98K-499-39, Suvita 2, IT93K-503-1 and Mouride were found to be highly resistant with mortality below 10%.

Comparing the studies, Oladimeji et al. (2012) screened five cowpea cultivars for resistance against *M. phaseolina*. The cultivar ITO4K-217-5 exhibited resistance to the pathogen under both methods, supporting the findings of the present study.

In the case of Tanzeel-u-Rehman (2015) screened seven varieties/lines of cowpea (SA dandy, Elite, Rawan-2003, White star, CP1, P-518, P-2127) against *M. phaseolina*. Among seven cultivars, White star and Elite showed resistant reactions and SA dandy, CP1 and Rawan-2003 showed tolerance against the disease.

Compared to the studies, Lamini et al. (2022) screened 49 cowpea lines against MRRD. A selection of eight resistant lines (Suvita 2, Abagbaala, IT97K573-1-1, IT93K-503-1-1, Hewale, AV2 3224, Nhyira and T2T4), and a susceptible check (Songotra) were evaluated against 10 isolates of *M. phaseolina* using a sick pot method.

Similarly, Sasode et al. (2023) screened nineteen cowpea genotypes against *M. phaseolina*. In this study, none of the entries were found free from dry root rot. Eleven entries were found moderate resistant.

5.2.2 Studies on biochemical alteration in cowpea post inoculation with *M. phaseolina*

Biochemical analysis of healthy and diseased leaves of four entries *viz*., using CPD 307 and CPD 347 (MR), RC 19 and CPD 249 (HS) showed susceptible to resistant reaction against *M. phaseolina*.

5.2.2.1 Phenol

The results showed that total phenol content increased after 24 hpi in all the inoculated entries, whereas a sharp increase after 72 hpi was found in resistant entries and declined after 72 hpi in susceptible entries of cowpea. Contrary to our study, Monaim et al. (2019) reported that the total phenols increased in cowpea plants inoculated with *M. phaseolina* and treated tested inducer resistance chemicals (IRCs). The maximum phenol compound was recorded in six days after the application then decreased gradually.

In case of Sharma et al. (2011), the phenol in *M. phaseolina* infected guar plants increased from 24 to 120 hours in all the genotypes of guar similar to our study. The maximum accumulation of phenolic acids was 23 per cent higher than in control.

Similar to our study, Jyothi et al. (2018), the highest phenol activity was recorded in chickpea roots inoculated with *M. phaseolina* at 96 hpi in moderately resistant genotypes, Phule G 12107 (1.43 µl/ml), NDG 13-21 (1.35 µl/ml) and IPC 2010-112 (1.03 µl/ml).

Compared to this study Kumar et al. (2019) reported that the effect of phenol content in mungbean against *M. phaseolina*. Maximum phenol content increased in diseased root of SML-668 (44.36%) followed by MH-2-15 (25.85%) and IPM-02- 03 (14.66%) as compared to respective healthy roots.

5.2.2.2 Proline

Proline content increased rapidly up to 48 hpi, followed by a gradual decline across all the studied entries. This indicates that proline accumulation is not a distinguishing factor between susceptible and resistant entries inoculated with *M. phaseolina*.

Similar finding was reported by Shruti et al. (2018), wherein, proline content for one month old, inoculated plant was highest at 48 h with 4.88, 5.55 and 9.41 mg/g fw for variety RMO-40, CZM-3 and FMM-96, respectively, which are 1.77, 1.92 and 3.14-fold higher in inoculated plants as compared to the control. From the data the proline content is higher in the pathogen inoculated samples compared to the control plants. Varietal differences are also observed from the data, the proline content is higher in resistant variety than the susceptible variety against pathogen *M. phaseolina.*

Compared to these results Chavan et al. (2019) that highest proline content was found in AMS MB 5-19 (24.42 g/100g) and lowest amount of proline was found in AMS 475 (21.42 g/100g) in soybean.

5.2.2.3 Total antioxidant enzymes activity

The total antioxidant enzymes activity showed a gradual increase up to 24 hpi in all entries, followed by a more pronounced rise to 48 hpi in the resistant entries compared to the susceptible ones. Thereafter, a decline was observed across all entries, with a notably sharper decrease in the susceptible entries CPD 249 and RC 19.

Compared to these studied, Ramanathan et al. (2001) studied green gram suspension-cultured cells were treated with the elicitor isolated from cell wall of *M. phaseolina*, a more than 8-fold increase in peroxidase activity was observed, compared to that of mock treated suspension-cultured cells 24 and

48 h after treatment. When green gram leaves were inoculated with *M. phaseolina*, a more than 3-fold increase in peroxidase activity over uninoculated control was observed 24 and 48 h after inoculation.

Similar results also observed by Shruti et al. (2018) that the peroxidase activity is highest in one-month old plants was obtained at 48 h with 1.20, 1.77 and 2.74 nkat.mg-1 protein after pathogen inoculation for variety RMO-40, CZM-3 and FMM-96, respectively, which is 3.15, 3.10 and 3.60-fold higher in inoculated plants as compared to the control plants. Initially a gradual increase in peroxidase activity was observed upto 48 h, thereafter a decrease was observed upto 168 h. The decrease in peroxidase activity is 1.10-fold in variety RMO-40, 1.23 in CZM-3 and 2.51 in FMM-96 at 168 h after pathogen inoculation when compared to the maximum activity.

Contrary to our study, Shoaib et al. (2018) total antioxidant activities in these varieties *i.e*. resistant (AJMERI), moderately resistant (PSC-60 and RAWAL-I), susceptible (NARC-II) and highly susceptible (NARC-I). POX, PPO and CAT activities were estimated in leaves from pathogen inoculated (positive control) and un-inoculated (negative control) soybean plants. POX activities significantly increased by 39% and 43% in susceptible and highly susceptible groups, PPO was significantly raised up to two folds in susceptible and highly susceptible groups by 114% and 124% and CAT was significantly high in susceptible groups by 30% and 60% about their relevant control.

Contrary to our study, Monaim et al. (2019) reported that the total peroxidase increased in cowpea plants inoculated with *M. phaseolina* and treated tested IRCs. The highest level of peroxidase was found in 8 days.

5.3 Management strategies using new molecules against charcoal rot of cowpea under field conditions

Eight fungicides and bioagents were tested in present studies *in vivo* conditions against *M. phaseolina*. Eight fungicides were tested *viz*., Carboxin + Thiram, Azoxystrobin + Thiophanate Methyl + Thiamethoxam, Thiophanate Methyl + Pyraclostrobin, Penflufen + Trifloxystrobin, Fluxapyroxad, *Trichoderma asperellum* and *Trichoderma harzianum* (KNT). Among

fungicides, Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS was most effective in disease reduction (76.54%) and grain yield (6.88q/ha). In this case, Deepthi et al. (2014) evaluated some fungicides at field condition and found that the carboxin + thiram gave highest seed germination and less mortality in sesame. Similarly, Gupta et al. (2018) and Khan et al. (2019) were worked on the different *Trichoderma* spp. under field conditions against root rot complex diseases, in which soil application of *T. viride* or *T. harzianum* effectively controlled the root-rot disease complex (5–15%) and improved the grain yield of mungbean (14–19%) in diseased plots and in our study, *T. harzianum* showed the disease reduction (53.39%)

Chapter - 6

SUMMARY AND CONCLUSION

The present investigations were undertaken to study the "Comprehensive Studies and Management of Charcoal Rot of Cowpea caused by *Macrophomina phaseolina* (Tassi.) Goid." The main objective was to assess the morphological and molecular characterization of pathogen, performance of cowpea varieties and genotypes against the disease, effect on phenol, proline and total antioxidant enzyme activity of cowpea plant and evaluation of fungicides against the charcoal rot under field conditions and findings were briefly summarized here under.

The fungus was successfully isolated on PDA medium from the diseased plant sample and the isolated culture was further purified using the hypal tip isolation method. The pathogen was identified as *M. phaseolina* based on the morphological and molecular characteristics. The mycelium was septate and hyaline, while the sclerotia were brown to black in colour and either rounded or irregular in shape with 10-20 um of length. The pathogen was confirmed as *M. phaseolina* based on molecular characterization. It exhibited high nucleotide identity with *M. phaseolina* isolate Mp31 and *Macrophomina* sp. isolate Mavin 01 and showed the closest evolutionary relationship to isolate Mavin 01.

Pathogenicity of *M. phaseolina* was confirmed in pots using the soil inoculation method. Clear and visible symptoms of charcoal rot included gradual yellowing or straw colouring of older leaves from the lower to upper parts of the plant. The root portion became black in colour.

The signs of *M. phaseolina* caused charcoal rot were first seen in the field at seedling stage in particularly susceptible variety and then spread to all other cowpea genotypes at blooming and podding stages. None of the 35 varieties/genotypes evaluated were found to be fully free and resistant against *M. phaseolina* infection. Only four genotypes of CPD 324, CPD 332, CPD 347 and CPD 307 are moderately resistant. 6 cowpea genotypes classified as moderately susceptible and the 12 genotypes classified as susceptible. 13

genotypes were identified as highly susceptible to *M. phaseolina* infection in cowpea.

The phenol content was also determined in cowpea leaf tissues of healthy leaves of moderately resistant entry CPD 307 had maximum phenol content at 96 hpi and it was increased in diseased leaves of same entry and it was followed by the other healthy moderately resistant (MR) entry of CPD 347 which increased in diseased leaves sample. Whereas the lowest amount of phenol content was recorded in healthy leaves of highly susceptible entry CPD 249 at 96 hpi.

The proline content was also determined in cowpea leaf tissues of healthy leaves of moderately resistant entry CPD 307 had maximum proline content at 48 hpi and it was increased in diseased leaves of same entry and it was followed by the other healthy moderately resistant (MR) entry of CPD 347 which increased in diseased leaves sample. Whereas the lowest amount of proline content was recorded in healthy leaves of highly susceptible entry CPD 249 at 48 hpi.

The antioxidant enzyme was also determined in cowpea leaf tissues of healthy leaves of moderately resistant entry CPD 307 had maximum antioxidant enzyme at 48 hpi and it was increased in diseased leaves of same entry and it was followed by the other healthy moderately resistant (MR) entry of CPD 347 which increased in diseased leaves sample. Whereas the lowest amount of antioxidant enzyme was recorded in healthy leaves of highly susceptible entry genotype CPD 249 at 48 hpi.

Among the eight treatments, the maximum per cent disease reduction was recorded with T_2 (Seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS). It was found significantly superior in all the treatments. T_5 (Seed treatment with Fluxapyroxad 33.3% w/v) was found least effective due to show minimum per cent disease reduction. Grain yield recorded per plot which was revealed that maximum yield was obtained with T_2 (Seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS). The minimum yield was found with T_5 (Seed treatment with Fluxapyroxad 33.3% w/v). All the treatments were significantly differing from each other and found superior over control.

Conclusion

- Mycelium was hyaline, septate and rise at the right angle of 90°. The sclerotia was brown to black in colour, round to irregular shaped and were 10-20 µm in size. The pathogen was confirmed as *M. phaseolina* based on molecular characterization. It exhibited high nucleotide identity with *M. phaseolina* isolate Mp31 and *Macrophomina* sp. isolate Mavin 01 and showed the closest evolutionary relationship to isolate Mavin 01.
- In screening, out of thirty-five varieties, no variety was found to be free and resistant, only 4 varieties were found to be moderately resistant against charcoal rot disease.
- The phenol content increased after 24 hpi in all the inoculated entries, whereas a sharp increase after 72 hpi was found in resistant entries (CPD 307, CPD 347) and declined after 72 hpi in susceptible entries (CPD 249, RC 19) of cowpea.
- Proline content increased rapidly up to 48 hpi, followed by a gradual decline across all the studied entries. This indicates that proline accumulation is not a distinguishing factor between susceptible and resistant entries inoculated with *M. phaseolina*.
- The total antioxidant enzyme activity showed a gradual increase up to 24 hpi in all entries, followed by a more pronounced rise to 48 hpi in the resistant entries compared to the susceptible ones. Thereafter, a decline was observed across all entries, with a notably sharper decrease in the susceptible entries CPD 249 and RC 19.
- Out of eight treatments comprising chemical and bioagents under field conditions, maximum per cent disease reduction and grain yield was recorded with T_2 treatment (Seed treatment with Azoxystrobin 2.5% + Thiophanate Methyl 11.25% FS + Thiamethoxam 25% FS) and among bioagents, both *Trichoderma harzianum* (KNT) and *Trichoderma asperelium* (PQ409413) showed more than 50% disease reduction.

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