SCREENING AND EVALUATION OF CHICKPEA (CICER ARIETINUM L.) GENOTYPES FOR WILT TOLERANCE AGAINST RACE 3 OF *FUSARIUM* OXYSPORUM USING ARBUSCULAR MYCORRHIZAL FUNGI (AMF) THROUGH PHENOLOGICAL, BIOCHEMICAL AND MOLECULAR ASSESSMENT

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

IN

MOLECULAR BIOLOGY AND GENETIC ENGINEERING

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2025

DECLARATION

I, hereby declare that the presented work in the thesis entitled "**Screening and evaluation of Chickpea (*Cicer arietinum* L.) genotypes for wilt tolerance against race 3 of *Fusarium oxysporum* using Arbuscular Mycorrhizal fungi (AMF) through phenological, biochemical and molecular assessment**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of **Dr. Sarvjeet Kukreja**, working as **Professor**, in the **Department of Agronomy** of **Lovely Professional University**, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled **Screening and evaluation of Chickpea (*Cicer arietinum* L.) genotypes for wilt tolerance against race 3 of *Fusarium oxysporum* using Arbuscular Mycorrhizal fungi (AMF) through phenological, biochemical and molecular assessment**" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the **School of Bioengineering and Biosciences**, is a research work carriedout by **Samiksha**, **11919255**, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

This study investigated the agronomical response of different genotypes of chickpea to *Fusarium oxysporum f. sp. Ciceris* (FOC) race 3 infection, with a focus on identifying resistant and susceptible genotypes. The study encompassed field and greenhouse screening of genotypes for wilt incidence, disease severity, biomass, and yield, with significant variations observed across genotypes and phenological stages. Resistant genotypes, such as HC1 and GNG 2144, exhibited minimal wilt symptoms, while susceptible genotypes like PUSA 547 and JG 62 showed severe symptoms. The molecular screening using STMS markers identified polymorphic alleles associated with resistance and susceptibility, corroborating agronomical research findings. Cluster analysis based on agronomical and molecular data further elucidated the genetic diversity among genotypes, providing valuable insights for breeding programs aimed at enhancing chickpea resistance to *Fusarium* wilt. The experiment further involved enhancement of defense mechanisms in chickpea genotypes by screening and selecting appropriate arbuscular mycorrhizal (AM) fungal species for which, FOC combined with various AM fungi (*Glomus mosseae, Glomus hoi, Glomus intraradices, Glomus fasciculatum,* and a commercial biofertilizer from ProVam Pvt Ltd) was applied to the susceptible chickpea JG 62. Several parameters were assessed, including biochemical changes, disease severity index (DSI), and root colonization percentage (RCP). With significant variations in biochemical measures across treatments, indicating potential improvements in plant resilience, ProVam was found to exhibit the highest phenol and protein content, antioxidant activity, and disease resistance, with successful mycorrhizal colonization. Morphological studies identified the predominant AMF species as *Glomus intraradices* in the ProVam. Through transcriptome analysis, we compared the gene expression profiles of chickpea genotype PUSA 547 under three conditions: co-inoculation with AMF and FOC, inoculation with FOC alone, and control. RNA sequencing generated 224.76 million reads, which were processed and mapped to the chickpea reference genome, revealing differential gene expression linked to plant defense mechanisms. Our analysis identified 20,602 high-quality genes, with 12,986 successfully annotated to Ensembl gene IDs. Differential expression analysis showed significant upregulation and downregulation of genes related to defense responses in co-inoculated plants compared to FOC-only inoculated and control plants. Functional classification highlighted key biological processes, cellular components, and molecular functions, suggesting that AMF enhances structural integrity and stress responses, particularly through pathways related

to polysaccharide biosynthesis and hydrogen peroxide catabolism. Pathway analysis revealed significant upregulation of defense-related processes in co-inoculated plants, while FOC-alone treatments showed downregulation of crucial metabolic pathways, indicating a compromised state. Validation through qRT-PCR of select genes corroborated transcriptome findings, emphasizing increased expression of various genes in AMF-treated plants. This study provides comprehensive insights into the molecular mechanisms by which AMF colonization modulates chickpea plant responses to FOC infection, potentially enhancing resistance and improving stress adaptation.

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AMF	Arbuscular Mycorrhizal Fungus
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CUSAT	Cochin university of Science and Technology
CMN	common mycorrhizal network
CTAB	cetyltrimethylamonium bromide
CZ	Central Zone
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
dNTP	Deoyribonucleotide triphosphate
DSI	Disease severity index
EDTA	Ethylenediaminetetraacetic acid
FAOSTAT	Food and agriculture Organization Statistics
FOC	*Fusarium oxysporum* f. sp. *Ciceris*
FPKM	Fragments per kilobase per million mapped fragments
H2SO4	Sulfuric acid
HCl	Hydrochloric acid
HR	Highly resistant
HS	Highly susceptible
IARI	Indian Agricultural Research Institute
IIPR	Indian Institute of Pulses research
ITCC	Indian type culture collection
ITS	Internal transcribed spacer
JA	Jasmonic acid
JNKVV	Jawaharlal Nehru krishi vishwa vidhyalya
LOX	Lipoxygenase
MAS	Marker assisted selection
MDA	Malondialdehyde
MPKV	Mahatma Phule Krishi vidyapith
MR	Moderately resistant
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEHZ	North eastern hill zone

NGS	Next generation sequencing
NWPZ	North-western plain zone
PAL	Phenylalanine ammonia lyase
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
QC	Quality check
QTL	Quantitative Trait Loci
RAPD	Random amplified polymorphic DNA
RAU	Royal Agricultural University
RCP	Root colonization percentage
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROC	Root organ culture
ROS	Reactive oxygen species
RT-PCR	Real time- Polymerase chain reaction
SA	Salicylic acid
SOD	Superoxide dismutase
SSR	Simple sequence repeats
STMS	sequence tagged microsatellites
SZ	South zone
TBE	Tris/borate/EDTA
TCA	Trichloroacetic acid
TE	Tris-EDTA
Tm	Annealing temperature
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization

CHAPTER-I INTRODUCTION

Grain legumes have a crucial role in the diet of people in developing countries, and for this reason, are sometimes referred to as the poor man's meat (Arbab *et al*., 2023; Saleem *et al*., 2023). Among the grain legumes, Chickpea is the second most cultivated crop in the world, trailing only behind common beans. It is an important food legume produced in more than 50 nations worldwide encompassing mainly arid and semi-arid areas across the Central Asia, Mediterranean basin, East Africa, Australia, Europe, and North and South America (Nunes *et al*., 2023; Phiri *et al*., 2023; Bansal *et al*., 2023). Chickpea cultivation is particularly prominent in regions where water scarcity limits the growth of other protein-rich crops, making it a strategic agricultural asset. It is suggested that the Fertile Crescent (presently South-eastern Turkey and Syria) is primary centre of chickpea origin (Guerra-Garcia *et al*., 2023). The Indian subcontinent alone (including India, Nepal, Pakistan, Bangladesh, and Myanmar) is the largest producer and consumer of chickpeas, and accounts for over 70% of global output (Majhi and Sikdar, 2023).

Chickpea (*Cicer arietinum* L.) is a self-pollinating crop, is diploid (2n = 2x = 16) and has a genome size of 738 Mb (Ambika and Kumar, 2023; Hamza *et al*., 2023). Chickpeas are classified within the Fabaceae (Leguminosae) family, the Faboideae subfamily (Papilionaceae), and the *Cicer*eae tribe. The genera comprise nine annual and around 34 perennial wild species (Yadav *et al*., 2023) wherein chickpea (*Cicer arietinum* L.) is the only commercially cultivated annual species. There are two varieties of chickpeas: Desi and Kabuli. The Desi variety has very low amount of endosperm (microsperma), has thick and pigmented seed coat whereas, the Kabuli variety has an ample amount of endosperm (macrosperma) and a thin and beigecoloured seed coat (Yadav *et al*., 2023; Qureshi *et al*., 2023). The crop is globally significant as it is a very good source of nutrients and essential sterols. The seeds of chickpea contain high amount of protein and carbohydrates and are used in vegetarian diets as well as cattle feed (Purewal *et al*., 2023; Sandhu *et al*., 2023). Additionally, its affordability and adaptability to low-input farming systems make it a preferred c*hoi*ce for smallholder farmers. The protein concentration in chickpea seeds ranges from 15% to 30% depending on genotype and environment, whereas carbohydrate content ranges from 60% to 65% (Sofi *et al*., 2023; Asati *et al*., 2024). The World Health Organization (WHO) recommends 80g of protein consumption per day in order to meet daily protein requirements of an individual (Moughan *et al*., 2024). Thus, chickpeas are employed as a source of healthy nourishment in impoverished nations due to their low cost (Rehm *et*

al., 2023; Kaur and Purewal, 2024). Although the crop has relatively low lipid levels, it does contain certain necessary fatty acids including linoleic and oleic acids. Apart from that, the sterols contained in the chickpea include campesterol, stigmasterol and sitosterol (Shevkani, 2023; Sabat *et al*., 2023). Chickpea crop not only provides benefits to human health but also impacts soil positively by fixing atmospheric nitrogen in association with the nitrogen fixing bacteria (Kumar *et al*., 2023). It is therefore considered a vital crop for its multiple benefits as a protein source as well as for improving soil health. This mutually beneficial partnership not only promotes chickpea growth but also improves the soil for future crops, making it a true powerhouse for human nutrition and sustainable agriculture. However, despite its vital role, current production levels fall short of demand, highlighting the urgent need for strategies to optimize chickpea yield, especially in areas like India.

Asia accounts for 80-85% of world chickpea output. India alone accounted for almost 70% of world chickpea output (Li *et al*., 2023; Zhang *et al*., 2024). However this requirement is not fulfilled because of less production and increasing population. Rising population demands have led to an increased need for chickpea production, resulting in the import of around 300,000 tonnes of chickpeas to India in 2023 (FAOSTAT 2024). Furthermore, low chickpea production accounts for biotic stressors, the most significant of which is *Fusarium* wilt produced by the fungal pathogen *Fusarium oxysporum* f. sp. *Ciceris* (FOC) (Yadav *et al*., 2023). Almost all the countries producing chickpea face the challenges instigated by FOC. Currently, eight pathogenic races of FOC (race 0, 1A, and 1B/C, 2–6) have been documented globally (Yadav *et al*., 2023; Ekwomadu and Mwanza, 2023). It is worth noting that the area under chickpea cultivation has shifted from north India to the central and south India after the 1900s. The area under chickpea cultivation in Northern India was substantially decreased due to its redirection towards more profitable and less risky fine grain crops (wheat and mustard). The chickpea crop is considered as a risky crop in many regions because of the regular occurrence of *Fusarium* wilt and heavy damages leading to financial losses (Knez *et al*, 2023; Yadav *et al*., 2023).

Hence, a gap has been created between the demand and supply of chickpea. This disease *Fusarium* wilt was first reported in 1918 from the Northwest region of undivided India (Yadav *et al*., 2023). Since then, it has been one of the major reasons of concern among scientists as this soil borne pathogen can survive in soil for about six years. The countries producing chickpea, including India, face losses of approximately 10%-90% in yield annually and about 100% if the pathogen gets favourable environmental conditions

(Mirchandani *et al*., 2023). The pathogen exhibits a very intense life-cycle in which it survives both as a parasite and a saprophyte. Due to the viability of its spores for a very long time, the pathogen is difficult to control. *Fusarium* wilt affects both desi and kabuli chickpeas, irrespective of growth stage (Singh *et al*, 2023; Ekwomadu and Mwanza, 2023). The wilt symptoms in the plants can be seen within 25 days after sowing and the symptoms of late wilting are observed at about 6-8 weeks of sowing (Yadav *et al*., 2023; Achari *et al*., 2023). *Fusarium* wilt symptoms begin with the infection entering the diseased plant's vascular system through the roots and spread to the younger leaves until the plant dies (Namisy *et al*., 2023). Various studies have shown that FOC is a seed and soil-borne pathogen, it generates chlamydospores and allows the fungus to establish the disease in the soil. The chlamydospores accumulate to such levels that the susceptible chickpea genotypes cannot be effectively planted for about 6 years in the same field (Srinivas *et al*., 2023, Sankari *et al*., 2023). To overcome this problem, the researchers have suggested many solutions and have contributed towards providing different disease controlling strategies.

In order to manage *Fusarium* wilt effectively, it is prudent and cost-effective to cultivate resistant varieties, however, because of their high pathogenic variability that limits their effectiveness, these varieties perform differently at different locations (Varala *et al*. 2023; Jamro *et al*., 2023). Hence, considering this situation, breeders must identify and produce highly productive chickpea genotypes using a mix of breeding procedures (Yadav *et al*., 2023). The field screening and marker assisted screening are the two most employed methods carried out to select the resistant and susceptible varieties (Yadav *et al*., 2023; Suleimanova *et al*., 2023). Field screening employs cultivation of the crop under observation in a sick field and exposing it to the pathogen. The disease severity and wilting percentage are the basic criteria that help in the selection process (Varala *et al*., 2023; Sharma, 2023). This type of screening on one hand provides an understanding on the vulnerable stages of the plant development and on other hand helps in selection of genotypes that are unharmed by the pathogen attack under the influence of environmental conditions (Yadav *et al*., 2024; Dey *et al*., 2023). The other technique, Marker Assisted Selection (MAS) has evolved as a result of advancement of technology. MAS is a credible and precise method that helps in identification of desired genetic traits in a plant. This technique exploits the use of molecular markers present in the vicinity of the desired genes. The molecular markers then detect the presence or absence of the genes thereby conveying the appropriate results (Jeon *et al*., 2023; Haider *et al*., 2023). Many DNA-

based molecular markers such as sequence tagged microsatellites (STMS), Random amplified polymorphic DNA (RAPD), Simple sequence repeats (SSRs) and Restriction fragment length polymorphism (RFLP) have been developed that are not only easy to use but also detect the polymorphism in the genetic makeup of an organism (Undal *et al*., 2023). This technique has not only expedited the selection process but also has eliminated the need of extensive field trials for selection of genotypes. However, both the field selection and MAS are crucial as they offer the researchers a palette of strategies that can be employed in agriculture for the development of resilient genotypes (Baloch *et al*., 2023; Venkatesan *et al*., 2023).

The use of resistant genotypes although sounds an appropriate and promising alternative to manage *Fusarium* wilt, it has several limitations. Many genotypes of chickpea having *Fusarium* wilt resistance have been developed, however, their response is highly variable depending upon the race of FOC and the environmental conditions (Samiksha *et al*., 2023; Jorben *et al*., 2023). Another reason for the inefficient usage of such varieties is the undesirable agronomical characteristics like narrow sowing time and excess of secondary metabolites etc., associated with the wild donor parent of chickpea, the new genotypes are not adapted usually. Moreover, due to the high pathogenic diversity and rapid evolution of FOC races, the new varieties cannot be deployed effectively (Venkataramanamma *et al*., 2023; Yadav *et al*, 2023). Besides this, many other conventional approaches like changes in time of seed sowing and irrigation are common among the farmers. The other approaches include application of fungicides to control the disease. However, the continuous application of these chemicals not only are ineffective in complete eradication of the pathogen but also affect soil's flora and fauna negatively (Kure *et al*., 2023; Yadav *et al*., 2023; Venkataramanamma *et al*., 2023). The awareness about environment and human health is increasing among the population due to which there is a huge demand of sustainable production of chickpea. Therefore, the focus of researchers has now shifted from chemical-based control methods towards the use of biocontrol methods (Gupta and Saxena, 2023; Sandhu *et al*., 2023; Anuar *et al*., 2023).

The biocontrol methods of controlling the *Fusarium* wilt include the utilization of the microorganisms that are non-pathogenic and beneficial for plants and soil. In the pursuit of sustainable strategies for managing diseases, the use of plant- beneficial microorganisms is a much promising technique (Boulahouat *et al*., 2023; Mishra *et al*., 2023). This method not only activates the defense-system, but also increases the disease

resistance of plant without compromising soil's flora and fauna (Khoshru *et al*., 2023). Many studies have recorded Arbuscular Mycorrhizal Fungi (AMF) as one of the most potent and efficient microorganisms that helps in reducing disease severity by regulating a variety of mechanisms. Hence, AMF has been recommended as a natural aid in maintaining and promoting sustainable agriculture (Wahab *et al*., 2023; Boyno *et al*., 2023). Multiple studies have shown that AMF species, particularly *Glomus mosseae* and *Rhizophagus irregularis*, can reduce disease incidence caused by *Fusarium* spp. through mechanisms such as improved nutrient acquisition, increased systemic resistance, and root colonisation that prevents pathogen entry (Kashyap *et al*., 2023; Khanum *et al*., 2024). The biocontrol capability of AMF in chickpea has been investigated under a variety of soil types and environmental circumstances, with similar results of boosting plant tolerance and production in the face of *Fusarium* wilt stress (Paravar *et al*., 2023; Wahab *et al*., 2023).

The mechanism underlying the activation of defense system and increase in disease resistance of a plant on AMF inoculation is the change in gene expression of the plant (Menge, 2023). Inoculation of plant with either FOC or AMF involves a complex molecular cross-talk which generates cascade of reactions, therefore, generating desired signals and responses (Kumawat *et al*., 2023; Singh *et al*., 2023). In order to understand such complex mechanisms, the researchers resort to the technological advancements. Biotechnology has endowed the researchers with new techniques like Next Generation Sequencing (NGS) that enables them to understand the plant-pathogen interactions at the genetic level. NGS is a revolutionary technique that helps in unravelling intricacies of the biological systems by rapidly sequencing the genome. This has helped researchers in looking at the various facets of a genome and gene expression while comprehensively studying the genetics (Joshi *et al*., 2023; Machado, 2023). The current research was therefore designed to focus on screening out the resistant genotypes of chickpea against FOC by using both morphological and molecular screening methods. This integrated approach will enable the researchers to sieve out the robust and resilient genotypes of chickpea along with enabling the farmers to have access to resistant genotypes that will minimize their yield losses. The research also focuses on screening out the suitable AMF species that can be used as a biocontrol for the management of *Fusarium* wilt. By selecting suitable AMF fungi, this research can help farmers maximize the effectiveness and sustainability of biocontrol against *Fusarium* wilt, contributing to healthier chickpea

yields and more resilient cropping systems. The research further delves into the use of NGS to understand the changes in gene expression of a plant co-inoculated with FOC and AMF. The research will help not only in getting a deeper understanding on the potential and significance of AMF in managing biotic stress but will also unravel the expression profile of various genes involved in various conditions that plants are exposed to.

CHAPTER-2 REVIEW OF LITERATURE

Chickpea is a major rabi-season legume crop that provides nutrition to the world's population. The crop is however, challenged by many stresses includingbiotic and abiotic, which limit its productivity (Mart, 2022). The major abiotic stresses affecting chickpea production include drought and salinity stress and the major biotic stresses affecting chickpea production include wilt and blight diseases. The wilting caused by the pathogen *Fusarium oxysporum* f.sp. *Ciceris*is a major constraint in the production of chickpea (Sharma *et al*., 2019). In India, the annual losses in yield due to *Fusarium* wilt usually range from as lowas 10% to as high as 100%, if the climatic conditions are favourable to the pathogen. The yield losses also depend upon the factors like severity level of thedisease, the susceptibility of cultivar and stage of infection. In India, the diseaseclaims 100% crop output, and as a result, chickpea acreage has decreased substantially from 4.7 to 0.7 million acres in northern regions such as Punjab, Haryana, and Uttar Pradesh.

Although the adoption of resistant genotypes is a promising method, it is not dependable for a long term since new pathotypes are constantly emerging (Mannur *et al*., 2019; Venkataramanamma *et al*., 2023). As a result, a comprehensive method to screen resistant and susceptible genotypes, as well asbiocontrol of *Fusarium* wilt, is required.

2.1. Chickpea, the host plant

2.1.1. The taxonomy and basic biology of chickpea

Chickpea is thought to have originated in the region between the southeast portion of Turkey and the region bordering Syria (Sajja *et al*., 2017). The genus*Cicer* belongs to the tribe *Cicereae*, subfamily *Papilionaceae*, and family *Leguminosae* (Kumar *et al*., 2021). The family consists of 33 perennial and 9 annual species of *Cicer*. Chickpea, deriving its name from the Greek word *Kikus* that means "force of strength", is known as *Cicer arietinum* L. The crop is an annual herb with short and erect growth pattern. Except for the petals ofthe flowers, the entire chickpea plant is coated in glandular and non-glandular hairs that defend it from sucking insects. The stem of the chickpea plant has main, secondary, and tertiary branches. The secondary and tertiary branches bearleaves. Although there are occasionally solitary leaves, compound leaves with 6-7 pairs of leaflets are more common. The flower of chickpea plant is typically papilionaceous with one large petal, two wings and two boat shaped keels.

The stamens are diadelphous (9+1) and the stigma has ovules number ranging from 1 to 4. To ensure the self-pollination, anther dehiscence is observed a day beforethe flower opening (Sajja *et al*., 2017; Kumar *et al*., 2021). Within four weeks of pollination, the pods mature containing about 1 to 3 seeds/pod. The crop is diploid (2n=16) and has a genome size of 732 Mb (Mohan and Thiyagarajan, 2019). The seed colour is a very important characteristic on the basis of which, chickpea has been differentiated into Desi and Kabuli types. The Desi chickpea seeds are comparatively smaller in size than the Kabuli seeds, and weigh around 0.2 gm per seed. The seed coat is thicker and has brown pigmentation. The plantof desi chickpea bears flower of pink colour and have stems containing anthocyanin pigmentation. The Kabuli chickpea seeds on the other hand, weighapproximately 0.4 gm, have a thinner seed coat and have beige colour. The plantbears white flowers and the stem lacks anthocyanins (Sahu *et al*., 2022).

2.1.2. Importance of chickpea

The edible seeds of family *Leguminoseae* are referred to as "Pulses". These are the nutrient-rich food that have been used by humans since decades as a nutritionsource. Chickpeas are one of the major varieties of pulses recognized by the Food and Agriculture Organization of the United Nations (Ferreira *et al*., 2021).It is well known that pulses containing important amino acids, particularly sulphur-containing amino acids, are required for the human diet. Although not used as a staple food, chickpea holds a vital place in the human diet as a significant source of dietary protein for the inhabitants of the nation (Sajja *et al*.,2017; Kumar *et al*., 2021). Chickpeas contain roughly 24% protein, however this can range from 15 to 30% depending on variety and environmental conditions. They serve as an excellent source of carbohydrates and one of the vital vitamins, Vitamin B. They come under gluten-free food category and therefore, have become the food of interest nowadays. Moreover, they contain avariable amount of fibre, carbohydrates, vitamins and other bioactive compounds and therefore, are associated with a lowered risk of cardiovascular diseases (Gupta *et al*., 2019).

The chickpea plant not only benefits humans by supplying sufficient amount of nourishment but, also enriches soil and plants by increasing fertility and fixing nitrogen. The roots of chickpea plants harbour the nitrogen-fixing bacteria, *Rhizobium* in the root nodules. The plants cannot utilize Nitrogen in the elemental form and therefore, the *Rhizobium* bacteria helps in converting nitrogen from elemental form to the utilizable

form i.e. NH_4^+ and NO_3^- (Sharma*et al*., 2023). These bacteria reside in the nodules of chickpea plant root forming a symbiotic association and contributing in Biological Nitrogen Fixation (BNF). BNF is an economically and ecologically sound way of increasing nitrogen inputs in soil thus leading to enrichment of soil with nitrogen compounds naturally (Soumare *et al*., 2020).

2.1.3. Chickpea yield and losses

According to the recent data of FAOSTAT, the world chickpea production is estimated to be 13.54 Megatonnes with India contributing to over 75% of this global chickpea production (FAOSTAT, 2024). However, due to India's growing population, the supply in terms of production is unable to keep up withthe rising demand, which resulted in the import of roughly 3 lakh tonnes. The FAOSTAT data shows many ups and downs in the yield of chickpea over the past 10 years (Figure 2.1).

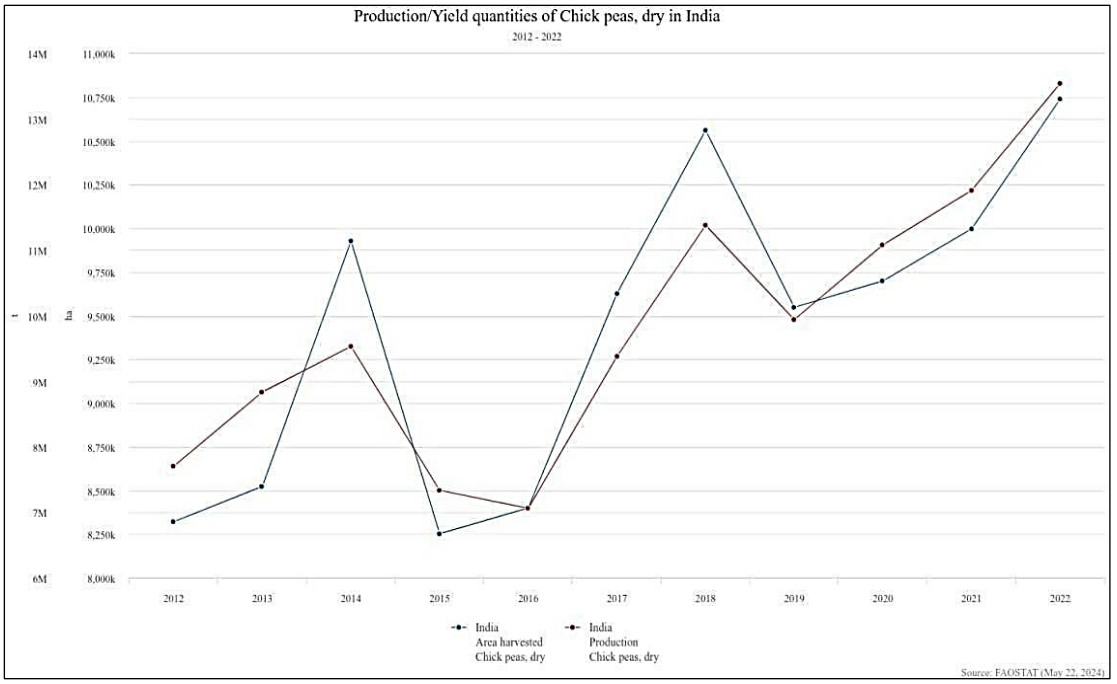


Figure 2.1: Production/Yield of Chick peas (dry) in India, FAOSTAT, 2024

The estimated yield of chickpea for the year 2021 is 10883 g/ha which is lower as compared to the yield during the year 2020 i.e. 11423 g/ha. There are many biotic and abiotic stresses that have a significant role in preventing the chickpeacrop from reaching its maximum yield potential. The major abiotic stresses include salinity and drought whereas the biotic stress include pathogen attacks, out of which the most dangerous is fungal pathogen attack caused by the fungus *Fusarium oxysporum* (Fo). The challenges caused by the fungus *Fo* has put the global legume production under a great risk. The researchers have put this pathogen at the fifth place among the top 10

pathogens that are of economic importance (Singh *et al*., 2022). The investigations led by Kumar *et al*., in 2021have revealed that the areas affected by the *Fusarium* wilt include Central India,Southern India, Northern India and the Eastern India. The losses caused by FO in India are around 10-15% per year. However, the percentage of these misfortunes shoot up to 60-70%of the crops are exposed to the pathogen for a long time period. In India, on getting a suitable environment, the pathogen may create a havoc causing 100% yield loss (Dhawale and Dhale, 2021).

2.2. The pathogen: *Fusarium oxysporum* f.sp. *Ciceris*

FOC is a fast growing pathogen with variable morphological growth characteristics viz. macroconidia, microconidia and mycelial growth. Different race isolates of the pathogens show differences in the colony growth and colours(Rani *et al*., 2022).

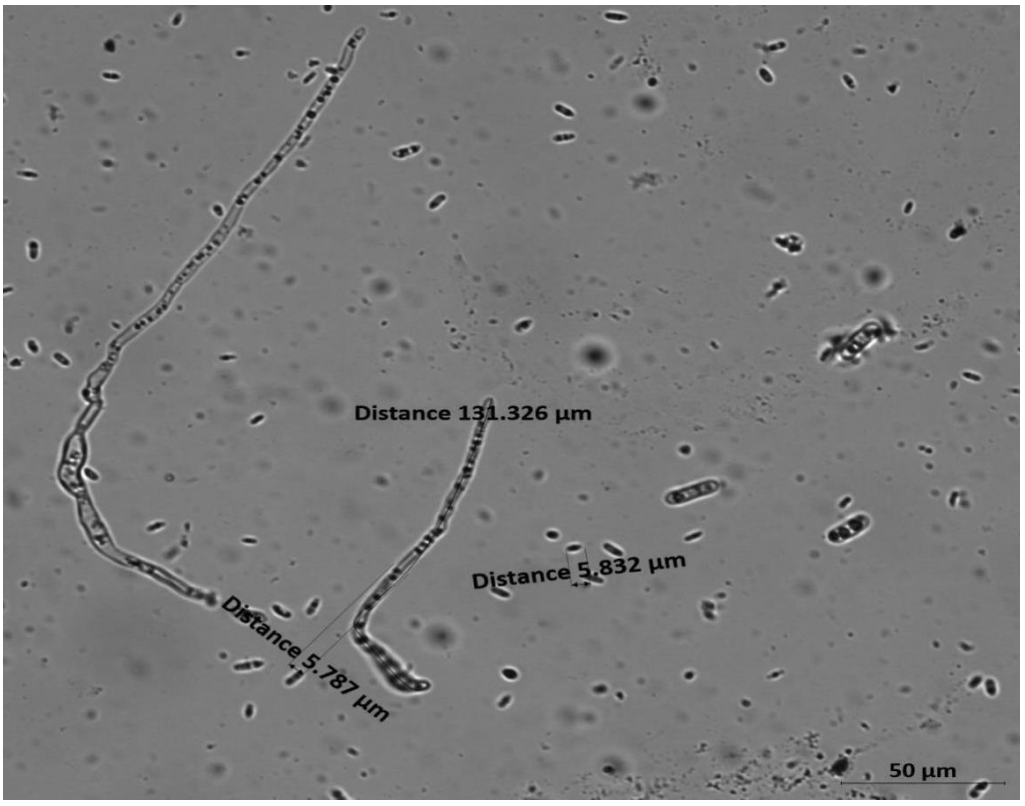


Figure 2.2: Mycelia of *Fusarium oxysporum* f. sp. *Ciceris* (captured by light microscope Axio Imager.M2m, Department of Plant Pathology, CCS HAU, Hisar)

2.2.1. Scientific categorization of *Fusarium oxysporum*

Fusarium oxysporum is a species complex that comprises of soil-borne fungi found in both cultivated and non-cultivated lands all over the world. It is a facultative parasite belonging to phylum *Ascomycete* and genus *Fusarium* (Astapchuk *et al*., 2020;

Jadhav *et al*., 2021; Jacobi *et al*., 2023).

Fusarium oxysporum is categorised by an unofficial rank known as formae speciales (f. sp.), which is typically given to the FOC based on its capacity to spread disease to a specific host plant. Although it was previously believed thatthese formae speciales descended from a single ancestor, genomic research of some species, such as *Fusarium oxysporum* f. sp. *cubense*, has shown that thesediseases have a polyphyletic origin (Samiksha and Kumar, 2021). The formae speciales are further sub-divided into races based on their virulence (Dandale *et al*., 2022).

2.2.2. Races

The concept of "races" within fungal pathogens holds historical significance. In 1913, the term was first used to categorize *Puccinia graminis* (Edel-Hermann & Lecomte, 2019). For *Fusarium oxysporum*, the term "race" refers to a "biotype" distinguishable from others based on its physiological characteristics and disease-causing abilities (pathogenicity). Research suggests the global distribution of eight physiological races of FOC (Bahadur *et al*., 2021). Races 1A, 2, 3, and 4 have been identified in India, while races 1B/C, 5, and 6 are prevalent in the USA and the Mediterranean region (Achari *et al*., 2023). This categorization stems from the varying disease severity and symptoms exhibited by different races on infected plants. Races 0 and 1B/C typically induce yellowing, while races 1A, 2, 3, 4, 5, and 6 cause wilting (Achari *et al*., 2023). Within India, all races are present, with races 2, 3, and 4 concentrated in the north (Lal *et al*., 2022). Notably, races 3 and 4 are particularly widespread in Punjab and Haryana (Bharadwaj *et al*., 2022). Dubey *et al*. (2009) successfully isolated various FOC races from diverse Indian regions. They studied the isolates' morphological features and pathogenicity. These isolates were subsequently deposited in the Indian Type Culture Collection (ITCC) at the Indian Agricultural Research Institute (IARI), Delhi, under unique accession numbers in 2014. For instance, the FOC culture with accession number ITCC 7679 belongs to race 3 and originates from Ludhiana, Punjab (ITCC catalogue, IX edition, 2019; Dubey *et al*., 2012). While previousstudies by Dubey *et al*. (2014) suggest high pathogenicity of the FOC strain ITCC 7679/ FOC-45, limited research exists on the specific impact of race 3 onchickpea cultivars.

2.2.3. Life cycle of *Fusarium oxysporum*

Fusarium oxysporum is an asexually reproducing fungus that causes severe vascular wilt in plants. The soil and air temperature ranging from 24^{o}C-28^{o}C is

considered as the optimum temperature for the disease progression by the pathogen (Huang *et al*., 2023; Khanna *et al*., 2022). This soil borne pathogen canspread to long distances via infected transplants or human interferences (Nazir *et al*., 2022; Kutama *et al*., 2022). It produces three different types of asexual spores known as micro-conidia, macro-conidia and chlamydospores. The pathogen stays dormant and immobile in the form of chlamydospores on the decaying plant tissues present in the soil for many years. These spores germinateinto macro and microconidia on getting a suitable host (Soni *et al*., 2023). The germinated hyphae penetrate through the roots, leaves, the stomatal openings orthe wounds on the plant. The leaves start showing symptoms of infection in laterstages after the elapse of incubation period (Tsror, 2023) (Figure 2.3). The pathogen requires both parasitic and saprophytic phases to complete its lifecycle. There are three phases in the life cycle of *Fusarium* (1) Determinative phase, (2) Expressive phase and (3) Saprophytic phase. During the determinative phase, the pathogen's colonisation extent is determined. During the expressive phase, symptoms emerge in the plant, and the saprophytic phase occurs when the pathogen develops spores and remains on dead and decaying plant waste (Upasani, 2017).

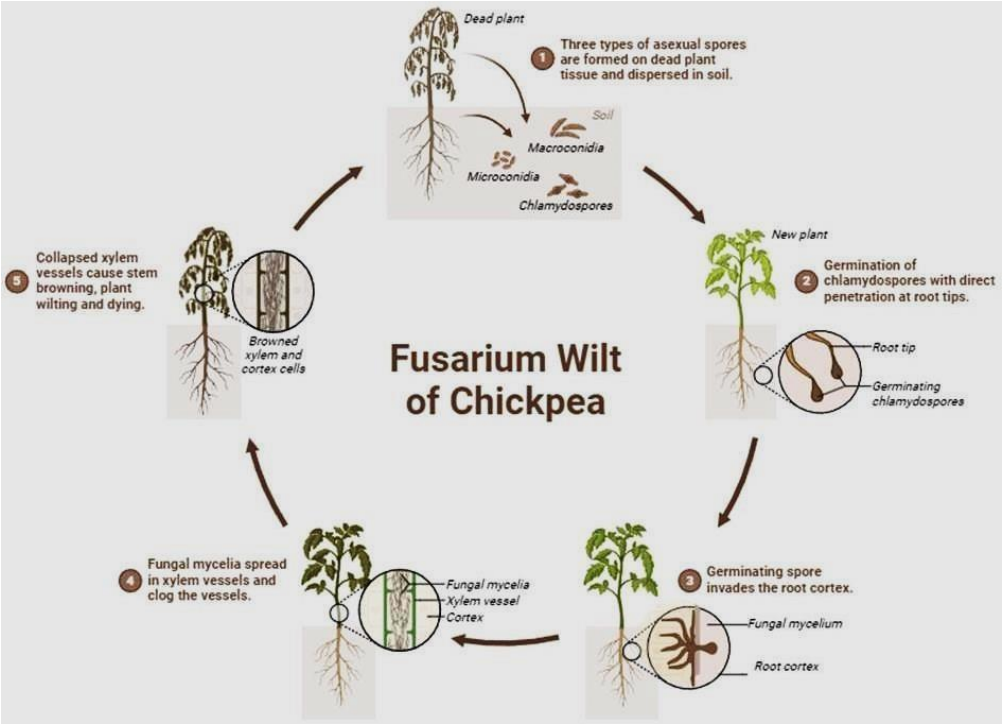


Figure 2.3: Life cycle of *Fusarium oxysporum*

2.2.4. Effect of plant-pathogen interaction

Plants and pathogens usually come in contact with each other through open wounds and pores. However, understanding the molecular and biochemical mechanisms behind pathogenesis and plant defense is of fundamental importance especially if the disease poses a threat to the food security. Establishment of disease in the host plant or acquisition of resistance against thepathogen depends on many factors including the race of the pathogen and genotype of the plant. The interaction between pathogen and plant is very complex and results in the expression of genes related to pathogenicity in the fungus as well as the defense-related genes of the host plant (Dandale *et al*., 2022). A variety of defense responses like suberization, formation of Reactive Oxygen Species (ROS), changes in the activity of enzymes like catalases, peroxidases, β-,3 glucanase, etc., have been observed in the plants after infectionwith FOC (Muche and Yemata, 2022).

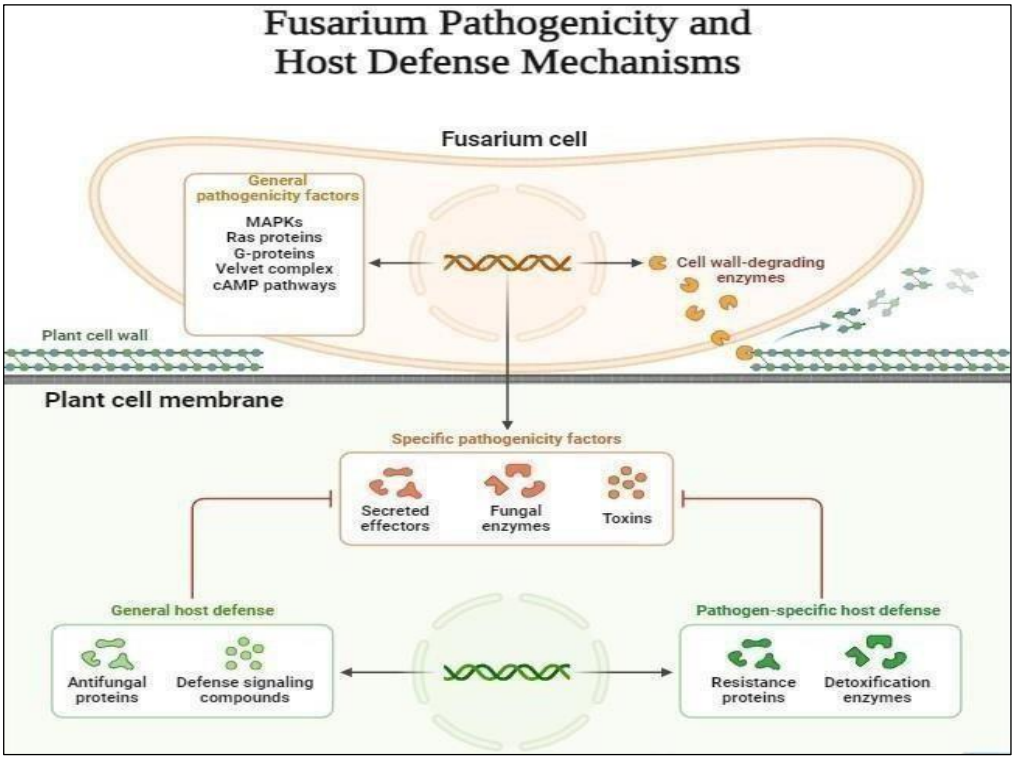


Figure 2.4: *Fusarium* Pathogenicity and Host Defense Mechanisms (Ma *et al*, 2013)

2.2.5. Symptoms

The term “wilt” typically suggests the wilting symptoms developed in a plant due to the drought conditions. The wilting plants show drooping leaves and branches, fading of the leaves to yellow and brown and ultimately falling off (usda.gov). In chickpea plants, the wilt symptoms appear within 25 days after sowing. This usually

happens in the case of susceptible genotypes and is knownas “Early wilting”. However in the case of “Late wilting”, the symptoms are shown 6-8 weeks after sowing. In the susceptible chickpea genotypes, the condition is worsened as the infection leads to a complete collapse of the plant (Chaithra *et al*., 2019). The infection can be diagnosed by observing the presenceof fungal hyphae and spores in the xylem tissue of the plant. A dark- brown vascular discoloration is observed when the plant roots are split open longitudinally (Hashem *et al*, 2020; Varala *et al*., 2023). This dark-brown discoloration is believed to be a consequence of accumulation of toxins releasedby FOC. The plants affected by FOC therefore do not receive the water due to the blockage of xylem tissue. This leads to the wilting of the plant. Moreover, the pathogen affects the seeds of the plants. Seeds collected from the wilt- infected plants have a lower weight, are blunt and wrinkled than the seeds collected from the uninfected plants (Hashem *et al*., 2020; Dhawale and Dhale,2021).

2.3. Disease management

Considering the havoc created by the pathogen, it is extremely necessary to control and manage the disease at an early stage. Many practices are followed to reduce the risk of crop loss due to FOC infection.

2.3.1. Cultural practices

The farmers follow many cultural practices to combat the disease. In many cases, the infection is spread due to the human intervention. Therefore, extensivehuman activities are checked and the use of infection-free seeds for sowing is encouraged. The pathogen-free seeds are kept quarantined for an effective disease control (Sampaio *et al*., 2020). The other methods include solarisation of soil and crop rotation. In the solarisation process, the soil containing mulchedplants is kept covered to increase the temperature. This helps in reduction of FOC inoculum. The process of solarisation together with crop rotation helps in reduction of disease (Dwivedi and Dwivedi *et al*., 2020; Sampaio *et al*., 2020). A successful crop rotation is possible only if the field has crop-rotation of the plants which are non-hosts of the *Fusarium oxysporum* for over six years as thechlamydospores of the pathogen stay in the soil for about six years. In spite of the availability of information about the host/non-host ranges of legume infecting FO f.sp. *Ciceris* optimization of promising legume crop rotation remains a challenge (Nikitin *et al*., 2023).

2.3.2. Use of chemicals

The management of fungal diseases is usually done by the use of agricultural chemicals. These chemicals are usually synthetic fungicides which are generallyused to treat seeds (Nagpal *et al*., 2020). There have been many reports of effective pathogen control using the fungicides like Carbendazim and Mancozeb, however, these are costly and undesirable due to their effect on the environment (Maurya *et al*., 2019; Khanna *et al*., 2021). The constant use of these synthetic products takes a toll on the soil's flora and fauna and degrades the environment in many ways (Singh *et al*., 2022). These chemicals not only pollute soil but also affect groundwater to a large extent and make it unsuitable for drinking and irrigation purposes. The use of chemicals is therefore discouraged globally and more emphasis is given on the use of other sustainableand environment friendly methods to reduce pathogen attack and symptoms (Pradhan *et al*., 2022).

2.3.3. Use of resistant genotypes

Due to difficulty in execution of complex cultural practices and side effects of usage of chemical treatments to control the disease, considerable emphasis is now being paid on the use of resistant chickpea genotypes (Mbasa *et al*., 2021).The cultivation of resistant plant genotypes is one of the most reliable and cost-effective methods to control the wilt caused by FOC (Thangavelu *et al*, 2020). Selection of wilt resistant cultivars has long been carried out on the basis of the response of plants against FOC. The experiments for identification of the wilt resistant varieties are carried out in fields and in greenhouses as well as in laboratories using markers (Yadav *et al*., 2023).

2.3.3.1. Phenological screening

This type of screening involves observing the effect of pathogen on crop at different time periods. This approach is crucial for understanding the influence of a disease on the different growth stages of a plant. The crops are sown in sickfields to assess their response against the pathogen and the resistant genotypes are selected (Lahlali *et al*., 2022). The phenotypic selection of the resistant chickpea genotypes include the characters like wilting percentage and the extent of disease severity (Yadav *et al*., 2023; Jorben *et al*., 2023). These studies are essential in providing an insight on the host-pathogen interactions and help in prediction of the vulnerable stages of a plant development. The phenological studies also enable researchers and farmers to develop disease management strategies prior to its outbreak. However, the phenological studies are very costlyand consume a lot of time and space (Karamidehkordi *et al*., 2023; Neyns,

2022).The recent advancements in technology has allowed the researchers to dive deep into the genetic basis of resistance and understanding the underlying mechanism of the responses of plants in presence of pathogen (Yadav *et al*., 2023; Kashyap*et al*., 2023).

2.3.3.2. Molecular Screening

Recent developments in genomics research have allowed for the discovery and use of molecular markers for crop improvement. Marker assisted selection (MAS) is a sophisticated technology that has aided in the targeting of desired genes. Markers have proven to be useful in improving selection efficiency and developing new cultivars (Yadav *et al*., 2023; Kashyap *et al*., 2023). With the advancement of technology, many novel methods have been developed which reveal the characteristics of a plant in a very concise manner. In this method, theplants are not influenced by any environmental stress or pathogen and therefore reveal the untamed genetic components to the researchers. The MAS allows to screen out a large number of genotypes in a very less time at a very precise location and without involvement of any labour (Samiksha *et al*., 2023; Salgotra and Chauhan, 2023). Moreover, with the use of MAS, the selection and inheritance of desirable genes in plants has become possible. Hence, MAS is a highly reliable technology that is employed in screening out of the resistant varieties. Screening of genotypes using MAS requires molecular markers to indicate the presence of resistance genes (Parveen *et al*., 2023; Suleimanova *et al*., 2023). Many DNA based markers have been identified in the host plant which are linked to resistance genes against the races 1-5 of FOC (Table 2.1). These markers are based on the polymorphisms which are associated with the resistance genes e.g. Single nucleotide polymorphisms (SNPs) and Simple sequence repeats (SSRs) (Suleimanova *et al*., 2023; Bahar and Esra, 2023).

Table 2.1: Markers associated with the genes for resistance against differentraces of *Fusarium oxysporum* f.sp. *Ciceris*

Race of FOC	Genes associated with resistance	Markers associated withgenes	Reference
0	*foc-01/FOC-01 foc-02/FOC-02*	TR-19, TA-194, and TA-660 OPJ20,600 and TR-59	Yadav *et al*., 2023; Lal *et al*., 2021; Choudhary *et al*., 2022
1	*h1, h2, H3*	TA-96, TA-110 and H3A12, CS 27	Sahu *et al*., 2020; Lal *et al*., 2021
2	*FOC-2*	TA-110, TR-19, TS-82, and CS27 TA-96, H3A12	Sahu *et al*., 2020; Lal *et al*., 2021
3	*foc-3/FOC-3*	TA-96, TA-27, TA-194	Caballo *et al*., 2019; Lal *et al*., 2021
4	*FOC-4*	TA-96, CS27	Sahu *et al*., 2020; Lal *et al*., 2021; Choudhary *et al*., 2022
5	*foc-5/FOC-5*	TA-59, TA-37, TA-96	Sahu *et al*., 2020; Caballo *et al*., 2019; Lal *et al*., 2021

The use of MAS technology can therefore help in screening of a large number of genotypes in a very less time and at a very less cost. However, these genotypes also do not perform well at all locations as the effectiveness of resistance is limited by high pathogenicity of the pathogen at different locations (Yadav *et al*., 2023; Dubey *et al*., 2012; Srivastava *et al*., 2021). The marker technology along with advanced breeding techniques serve as a tool in the development of high yielding and disease-free genotypes.

2.3.4. Advanced breeding techniques

Identification of desirable genes through marker assisted approach has revolutionized the development of plants with desired traits (Bacha and Iqbal, 2023). Advanced breeding techniques with a focus on genomics has considerably increased the efficiency of crop improvement. The genomic science unravels the organization of genes and gives details on how these genes are expressed to produce complex phenotypes. This approach undertakes marker assisted selection, gene mapping and genome sequencing (Bacha and Iqbal, 2023; Yadav *et al*., 2023). The traits like resistance against a disease and yield of the crops have been considered to be complex and as they are usually presentat different genomic regions with very small genetic contributions. Marker assisted selection therefore poses limitations as it only targets

only significant genes or Quantitative Trait Loci (QTLs) (Mishra *et al*., 2021). Hence, a successful genomic selection and development of genotypes having all the desirable traits requires a high throughput genotyping, an accurate phenotyping data combined with RNA-seq (Mishra *et al*., 2021; Yadav *et al*., 2023; Garg *et al*., 2023). RNA-seq using Next Generation Sequencing (NGS) is a very powerful and reliable tool that helps in identification of trait related genes and reveals how these genes express themselves under different environmental conditions. The analytical studies can therefore help in development of high yielding and biotic and abiotic stress resistant genotypes (Yang *et al*., 2023; Kumar *et al*., 2022).

2.3.5. Biological Control

Soil is niche to a large number of non-pathogenic microorganisms which help in maintaining a good soil health and ecology. In an ecosystem, the microbial communities are considered as the bioreactors that help in reduction of plant diseases by a variety of mechanisms. These mechanisms include induction of resistance response in plants, producing antifungal effect, enhancement of soil microbial activities to suppress pathogen (Hao and Ashley, 2021). Hence, usingmicroorganisms beneficial for both soil and plants is one of the most effective and eco-friendly way to manage the soilborne plant diseases (Elbouazaoui *et al*.,2022). There have been numerous reports suggesting that a reduction in beneficial microbial diversity of soil has led to an outburst of soil-borne diseases(Ye *et al*., 2020). The reports have also suggested that these microorganisms actas antagonist against the pathogens and have potential to reduce the growth of *Fo* (Singh *et al*., 2020). Arbuscular Mycorrhizal Fungi is one such microorganism that helps in increasing the disease tolerance of the plant and improving the uptake of nutrients and phosphate by the plants (Kumari and Prabina, 2019).

2.4. Arbuscular Mycorrhizal Fungi

AMF are commonly occurring species of non-pathogenic fungus that are found in the rhizosphere. The fossil and molecular records suggest that they first appeared on earth about 400 to 450 million years ago. AMF are well known since decades to play a significant role in maintaining plant's health by providing nutrition (Diagne *et al*., 2020).

2.4.1. Classification of AMF

Arbuscular Mycorrhiza is a ubiquitous microorganism and well known to establish a symbiotic relationship with approximately 80% of the vascular plantsof the

world (Cofré *et al*. 2019). In early classifications, the species were classified on the basis of the spore morphology and were grouped under the order Glomerales of phylum Glomeromycota. However, the researchers Schüßler and Walker rearranged the phylum on the basis of small and large subunits rRNA genes, β-tubulin and ITS region. This led to the formation of twofamilies *Glomeraceae* and the *Claroidoglomeraceae* under the order Glomerales(Gough *et al*., 2020).

2.4.1.1. Characteristics of AMF

The term "mycorrhiza" has Greek origins, combining "mykos" meaning fungus and "rhiza" meaning root. This aptly describes the symbiotic association formed between these fungi and plant roots (Ganugi *et al*., 2019). Within this partnership, the fungus develops intricate, tree-like structures called arbuscules that penetrate the plant's root cells. These arbuscules play a critical role in facilitating nutrient exchange, enhancing the plant's ability to acquire essential elements from the soil (Malar *et al*., 2022). Arbuscular mycorrhizal fungi (AMF)encompass a diverse group of fungi that form these mutualistic relationships with a vast majority of land plants. AMF play a significant role in plant health and ecosystem function by promoting nutrient uptake, improving plant growth, and enhancing survival rates.

2.4.2. About AMF

The morphology of AMF is characterized by the presence of specialized structures called sporocarps. These sporocarps are the reproductive structures ofAMF and are typically found in the soil surrounding the plant roots (Mitra *et al*.,2022).

2.4.2.1. Sporocarp

Sporocarps are small microscopic bodies which are and often difficult toobserve with naked eyes. With variable shape and size, the sporocarps appear tobe white or pale in color. The sporocarps contain spores, which are the reproductive cells of the fungi. These spores are responsible for the dispersal and propagation of AMF. The morphology of the sporocarps and spores can vary between different species of AMF, allowing for the identification and classification of these fungi (Vogt-Schiib *et al*., 2022). Sporocarps therefore, are crucial in understanding the life cycle of AMF.

2.4.2.2. Arbuscules

Arbuscules are the specialized structures formed by AMF inside the root cells of the host plant. These structures are responsible for the exchange of nutrients between the fungus and the plant (Monika *et al*., 2022). These are highly branched, tree-like structures that penetrate the host plant's cells. They have a large surface area, which allows for

efficient nutrient transfer. The exchange of nutrients occurs through numerous intimate contacts between the arbuscules and the plant cells (Shi *et al*., 2023).

2.4.2.3. Hyphae

The hyphae of AMF are slender, thread-like structures that extend from the arbuscules into the surrounding soil (Taulera, 2023). These hyphae play a crucialrole in the acquisition of nutrients from the soil. They have a high surface area to volume ratio, which enables them to explore the soil efficiently and absorb nutrients such as phosphorus, nitrogen, and water. The hyphae also facilitate the transport of these nutrients back to the host plant (Wen *et al*., 2023; Feng *et al*.,2022).

2.4.3. Life cycle

Arbuscular mycorrhizal fungi (AMF) exhibits a complex life cycle involving both sexual and asexual reproduction. Asexual reproduction, the dominantmode, relies on spores for propagation (Tzng, 2022). These spores develop within soil-borne fungal structures called vesicles. Spores remain dormant untilencountering suitable host plant roots. Upon contact, the spores germinate and form hyphae that colonize the root, establishing a symbiotic connection through the formation of arbuscules (Branco *et al*., 2022). The fungal network expands outward via hyphal growth, exploring the surrounding soil for nutrients. As the host plant matures, the fungal network keeps pace, facilitating a continuous exchange of resources (Das *et al*., 2022). During the asymbiotic phase, spores germinate under favorable moisture and temperature conditions, producingshort-lived hyphae. These hyphae exhibit a remarkable ability to reorient their growth towards root exudates released by potential host plants, triggering a distinct branching pattern (Giovannini *et al*., 2020). This intricate interplay between arbuscules, hyphae, and the overall life cycle of AMF underscores theirefficiency. The presence of both sexual and asexual reproduction strategies ensures the successful spread and persistence of these beneficial fungi in the ecosystem (Giachero *et al*., 2022; Sudharsan *et al*., 2023).

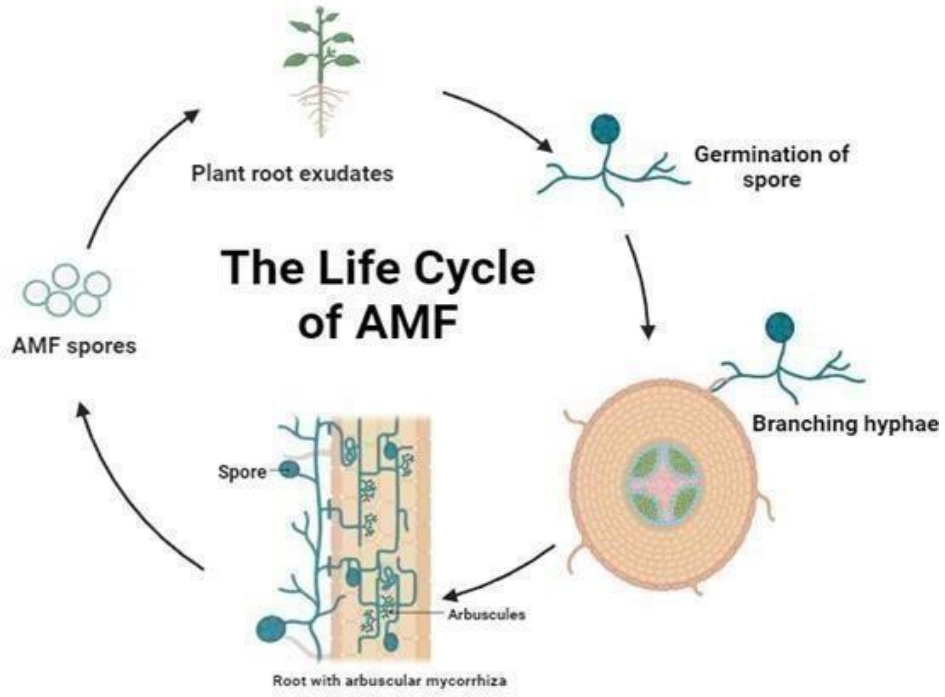


Figure 2.5: Life cycle of AMF

2.4.4. Mass Production of AMF

While isolating and selecting AMF species is a powerful tool for plant growth promotion, cultivating them in pure culture remains a challenge. This process relies on a compatible host plant to maintain the AM inoculum's viability (Gelvez-Pardo *et al*., 2023; Juntahum *et al*., 2022). Two critical factors influence successful inoculum generation: 1) selecting fungal isolates that effectively promote the target plant's growth and 2) choosing an inoculum that thrives on the host plant, not just benefiting the targeted plant (Sharma *et al*., 2017; Bender *et al*., 2019). The inoculum should be effective in field circumstances and demonstrate utility in the target plant. The plants that naturally promote increasein AM colonisation are sometimes referred to as stock plants (Sharma *et al*., 2017). To fully realise the promise of AMF, efficient ways for mass producing AM fungus are necessary. *In-vitro* root organ culture (ROC) is one of the most effective methods, as it nurtures the symbiosis between AMF and host plant roots in pots or chambers (Sharma *et al*., 2023). The controlled lighting, temperature, and nutrition regimes provide optimal circumstances for specific AMF strains and appropriate host plants, resulting in maximum colonisation andsporulation (Chandarana *et al*., 2023). These control conditions during the experiment make the symbiotic connections easier, paving the possibility for animproved and efficient performance of both plants and AMF. Despite the constraints of slow sporulation and the risk of contamination, ROC's focus to imitating natural systems and cultivating diverse, high-

quality inoculum has enormous promise. The widespread multiplication of AMF under ROC is primed to move sustainable agriculture forward, promising a future in which plants and their fungal friends thrive in harmony (Khan *et al*., 2020; Perera Garcia *et al*., 2022).

2.4.5. Transformed and non-transformed root organ cultures

The transformed ROC technique involves development on AMF on the hairy roots produced by the bacteria *Agrobacterium tumefaciens* whereas a non- transformed ROC technique does not involve the transformation of roots of hostplants (Su, 2021; Ghorui *et al*., 2023). ROC of AMFs a specialised technique for investigating the symbiotic connection between these fungi and plant roots under controlled conditions (Richter *et al*., 2023). Unlike transformed root cultures, which involve genetic alteration, non-transformed ROC uses unaltered plant roots to preserve the natural genetic makeup and physiological responses.This method often entails growing excised root segments of a host plant in a sterile, nutrient-rich media alongside AMF spores or mycelium (Ghorui *et al*., 2023). The roots supply the fungi with vital carbohydrates, while the fungi improve nutrient uptake by the roots, notably phosphorus. Non-transformed ROC is useful because it preserves the natural interactions between plants and fungi, allowing for comprehensive insights into the biochemical and molecular mechanisms underlying mycorrhizal symbiosis (Kumar and Saxena, 2017; Selvakumar *et al*., 2018). This system enables real-time observation of root colonisation, fungal development, and metabolite exchange, without the complications associated with whole-plant systems (Zhao *et al*., 2024). Furthermore, non-transformed ROC is useful for assessing the efficacy ofdiverse AMF strains, investigating the effect of varied environmentalcircumstances on mycorrhizal association, and investigating the potential of AMF in increasing plant health and production in sustainable agriculture. The capacity to grow and examine these cultures *in-vitro* is a valuable tool for furthering our understanding of AMF biology and its implications in crop resilience and soil health (Ghorui *et al*., 2023; Zhao *et al*., 2024).

2.5. AMF mediated suppression of *Fusarium* wilt

AMF helps in managing the *Fusarium* wilt by influencing the plant's health and improving the defense mechanism. It plays a multifaceted role by enhancing nutrient flow and conferring tolerance against the biotic and abiotic stresses (Sharma *et al*., 2023). The studies also reveal that the AMF competes with the pathogen for nutrients and space leading to a suppression of pathogen. The mechanism behind the AMF

mediated suppression of *Fusarium* wilt involves both molecular and biochemical changes in the plants (Sarkar and Sadhukhan, 2023).

2.5.1. Morphological changes

The colonization of AMF into the roots of plants show significant changes in the roots by inducing the changes in root architecture (Liu *et al*., 2023). The profound changes visible in the roots include increase in the length of the roots and increase in the degree of branching. Studies have also revealed that there is an increase in the diameter of the roots after colonization with AMF (Peighami Ashnaei, 2019; Sulaiman *et al*., 2020). The increase in branching of roots is accompanied by establishment of common mycorrhizal network (CMN) that plays a key role in the facilitation of belowground AMF network (Goicoechea, 2020). CMN fosters the biocontrol effects where allelochemicals influence the soil environment and protect against pathogens (Kalamulla *et al*., 2022).

2.5.2. Biochemical changes

When plants encounter biotic stresses, such as fungal pathogen attacks, they unleash a chain of biochemical reactions. This response includes the production of reactive oxygen species (ROS) and, alongside ROS, the release of reactive nitrogen species (RNS) (Sahu *et al*., 2022). It is also accompanied by significant changes in the levels of secondary metabolites like proline and phenols that have antimicrobial properties and help in inhibiting the fungus (Kaur *et al*., 2022). Mycorrhization enhances the resistance of plants against pathogens by altering the levels of antioxidants and enzymes (Kalamulla *et al*., 2022). This change in the levels of enzymes including peroxidases and catalases etc. which potentially help in scavenging the reactive oxygen species. The other changes involve alterations in the levels of phytohormones like Jasmonic acid (JA), Salicylic acid (SA) and Ethylene. These changes induced in a plant as a result of pathogen attack allow plants to collectively generate a dynamic defense response against the stress (Gupta *et al*., 2022; Mansoor *et al*. 2022).

2.5.3. Molecular changes

Recent researches have unraveled the mechanisms governing resistance against *Fusarium oxysporum* in chickpea plants. RNA-seq using Illumina platform is the technique employed to identify the differential expression of genes in a sample exposed to different treatments. Garg *et al*. in 2023, have identified 382 transcription factors that encode genes exhibiting differential gene expression (DEGs) out of which 287 genes were associated with the *Fusarium* wilt resistance. The group of scientists have reported

a downregulation in the genes linked with the leaf development (Ca_v2.0_01980), photosynthesis (Ca_v2.0_10872 and Ca_v2.0_23888), chlorophyll biosynthesis (Ca_v2.0_03879), and flower development (Ca_v2.0_11517). However an upregulation was observed in the case of several other genes linked withaccelerated cell death 11 (Ca_v2.0_00854), senescence-associated protein (Ca_v2.0_00473 and Ca_v2.0_03058). Some other genes related to heat shock proteins (HSPs) have also shown to play a crucial role in the development of resistance against pathogens in plants. In 2017, Upasani *et al*., provided an insight into the interplay mechanism between *Fusarium* and the host plant usingLongSAGE. The pathogen responsive genes were identified by comparing the DEGs in the pathogen inoculated plants were compared with the differentially expressed genes (DEGs) of control plants. The genes related to protein metabolism like Ubiquitin conjugating enzyme E2 28, peroxidase 42, glutathione S-transferase etc., were found to be highly upregulated in theresistant genotype DVI. However, the genes related to protein degradation and stress were found to be degraded in the susceptible genotype JGI. The authors have also proposed the potential biochemical pathway deducing the interconnections of numerous biological processes during the pathogen-host interaction. The recent researches on chickpea reveal that the stresses induced in the plants can be ameliorated by judiciously using AMF. The symbiotic fungihas proved its potential by enhancing the K^+/Na^+ ion uptake and efficiently scavenging the ROS generated during the stresses (Hsieh *et al*., 2022).

While substantial progress has been made in understanding chickpea resistance pathways to the formidable *Fusarium oxysporum* (FOC), most current researchfocuses on single infections. An important gap remains: understanding how chickpea responds to the combined challenge of FOC and arbuscular mycorrhizal fungus (AMF). Therefore, diving into the complex relationship between chickpea, FOC, and AMF at the transcriptional level holds enormous promise. Unravelling the dynamic reprogramming of gene expression in this tripartite connection may reveal fresh ways for increasing chickpea resilience and maintaining long-term crop production in the face of biotic and abiotic stresses.

2.5.4. Hypothesis

Integrating agronomic and molecular approaches will identify chickpea genotypes with enhanced tolerance to *Fusarium* wilt, elucidate the underlying molecular mechanisms of resistance, and optimize the application of arbuscular mycorrhizal fungi (AMF) as a biocontrol agent for sustainable *Fusarium* wilt control in chickpea.

2.5.5. Objectives

1. Screening and evaluation of chickpea genotypes against FOC usingagronomical and molecular traits
2. Screening and selection of suitable AM fungal species against *Fusarium*wilt
3. Mass multiplication of specific AMF fungi under root organ culture
4. Expression study of selected chickpea genotype during FOC infection and FOC+AMF infection using Next Generation Sequencing (NGS) approach

CHAPTER-3 MATERIALS AND METHODS

Fusarium wilt of chickpea, caused by FOC, is one of the most damaging diseases of chickpea, resulting in significant losses in the chickpea growing areas around the world. The screening and *in-vitro* evaluations of biocontrol fungus AM, against FOC were conducted at the College of Basic Sciences and Humanities and College of Agriculture in CCS Haryana Agricultural University, Hisar. Screening studies, mycorrhizal multiplication, as well as assessments, against *Fusarium* wilt were conducted in the green house and experimental area of CCS Haryana Agricultural University, Hisar (Latitude: 29°14'92" N, Longitude: 75°72'17" E, Altitude: 215 m AMSL). The methodology used to conduct the investigations is described in detail below.

3.1 Materials

3.1.1. Chemicals

All the chemicals used in the present investigation were of analytical grade. The chemicals utilised in molecular research were obtained from HiMedia (molecular grade), Promega, Thermo Fisher SCIENTIFIC, and BioRad. Borosilicate quality glassware and Tarsons plasticware were utilised and were sourced from Borosil India Limited, Corning Glass Company, and Tarsons Products Pvt. Limited, respectively.

3.1.2. Seeds

Seeds of some of the chickpea genotypes were obtained from Pulses Section, Department of Genetics and Plant Breeding, CCS HAU, Hisar, while some were obtained from LPU. In total, 52 genotypes were subjected to the experiment. The Table 3.1 shows the details of all the genotypes and their source of collection.

Table 3.1: Origin of genotypes and pedigree

Genotypes	Type	Pedigree	Recommended for cultivation in	Source of origin
GNG 2144	Desi	CSJD 901 × CSG 8962	NWPZ (HR, PN, RJ, UP, UK, DL)	RAU, Sriganganagar
RSG 888	Desi	RSG-44 X E100Y	Punjab, Haryana, Delhi, North Rajasthan& West U.P)	RAU, Durgapura
ICC 5335	Desi	Landrace	Maharashtra	ICRISAT
ICCV 96029	Kabuli	ICCV2xICCV93929	NEHZ (Assam)	ICRISAT
PHULe G 0517	Kabuli	Selection from local germplasm	CZ (MH, MP) SZ (KA)	MPKV
PUSA 547	Desi	Mutant of BG 256	NWPZ	IARI
GLK 16063	Kabuli	-	Punjab	Ludhiana
CSJ 513	Desi	-		ARS, Durgapura
HC 7	Desi	-	NWPZ (PN, HR, WUP, Delhi, N-Raj., J&KHP, UK)	CCS HAU
GNG 2477	Desi	-	CZ (RJ), NHZ (J&K)	RAU, Sriganganagar
GNG 1581	Desi	GPF2xH82-2	NWPZ (HR, PN, UP) CZ (RJ), NHZ (J&K)	RAU, Sriganganagar
CSJ 515	Desi	FG712xCSJ146	NWPZ	ARS, Durgapura
HC 1	Desi	-	NWPZ	CCS HAU
ICCV 10	Kabuli	PI231xP126	SZ (A.P., Karnataka, Orissa & Tamil Nādu) CZ(MP, Maharashtra, Gujarat)	ICRISAT
HC 5	Desi	H 89-78 × H 89-84	Haryana	CCS HAU
ICCV 07107	Kabuli	ICCV10xICCC37	-	ICRISAT
GNG 2477	Desi	-	-	RAU, Sriganganagar
DCP 92-3	Desi	Selection from germplasm	-	IIPR

PHULeG 0127	Desi	[(ICCV 95412 x Phule G 92307) x ICCV 95412]	NWPZ (HP,UK) NHZ (J&K	MPKV
KPG 59	Desi	RadheyxK468	NEPZ	CASUAT
BG 212	Desi	-	Bangladesh	IARI
DIGVIJAY	Desi	Phule G 91028 × Bheema	Maharashtra R	MPKV
RSG 807	Desi	-	Rajasthan	RAU, Durgapura
GNG 2459	Desi	-	-	RAU, Sriganganagar
Local variety 2	Desi	Punjab- 1	Punjab	PAU
ICC 07304	Kabuli	ICCV98502xICCV98004	-	ICRISAT
HC 3	Desi	L 550 x E 100 Ym	Haryana	CCS HAU
RVG 203	Desi	ICCV10 x ICCL8732	CZ (MP,MH,GJ) NWPZ (UP, RJ)	RVSKVV
RSG 963	Desi	RSG524xPD84-10	Haryana, Punjab, Delhi, UP, Rajasthan, Jammu& Uttaranchal	RAU, Durgapura
ICCV 6	Kabuli	Selection from germplasm	-	ICRISAT
GNG 2418	Desi	-	-	RAU, Sriganganagar
PBG 5	Desi	BG-257 X, Narsinghpur	Punjab	PAU
WR 315	Desi	-	-	ICRISAT
GNG 2171	Desi	GNG 663 × BG 1044	NWPZ (HR, PN, UK, DL, RJ.UP) NHZ(HP,J&K)	RAU, Sriganganagar
ICC 3020		Landrace		ICRISAT
RSG 931	Desi	RSG-44XRSG-524	NWPZ	RAU, Durgapura
PUSA 372	Desi	-	CZ	IARI
BG 4011	Desi	F1[F1(ICC4958 x ICCV10) x F1 (Pusa372 x Pusa 256)] x F1(Pusa 547 x JAKI 9218)	-	IARI

HC 6	Desi	-	Haryana	CCS HAU
ICCV 512		Selection from germplasm	Hyderabad	ICRISAT
SADABAHAR	Desi	Hima × L 245	Uttar Pradesh	CSAUAT
GNG 1958	Desi	GNG 1365 × SAKI 9516	NWPZ (HR, PN, RJ, UP, UK, DL)	RAU, Sriganganagar
RSG 945	Desi	RSG-668 X RSG-617	Rajasthan	RAU, Durgapura
C 235	Desi	IP 58 × C 1234	Punjab and Haryana	CCS HAU
RSG 991	Desi	RSG289X BG1053	Rajasthan	RAU, Durgapura
HK 4	Kabuli	HK92-94xHK1	NWPZ	CCS HAU
HK 2	Kabuli	(H82-2XE100ym)XBhima	NWPZ	CCS HAU
HK 1	Kabuli	(L 550 x E 100 Ym) x (ICCC 32 X ICC 820001)	Haryana	CCS HAU
KAK 2	Kabuli	ICCC X Sunetato-77	CZ (MP, CG, MS, Gujarat)	LPU
GLK 28127	Kabuli	GLK 88016 x FLIP 88-34C	NWPZ (HR, PN, RJ, UP, UK)	Ludhiana
GLK 17301	Kabuli	-	Punjab	Ludhiana
JG 62	Desi	Selection from germplasm	-	JNKVV

3.1.3. The pathogen

The pathogen FOC, race-3 with accession number ITCC 7679 was procured from the ITCC, IARI.

Figure 3.1: The pathogen FOC, race-3 with accession number ITCC 7679 was procured from the ITCC, IARI.

3.1.4. The primers for molecular screening

The STMS markers TA-194, TA-97 and TA-27, linked to *foc-3,* the wilt resistance gene, were procured from Eurofins Genomics India Pvt Ltd, Bangalore. The details of the forward and reverse sequence of primers are given in the Table 3.2. (Sharma *et al*., 2007; Lal *et al*., 2021).

Table 3.2: List of markers used for the detection of resistance genes linked to *foc-3.*

Name of Marker	Forward (F) and Reverse (R) sequence	Tm
TA-96	F-GATAAAATCATTATTGGGTGTCCTTT	50.5^{o}C
	R-TTCAAATAATCTTTCATCAGTCAAATG	
TA-27	F-TGTTTTGGAGAAGAGTGATTC	55^{o}C
	R-TGTGCATGCAAATTCTTACT	
TA-194	F- TTTTTGGCTTATTAGACTGACTT	57.1^{oC}
	R- TTGCCATAAAATACAAAATCC	

3.1.5. AMF

The mycorrhizal fungus *G. fasciculatum, G. mosseae, G. intraradices,* and *G. hoi* were obtained from the Department of Plant Pathology, CCS HAU, Hisar. These

cultures of respective AMF were maintained on pearl millet plants (*Pennisetum typhoides*) in 20 cm wide earthen pots, separately. The ProVAM consortium was however, obtained from ProVam Industries in Nasik in a sealed 200 gm container with an IP value of 100 propagules/gm.

Figure 3.2: Maintenance of mycorrhizal fungi *Glomus* species on pearl millet plants

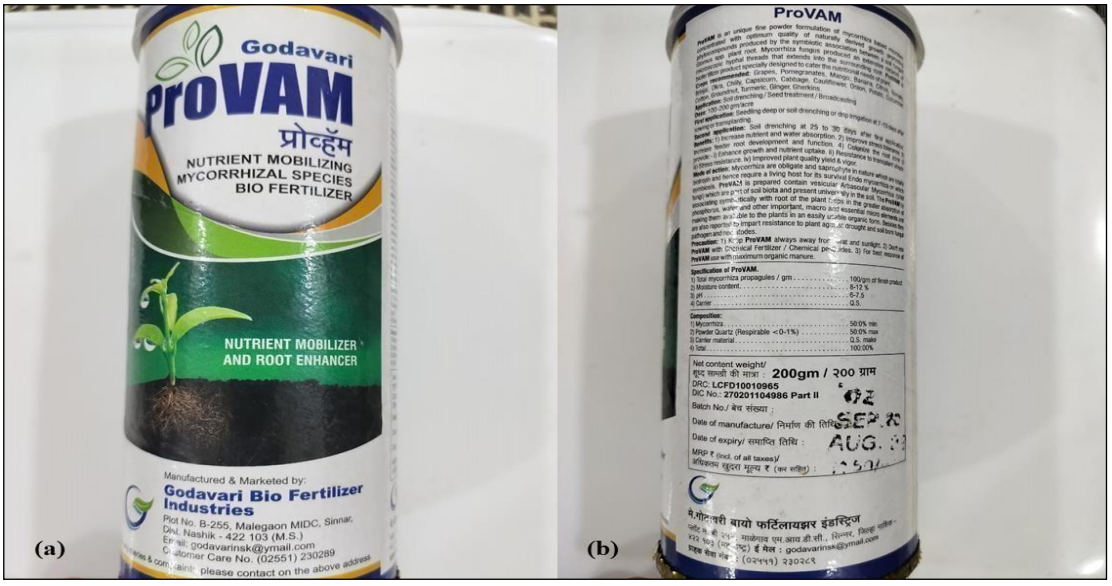


Figure 3.3: The AMF consortia obtained from the ProVam PvtLtd

3.1.6. Statistical analysis

The software R studio was used for all the statistical analyses and graphical presentations. The packages used for these analyses were Multcomp, Bioconductor, Metan, Emmeans, ggplot, ggpattern, semPlot, LavaanPlot, corrplot and hclust. The analysis of transcriptome data was done using iDep2.0.

3.2. Methods

3.2.1. The pathogen and its mass culturing

Potato dextrose agar (PDA) was utilized as the initial growth medium for the pathogen. PDA preparation involved boiling thinly sliced and peeled potatoes in500 ml of distilled water until softened. The potato extract was then filtered through cheesecloth or muslin cloth, and the filtrate was collected in a beaker. Twenty grams each of agar and dextrose powder were added to the filtrate and dissolved to create a clear solution. The volume was adjusted to 1 liter using distilled water. The solution was dispensed into conical flasks plugged with cotton and sterilized in an autoclave at 121°C and 15 psi for 20 minutes. Potato dextrose broth (PD broth) was prepared similarly, omitting the agar (Table 3.3).Table 3.3: PDA media

Peeled and cut potato	200 g
Dextrose	20 g
Agar	20 g
Distilled Water	1000 ml
pH (Adjusted)	6-6.5

For field experiment, FOC was cultured on wheat seeds (wheat spawn) in polypropylene bags. The bags were filled with non-absorbent cotton and autoclaved for 20 minutes at 15 psi. The bags were infected under sterilized circumstances with a seven-day-old culture of FOC and thoroughly mixed.

The bags were stored in an incubator at 27±1˚C. After ten days of incubation, the culture was employed as an artificial inoculation in field screening experiment (Bai *et al*., 2018). For pot screening, a hemocytometer was employed to quantify the concentration of FOC spores. A spore suspension of 10^6 spores/ml was prepared using serial dilutions (Li *et al*., 2021). Prior to planting, chickpea seeds from all genotypes were sterilized. The sterilized seedswere then sown in individual clay pots filled with autoclaved soil, with each genotype replicated three times per pot. After seedling establishment, three- week-old plants were submerged in the FOC suspension culture for 15 minutesto facilitate disease establishment (Patidar *et al*., 2022).

Figure 3.4:(a) Wheat spawn harbouring the pure culture of FOC; (b) Chickpea plants treated with the pathogen FOC cultured over wheat spawn

3.2.2. Screening

The screening of genotypes in the field was done in Randomised Block Design (RBD) in three replications. The data observations were taken at two different phenological stages i.e. 28 DAS and 45 DAS stage.

3.2.2.1. Wilting percentage

The wilting percentage of the plans was determined by the formula(Saleh *et al*., 2016):

Wilting Percentage =

(Number of plants showing wilt symptoms/Total number of plants observed) x 100

3.2.2.2. Disease Severity Index

The disease severity index (D.I.) was calculated using the formula given and described by Keote *et al*., 2019.

$$D.I. = \frac{0\,(Hn) + 1\,(Sn) + 2\,(Hn^*) + 3\,(Dn)}{\text{Total number of plants examined}} \times 100$$

Where the terms Hn, Sn, Hn* and Dn were given as:

(Hn) = Number of healthy plants = plants showing no wilt symptoms (Sn) = Number of slightly infected plants = Plants showing slight wilt symptoms

(Hn*) = Number of heavily infected plants = Plants showing heavy wiltsymptoms

(Dn) = Number of dead plants = Dead plants

3.2.2.3. Categorization of the plants

The plants were categorized on the basis of their wilting percentage as HR, R, MR, S, and HS. The basis of the categorization is given in the Table:

Table 3.4: Disease reaction of the chickpea plants based on the Wilting Percentage

Wilting Percentage	Disease reaction
0-10 %	HR
10.1-20%	R
20.1-30%	MR
30.1-50	S
>50.1%	HS

Figure 3.5: Experimental view and randomly clicked photograph during experimental work

3.2.3. Molecular screening of the genotypes

For molecular screening, the total DNA was extracted from the leaves of 30day-old seedlings using the CTAB technique.

3.2.3.1. Genomic DNA isolation

The plants' genomic DNA was extracted from young leaf samples using Murray and Thompson's (1980) CTAB DNA extraction technique, which was improved by Saghai- Maroof *et al*. (1984). All buffers and solutions used in the isolation procedure were made from chemicals/reagents in autoclaved glassware (Table 3.5). The buffer was chilled to room temperature before adding β- mercaptaethanol. Approximately 2 grams of fresh leaf tissue were ground into afine powder using a sterilized mortar and pestle aided by liquid nitrogen. The homogenized material was then transferred to a 5 ml centrifuge tube containing 2 ml of extraction buffer. This mixture was incubated in a water bath set at 65°C for 1.5 hours with occasional gentle mixing. Thereafter, the contents were cooleddown to room temperature. Next, 800 µl of a chloroform : isoamyl alcoholmixture (24:1 v/v) was added to the sample and mixed gently for several minutes and centrifuged at 10,000 rpm to promote organic phase separation. This mixturewas then centrifuged at 10,000 rpm for 20 minutes to separate the phases. The upper,

aqueous phase containing the purified DNA was carefully transferred to a new centrifuge tube. This process, known as chloroform- isoamyl alcohol (CI)washing, was repeated once to further enhance DNA purity by removing protein and lipid contaminants. Following a second centrifugation at 8,000 rpm for 10 minutes, the resulting clean supernatant containing purified DNA was transferred to a fresh tube for further processing. To recover the DNA pellet, an equal volume of pre-chilled isopropanol was added to the supernatant. The mixture was thoroughly mixed and centrifuged again at 10,000 rpm for 10 minutes. This centrifugation step pelleted the DNA at the bottom of the tube. The supernatant was then carefully discarded, and the tubes were inverted on absorbent paper for 10 minutes to eliminate any remaining traces of alcohol. TheDNA pellet was air-dried for 30 minutes to remove residual solvent. Finally, thepurified DNA pellet was resuspended in 200 µl of TE buffer (Table 3.5) and stored at 4°C for subsequent use.

Table 3.5: Composition and preparation protocol of the solutions, buffers and medium used in DNA isolation

S.No.	Buffer/Solution	Composition
1	1M Tris HCl	A final volume of 1000 ml of the solution was obtained by dissolving 121.1 g of Tris base in 800ml distilled water and then adding concentrated HCl to the solution to bring the pH to 8 by addingconcentrated HCl.
2	0.5 M EDTA	In 800 ml of distilled water, 186.1 g of sodium ethylene diamine tetra acetate was dissolved in 1 M NaOH to adjust the pH to 8.
3	5M NaCl	In 800 ml of distilled water, 292.1 g of NaCl weredissolved and a final volume of 1000 ml wasobtained.
4	CTAB buffer	2% CTAB buffer was prepared by dissolving 20 ml of Tris (200 mM), 4 ml of EDTA (20 mM), 28ml of NaCl (1.4M), 2g CTAB (2%) and 1g PVP (1%) by making a final volume of 100 ml out of distilled water. Further, 100 µg β-mercaptaethanolwas added prior to use.
5	Ethidium bromide (10 mg/ ml)	1 g of ethidium bromide was dissolved in 100 mlwater and stirred vigorously on a magnetic stirrer
6	10X TBE Buffer	In a beaker, 108 g of Tris base, 55 g Boric acid, and 40 ml 0.5 M EDTA were mixed with 1000 ml distilled water and dissolved on a magnetic stirrer.
7	TE Buffer	In order to prepare 100 ml of TE Buffer, 1 ml of 1M Tris was mixed with 0.2 ml of 0.5 M EDTA, with 100 ml of distilled water being the finalvolume.
8	6X loading dye	0.25% (w/v) Bromophenol blue 0.25% (w/v) Xylene cyanol 60% (v/v)Glycerol.

3.2.3.2. Quality assessment of extracted DNA

The quality of DNA samples was assessed using both UV spectrophotometers (Nanodrop) and agarose gel electrophoresis. The absorbance ratio at 260 and 280 nm was determined using a spectrophotometer. The genomic DNA was runon a 0.8% agarose gel for 45 minutes to determine the strength of bands, the presence of RNA, and protein.

3.2.3.3. Quantification of isolated DNA

The DNA was quantified by depositing 1 µl of DNA on the sensor of a UV spectrophotometer and recording the concentration of DNA contained in the testsample (in ng/µl).

3.2.3.4. PCR amplification using STMS primers

The experiment utilized a BioRad Thermocycler for PCR amplification of target DNA. A master mix containing all necessary reaction components was preparedand was then aliquoted into PCR tubes, followed by the addition of individual DNA samples. To optimize the PCR reaction, we systematically varied the concentrations of key components: template DNA (50, 100, and 150 ng/µl), dNTPs mix (10, 100, and 200 µM), primers (0.2, 0.3, 0.4, and 0.6 µM), and TaqDNA polymerase (0.5 and 1 unit) within a total reaction volume of 10 µl (Table3.6).

Table 3.6: Composition of the 10.00 µl of PCR reaction mixture

Component used (concentration)		**Amount**
DNA template (50 ng)	:	1.00 µl
10 X PCR buffer	:	1.00 µl
$MgCl_2$ 25 mM	:	1.00 µl
dNTPs mix (10 µM)	:	0.50 µl
Forward primer (10 µM)	:	0.50 µl
Reverse primer (10 µM)	:	0.50 µl
Taq DNA Polymerase (5U/µl)	:	0.20 µl
Sterile distilled water	:	5.30 µl
Total volume	:	10.00 µl

Thin walled, sterile PCR tubes were used in BioRad Thermocycler and the following reaction conditions were given:

Table 3.7: The reaction conditions and steps followed for the PCR

Steps	Cycles	Temperature	Duration
Initial denaturation	1	94	4 min
Denaturation	40	94	1 min
Primer annealing		45-65	1 min
Primer extension		72	1 min
Final primer extension	1	72	7 min

3.2.4. Agarose gel electrophoresis

In this, the PCR products were analyzed using a 2% (w/v) agarose gel. Following standard procedures, the gel tray was cleaned and dried. Agarose wasthen dissolved in 1X TBE buffer using a microwave oven while following safetyguidelines to avoid overheating. After the solution cooled, ethidium bromide (ata final concentration of 0.02 μg/ml) was added as a DNA stain. The gel tray wasequipped with appropriate combs to create sample wells, and the agarosesolution was poured. The gel was allowed to solidify for 15-20 minutes at a thickness of 0.4-0.6 cm. Once solidified, the combs were carefully removed, andthe gel tray was submerged in 1X TBE buffer within the electrophoresis chamber. PCR products were mixed with loading dye (at a ratio of 1:3) and centrifuged briefly to ensure proper mixing. Using a micropipette, samples were loaded into the wells. Electrophoresis was then conducted for approximately 2.5hours at a constant voltage of 90 V. The separated DNA fragments werevisualized under UV light using a transilluminator and documented using a gel documentation system. A 100 bp molecular weight ladder was included in the gel to determine the size of the amplified PCR products

3.2.4.1. Screening of suitable AMF species against Fusarium wilt

The experiment was carried out to determine the efficacy of managing *Fusarium* wilt of chickpea using the bio-control fungus AMF in greenhouse conditions at the Experimental Area of the department of Plant Pathology, CCSHAU, Hisar, using the susceptible check chickpea genotype JG 62.

3.2.4.2. Experimental design

The experiment used various *Glomus* species and *in-vitro* consortia of ProVam, with the following treatments applied.

1. JG 62 (control)
2. FOC + JG 62
3. FOC+ *Glomus mosseae* + JG 62
4. FOC + *Glomus intraradices* + JG 62
5. FOC + *Glomus fasciculatum* + JG 62
6. FOC + *Glomus hoi* + JG 62
7. FOC + ProVam + JG 62

The experiment employed ten-day-old cultures of AMF and FOC. The test pathogen (FOC at 1 g/kg soil) and mycorrhizal fungus (400-450 chlamydosporesand 1-1.5 g roots per kg soil) were combined in the top 5 cm of soil. All treatments were applied by seed treatment. The biochemical analysis was done at 7^{th} day post infection.

3.2.4.3. Total Phenol Content

In this experiment, one milliliter of the extract was diluted with 7.5 milliliters of distilled water. Further, 0.5 milliliters of a diluted Folin-Ciocalteu reagent was added and the mixture was stirred well for 3 minutes. Subsequently, 1 milliliterof saturated sodium carbonate solution was added, and the final volume was adjusted to 10 milliliters with distilled water. Following a one-hour incubation period, the absorbance of the solution was measured at 725 nm using a spectrophotometer. Total phenolics were quantified by referencing a standard curve generated using tannic acid as the standard phenol. The final data were expressed as milligrams of total phenolics per gram of dry weight sample (Dhillon *et al*., 2012).

3.2.4.4. Total Proline Content

To determine proline content, leaf sample of 2mg was crushed with a mortar pestle in 1.0 ml of 3% homogenised sulphosalicylic acid, following the procedure described by Bates *et al.,* 1973. The sample was centrifuged at 1000 rpm for 10 minutes and the supernatant was transferred to a 15.0 ml Falcon tube.After adding 500µl of ninhydrin and 500µl of glacial acetic acid, samples were heated in a water bath at 100°C for 60 minutes and then cooled to -80 °C for 2 minutes. Next, add 750 µl of toluene and vortex for 1 minute. After adding the solution, a pink layer developed. The upper layer was then measured forabsorbance at 520 nm using a spectrophotometer (Abraham *et al*., 2010).

3.2.4.5. Total Protein Content

The Folin-Ciocalteau reagent, which contains sodium tungstate, molybdate, and phosphate, was used to create a blue-purple colour complex with the highest absorption at 660 nm. The method's sensitivity extends down to roughly 10 g/ml,making it one of the most often used protein assays. The chemicals used were Na_2CO_3 in 0.1 N NaOH, a 1% solution of $CuSO_4.5H_2O$, a 2% solution of sodiumpotassium tartrate, a freshly made mixture of reagents B and C, a combination of reagents A and D, 1 N Folin-Ciocalteau reagent, and 20% trichloroacetic acid. The process included precipitating soluble proteins with 20% TCA, re- dissolving the residue in 0.1N NaOH, and adding reagent E. The processincluded precipitating soluble proteins with 20% TCA, redissolving the residuein 0.1N NaOH, adding reagent E to the protein sample, leaving it to stand for 10 minutes, then adding reagent F, mixing, and measuring the colour intensity at 660 nm after 30 minutes. The corresponding amount of protein was determinedusing a standard curve made from bovine serum albumin (20-200 g ml^{-1})(Dhillon *et al*., 2012).

Reagents:

Reagent A : 2% Na_2CO_3 in 0.1% NaoH.

Reagent B : 0.1% $CuSO_4$ in 1% Na--k tartarate.

Reagent C : 50ml of A + 1ml of B is mixed.

Reagent D : Folin & Ciocalteu's reagent 50% diluted with distilled water).

Reagent E : BSA for standard curve.

Reagent F : 1X phosphate buffer

3.2.4.6. Total Soluble Sugars

This method involved dehydration of glucose to hydroxymethyl furfural in a hot acidic solution, leading to a yellow complex with phenol that absorbs light at 490 nm. Briefly, 1 ml of diluted sugar extract (1:9 dilution with distilled water)was added to a 25x200 mm test tube. Sequentially, 2 ml of a 2% phenol solution and 5 ml of concentrated sulfuric acid were added, with the acid poured directlyonto the solution. After shaking, the tubes were cooled for 30 minutes.

Absorbance at 490 nm was measured using a spectrophotometer. A standard curve of glucose prepared concurrently was used to estimate the concentration of total sugars, which were expressed as mg g^{-1} dry weight or as a percentage ofdry weight (Dhillon *et al*., 2012).

3.2.4.7. Catalase activity

The enzyme extract was produced using a method modified from Sinha (1972) for activity assessment. The assay used a combination of 0.55 ml 0.1 M potassium phosphate buffer (pH 7.0), 0.4 ml 0.2 M H2O2, and 50 µl enzyme extract. After one minute of incubation, 3.0 ml of 5% potassium dichromate: acetic acid solution (1:3) was added. Controls containing simply assay buffer and H_2O_2 were run alongside the samples. The H_2O_2 levels might be calculated by boiling the tubes for 10 minutes, chilling them, and measuring their absorbance at 570 nm. Catalase units were defined as the enzyme amount required to consume one µmol of H_2O_2 per minute or per mg of protein, based on the standard curve. Extinction coefficient for H_2O_2 was 0.0394 $mM^{-1}cm^{-1}$ (Dhillon *et al*., 2012).

3.2.4.8. LOX activity

To assess lipoxygenase involvement, an enzyme extract was prepared. Its activity was then measured spectrophotometrically at 234 nm using a method adapted from Catherine *et al*. (1998). The reaction mixture formed by adding15µl of 30 mM linoleic acid in methanol, 2.785 ml of 100 mM phosphate buffer (pH 6.8), and 200 µl of the enzyme extract. The increase in absorbance at 234 nm was monitored over 2 minutes at room temperature to determine enzyme activity. This activity of LOX was expressed as µmol hydroperoxide/min/mg protein (Dhillon *et al*., 2012)**.**

3.2.4.9. PAL activity

To measure PAL activity, the conversion of L-phenylalanine to transcinnamic acid at a 290 nm wavelength was monitored. One gram of fresh plant material was crushed with 5 ml of 0.1 M sodium phosphate buffer (pH 7.0) and 0.1 g of polyvinyl pyrrolidone (PVP) in a chilled mortar to minimize interference. A control sample, lacking the substrate (L-phenylalanine), contained only the enzyme extract and borate buffer. The PAL activity, represented by the amountof trans-cinnamic acid produced, was calculated using an extinction coefficient of 9630 M^{-1} cm^{-1} and expressed as nmol cinnamic acid/min/g fresh weight (Saikia *et al*., 2006).

3.2.4.10.SOD activity

Superoxide dismutase (SOD), a critical metalloenzyme, plays a vital role in cellular defense against oxidative stress. It catalyzes the dismutation of superoxide radicals into hydrogen peroxide (H_2O_2) and molecular oxygen, acting as a key antioxidant in aerobic organisms. This experiment measured SOD activity using its

ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT). The assay followed the principles established by Beauchampand Fridovich (1971). The reaction mixture contained components that facilitatethe generation of superoxide radicals and monitor their reduction by NBT. Specifically, it included a buffer (60 mM Tris-HCl, pH 7.8), a reducing agent (420 mM L-methionine), NBT (1.80 mM), a photosensitizer (90 mM riboflavin),a metal chelator (3.0 mM EDTA), and the enzyme extract being analyzed. Light exposure initiated the reaction, which was terminated after 40 minutes. The amount of NBT reduced was determined by measuring absorbance at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the photoreduction of one mole of NBT. For studies focusing on enzyme kinetics and regulation, activity was expressed in units per gram of fresh weight following the formula provided by Giannopolitis and Rie (1977) (Dhillon *et al*.,2012). The percent inhibition was calculated by the following formula of Asada *et al*. (1974):

SOD Percent Inhibition (%) =

[(Control absorbance−Sample absorbance)/Control absorbance]x 100

3.2.4.11. AMF root colonization percentage

The disease incidence was determined using the formula provided. The number of sporocarps were estimated using Gerdemann and Nicolson's (1963) wet sieving and decantation procedure. Freshly acquired soil sample, rich in AMF spores (10 g) was placed in a plastic beaker. After crushing the soil macro- aggregates by hand, the soil was suspended in approximately 500 ml of tap water. After 10-30 seconds of settling, the upper layer of soil suspension was emptied onto the sieve (Figure 3.6). The same protocol was repeated until the soil suspension's upper layer became translucent. The fine mesh sievings were gathered in a tiny beaker and observed under microscope for sporocarp count.

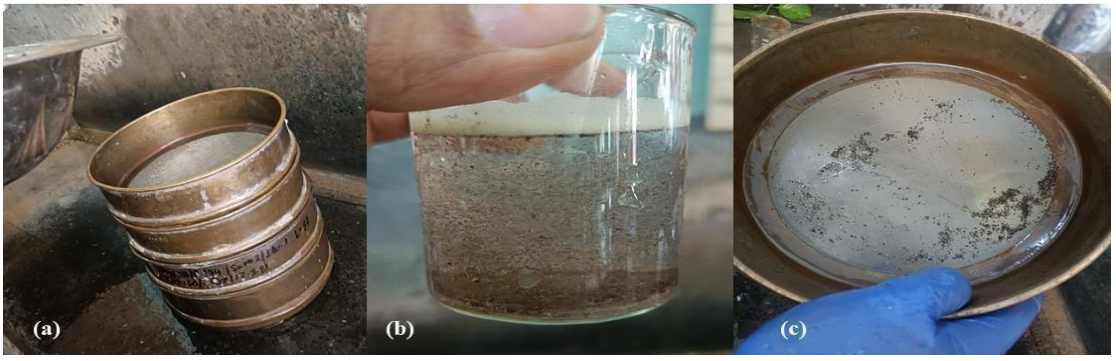


Figure 3.6 The procedure of wet-sieving and decantation; (a) emptying the upper layer of soil suspension into the sieve, (b) translucent upper layer after fine sieving, (c) fine mesh sievings

The mycorrhizal colonisation from root samples were stained according to Phillips and Hayman's (1970) procedure. Roots were chopped into 1 cm portionsand placed loosely in rectangular cassettes with tiny holes (0.9mm), which are suitable for preventing root loss. The roots were then heated in 10%

KOH at 90˚C for one hour. Following that, the roots were rinsed with a fresh 10% KOH solution and submerged in alkaline hydrogen peroxide (H_2O_2) for 30minutes. To eliminate excess H_2O_2, the roots were rinsed with distilled water and then acidified with 5 N HCl for half an hour. The roots were boiled in trypanblue and lactophenol (0.05%) for 5 minutes. Finally, the roots were placed in lactophenol to remove the excess colour and viewed under a microscope to determine the root colonization percentage (Jalali and Domstch, 1975).

Root colonization percentage= (Number of root segments colonized/ Total number of root samples observed) x 100

3.2.5. Mass production of AMF under root organ culture

Mass production of root culture was done using the protocol described by Kumar and Saxena, 2017, Selvakumar *et al.*, 2018. The spores were obtained from the ProVam AMF infected soil using the wetsieving processand were examined under a microscope to verify that only healthy and undamaged spores were used for AMF *in-vitro* propagation. Sorghum seeds were surface sterilised by immersing in 70% ethanol for 2 minutes, then in a 1%sodium hypochlorite solution (NaOCl) for 3 minutes, and finally rinsing with sterile distilled water. These surface-sterilized seeds were then placed in Petri plates with sterile wet filter paper and incubated in a growth chamber in the Department of Botany and Plant Physiology, CCS HAU, Hisar for three days to induce germination under conditions of 12 hours of light at 25°C, 12 hours of darkness at 20°C, and 70% humidity. After successful germination, the seedlingswere placed on folded filter paper, and healthy spores were placed near the roots.The assembly was then placed in a tray containing sterilised vermiculite and waswatered daily for 15 days. After this time, root staining was used to measure AMF colonisation, and the germinated spores that had colonised the roots weretransplanted into 200 mL pots filled with sterilised soil, where new pre- germinated Sorghum grass seedlings were planted. This arrangement was maintained for one month to promote fresh seedling establishment and root colonisation. Following this interval, the contents were moved to 1 kg size pots with little interruption. For 90 days, these pots were kept in a greenhouse with controlled

circumstances (25°C, and 70% relative humidity).

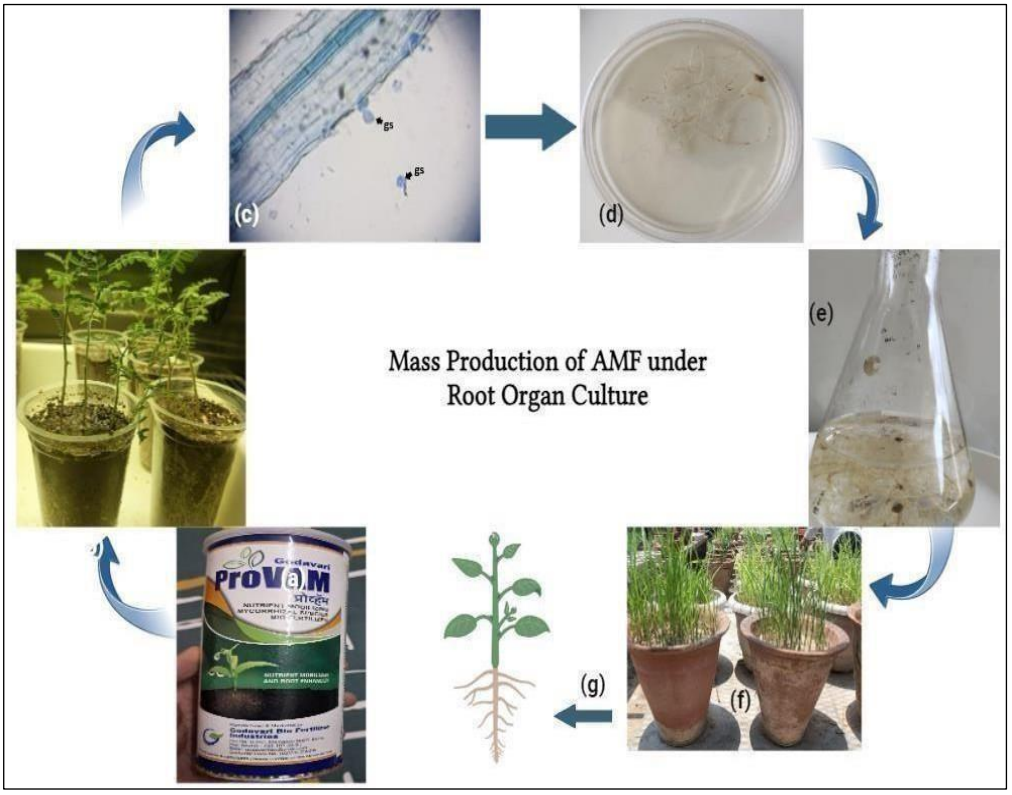


Figure 3.7: Axenic mass multiplication of AMF under Root Organ Culture: (a,b) Inoculation of plants sowed in sterilized soil with consortia obtained from Godavari bio fertilizers; (c, d) Isolation of single spores of AMF and placing pre-germinated spores near roots

3.2.5.1. Root biomass

Harvested roots were washed and dried. The root biomass yield was determined by dividing dry biomass (g) by per /kg soil and reported as weight per kg of soil(g /kg) (Ghorui *et al*., 2023).

3.2.5.2. Spore germination percentage

10 spores from each age group (5, 10, 15, 20 and 25 days) were collected from the in-vitro cultures and placed on an agar medium having a pH of 6.0. The spores were then cultured in dark conditions at a temperature of 27^{o}C for 30 days. Spores having hyphae longer than with 150 µm were considered to be germinated (Ghorui *et al*., 2023).

3.2.5.3. Spore viability

To assess spore viability, a 1:1 mixture (1 ml each) of MTT stock solution and an aqueous spore suspension was prepared in a screw-cap tube. The tube was tightly sealed and incubated for 40 hours at 27°C in the dark. Following incubation, the spores were separated and examined under darkfield illumination using a stereomicroscope. Viable spores stained a bright red color with MTT, while autoclaved or ethanol-killed

spores remained unstained throughout the observation period (Varga *et al*., 2015).

3.2.6. Selection of susceptible genotype for RNA-seq

The four susceptible genotypes selected from the field screening were placed in a growth chamber to standardise the environmental conditions. Biochemical changes, disease severity, and AMF root colonisation percentage were all measured throughout three weeks of observation. The severity of the disease was assessed based on the methodology described previously. The percentage of AMF root colonisation was determined using microscopy on root samples. The treatments given to the plants were as follows:

1. Susceptible genotype under observation (control)
2. *F. oxysporum* f. sp. *Ciceris* + genotypes under observation
3. *F. oxysporum* f. sp. *Ciceris* + selected AMF + genotypes under observation

3.2.6.1. Plant material

Two different treatment samples (FOC treated and FOC+AMF treated) were chosen from the experiment and raised in sterilised coco peat: vermiculite: sand(1:1:1) mixture in the growth chamber at the Molecular Biology and Biotechnology, CCS, HAU, Hisar. The experiment included two differential treatments and one set of control (distilled water). The experiment was carried out in three replicates. After the confirmation of infection using staining techniques as mentioned previously, the plants were analysed for their biochemical response as well as disease severity. The genotype showing the least disease severity as well as biochemical responses in presence of AMF, was subjected to further trascriptome analysis.

The selected genotype was further cultivated in sterile and controlled conditions in growth chamber with two replicates of FOC+AMF treated plants, two replicates of FOC treated plants and one replication of control plant. After infection confirmation, the samples were prepared for RNA extraction (Kashyap *et al*., 2016).

3.2.6.2. RNA Extraction and Quality Check

The RNA extraction was done using the standard Trizol method. The plant tissues were homogenized in Trizol using a pestle, followed by a room temperature incubation for 5 minutes. Chloroform was then added, and the mixture was vortexed and centrifuged at 11,300 rpm for 15 minutes at 4°C. Thisresulted in a three-phase separation: a clear aqueous phase containing RNA, a white interphase with DNA, and a red organic phase with proteins. The aqueousphase, containing the desired RNA, was carefully transferred to a new tube, leaving behind a portion of the interphase. Isopropanol was subsequently added to the Trizol to precipitate the RNA. After

vortexing and incubation, the supernatant was discarded, and the RNA pellet was washed with cold 75% ethanol. The washed pellet was then air-dried and resuspended in elution bufferwith a 55°C incubation for 5 minutes. Following DNAse treatment and removalof the DNA-containing pellet, the RNA was further concentrated using linear acrylamide, ammonium acetate, and ethanol. The concentrated RNA pellet was then air-dried, resuspended in elution buffer, and stored on ice for downstream analysis. RNA concentration and quality were assessed using the NanoDrop ND-100 Spectrophotometer, Qubit 4 Fluorometer, and Tapestation 4150 instruments. The sample was then sent to Redcliffe Genetics Pvt Ltd, Noida.

3.2.6.3. RNA Libraries

The prepared libraries were sequenced on an Illumina Nextseq 2K to obtain 40 million, 2x150bp reads per sample. The sequenced data was processed to generate FASTQ files, which were then analysed. The quality of raw data was examined using FASTQC (https://www.bioinformatics. babraham.ac.uk/projects/fastqc/). The quality assessment includes examining paired-end raw sequence reads to determine base quality and identify potential contamination from sequencing artefacts. AdapterRemoval v2.3.2 was used to trim, filter, and remove low-quality sequences from paired reads. The trimmed sequence readings were then aligned with a reference using the alignment programme HISAT2 v2.1.0 (https://ftp.ncbi.nlm.nih.gov/genomes/all/ GCF/000/331/145/GCF_000331145.1_ASM 33114v1/GCF_000331145.1_ASM33114v1_genomic.fna.gz). For assigning feature reads to the genomic sequences, the tool FeatureCounts v2.0.1 was used. The Bioconductor package EdgeR was used for differential expression analysis of digital gene expression data.

3.2.6.4. Differentially expressed genes

Differentially expressed genes (DEGs) were identified by comparing different samples under control (non-inoculated) and treatment conditions.

DEGs with a log2 fold change ≥ 2 (upregulated) or < -2 (downregulated) and an FPKM > 2 in each pair-wise comparison were considered significantly differentially expressed.

3.2.6.5. Clustering

The expression estimation file was used to carry out k-mean clustering, EnsembL IDs assignment, pathway enrichment analysis using iDEP 2.0 with a p-value of 0.05 as the significance criterion (Ge *et al*., 2018).

3.2.6.6. Principal Component Analysis

The principal component analysis (PCA) for all the combinations were

generated with and without normalization.

3.2.6.7. Work flow

A brief workflow of the process is depicted in Figure. The workflow chart depicts the stops made throughout the investigation. After RNA sequencing, sequence reads were acquired in FASTQ format. The QC report was generated following adapter cutting and FASTQ quality filtration. The readings were aligned with chickpea's reference genome to provide alignment statistics. The differentially expressed genes were removed from the aligned data before the gene count matrix was generated. The functional annotated genes were isolatedfrom the differential analysis and then used to create the functional annotation report.

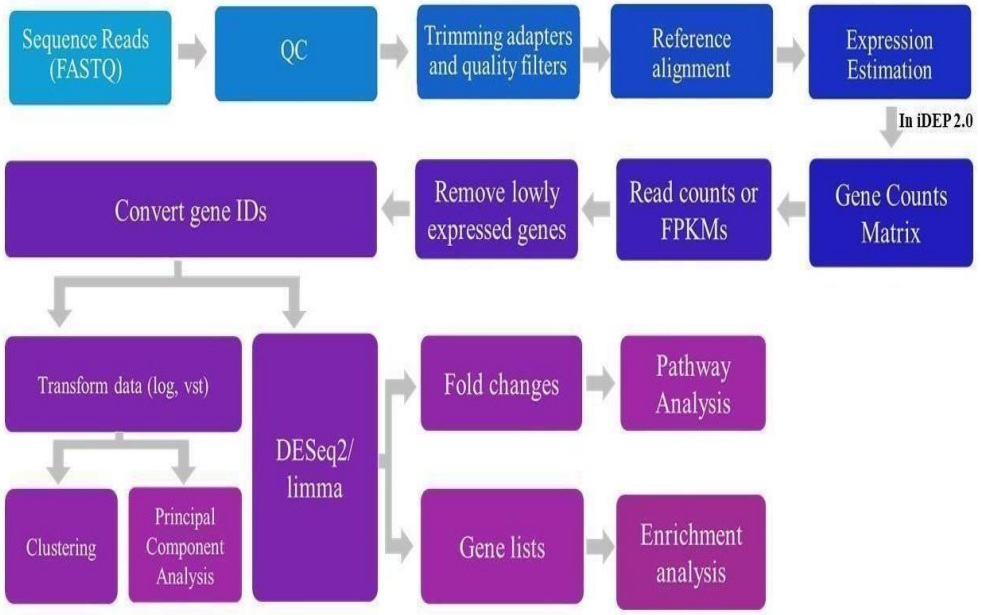


Figure 3.8: The workflow of the experiment

3.2.7. Gene expression studies

3.2.7.1. Analysis of Integrity, Quality and Quantity of RNA

The integrity of RNA was assessed using a 1% agarose gel. The Nanodrop (Denovix DS-11 Spectrophotometer) was used to measure the quantity and quality of RNA. RNA samples with 260/280 ratio 2 underwent DNase I treatment at a concentration of 1µg/µl.

3.2.7.2. cDNA Synthesis

The initial step in cDNA synthesis was to produce the first strand. Random hexamer measuring 1 µl and nuclease free water measuring 12 µl were added to1 ng of total RNA in a 2 ml Eppendorf tube. The tube contained 4 µl reaction buffer, 2 µl 10 mM dNTP mix, 1 µl Ribolock RNase inhibitor and 1 µlRevertAid M-MuLV RT. The

mixture was gently mixed and centrifuged before being incubated at room temperature (25ºC) for 5 minutes, followed by another 60 minutes at 42ºC. After incubation, the sample was heated to 70ºC for 5 minutes and used for the PCR reaction. The procedure was carried done in a thermal cycler using the following methodology:

1. Priming (at 25°C for 5 minutes)
2. Reverse transcription (at 46°C, for 20 minutes)
3. Reverse transcriptase inactivation (at 95°C for 1 minute)
4. Hold at 4°C (optional)

3.2.7.3. Gradient Polymerase Chain Reaction to Check the Best-Suited Annealing Temperature for the Primers

Gene sequences were sourced from the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/). Primers (details in Table 3.9) were constructed using Integrated DNA Technologies' Primer Quest programme (https://sg.idtdna.com/PrimerQuest/Home/Index). Todetermine the optimal annealing temperature, the gradient PCR reaction was carried out according to the procedure outlined below.

Step 1 : Denaturation (at 94°C for 3 minutes)

Step 2 : Denaturation (at 94°C for 45 seconds)

Step 3 : Annealing (variable according to the primer) (for 45 seconds)

Step 4 : Extension (at 72°C for 45 seconds)

Step 5 : Extension (at 72°C for 5 minutes)Step 6: Hold (4°C for infinity)

Gene Expression Studies by Real-Time PCR

BIO-RAD's iTag™ Universal SYBR Green Supermix was used for quantitative RT-PCR. The reaction mixture was created in three technical replicates. A 20 µlreaction was produced with iTag Universal SYBR Green Supermix (10 µl), forward primer (1 µl), reverse primer (1 µl), and template (Table 3.8). The final volume (20 µl) was adjusted using water (nuclease-free) from the kit. Actin wasutilised to normalise. The CFX96T™ Real-Time System was used to measure gene expression levels. The data was calculated using the 24ºC equation (Livak & Schmittgen, 2001).

Table 3.8: The protocol mentioned below was provided along with the iTaqTM Universal SYBR Green Supermix (BIO-RAD) was followed

RT-PCR used	Mode	Polymerase activation and DNA denaturation at95^{o}C	Amplification			Melt- curveanalysis
			Denaturationat 95^{o}C	Annealing extension plate read at60^{o}C	Cycles	
BioRad^{R}CFX96^{TM}	SYBR^{R} only	20-30 seconds	2-5 seconds	15-30 seconds	35-40	Instrument default setting

Table 3.9: The list of primers of selected genes for validation by RT PCR

Gene name	Function	Sequence	Length
LOC101499357	Peroxidae- 3	F:CAAAGCAACACGGCTGAAAG	20
		R:GAGCAGGAAGGCCAAGTAAA	20
LOC101502158	ethylene-responsive transcription factor 9	F:GGTTCACTTCAGAGGCGTTAG	21
		R:CTGGCTTGGGCTCTGATTATT	21
LOC101491788	protein NRT1/ PTR FAMILY	F:TCGCTTCAGTCCATTCCATAC	21
		R:TTCCCTGGCATTGGGATTAG (	20
LOC101505466	ATP phosphoribosyltransferase 2, chloroplastic-like	F:CTGTTCAGCTGGGTCCTAAAT	21
		R:GCACTCACTCATGGGAAGAA	20
LOC101513079	potassium channel AKT2/3	F:TGTGCTGGGTGCCTTTATT	19
		R:GGTATGTACTCTGCCGTCATTT	22
LOC101515358	UDP-glucose:glycoprotein glucosyltransferase	F:CAAGCGGTAACAGACTCCATA G	22
		R:CCAACTGAGAGACAACCCTAA TC	23
LOC101506978	photosystem II 22 kDa protein, chloroplastic	F:GGTGTTATTCCTCCCGGTAAAG	22
		R:GATTCAAAGCAGCAACGAAGA A	22
LOC101507594	phenylalanine ammonia-lyase 1-like	F:CTGGCGTGAATGGTGAACTA	20
		R:GGGTCTTCAACTCATCCTCAAA	22
LOC105851638	cytochrome P450 CYP736A12-like	F:CCGTGATTGAGTGGGCTTTA	20
		R:GCCTACGACCAGAACCAAAT	20
LOC101501314	catalase activity	F:CAAAGAAGGAGCTGAGGTACAA	22
		R:AAGGTGGCAGGAAAGGATTAG	21

CHAPTER-4 RESULTS AND DISCUSSION

4.1. Morphological characterization of FOC

Seven days post inoculation, the FOC ITCC 7679 inoculum exhibited rapid growth, completely covering the plates. Microscopic examination revealed oval to cylindrical microconidia and macroconidia with thin walls and pointed ends,averaging 8–10 µm × 3–5 µm with 1–2 septa per conidia. In contrast, the macroconidia lacked septa and measured 20.5–24 µm x 3-4.5 µm. Morphological traits, including colony diameter, macroconidia, microconidia dimensions, and mycelial growth patterns, closely resembled those described by Dubey *et al*. (2009) and Rani *et al*. (2022) (Figure 4.1 A and B).

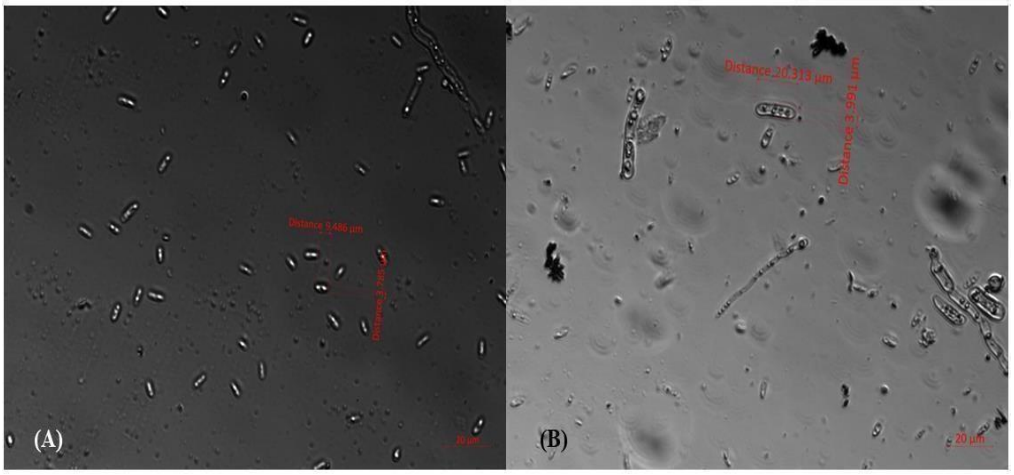


Figure 4.1: The (A) microconidia and (B) macroconidia of FOC observed under microscope.

Confirmation of plant infection involved microscopic examination of stained roots. Infected plants exhibited the presence of chlamydospores of pathogen within the tissues (Figure 4.1 A, B). These results were found to be consistent with the previous researches done on FOC (Tintor *et al*., 2020; Mendu *et al*., 2022). Subsequent isolation of the pathogen from diseased stems confirmed that the isolate retained the same morphological traits as the original pathogen, thus confirming the identity of the infecting pathogen as ITCC 7679 (Ponnusamy *et al*., 2020; Xu *et al*., 2022).

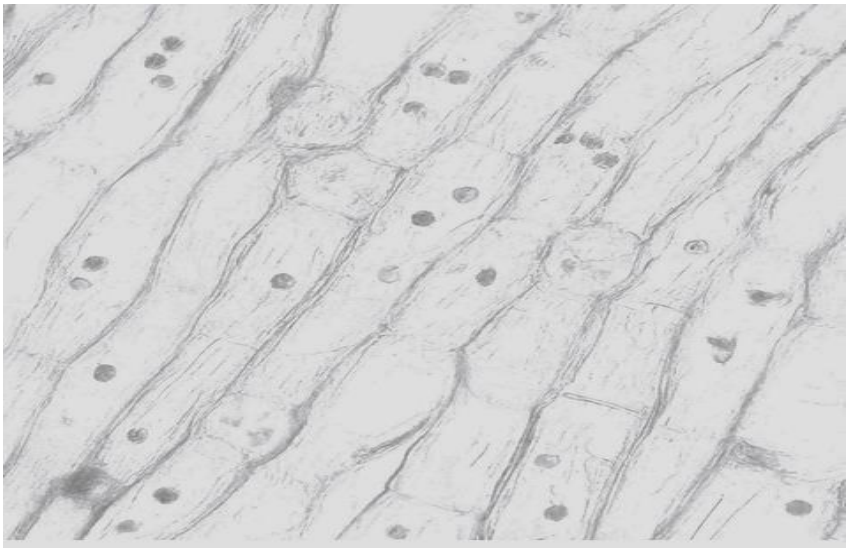


Figure 4.2: Light micrographs of transverse sections of plants, showing the root colonization of chickpea tissues by *Fusarium oxysporum* f. sp. *Ciceris*

4.2. Agronomical assessment of Chickpea Genotypes under FOC infection The chickpea genotypes were subjected to field and greenhouse screening against the race 3 of FOC. The plants infected with FOC were analysed for different agronomical parameters.

4.2.1. Wilt incidence

Wilt incidence was observed in plants exposed to the pathogen under both field and controlled greenhouse conditions and the phenological characteristic of pre-and 45 DAS stages was considered. The infected plants exhibited darkening of the xylem tissue (Figure 4.3) and wilting symptoms.

Figure 4.3: Transverse section of an infected chickpea plant stem showing darkening of xylem.

Symptoms first appeared in the susceptible check genotype JG 62, eight days after inoculation of FOC. The symptoms mirrored patterns reported by other researchers (Jorben *et al*., 2023). Typical signs of wilting included leaflet drooping and yellowing, starting at the leaf apex and progressing downward until the entire plant wilted. Splitting open affected plant roots and stems revealed discolored internal tissues and these results were consistent with wilt studies. Plants displaying wilting symptoms were categorized as Highly Resistant (HR), Resistant (R), Moderately Resistant (MR), Susceptible (S), or Highly Susceptible (HS) (Figure 4.4). These observations were in line with previous chickpea screening studies against FOC (Rathod *et al*., 2020; Achari *et al*., 2023). Re-isolation of cultures from infected plants exhibitedmorphological resemblance to the parent strain. Two-way analysis of variance was performed to assess genotype reactions under both field and pot conditions (Table 4.1). The mean wilting percentage of genotypes under field and pot conditions is mentioned in Table 4.2.

Table 4.1: Two-way ANOVA for wilting percentage of different genotypes at pre and 45 DAS stages under pot and field conditions

Source of Variation		**Pot conditions**	**Field Conditions**	**Pot conditions**	**Field Conditions**
ANOVA Table	df	F (dfn, dfd)		P value	
Genotypes	51	F = 91.22	F = 27.82	P<0.0001***	P<0.0001***
28 DAS and 45 DAS stages	1	F = 602.5	F = 144.3	P=0.0017**	P=0.0069**
Interaction: Genotypesx 28 DAS and 45 DAS stages	51	F = 9.327	F = 6.782	P<0.0001***	P<0.0001***

The ANOVA results revealed significant effects of both genotypes and stages (pre- and 45 DAS) on the response of chickpea to *Fusarium* wilt under both pot and field conditions. In terms of response, significant differences wereobserved between the 52 chickpea genotypes in both pot ($p < 0.0001$) and field ($p < 0.0001$) conditions, indicating considerable variability in susceptibility and resistance to FOC infection across genotypes. The stages of plant developmentalso significantly influenced the wilt response under both pot ($p = 0.0017$) and field ($p = 0.0069$) conditions, suggesting that the severity of wilt symptoms varied depending on the stage of growth.

Table 4.2: Mean wilting percentage of chickpea genotypes in response to *Fusarium oxysporum* f.sp. *Ciceris* race 3 under field and pot conditions

	Mean wilting percentage			
	Field		Pot	
Genotypes	**28 DAS**	**45 DAS**	**28 DAS**	**45 DAS**
GNG 2144	0.00±0.00	1.30±0.07	1.83±0.82	7.72±1.70
RSG 888	28.96±1.23	47.35±6.78	45.31±4.66	74.56±7.45
ICC 5335	25.50±1.11	28.45±2.56	21.50±3.23	41.12±5.04
ICCV 96029	60.00±4.04	60.31±5.75	60.00±9.01	84.91±7.77
PHULe G 0517	3.03±0.05	7.99±1.56	3.03±1.46	11.98±2.04
PUSA 547	39.34±6.78	52.99±7.43	39.34±5.45	79.49±6.69
GLK 16063	10.43±1.56	11.44±1.45	9.76±2.33	15.83±3.01
CSJ 513	38.65±3.54	56.02±7.43	38.65±6.2	84.03±7.89
HC 7	10.31±1.70	11.22±2.65	14.61±2.23	19.06±2.65
GNG 2477	36.67±4.44	38.14±5.32	34.00±3.23	68.09±5.78
GNG 1581	28.57±3.76	32.27±2.54	21.57±1.34	33.58±3.69
CSJ 515	14.53±2.75	16.60±1.77	12.03±2.22	23.14±2.45
HC 1	0.00±0.00	1.85±.12	0.00±0.00	2.78±0.22
ICCV 10	11.67±1.11	19.06±2.57	30.19±4,96	34.84±3.45
HC 5	3.70±1.04	4.93±1.66	3.03±1.01	7.03±1.22
ICCV 07107	14.64±2.55	19.93±2.64	13.31±1.13	24.59±2.34

GNG 2477	8.78±2.61	9.03±0.54	8.78±1.13	15.08±1.67
DCP 92-3	5.12±0.47	14.10±2.88	4.46±0.14	22.17±2.35
PHULeG 0127	7.03±1.77	7.46±0.23	7.03±1.44	14.53±1.2
KPG 59	26.10±3.45	57.21±6.43	24.19±3.98	24.43±2.34
BG 212	22.52±2.60	22.65±3.33	21.19±2.67	24.95±2.37
DIGVIJAY	6.67±1.78	7.61±2.45	3.33±1.13	6.67±1.11
RSG 807	23.34±4.67	25.55±2.46	21.67±4.55	60.00±5.97
GNG 2459	24.36±3.89	24.85±4.64	15.04±2.67	19.92±1.87
Local variety 2	20.88±3.22	29.06±3.67	24.20±3.06	58.80±.12
ICC 07304	19.52±2.15	29.61±4.22	16.31±3.33	24.42±2.55
HC 3	5.56±1.66	19.02±1.88	4.55±1.12	17.67±1.56
RVG 203	2.23±0.33	12.18±2.55	3.34±0.34	18.86±1.59
RSG 963	45.31±6.34	52.48±.82	41.61±4.68	44.21±3.68
ICCV 6	26.13±3.43	36.15±4.64	36.50±4.33	54.23±4.95
GNG 2418	11.69±1.76	13.23±1.55	13.55±1.23	19.84±1.84
PBG 5	8.58±1.34	15.31±2.88	7.58±0.33	17.67±1.65
WR 315	16.62±2.58	17.74±2.45	11.65±1.23	12.59±.98
GNG 2171	2.56±0.43	12.22±1.43	2.56±0.55	18.33±1.76
ICC 3020	21.13±1.82	28.61±4.66	17.80±3.34	32.67±2.89
RSG 931	53.32±4.23	58.51±8.36	53.33±6.56	87.78±7.85
PUSA 372	37.26±6.26	39.97±4.76	35.55±7.08	38.55±2.98

BG 4011	24.07±2.14	26.85±2.23	10.43±2.56	17.16±2.18
HC 6	15.23±2.34	22.72±3.67	12.25±2.45	17.71±2.44
ICCV 512	32.32±4.34	48.98±5.33	32.32±5.67	73.48±4.77
SADABAHAR	22.08±4.34	26.05±3.45	22.08±1.56	39.08±2.34
GNG 1958	21.127±3.54	23.82±2.67	21.12±5.33	35.73±2.98
RSG 945	57.55±6.34	57.78±4.67	36.67±4.33	52.56±.65
C 235	2.38±0.13	6.07±2.78	2.38±0.29	9.11±0.55
RSG 991	17.72±2.24	18.69±1.56	15.87±2.45	26.45±1.98
HK 4	2.78±0.04	10.87±1.88	3.61±1.12	12.78±1.02
HK 2	16.67±1.54	21.11±2.66	16.67±3.34	23.33±1.79
HK 1	9.72±2.86	16.82±1.98	9.05±2.22	23.06±1.68
KAK 2	40.90±8.34	41.41±6.56	38.52±.34	50.30±4.25
GLK 28127	16.38±1.89	30.55±4.33	15.27±2.23	41.67±3.54
GLK 17301	30.63±2.45	31.46±5.67	29.04±3.42	42.96±3.12
JG 62	86.11±7.01	97.91±9.34	87.17±8.09	99.91±8.36
Average	21.47	27.37	20.67	34.83
t-test (field vs pot)	**0.043****			

Additionally, a significant interaction between genotypes and stages was observed in both pot ($p < 0.0001$) and field ($p < 0.0001$) conditions, indicating that the response of chickpea genotypes to *Fusarium* wilt was influenced by thedevelopmental stage of the plant, regardless of the growth conditions. These findings emphasize the importance of considering both genotype and stage of growth when evaluating chickpea resistance to *Fusarium* wilt, as they significantly impact the observed wilt response across different growing environments.

The results indicated that, on average, the 28 DAS wilting percentage of genotypes infected with FOC under field conditions was 21.4%, which increased to 27.3% 45 DAS. Similarly, under pot conditions, the average28 DAS wilting percentage was 20.6%, rising to 34.8% 45 DAS. The genotypes GNG 2144 and HC 1 did not show any wilt symptom during the 28 DAS stages under field conditions. Even in pot conditions, this wiltingpercentage remained below 10%. Therefore, can be considered as highly resistant to the FOC wilt. However, rest of the genotypes started showing wilting symptoms at early stages of 28 DAS and these symptoms increased progressively in 45 DAS stages also. These results were consistent with other researches also in which no genotype was found to be completely resistant to the FOC (Yadav *et al*., 2023). The t-test demonstrated significant differences between the wilting percentages of genotypes under fieldand pot conditions, indicating that the growth environment significantly impacts the severity of *Fusarium* wilt symptoms. These findings align with previous researches on chickpea screening, which noted higher wilting percentages in chickpea genotypes under pot conditions due to greenhouse conditions favouring fungal sporulation and mycelium clogging in the roots (Kumar *et al*., 2017; Patil *et al*., 2024).

4.2.1.1. Categorization of genotypes on the basis of mean wilting percentage

The response of genotypes was assessed and categorization of genotypes wasdone on the basis of percent wilt incidence (Table 4.3 and 4.4).

Table 4.3: Categorization of genotypes of chickpea based on their wilting response against *Fusarium oxysporum* f.sp. *Ciceris* race 3 under fieldconditions

Wilting percentage	Response	Genotypes	Total
1-10%	**HR**	HC1, C235, GNG 2144	3
11-20%	**R**	**WR 315**, HC 5, PHULeG0517, GNG 2171, PHULeG 0127, GNG 2477, HC 3, HK 4, HC 7, GLK 16063, GNG 2418, HC 6, PBG 5	13
21-30%	**MR**	HK 1, DCP 92-3, KPG 59, RVG 203, GNG 2459, DIGVIJAY, CSJ 515, BG 212, RSG 991, ICCV 07107	10
31-50%	**S**	HK 2, GNG 1581, ICC 3020, GNG 1958, SADABAHAR, ICC 5335, PUSA 372, BG 4011, ICC 07304, RSG 963, GLK 28127, KAK 2, GLK 17301	13
>50%	**HS**	RSG 931, ICCV 10, ICCV 6, PUSA 391, PUSA 547, RSG 945, RSG 807, Local variety 2, RSG 888, CSJ 513, **JG 62**, ICCV 512, ICCV 96029	14
	Total		52

Table 4.4: Categorization of chickpea genotypes of chickpea based on their wilting response against *Fusarium oxysporum* f.sp. *Ciceris* race 3 onthe basis of pot screening

Wilting percentage	Response	Genotypes	Total
1-10%	**HR**	HC1, GNG2144, C235, Digvijay, HC5	5
11-20%	**R**	GNG 2459, GNG 2418, HC 7, RVG 203, GNG 2171, HC 6, HC 3, PBG 5, BG 4011, GLK 16063, GNG 2477, PHULeG 0127, HK 4, WR 315, PHULe G 0517	15
21-30%	**MR**	RSG 991, BG 212, ICCV 07107, ICC 07304, KPG 59, HK 2, CSJ 515, HK 1, DCP 92-3,	9
31-50%	**S**	RSG 963, GLK 17301, GLK 28127, ICC 5335, SADABAHAR, PUSA 372, GNG 1958, ICCV 10, GNG 1581, ICC 3020	10
>50%	**HS**	**JG 62**, RSG 931, ICCV 96029, CSJ 513, PUSA 547, RSG 888, ICCV 512, PUSA 391, RSG 807, Local variety 2, ICCV 6, RSG 945, KAK 2	13
	Total		52

The categorization of genotypes based on their wilting percentage indicated distinct responses among different genotypes. Specifically, genotypes HC1, C235, and GNG 2144 demonstrated a highly resistant response, exhibiting minimal wilting under both field and pot conditions. Conversely, genotypes RSG 931, ICCV 6, PUSA 391, PUSA 547, RSG 807, Local variety 2, RSG 888, CSJ 513, JG 62, ICCV 512, and ICCV 96029 displayed a highly susceptible response, showing significant wilting percentages under both conditions. Thesefindings suggest that certain genotypes exhibit consistent resistance or susceptibility to wilting even under different environmental conditions.

4.2.2. Disease Severity Index, Biomass and Yield

The plants in the field conditions were evaluated for Disease Severity, Biomass, and Yield. Following infection, the plants were classified into categories: healthy, moderately infected, severely infected, and deceased and scored basedon morphological characteristics (Figure 4.4) (Table 4.5).

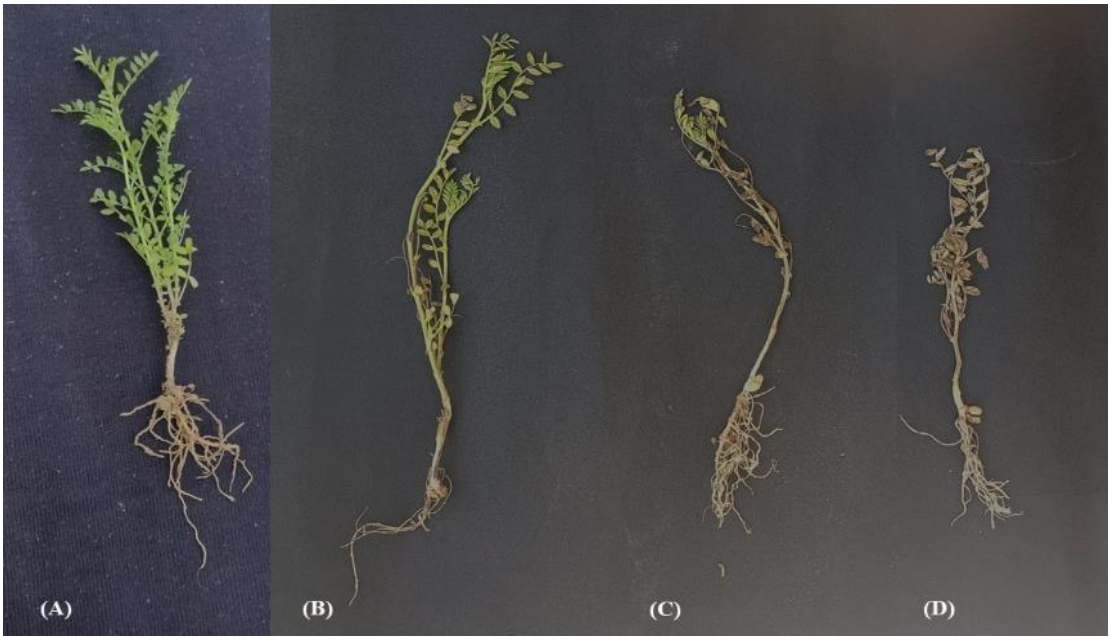


Figure 4.4: Categorisation of infected chickpea plants based on varying disease severity: (A) Healthy plant; (B) Moderately infected; (C) Severely infected; and (D) Dead.

Table 4.5: Mean performance of the chickpea genotypes under field conditions

Genotypes	Wilting (%)	DSI	Biomass(q/ha)	Yield(q/ha)
GNG 2144	8.50±2.02	0.00±0.00	15.49±0.78	9.16±071
RSG 888	66.03±2.41	25.44±4.38	19.33±2.06	8.92±0,17
ICC 5335	28.12±2.43	11.86±0.56	26.06±0.43	10.04±0.6
ICCV 96029	90.47±4.76	38.44±5.24	20.67±0.69	7.13±0.63
PHULe G 0517	10.31±1.91	4.61±1.20	24.15±0.58	9.46±0.86
PUSA 547	60.65±1.56	29.83±2.43	18.39±1.41	7.46±0.8
GLK 16063	17.16±4.10	7.11±2.72	20.55±1.87	8.75±0.27
CSJ 513	71.41±1.80	29.71±1.15	21.58±0.88	10.56±0.22
HC 7	16.83±2.56	2.83±1.5	28.00±1.15	8.56±0.11
PUSA 391	59.44±3.88	25.94±2.52	31.36±0.86	6.83±0.94
GNG 1581	34.92±0.79	14.32±0.26	25.21±0.70	11.10±1.13
CSJ 515	25.74±2.27	9.69±1.20	27.97±1.54	9.44±0.29
HC 1	2.78±3.84	0.92±0.92	28.45±1.27	8.51±1.20
ICCV 10	53.84±6.42	21.48±1.69	25.09±0.92	9.37±0.30
HC 5	10.74±5.02	3.58±2.14	22.71±2.46	10.27±0.89
ICCV 07107	29.89±4.48	12.82±3.11	25.08±2.55	6.88±0.06
GNG 2477	13.54±2.93	7.21±2.87	24.39±3.38	10.22±0.43
DCP 92-3	21.15±0.72	7.05±0.97	24.46±2.07	9.71±1.07
PHULeG 0127	11.20±0.88	3.73±1.71	23.21±3.87	9.44±1.51
KPG 59	21.50±2.70	7.91±0.82	26.65±2.17	9.64±1.36
BG 212	26.28±2.48	9.51±4.81	26.04±2.46	9.25±147
DIGVIJAY	24.52±7.26	8.17±0.80	21.36±2.96	9.71±0.70
RSG 807	28.33±1.54	31.05±3.51	20.15±3.55	7.30±0.36
GNG 2459	24.36±8.46	9.65±1.79	21.18±3.53	9.51±1.74
Local variety 2	25.56±1.39	29.81±1.09	26.10±2.21	7.57±0.71
ICC 07304	44.42±3.06	22.37±0.83	22.93±1.87	9.38±1.05
HC 3	14.39±1.10	3.87±1.51	24.73±9.58	8.63±0.49
RVG 203	22.20±1.00	10.30±3.26	22.08±3.74	8.37±0.45
RSG 963	18.41±2.16	19.23±2.54	26.27±3.21	8.87±1.10

ICCV 6	37.56±5.09	28.39±2.41	28.45±2.38	6.03±0.23
GLK 28127	45.83±2.56	9.03±0.69	25.41±1.33	9.77±0.96
PBG 5	19.94±2.09	12.15±1.19	24.81±1.17	9.55±1.06
WR 315	10.23±3.59	3.41±0.37	26.81±6.00	10.39±1.11
GNG 2171	11.00±0.92	3.66±0.60	28.77±0.83	10.25±0.30
ICC 3020	35.13±3.33	12.55±1.14	25.87±3.63	9.22±0.61
RSG 931	53.33±2.01	23.97±3.13	22.31±0.63	8.28±1.14
PUSA 372	40.26±5.00	14.31±1.15	25.21±0.70	4.10±1.01
BG 4011	13.61±1.12	18.82±1.45	24.95±4.11	9.07±0.69
HC 6	19.27±5.30	6.84±3.01	25.32±0.96	9.84±1.12
ICCV 512	73.48±3.51	20.18±0.84	21.81±1.72	7.95±0.21
SADABAHAR	39.08±1.24	18.26±1.76	24.94±2.82	5.75±1.16
GNG 1958	35.73±5.77	18.90±0.56	23.38±0.91	8.58±0.67
RSG 945	40.00±1.70	28.77±1.05	20.10±0.85	9.13±0.81
C 235	9.11±1.77	3.03±2.04	26.33±1.02	10.41±1.16
RSG 991	28.042±3.21	12.16±2.70	26.00±1.94	9.22±0.6
HK 4	16.31±5.66	7.18±1.04	29.19±0.80	9.83±0.81
HK 2	31.67±9.27	10.56±3.09	26.06±0.43	9.35±1.61
HK 1	21.06±1.15	9.05±	22.99±1.37	9.59±0.61
KAK 2	13.63±5.46	21.96±2.04	20.83±1.57	8.43±1.38
GNG 2418	18.62±4.16	21.85±2.89	21.77±0.64	8.52±2.00
GLK 17301	47.19±5.13	21.91±2.36	23.04±0.3	8.13±1.19
JG 62	73.47±2.08	30.74±2.61	21.35±0.88	7.99±1.52

*DSI= Disease Severity Index

The results demonstrated significant variability in wilt incidence, Disease Severity Index (DSI), biomass, and yield among the tested chickpea genotypes. HC1 exhibited the lowest wilt incidence at 2.7%, while ICCV 96029 displayed the highest incidence at 90.4%, indicating a wide range of susceptibility levels.

Similarly, DSI values varied from 0 to 38.4, with GNG 2144 having the lowest DSI and ICCV 96029 showing the highest, further highlighting the diverse responses to *Fusarium* wilt. In terms of biomass, PUSA 391 exhibited the highest mean biomass production of 31.69 q/ha, contrasting with GNG 2144, which had the lowest biomass

of 15.4 q/ ha. Furthermore, GNG 1598 demonstrated the highest mean yield at 11.1 q/ha, while PUSA 372 had the lowest yield at 4.10 q/ha.These observations were further analysed to assess the ANOVA, correlations and to cluster genotypes into the most resistant and susceptible groups, to determine which genotypes performed best under field conditions.

Table 4.6: One-way ANOVA for various traits under study

Source of Variation	df	Wilt (%)	DSI	Biomass	Yield
Treatment		2			
Mean sum of squares		257.45	73.134	17.587	20.384
Treatment	51	**2.2e-16 *** **	**0.007366 ** **	**0.02639 * **	**0.0002584 *** **
Error	51	3.6902	2.1746	2.3785	0.8704
F value		31.8339	19.2611	1.5760	2.3234

Following the FOC infection, significant differences were found between the chickpea genotypes for all four parameters assessed. The p-value < 0.05 suggested that the reaction of genotypes to the fungus infection varied, validating considerable genetic differences among the genotypes, leading to a differential response to the infection and its impact on different parameters (Table 4.6). Although the biomass showed a less significant response than the other parameters, the results suggested that the different genotypes have different biomass allocation genetically and are influenced by the infection. These results align with earlier research, emphasizing the importance of integrating genetic factors in understanding plant-pathogen interactions(Srivastava *et al*., 2021; Yadav *et al*., 2023).

4.2.3. Correlation coefficient analysis

The correlation matrix demonstrates strong relationships between the various studied variables (Table 4.7).

Table 4.7: Pearson correlation coefficient of the disease and agronomical parameters observed under FOC infection of chickpea genotypes

	WI	DSI	BI	YL
WI	1.000**			
DSI	0.944^{**}	1.000^{**}		
BI	-0.372^{*}	-0.415^{*}	1.000^{**}	
YL	-0.461^{**}	-0.473^{**}	0.050^{NS}	1.000^{**}

*WI : Wilt incidence; DSI: Disease Severity Index; BI: Biomass, YL: Yield.

The strong positive correlation between wilt incidence (WI) and disease severity index (DSI) (r = 0.944, p < 0.01) revealed that as there is an increase inthe disease

severity with increased wilt incidence. These findings corroborated with the findings from other experiments on plant-pathogen interactions (Wafa*et al*., 2022; Jagre *et al*., 2022). Moreover, the significant negative correlations between Wilt incidence and yield (Y) ($r = -0.461$, $p < 0.01$) and between diseaseseverity Index and yield ($r = -0.473$, $p < 0.01$) indicate the detrimental impacts of FOC infection on chickpea yield, aligning with prior research on fungal diseases affecting crop productivity (Wafa *et al*., 2022; Keote *et al*., 2019; Jagre *et al*., 2022). The moderate negative correlation between Wilt incidence and biomass ($r = -0.372$, $p < 0.05$) suggests that higher wilt incidence correlates with lower biomass production, underscoring the significance of disease management strategies in mitigating yield losses in chickpea cultivation. However, the non-significant association between BI and YL ($r = 0.050$, $p > 0.05$) implies that while biomass may exert an indirect influence on yield, otherfactors likely play more crucial roles in determining final yield outcomes (Mufti*et al*., 2023; Amine *et al*., 2022).

4.2.4. Divergence analysis

Using the clustering approach, chickpea genotypes were systematically organised based on their agronomical qualities, providing significant insights into their performance and resistance/susceptibility characteristics. The Kmean clustering revealed five as the optimum number of clusters required to categorize the data. A representation of cluster division is given in Table 4.8 andFigure 4.5.

Table 4.8: Distribution of genotypes among 5 clusters on the basis of D^2 statistics

Cluster No	Members	Total
1	GNG2144, HC 3, GNG 2477, DCP 92-3, HC 6, HC 5, PHULeG0517, PHULe G 0127, HK 1, **Digvijay**, GNG 2459, GLK 16063, RVG 203	13
2	HC 7, HC 1, **WR 315**, C 235, GNG 2171, HK4, RSG 963, BG 4011, CSJ 515, KPG 59, ICC 3020, BG 212, RSG 991, HK 2, ICC 5335, PBG 5, GNG 1581, GLK 28127	18
3	PUSA 372, ICCV 07107, Sadabahar, PUSA 391, Local variety 2, ICCV 6	6
4	CSJ 513, ICCV 96029, ICCV 512, **JG 62**, RSG 888, PUSA 547	6
5	RSG 931, GLK 17301, ICCV 10, ICC 07304, GNG 1958, KAK 2, GNG 2418, RSG 807, RSG 945	9
	Total	52

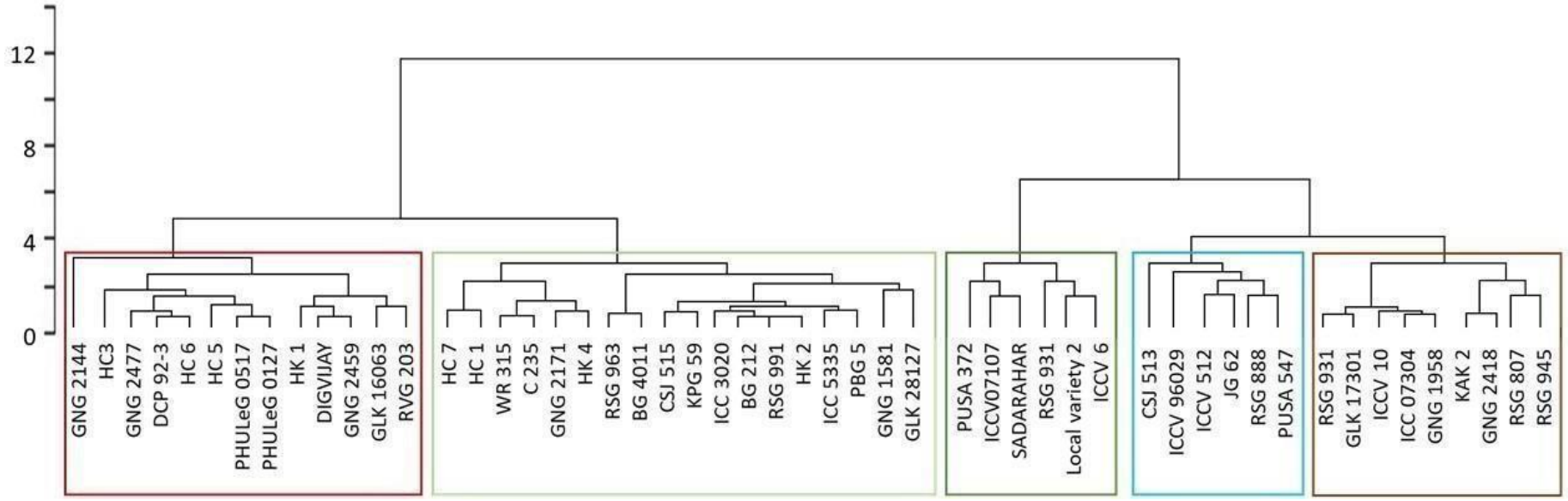


Figure 4.5: Dendrogram representing the response of chickpea genotypes in a clustering pattern

Based on how the 52 chickpea genotypes responded to *Fusarium* wilt infection and agronomical parameters, the cluster analysis divided them into five differentgroups. WR 315 and Digvijay, two resistant check genotypes, were clustered inGroup 1 and 2 respectively, suggesting that genotypes with substantial resistance to FOC are present in this cluster. On the other hand, JG 62, the susceptible check genotype, belonged to Cluster 4 along with other susceptible genotypes. This pattern implies that genotypes were successfully segregated according to their susceptibility levels by the clustering. Additionally, thegenotypes belonging to Groups 1 and 5 exhibited the most genetic separations from one another, which suggest variations in the resistance levels among theseclusters. The genotypes clustered in cluster 4 were highly susceptible whereas, in cluster 5 were moderately resistant.

4.2.5. Inter cluster analysis

The genotypes were grouped differently according to the inter cluster distance table (Table 4.9). The distance between two clusters is represented by each value in the table. The distance of 56.274 between Clusters 1 and 2 showed that the genotypes in those clusters are comparatively distinct (Table 4.9). Greater dissimilarity between clusters is shown by higher values farther away from the diagonal, whereas lower values closer to the diagonal signal more comparable genotypes within a cluster. Based on how the genotypes responded to *Fusarium* wilt infection, this pattern indicates that the clustering successfully divided the genotypes into five different groups.

Table 4.9: Inter cluster distances of the genotypes distributed among clusters.

Cluster No	**1**	**2**	**3**	**4**	**5**
1	0.000				
2	56.274	0.000			
3	33.048	23.317	0.000		
4	86.135	29.918	53.182	0.000	
5	13.446	42.831	19.617	72.704	0.000

The inter-cluster distance table reveals distinct groupings among the genotypes based on their response to *Fusarium* wilt infection. A distance of 56.274between Clusters 1 and 2 suggests significant dissimilarity, indicating that the genotypes within these clusters exhibit markedly different responses to the infection.

Similarly, the distances of 33.048 and 23.317 between Clusters 1 and 3, Clusters 2 and 3 respectively, illustrate varying degrees of dissimilarity among these clusters. Conversely, the lower distances closer to the diagonal, such as 13.446 between Cluster

1 and Cluster 5, suggest more comparable genotypes within these clusters. Overall, these results suggest that the clustering effectivelystratified the genotypes into five distinct groups based on their wilt infection responses.

4.3. Assessment of chickpea genotypes based on molecular response

4.3.1. Qualitative and quantitative assessment of DNA

The extracted DNA was subjected to quality assessment and quantification employing both agarose gel electrophoresis and UV spectrophotometry techniques. DNA concentrations ranged from 480 to 2300 ng/µl, indicating sufficient DNA yield across samples. The ratio of absorbance at wavelengths A260 and A280 fell within the range of 1.69 to 1.87, suggesting the absence of impurities like polyphenols, polysaccharides, proteins, RNA, and ensuring the purity of the DNA (Table 4.10).

Table 4.10: Concentration of DNA (ng/µl) of fifty two chickpea genotypes and their A260/280 ratio.

Sample no.	Concentration (ng/µl)	A260/280	Sample no.	Concentration (ng/µl)	A260/280
1	1128	1.71	27	1484	1.85
2	1546	1.82	28	1263	1.80
3	520	1.75	29	1436	1.76
4	1145	1.81	30	1070	1.72
5	1320	1.85	31	2015	1.85
6	2105	1.74	32	1964	1.84
7	2210	1.69	33	1872	1.75
8	1105	1.70	34	1964	1.69
9	954	1.77	35	1118	1.76
10	1487	1.74	36	1277	1.85
11	856	1.78	37	1486	1.82
12	1463	1.87	38	1752	1.76
13	1597	1.72	39	1566	1.84
14	1693	1.74	40	1080	1.71
15	2170	1.70	41	2090	1.75
16	489	1.84	42	2149	1.70
17	752	1.85	43	1547	1.72
18	694	1.69	44	754	1.83
19	842	1.84	45	951	1.74
20	890	1.75	46	1420	1.79
21	1258	1.70	47	1574	1.86
22	1654	1.82	48	1860	1.72
23	1746	1.73	49	2181	1.78
24	1594	1.86	50	2290	1.75
25	1357	1.77	51	2171	1.84
26	1564	1.79	52	1564	1.81

Additionally, agarose gel electrophoresis (0.8%) was employed as another method to assess DNA quality. The presence of distinct bands in the genomic DNA confirmed its integrity, with no evidence of contamination, thereby affirming the reliability of the extracted DNA for further molecular analyses.

4.3.2. Genetic analysis of the genotypes using STMS markers

The molecular screening of the genotypes was conducted using STMS markers TA-194, TA96, and TA-27, to check their responses to race 3 of FOC, based onallelic differences detected by these markers. Each marker exhibited polymorphism and two alleles were identified with each of the marker. The STMS primers used, yielded distinct bands which distinguished between susceptible and resistant genotypes.

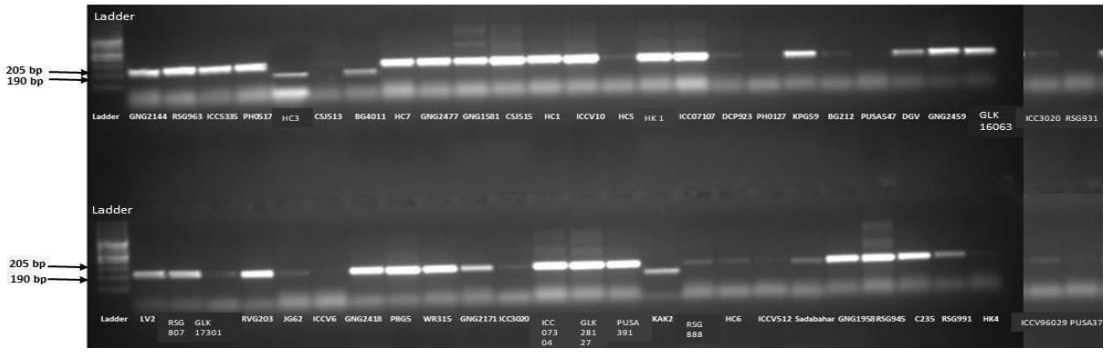


Figure 4.6 (a): Amplification of *foc-3* gene in chickpea genotypes using STMS marker TA194.

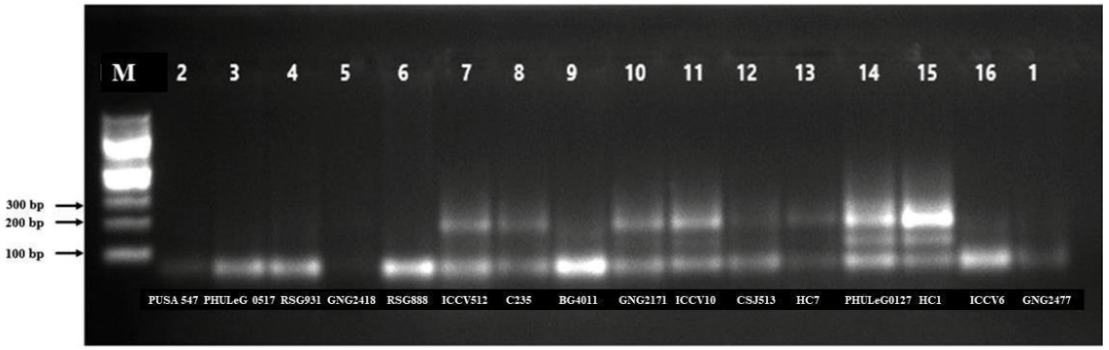


Figure 4.6 (b): Amplification of *foc-3* gene in chickpea genotypes using STMS marker TA-27

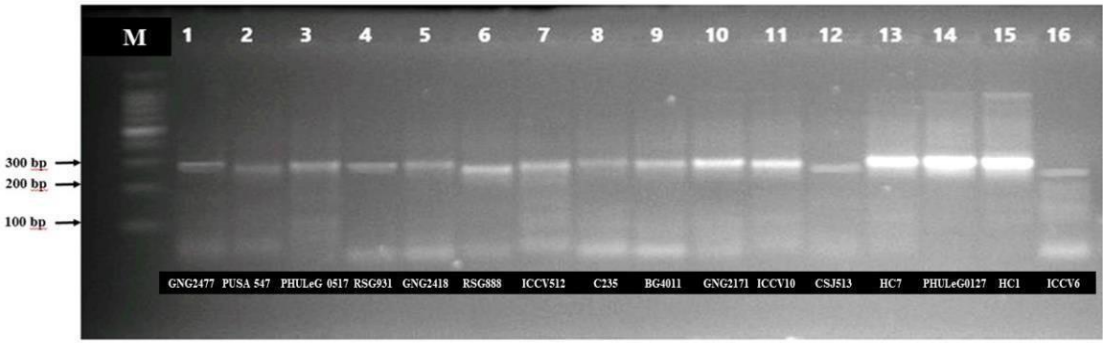


Figure. 4.6 (c): Amplification of *foc-3* gene in chickpea genotypes using STMS marker TA-96

Based on the allelic differences of three markers, the study showed the resistance and susceptibility of the genotypes against FOC. For the marker TA-96, an amplicon of size 280 bp was amplified for the genotypes exhibiting FOCresistance (GNG 2144, ICC 5335, HC 7, CSJ 515) whereas an amplicon of 260bp was produced by the genotypes exhibiting FOC susceptibility (RSG 888, ICCV 96029, PUSA 547). When amplified by TA-27, the resistant genotypes produced an amplified product of size 208 bp (HC 7, ICCV 10, DCP 92-3, HC 3), while susceptible genotypes produced an amplified product of size 190 bp (PHULe G 0517, PUSA 547, RSG 807). However, the genotypes PHULe G 0127 and HC 1, amplified alleles of both the sizes and were therefore considered heterozygous for the genes of resistance and susceptibility. Similarly, in case ofthe marker TA-194, the amplicons were of size 205 bp and 190 bp for theresistant (PHULe G 0517, CSJ 515, KPG 59, Digvijay) and susceptible genotypes (RSG 888, GLK 16063, BG 212, PBG 5), respectively. Conversely, some genotypes showed unclear amplification or no amplification at all, indicating susceptibility to FOC for those specific markers. Similar patternswere observed in previously done researches with markers TA-27 and TA-194, corroborating recent research findings (Tatte *et al*., 2018; Suleimanova *et al*., 2023; Amadabade *et al*., 2016). The amplification summary of all the markers against the genotypes is presented in Table 4.11.

Table 4.11: Alleles generated by STMS markers in response to presence or absence of *foc-3* gene.

Genotypes	TA-96	TA-27	TA-194
GNG 2144	HR	HS	HR
RSG 888	HS	HS	HS
ICC 5335	HR	HS	HR
ICCV 96029	HS	HS	HS
PHULe G 0517	HR	HS	HR
PUSA 547	HS	HS	HS
GLK 16063	HR	HS	HR
CSJ 513	HS	HS	NA
HC 7	HR	HR	HR
PUSA 391	HR	HR	HR
GNG 1581	HS	HR	HR
CSJ 515	HR	HS	HR
HC 1	HR	HTR	HR
ICCV 10	HR	HR	HR

HC 5	HS	HR	HS
ICC 07304	HS	HS	HR
GNG 2477	HR	HS	HR
DCP 92-3	HS	HR	HS
PHULeG 0127	HR	HTR	NA
KPG 59	HR	HS	HR
BG 212	HR	HS	HS
DIGVIJAY	HS	HR	HR
RSG 807	HR	HS	HR
GNG 2459	HR	HS	HR
LOCAL VARIETY 2	HS	HS	HR
ICCV 07107	HS	HS	HR
HC 3	HS	HR	HS
RVG 203	HR	HS	HR
RSG 963	HS	HS	HR
ICCV 6	HS	HS	HS
GLK 28127	HS	HS	HR
PBG 5	HS	HR	HS
WR 315	HS	HR	HR
GNG 2171	HR	HR	HR
ICC 3020	HS	HS	HS
RSG 931	HS	HS	HS
PUSA 372	HS	HR	HS
BG 4011	HR	HS	HS
HC 6	HS	HR	HS
ICCV 512	HR	HR	HS
SADABAHAR	HS	HS	HR
GNG 1958	NA	HS	HR
RSG 945	HS	HS	HR
C 235	HR	HR	HR
RSG 991	HS	HR	HR
HK 4	HR	HS	HS
HK 2	HR	HS	HS
HK 1	HS	HR	HR
KAK 2	HS	HR	HS
GNG 2418	HS	HS	HR
GLK 17301	HR	HS	HS
JG 62	HS	HS	HS
GNG 2144	HR	HS	HR
RSG 888	HS	HS	HS

ICC 5335	HR	HS	HR
ICCV 96029	HS	HS	HS
PHULe G 0517	HR	HS	HR
PUSA 547	HS	HS	HS
GLK 16063	HR	HS	HS
CSJ 513	HS	HS	NA
HC 7	HR	HR	HR
PUSA 391	HR	HR	HR
GNG 1581	HS	HR	HR
CSJ 515	HR	HS	HR
HC 1	HR	HTR	HR
ICCV 10	HR	HR	HR
HC 5	HS	HR	HS
ICC 07304	HS	HS	HR
GNG 2477	HR	HS	HR
DCP 92-3	HS	HR	HS
PHULeG 0127	HR	HTR	NA
KPG 59	HR	HS	HR
BG 212	HR	HS	HS
DIGVIJAY	HS	HR	HR
RSG 807	HR	HS	HR
GNG 2459	HR	HS	HR
LOCAL VARIETY 2	HS	HS	HR
ICCV 07107	HS	HS	HR
HC 3	HS	HR	HS
RVG 203	HR	HS	HR
RSG 963	HS	HS	HR
ICCV 6	HS	HS	HS
GLK 28127	HS	HS	HR
PBG 5	HS	HR	HS
WR 315	HS	HR	HR
GNG 2171	HR	HR	HR
ICC 3020	HS	HS	HS
RSG 931	HS	HS	HS
PUSA 372	HS	HR	HS
BG 4011	HR	HS	HS
HC 6	HS	HR	HS
ICCV 512	HR	HR	HS
SADABAHAR	HS	HS	HR
GNG 1958	NA	HS	HR

RSG 945	HS	HS	HR
C 235	HR	HR	HR
RSJ 991	HS	HR	HR
HK 4	HR	HS	HS
HK 2	HR	HS	HS
HK 1	HS	HR	HS
KAK 2	HS	HR	HS
GNG 2418	HS	HS	HR
GLK 17301	HR	HS	HS
JG 62	HS	HS	HS

* HR: homozygous resistant; HTR: heterozygous resistant; HS: homozygous susceptible.

To evaluate the differential response of genotypes based on molecular markers, cluster analysis was conducted using the computer program "Simqual NTSYS PC" (version 2.0), which constructed a dendrogram from data obtained from STMS markers. The dendrogram in Figure 4.7 represents the division of genotypes across different clusters according to their resistance and susceptibility. The analysis revealed two primary clusters, each further subdivided into seven sub-clusters, indicating the genetic diversity among the genotypes. The resistant and susceptible check genotypes were segregated into distinct clusters, affirming their contrasting genetic profiles. Genotypes exhibiting resistance reaction i.e., HC 7, HC 1, ICCV 10, PHULe G 0127, GNG 2171, ICCV 512, and C 235, were grouped in cluster 2 based on marker analysis.Conversely, susceptible genotypes RSG 888, ICC 96029, PUSA 547, CSJ 513, ICCV 6, ICC 3020, RSG 931, and JG 62, formed cluster number 5.

The results of pot and field screenings corroborated with the findings of molecular results. Despite the fact that the majority of the pot screening results are supported by the molecular screening conducted in the current experiment, the reaction of genotypes GLK 17301, GLK 28127, ICCV 07107, PUSA 372, RSG 945, Sadabahar, ICCV 6, GNG1958, ICC 3020, GNG 1581, HK 2, PUSA 391, ICC 512 and ICCV10 was found to be different. The centre and southern regions of India received the release of the ICCV 10 genotype and hence, there are no reports of its resistance in the northern zones. Conversely, ICC 512 is a landrace that was obtained from Hyderabad (Gowda *et al*., 1995; GIS).

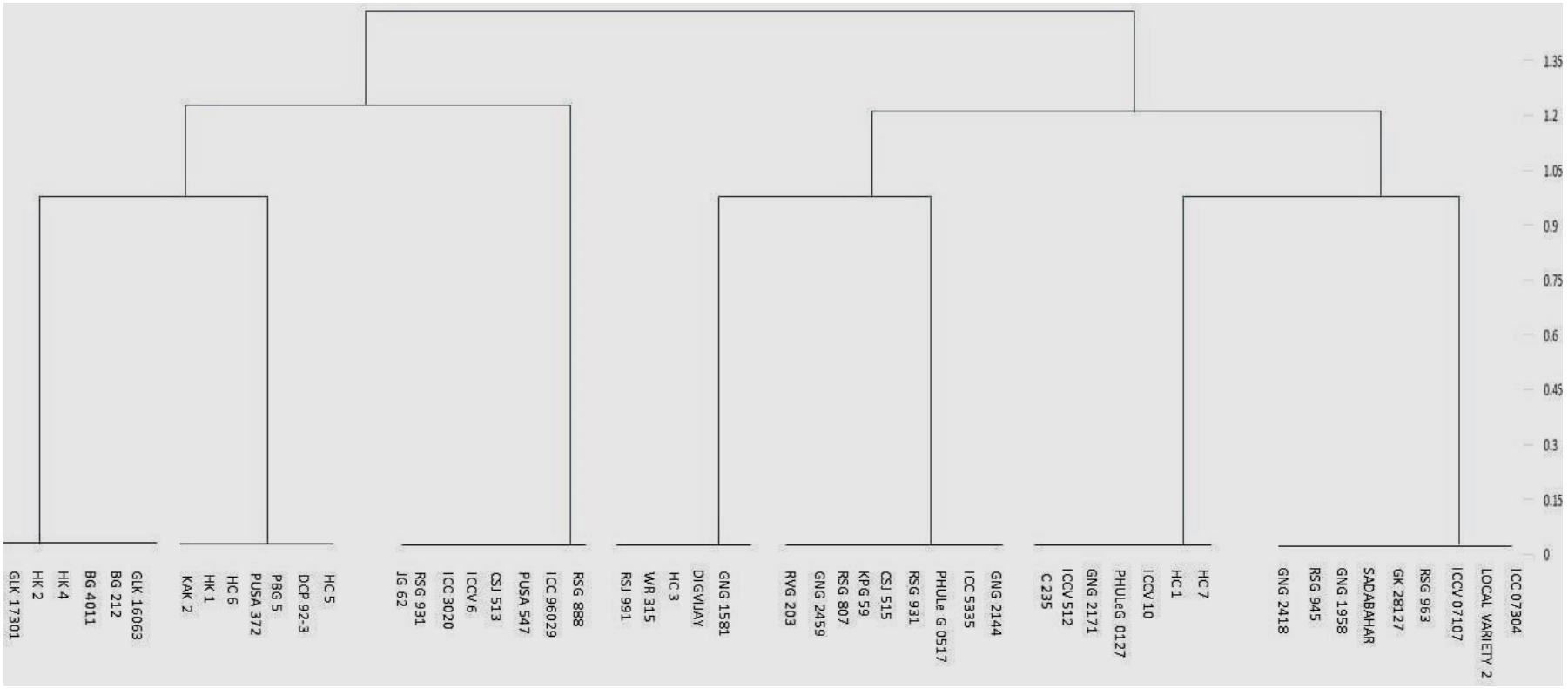


Figure 4.7: Dendrogram showing the clustering pattern of 52 genotypes based on STMS marker

Unlike in the current investigation, the genotype PUSA 391 was found to be susceptible against many races of *Fusarium* according to previous reports (Bharadwaj *et al*., 2022). The climate and soil characteristics of these regions differ greatly from those of the Northern zone, wherethe experiment was conducted. Thus, a notable variation in the environmental circumstances could be the cause of this divergence in the results. In case of chickpea, as the resistance to several races of FOC is controlled by different genes, the markers specific to *foc-3* genes were used. However, these markers were reportedto be associated with the other races of FOC also (Rani *et al*, 2022, Yadav *et al*., 2022; Sharma and Muehlbauer, 2017). This is one of the causes of the differences inthe results of current study. Another reason of this difference in disease reaction is the inconsistent expression of resistance gene that has been documented for other resistance genes. There are reports where the *I1* gene linked to the resistance againstthe race 4 of *Fusarium oxysporum* in tomato was incompletely expressed (Swett *et al*., 2023). Hence, considering both agronomical and molecular results, the genotypes HC 1, GNG 2144, and C 235 were found to be highly resistant against FOC race 3 and the genotypes PUSA 547, ICCV 96029, CSJ 513, RSG 888 were found to be most susceptible to race 3 of FOC.

*4.4.*Screening and selection of suitable AM fungal species against *Fusarium* wilt

In order to strengthen the plant defense mechanisms and encourage growth in genotypes that are sensitive to the disease, the screening and selection of appropriate arbuscular mycorrhizal (AM) fungal species against *Fusarium* wilt is critical. In this experiment, FOC and FOC in combination with AMF treatments were applied to the susceptible check genotype JG 62. A number of parameters, such as biochemical changes, growth parameters, disease severity index, and root colonization percentage of various AMF species, including *Glomus mosseae, Glomus hoi, Glomus intraradices,* and *Glomus fasciculatum,* and an *in-vitro* mycorrhizal biofertilizer from ProVam Pvt Ltd, Nasik were assessed.

4.4.1. Impact of AM Fungal Inoculation on Susceptible Chickpea Genotype

After analysis, it was observed that significant variations were there across treatments in a number of biochemical measures, suggesting that some treatments may be useful in enhancing plant resilience (Table 4.12).

Table 4.12: Mean performance of susceptible check genotype JG 62 under differential treatments.

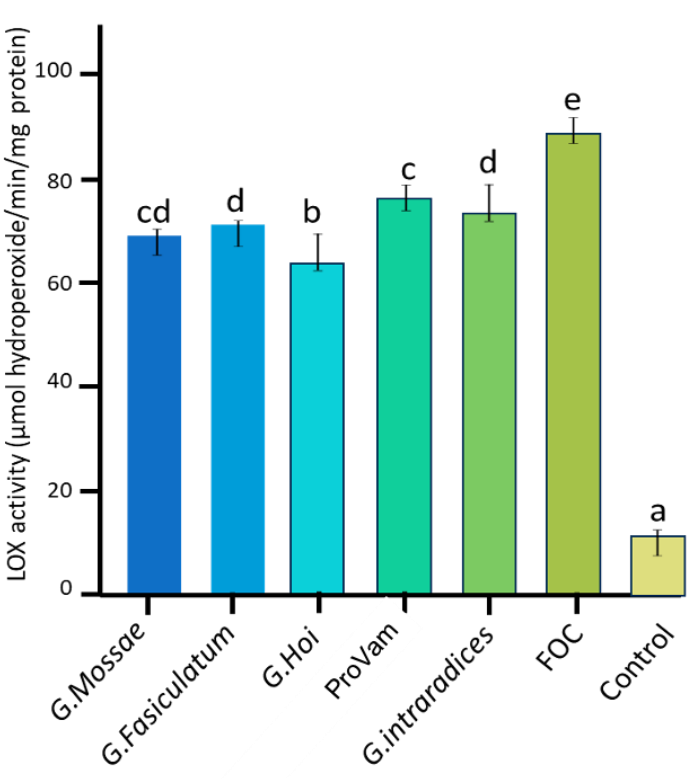
Treatments	Phenol (mg/g dw ofGAE)	Proline (µg/g dry weight)	Protein (mg/g fw)	PAL (Units/g fresh weight)	TSS (mg/gfw)	Catalase (Units/g fw)	LOX (Units/mg protein)	SOD (Units/mg protein)	DSI	RCP (%)
G.mosseae	3.37±0.28ab	0.62±0.04c	3.28±0.37bc	73.52±4.00cd	11.96±0.54bc	36.41±4.91d	68.53±2.62cd	16.33±5.22c	53.33±5.54c	21.34±1.34b
G.fasciculatum	3.68±0.13b	0.90±0.04bc	3.93±0.12c	89.22±5.63b	12.01±3.11b	40.39±1.11c	70.36±5.64d	18.07±3.46d	60.00±11.47bc	18.67±1.34bc
G.hoi	3.54±0.21b	1.17±0.2b	3.16±0.30b	89.36±5.81b	13.02±0.82b	35.20±2.00d	63.22±4.55b	10.25±0.85ab	66.67±17.63b	20.00±2.34bc
ProVam	4.43±0.32c	1.50±0.02a	5.91±0.22d	95.88±3.23c	14.56±0.32a	49.16±3.55b	75.42±4.34c	19.69±2.68e	33.34±6.67d	33.34±1.34a
G.intraradices	4.14±0.3b	1.16±0.02b	3.16±0.2b	38.03±3.23e	12.20±1.02b	42.57±3.21bc	72.63±6.26d	16.19±0.38cd	53.34±6.67bc	16.00±2.34c
FOC	2.48±0.09a	2.60±0.19d	1.66±0.29a	117.74±4.71a	11.34±0.38bc	25.54±3.81a	88.20±3.65e	11.83±6.34b	180.00±11.54a	0
Control	1.67±0.12d	0.79±0.21c	2.97±0.61b	48.4±3.23d	10.80±0.65c	17.47±2.11e	11.38±1.65a	8.33±0.35a	0	0

*PAL: Phenylalanine-ammonia lyase; TSS: Total Soluble Sugars LOX: Lipoxygenase; SOD: Superoxide Dismutase; GAE: Gallic Acid Equivalent; fw: fresh weight; CD: µmol conjugated diene produced; Catalase Units: µmol H_2O_2 decomposed/min; LOX units: µmol H_2O_2/min; PAL units: nmol cinnamic acid/min

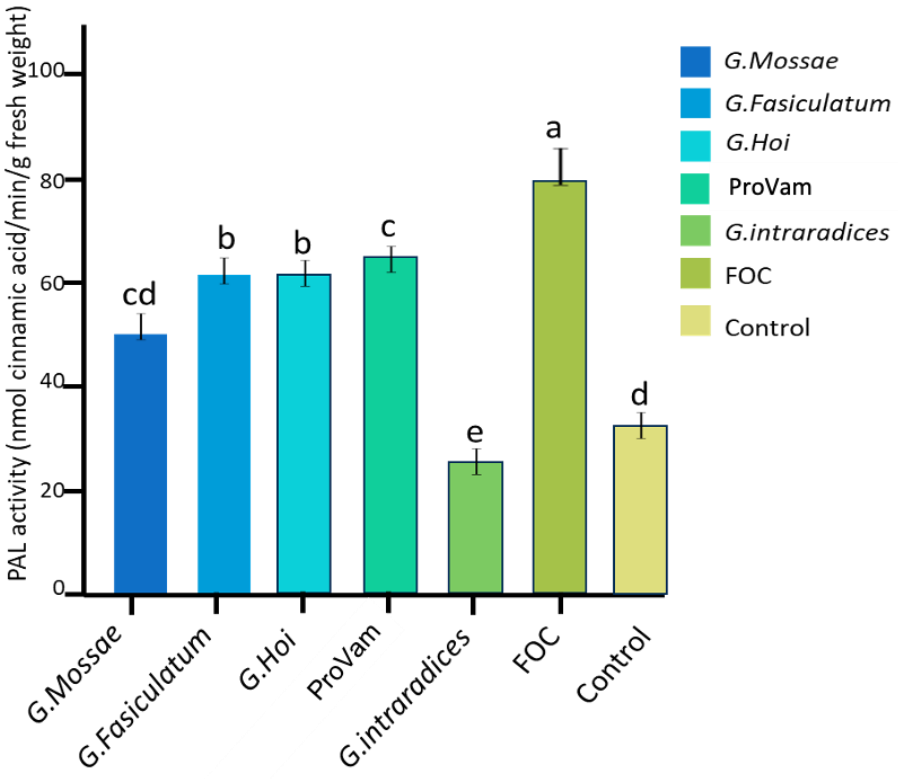
Table 4.13: One way ANOVA summarising the effects of treatments on biochemical parameters and stress response enzymes in susceptible checkJG 62

	df	Phenol	Proline	Protein	PAL	TSS	Catalase	LOX	SOD
MS		2.01	1.40	3.77	3755	42.5	230.99	4113	45.95
Treatment	6	<0.005***	<0.005***	<0.005***	<0.005***	0.0026**	<0.005***	<0.005***	0.01***
Residuals	14	0.14	0.08	0.16	51	6.2	28.55	75	4.78

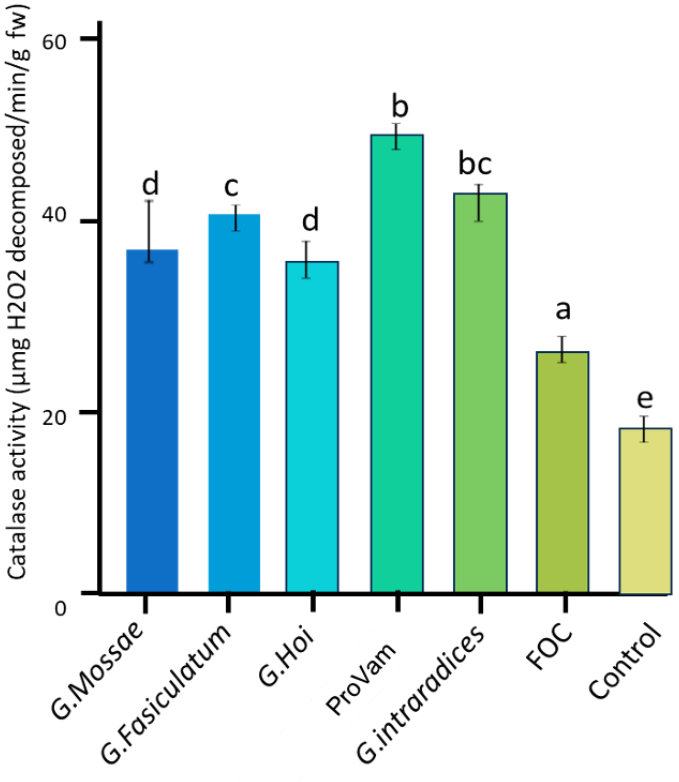
The ANOVA table indicated significant effect of treatment on various biochemical parameters in the study. Treatment significantly influenced the levels of phenol, proline, protein, PAL, LOX, SOD, TSS and catalase ($p < 0.001$). The residuals indicated the variability within treatment groups, with higher mean squares for some parameters compared to others, suggesting variability around the treatment means. These results underscored the significant influence of treatment on biochemical responses in the experiment, highlighting the importance of treatment conditions in plant responses to stress.


LOX activity (µmol hydroperoxide/min/mg protein)
100
80
60
40
20
0
cd
d
b
c
d
e
a
G.Mossae
G.Fasiculatum
G.Hoi
ProVam
G.intraradices
FOC
Control

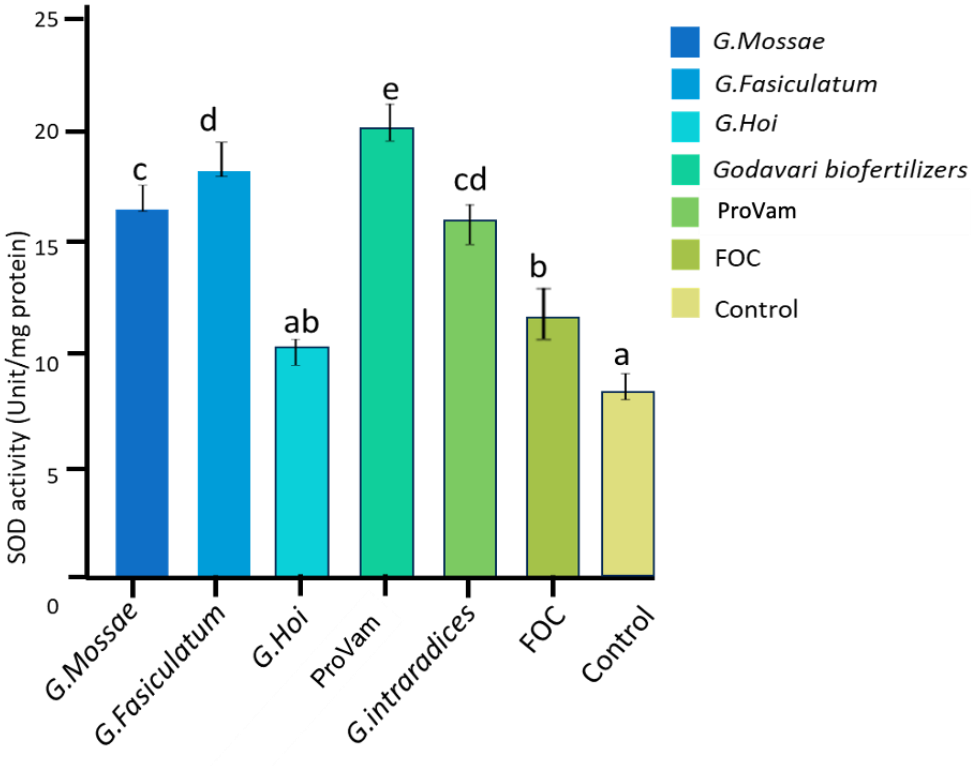
(a)


PAL activity (nmol cinnamic acid/min/g fresh weight)
100
80
60
40
20
0
cd
b
b
c
e
a
d
G.Mossae
G.Fasiculatum
G.Hoi
ProVam
G.intraradices
FOC
Control

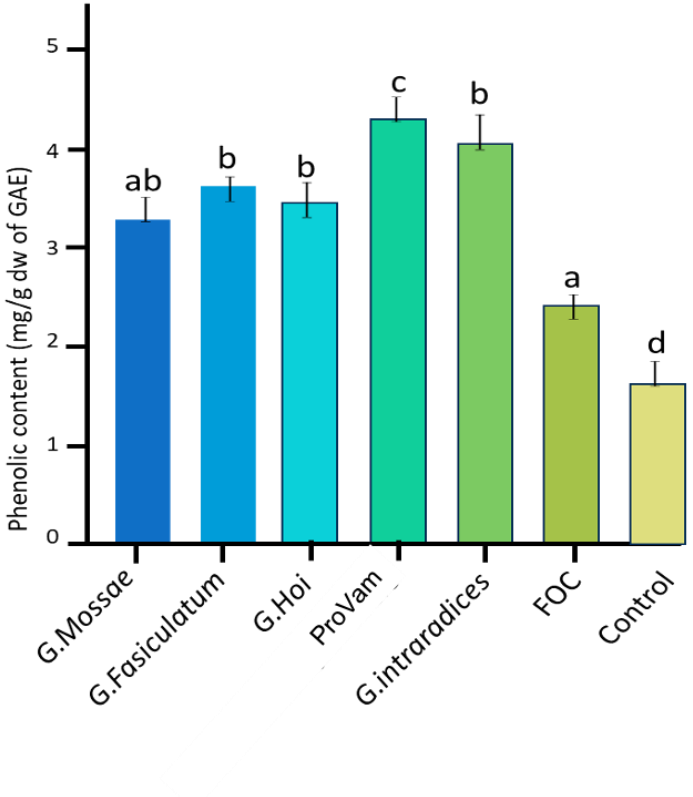
(b)


Catalase activity (µmg H2O2 decomposed/min/g fw)
60
40
20
0
d
c
d
b
bc
a
e
G.Mossae
G.Fasiculatum
G.Hoi
ProVam
G.intraradices
FOC
Control

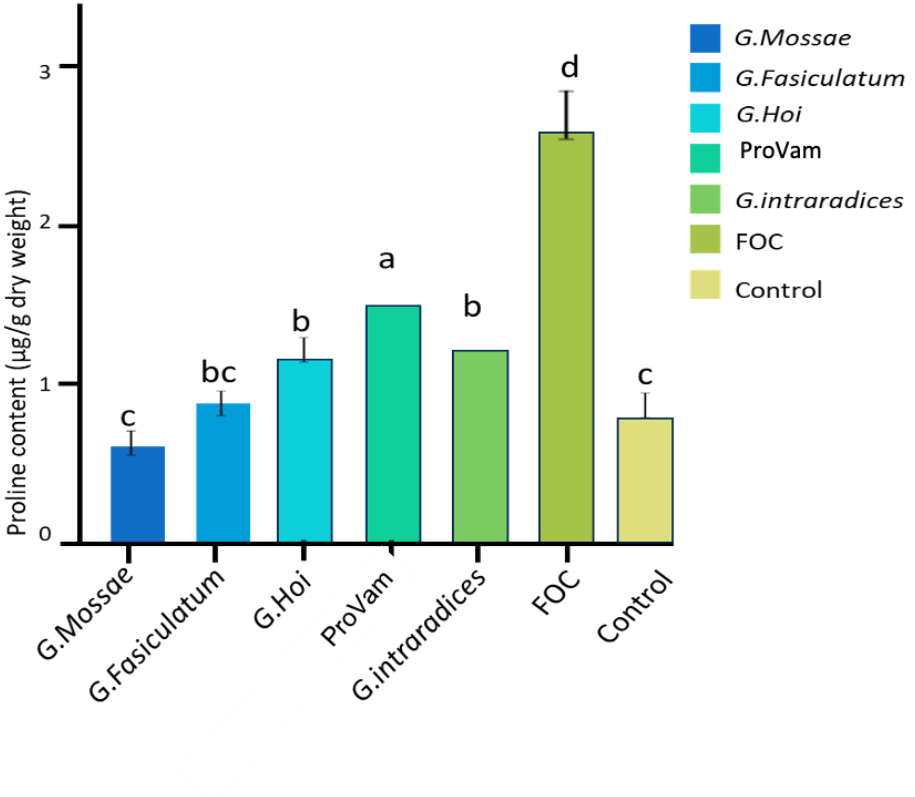
(c)


SOD activity (Unit/mg protein)
25
20
15
10
5
0
c
d
ab
e
cd
b
a
G.Mossae
G.Fasiculatum
G.Hoi
ProVam
G.intraradices
FOC
Control
G.Mossae
G.Fasiculatum
G.Hoi
Godavari biofertilizers
ProVam
FOC
Control

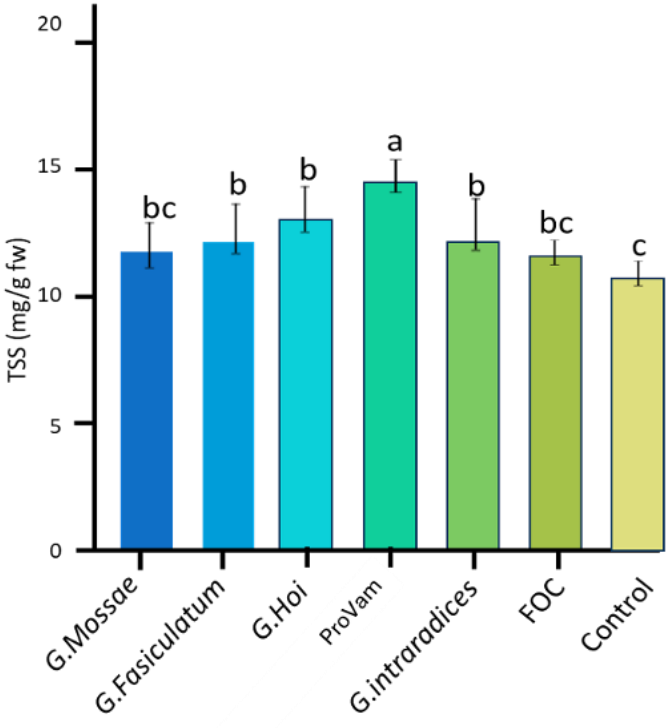
(d)


Phenolic content (mg/g dw of GAE)
0
1
2
3
4
5
ab
b
b
c
b
a
d
G.Mossae
G.Fasiculatum
G.Hoi
ProVam
G.intraradices
FOC
Control

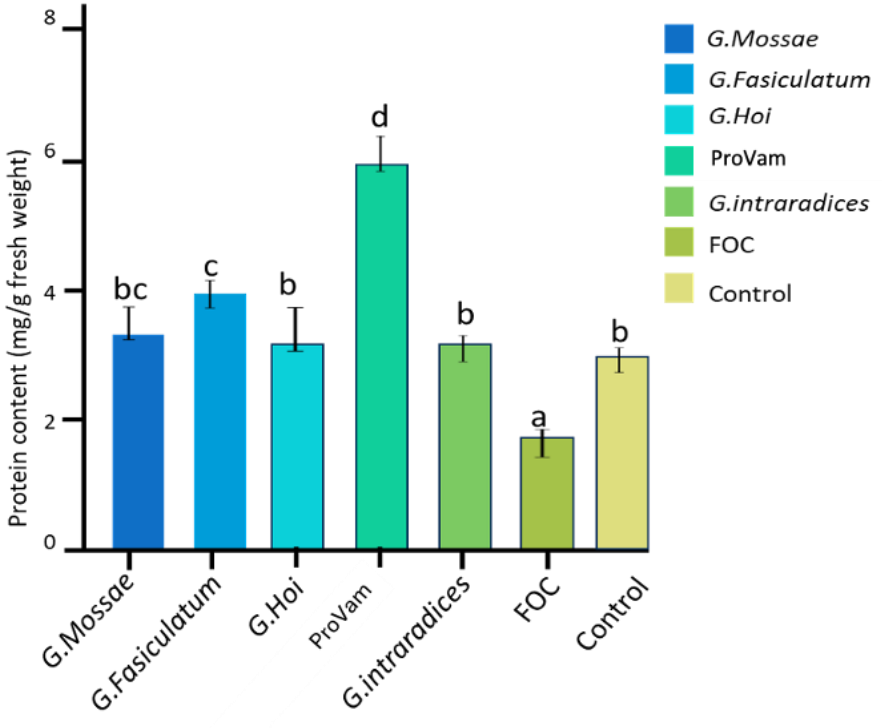
(e)


Proline content (µg/g dry weight)
0
1
2
3
c
bc
b
a
b
d
c
G.Mossae
G.Fasiculatum
G.Hoi
ProVam
G.intraradices
FOC
Control

(f)


TSS (mg/g fw)
20
15
10
5
0
bc
b
b
a
b
bc
c
G.Mossae
G.Fasiculatum
G.Hoi
ProVam
G.intraradices
FOC
Control

(g)


Protein content (mg/g fresh weight)
8
6
4
2
0
bc
c
b
d
b
a
b
G.Mossae
G.Fasiculatum
G.Hoi
ProVam
G.intraradices
FOC
Control
G.Mossae
G.Fasiculatum
G.Hoi
ProVam
G.intraradices
FOC
Control

(h)

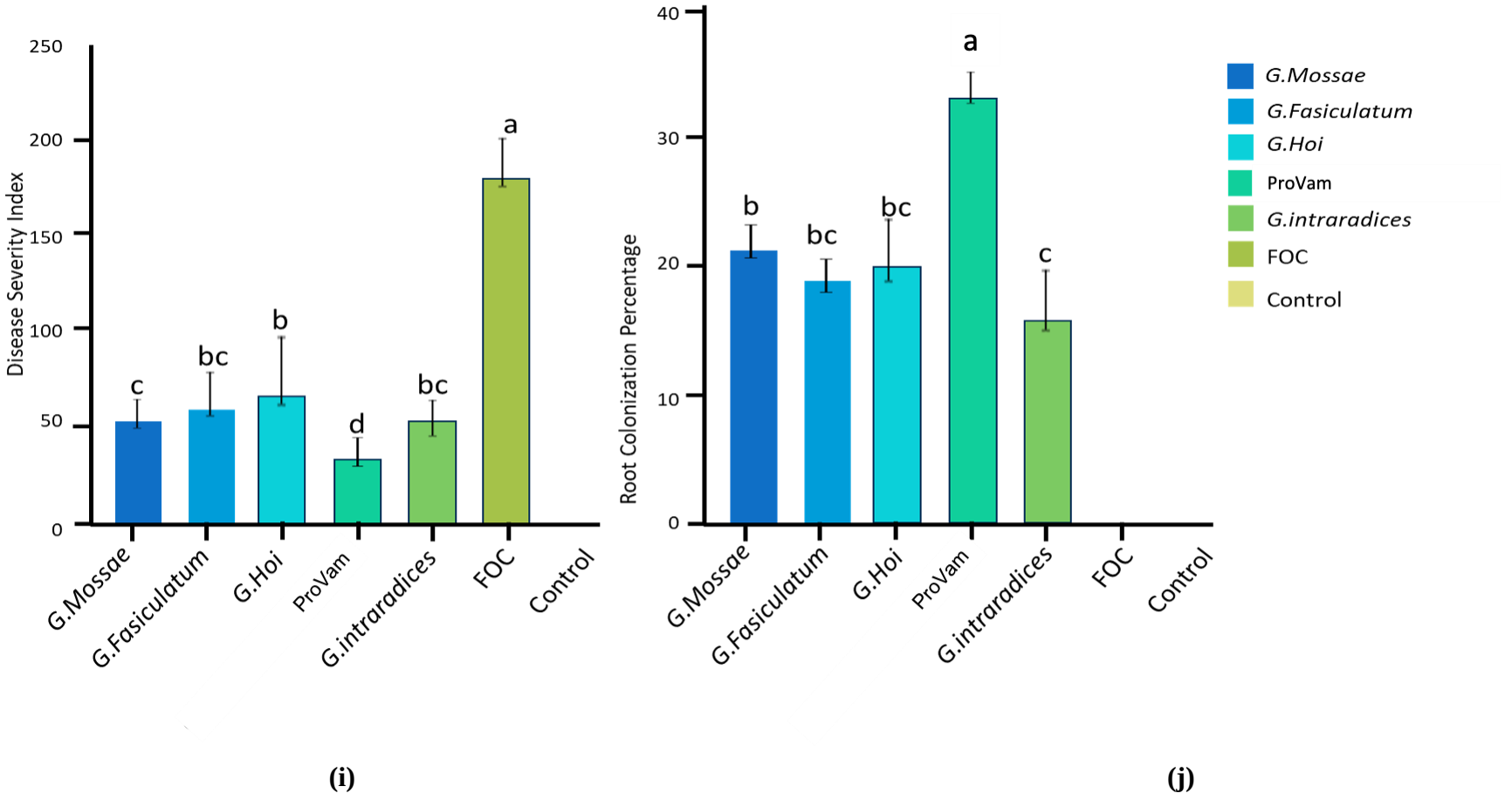


Figure 4.8: **Graphs representing biochemical response of genotype JG 62 under Control, FOC-treated and FOC+AMF treated conditions; (a)PAL activity, (b) LOX activity, (c) Phenolic content, (d) Proline content, (e) Catalase activity, (f) SOD activity (g) Total SolubleSugars (h) Protein Content, (i) Disease severity index, (j) Root colonization percentage**

The FOC-treated plants exhibited the lowest levels of LOX activity, hinting at a potentially milder stress response in the susceptible genotype. Similar findings have been reported in studies where plants treated with both pathogen and AMFas biocontrol exhibited increased expression of the LOX gene. A correlation wasobserved between elevated levels of methyl jasmonate (MeJA) in AM treated plants and increased LOX activity, an enzyme involved in Jasmonic Acid biosynthesis (Wang *et al*., 2022; Zhang *et al*, 2022). The phenylalanine ammonia-lyase (PAL) which is involved in phenolic compounds synthesis and defense related metabolites, was highest in plants treated with AMF and lowest in those treated with FOC. This suggests that PAL-related defense pathways may have been more strongly activated due to AMF treatment. Increased PAL activity in susceptible plants on AMF addition following pathogen attack has been documented in other reports as well (Jiang *et al*., 2019; Lavanya *et al*., 2019; Zhang *et al*., 2022).

With highest phenol content in ProVam treated plants, the treatments involving FOC and FOC+AMF, exhibited significantly higher phenol content as compared to control plants. Phenols are renowned for their antibacterial and antioxidant properties, suggesting that plants treated with AMF had an enhanced defense mechanisms due to their increased phenol content. These findings align with similar research conducted by other authors (Abdelrhim *et al*., 2023). The highest proline content was observed in FOC treated plants, indicating increased osmolyte accumulation owing to biotic stress. However, the AMF treated plants showed a reduction in the proline levels with *Glomus mosseae* treated plant showing the least proline content. Additionally, plants treated with ProVam and *Glomus intraradices* exhibited the second lowest levels of proline content. This indicatesthat while stress induced by FOC treatment was present, it may have been mitigated by other treatments, resulting in diminished proline accumulation. Theresults were found to be consistent with the results of Zakaria *et al*., 2023.

Glomus hoi and *Glomus fasciculatum*-treated plants exhibited the highest levels of superoxide dismutase (SOD) activity, an enzyme crucial for antioxidant defense, indicating enhanced antioxidant capability in these treatments. This suggests that these treatments effectively mitigated the oxidative stress inducedby *Fusarium* infection (Cheema and Garg, 2024; Bushra *et al*., 2024). However,the total soluble sugar content did not significantly differ between the treatments, except for the ProVam implying that the investigatedtreatments may not have exerted a major impact on sugar metabolism. In FOC+AMF treated plants, the inoculation with ProVamdisplayed the highest levels

of LOX activity suggesting heightened response to stress and involvement in the generation of signalling molecules in response to the stress.

Furthermore, the highest protein content was found in the plants treated with ProVam, indicating improved protein synthesis in the presence ofAMF. Since defense systems and other physiological activities rely on protein, this finding is significant. Conversely, plants treated with FOC exhibited reduced protein levels, suggesting a stress led protein degradation. This outcomealigns with the study conducted on wheat, where the susceptible variety showeddecreased protein concentration upon fungal infection (Manghwar *et al*., 2021).Whereas, introduction of biocontrol agents like different AMF species, enhanced protein content in plants (GonzálezGonzález *et al*., 2020; Laranjeira *et al*., 2021).

Moreover, the percentage of root colonization was highest in plants treated with ProVam, indicating successful establishment of beneficial mycorrhizal associations. These associations are known to enhance nutrient uptake and stress tolerance in plants. Notably, plants treated with ProVam exhibited the lowest disease severity, implying that this treatment conferred the highest level of disease resistance. These results suggest that treatment with ProVam not only fostered beneficial interactions with mycorrhizal fungi but also effectively mitigated the adverse effects of *Fusarium* infection.

4.4.2. Correlation analysis of biochemical responses of the susceptiblegenotype with the DSI and RCP

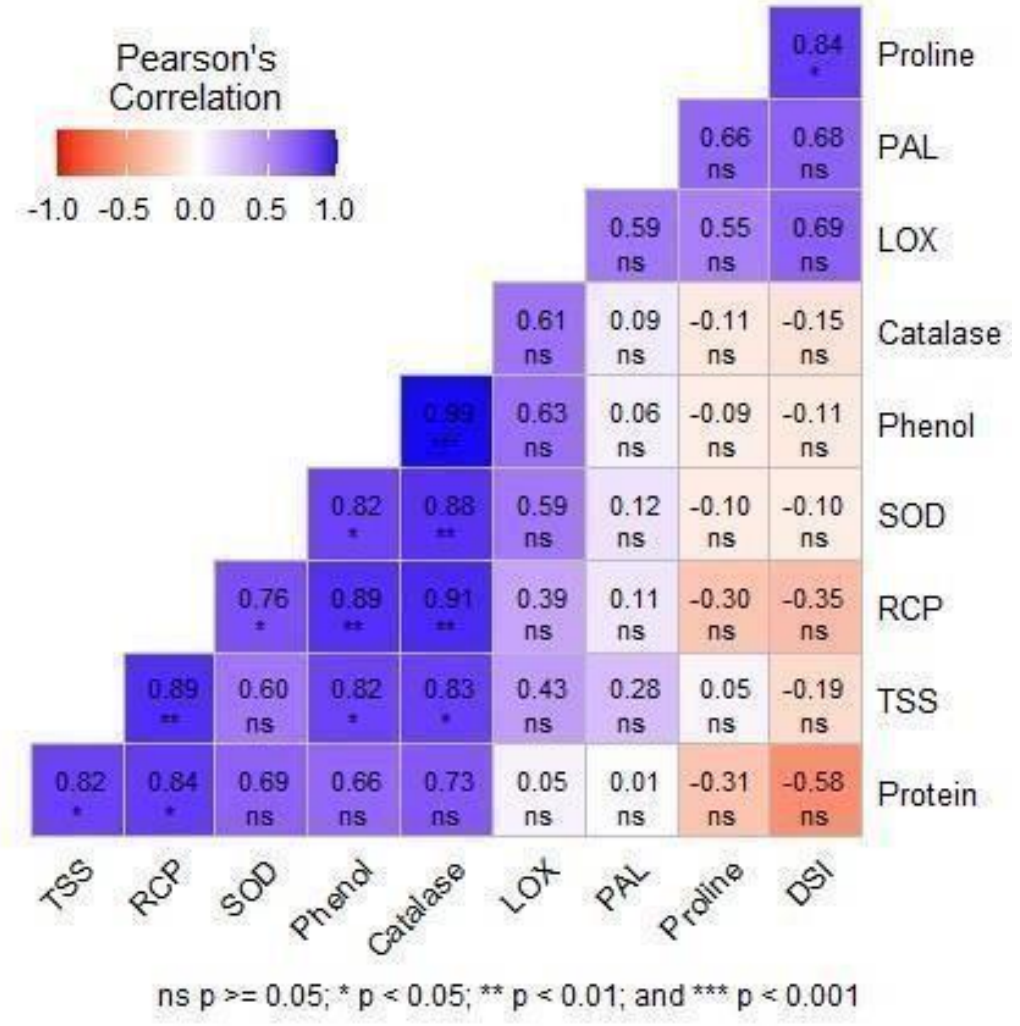


Figure 4.9: Pearson correlation matrix for different biochemical parameters corresponding to the disease severity index and AMF root colonization percentage.

Correlation analysis was also carried out to determine the relationship between different biochemical parameters, DSI and RCP. The findings showed that a number of metabolic indicators have substantial connections with both DSI and RCP. Higher phenol levels were linked to less severe disease, as demonstrated by the non-significant negative correlation between phenol content and DSI (r= -0.11), and a significant positive correlation with RCP (r=0.89) which is consistent with other research showing the function of phenolic compounds in plant defense against pathogens (Wallis *et al*., 2020). As proline is involved in stress tolerance and osmotic adjustment, it exhibited a significantly positive association with DSI (r = 0.84) and negative with RCP. These

results were in line with other similar studies (Manghwar *et al*., 2021; Attia *et al*., 2022). Therewas a negative association between protein content and disease severity (r = -0.58) potentially because of protein disintegration under stress (Kaur *et al*., 2022). However, the positive correlation of protein content with RCP indicatedan enhanced protein production. These results aligned with the other AMF related studies (Pereira *et al*., 2020). However, the correlation of protein with DSI as well as RCP was non-significant in our study.

PAL activity had a very weak non-significant positive correlation with RCP (r=0.11) and non-significant positive correlation with DSI (r=0.68) also suggesting that higher PAL activity is increased with the increase in disease severity (Kiani *et al*., 2021). The total soluble sugar (TSS) content showed a non-significant negative correlation (r =-0.19) with disease severity, indicating that higher TSS levels were linked to a less severe disease (Hembade *et al*., 2022). A non-significant negative correlation between catalase activity and DSI(r = -0.15) indicated that higher catalase activity was linked to decreasing disease severity (Al-Surhanee *et al*., 2021). The non-significant positive correlation observed between lipoxygenase (LOX) activity and disease severity(r = 0.69) and RCP (r=0.39) indicating an increase in LOX activity with increasing infection. However, the rise in LOX activity is linked with the synthesis of Jasmonic acids which are further triggered by the AMF root colonization. These correlations therefore justify the positive relationship between LOX, DSI and RCP. This observation was in line with previous researches which suggested that LOX's function in lipid peroxidation and the generation of signalling molecules during stress responses (Trindade *et al*., 2022). SOD activity was found to have a non-significant negative correlation with DSI (r = -0.10) and significant positive correlation with RCP (r=-0.76) indicating that the inoculation of plants with AMF helped in increased production of antioxidant enzymes which are responsible for a reduction in the disease severity. These results find similarity with the results of other researches (Batool *et al*., 2020). Furthermore, a significant negative correlation was observed between RCP and DSI (r = -0.35), suggesting that a higher degree of AMF root colonisation was linked to a reduced severity of disease. This finding is in line with AMF's protective function in strengthening plant defense mechanisms (Spagnoletti *et al*., 2021; Cunha *et al*., 2023).

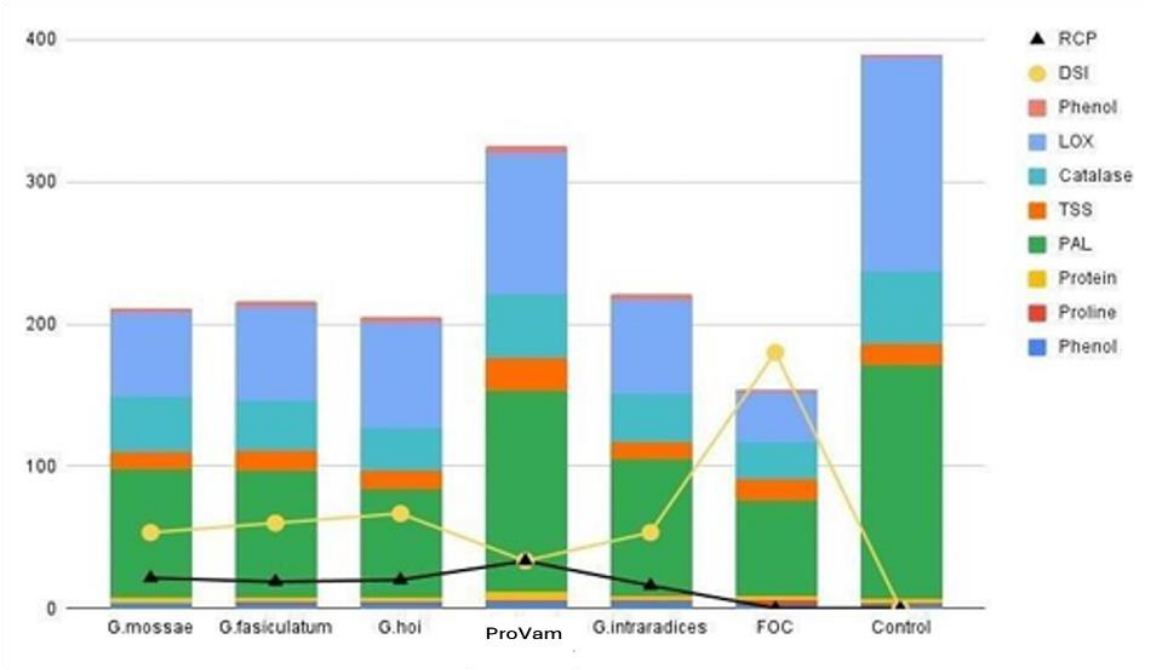


Figure 4.10: Summary of JG62 data analysis: Relationship between disease severity, RCP and other biochemical parameters

Hence, the FOC susceptible was found to respond best to the ProVam treatment, which increased the plants' phenol content, proteinsynthesis, antioxidant activity, and disease resistance while also successfully forming mycorrhizal associations. Hence, ProVam was selected for the further research.

4.5. Morphological description of the AMF isolated from the ProVam

The most promising treatment, ProVam as assessed in previous experiment, was further investigated for mass production of AMF using Root organ culture.

4.5.1. Morphological description of *Glomus intraradices* isolated fromconsortia of ProVam

Based on morphological characteristics such as size, shape subtending hyphae, and unique characteristics of the spores, the spores were tentatively identified as *Glomus intraradices* (Figure 4.11).

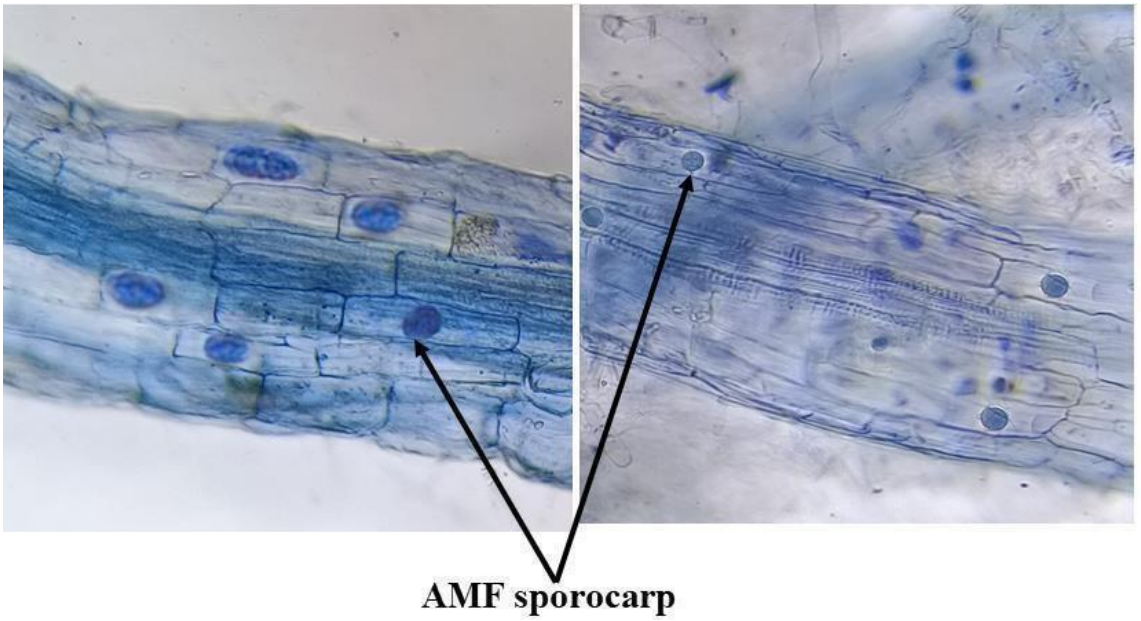


Figure 4.11. The sporocarp of AMF isolated from ProVamcolonizing roots of host plant

Table 4.14: Morphological description of the isolated AMF species from ProVam

Shape ofspores	Size of spore	Special feature	Tentative identification
Globose and Subglobose	123 µm	Spore Surfacepitted found with small notches	*Glomus intraradices*

4.5.2. Inoculation of root segments with pre-germinated spores

The pre-germinated spores placed near the roots started to exhibit hyphae, a characteristic feature of arbuscular mycorrhizal fungus (AMF), approximately 5 days after infection (DAI).

4.5.3. Germination percentage of AMF spores

The germination percentage of AMF spores was evaluated and the results indicated that the initial spore germination was only 13.3 percent indicating a very low spore germination. However, the spore germination increased gradually as the days passed and reached up to the maximum spore germination(80 %), at 25 DAI (Table 4.15) (Figure 4.12a).

Table 4.15: Germination percentage, spore viability, root colonization percentage,root biomass and spore density at different days after inoculation

	Percent spore germination at days after inoculation				
DAI	5 DAI	10 DAI	15 DAI	20 DAI	25 DAI
Mean spore germination	13.34±3.34	30.00±5.77	50.00±5.77	66.67±6.67	80.00±5.77
Mean spore viability	98.67±1.15	85.00±13.22	81.67±5.77	81.67±5.77	78.33±2.88
Mean RCP	16.67±1.15	20.67±3.05	24.67±1.15	27.34±1.15	29.34±1.15
Mean root biomass	1.67±0.31	1.86±0.06	2.17±0.03	2.44±0.04	2.47±0.09
Mean spore density	2.67±0.34	5.34±1.34	9.34±2.67	10.67±1.34	16.00±00

*RCP: Root Colonization Percentage

4.5.4. Viability of the AMF spores

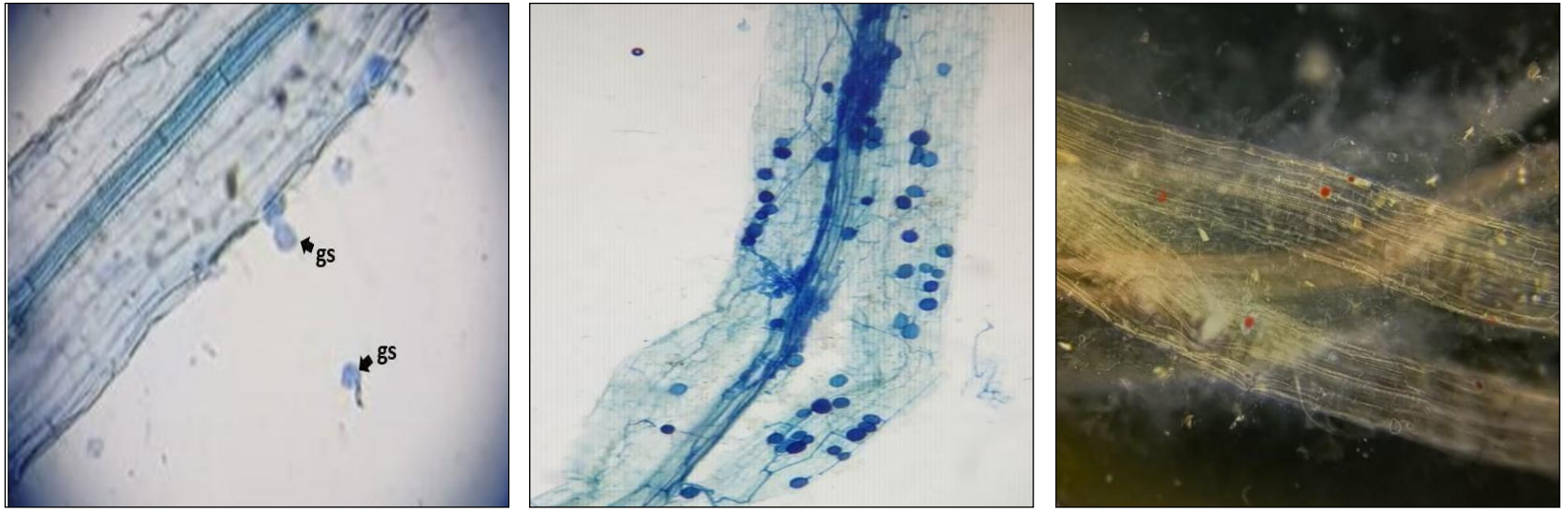
The experiment examined the AMF spores' viability up to 25 days after inoculation (DAI). The outcomes showed that range of viability varied from 98.66% to 78.33% the up to 25-day observation period. Although minor fluctuations were observed over this time period, these results suggest that the spores were able to survive and germinate to initiate mycorrhizal colonisation within the host roots (Table 4.15) (Figure 4.12 c). These results were in line with researches carried out previously (Rajpurohit *et al*., 2022; Ghorui *et al*., 2023).

4.5.5. Root colonization Percentage

The first AMF establishment on the roots was indicated by the mean percent root colonisation of 16.67% at 5 DAI. A consistent rise in root colonisation was seen with time, with the mean percent colonisation of 20.67%, 24.67%, 27.34% and 29.34% at 10, 15, 20 and 25 DAI, respectively. This steady rise in colonisation indicated a progressive establishment of AMF in the roots (Figure 4.12 b, Table 4.15). Similar results were observed in the experiments done on AMF by Selvakumar *et al*., 2018 and Hussain *et al*., 2021.

4.5.6. Root biomass

The results revealed that at 5 DAI, the mean biomass of the roots was 1.67 g which increased noticeably throughout the course of the experiment, reaching 1.86 g at 10 DAI, suggesting early growth and development. At 15 DAI, the mean root biomass increased significantly to 2.74 g, suggesting that AMF colonisation contributed to the notable rise in root development (Table 4.15). This trend persisted till the last observation taken at 25 DAI. The results were consistent with the results of Wu *et al*., 2021.



(a) **(b)** **(c)**

Figure 4.12 (a) Pre-germinated spores placed near the root for root colonization, gs: germinating spore; (b) Root Colonization; (c)Viable spores (stained in red colour)

4.5.7. Spore density

The spore density was calculated as the number of spores per g of soil which was found to increase gradually with the days after inoculation, starting from 2.66 spores per g of soil at 5 DAI and reaching 162 spores per g of soil at 25 DAI. This increase in the number of spores of AMF with increasing time in soil indicated the multiplication of AMF (Table 4.15). These results were consistentwith the findings of Fasusi *et al*., 2021 and Shrestha *et al*., 2023.

4.5.8. Impact of DAI and root colonization on different aspects of sporesand roots

Correlation analysis was done to assess the relation between different parameters and the root colonization percentage with the increasing number of days post inoculation. The results are summarized in Figure 4.13.

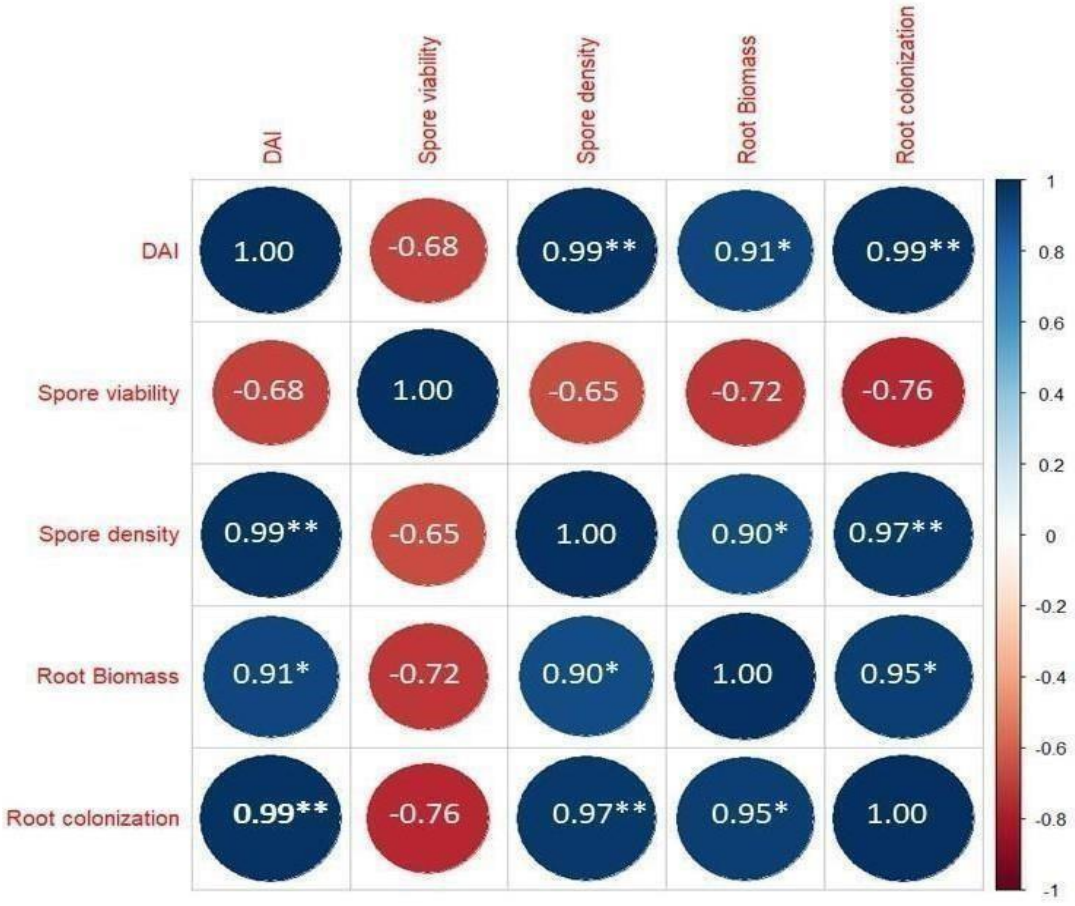


Figure 4.13: Pearson correlation matrix showing correlation between various variable and DAI

DAI exhibited a highly significant positive association (0.99) with spore density and a moderate positive correlation (0.91) with root biomass, indicating that both parameters tend to increase with increasing DAI. Root biomass is increased with these variables, as seen by its positive correlation (0.90) with spore density and positive correlation (0.91) with DAI. The data indicates that there is a non-significant negative correlation between spore viability and DAI.The relationship between colonisation and spore density is significantly positive(0.97), suggesting that higher spore density are related to increased colonisation(Rajpurohit *et al*., 2022; Ghorui *et al*., 2023).

4.6. Gene expression study of selected chickpea genotype during FOC infection and FOC+AMF

4.6.1. Selection of the susceptible chickpea genotypes for transcriptome analysis

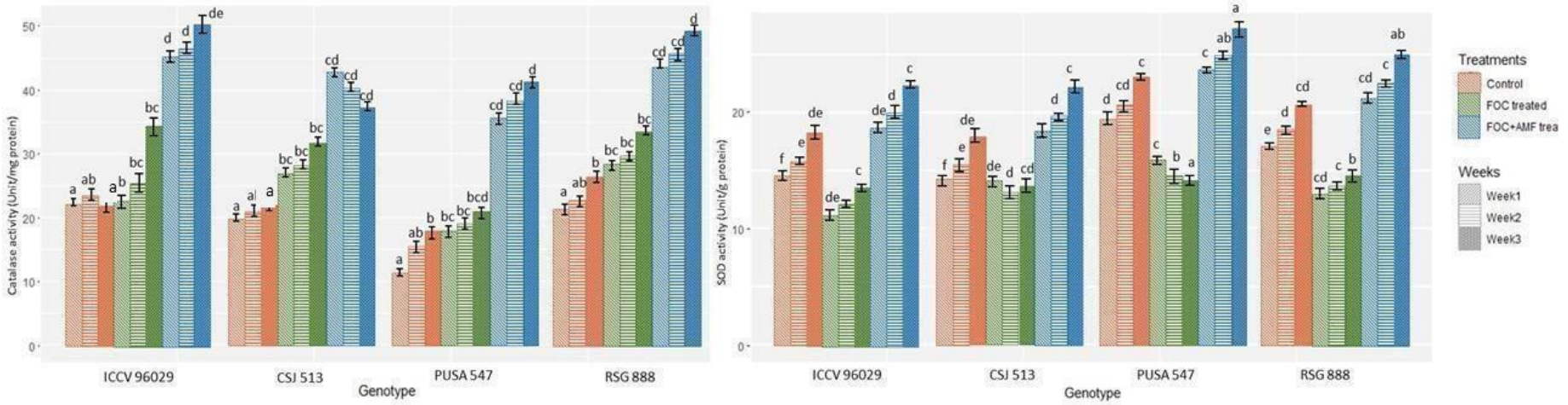
Fusarium oxysporum f. sp. *Ciceris* (FOC) is a fungal wilting pathogen. Four candidate genotypes were assessed for their reaction to FOC and its association with arbuscular mycorrhizal fungi (AMF) in order to determine a highly vulnerable genotype for transcriptome analysis. For three weeks in a row, the plants were treated with FOC, control, and FOC+AMF (Table 4.16, Figure 4.14). Throughout the investigation, the disease severity index and a number of biochemical indicators were tracked. For additional transcriptome analysis, the genotype that showed the greatest percentage of illness decrease after receiving both FOC and AMF was found to be the most promising.

Table 4.16: Biochemical response of four susceptible genotypes over 3 weeks on treatment with FOC and FOC+AMF

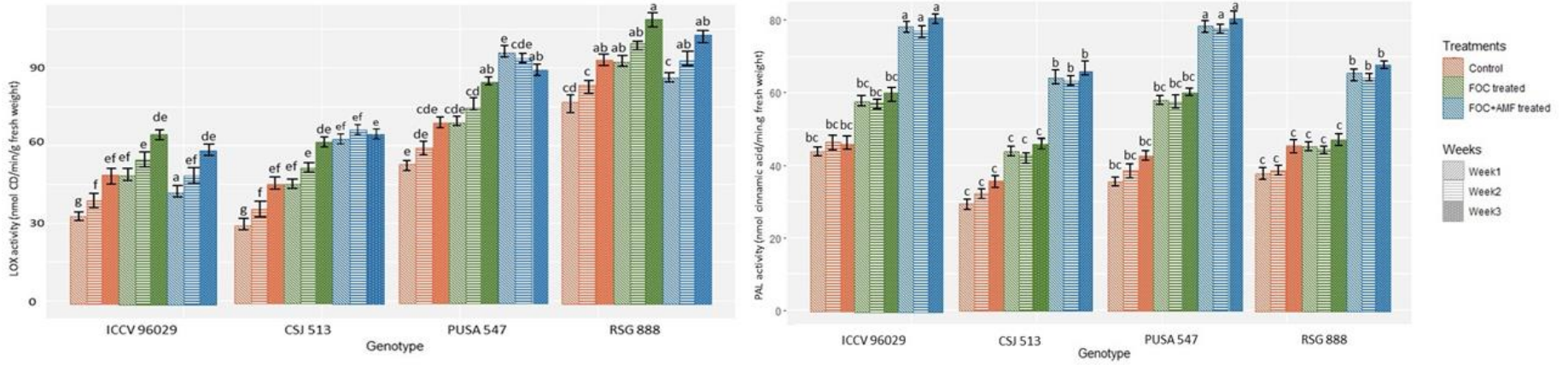
Treatments	Genotype	Week	Phenol (mg/g dw of GAE)	Proline (µg/g dry weight)	Protein (mg/g fw)	PAL (Units/g freshweight)	TSS (mg/g fw)	Catalase (Units/g fw)	LOX (Units/mg protein)	SOD (Units/mg protein)	DSI	RCP(%)
Control	ICCV 96029	1st	2.13±0.09	0.80±0.03	1.90±0.15	45.58±2.87	14.25±0.87	22.97±1.60	35.45±1.65	14.41±0.89	0.00	0.00
	ICCV 96029	2nd	2.21±0.17	1.17±0.03	2.10±0.08	48.00±1.73	15.61±2.82	23.02±1.14	37.42±0.59	16.64±0.91	0.00	0.00
	ICCV 96029	3rd	2.48±0.13	1.49±0.04	2.43±0.03	47.29±1.81	16.75±0.73	21.10±0.95	39.62±0.68	17.98±0.60	0.00	0.00
Control	CSJ 513	1st	1.73±0.06	0.51±0.053	1.89±0.09	31.54±1.24	13.52±1.04	18.54±0.77	37.50±0.41	14.30±0.99	0.00	0.00
	CSJ 513	2nd	1.77±0.06	0.9±0.05	2.10±0.01	32.24±0.75	14.66±0.98	18.29±0.17	30.57±2.03	15.78±0.62	0.00	0.00
	CSJ 513	3rd	1.78±0.04	1.31±0.05	2.36±0.02	35.78±0.81	14.84±0.99	21.65±0.21	37.87±0.97	17.25±0.60	0.00	0.00
Control	PUSA 547	1st	1.55±0.08	0.63±0.013	1.44±0.04	35.89±3.66	14.94±0.41	12.87±1.06	47.82±10.94	18.14±1.03	0.00	0.00
	PUSA 547	2nd	1.62±0.06	1.16±0.07	1.65±0.07	37.52±2.78	13.29±0.35	16.28±0.97	50.95±9.18	21.45±1.29	0.00	0.00
	PUSA 547	3rd	1.76±0.06	1.45±0.12	1.99±0.07	42.95±3.67	15.58±0.40	17.91±0.40	60.62±6.87	23.58±1.6	0.00	0.00
Control	RSG 888	1st	1.53±0.16	0.61±0.04	1.62±0.09	37.84±1.38	13.97±0.56	22.43±0.60	77.23±7.10	16.45±1.36	0.00	0.00
	RSG 888	2nd	2.02±0.20	0.77±0.04	1.60±0.09	39.52±1.12	13.87±0.57	23.82±0.76	82.89±6.99	17.80±1.25	0.00	0.00
	RSG 888	3rd	2.15±0.37	1.38±0.08	1.76±0.05	42.18±2.78	15.05±0.60	24.44±0.68	94.25±4.64	20.41±1.23	0.00	0.00
FOC	ICCV 96029	1st	2.65±0.23	1.10±0.18	2.33±0.13	58.11±1.19	16.07±0.84	27.26±1.37	43.72±2.04	12.31±0.73	46.67±13.33	0.00
	ICCV 96029	2nd	2.90±0.07	1.24±0.09	2.03±0.19	56.93±2.90	16.77±0.86	30.20±0.41	50.59±2.05	12.85±0.77	66.67±6.67	0.00
	ICCV 96029	3rd	3.10±0.09	1.40±0.29	1.90±0.28	61.09±8.45	15.28±0.59	34.95±9.80	68.38±12.74	13.58±3.79	106.67±6.67	0.00
FOC	CSJ 513	1st	2.20±0.03	1.09±0.21	2.23±0.15	42.03±3.54	14.74±1.11	22.79±2.33	38.16±1.62	13.39±1.45	26.67±13.33	0.00
	CSJ 513	2nd	2.35±0.06	1.30±0.09	2.04±0.05	40.70±1.34	13.54±0.38	24.81±2.49	52.83±3.19	12.09±0.96	46.67±13.33	0.00
	CSJ 513	3rd	2.43±0.00	1.24±0.26	1.75±0.35	54.05±7.85	14.89±0.15	33.27±8.91	65.93±8.07	12.45±5.07	106.67±11.54	0.00
FOC	PUSA 547	1st	2.47±0.07	0.72±0.37	1.45±0.10	58.27±1.30	14.80±0.19	21.22±0.86	70.30±2.50	17.51±1.37	40.00±6.67	0.00
	PUSA 547	2nd	2.61±0.01	0.62±0.32	1.38±0.03	60.94±1.50	13.13±0.67	24.26±1.46	74.90±1.63	16.12±0.80	86.67±6.67	0.00
	PUSA 547	3rd	2.77±0.32	0.53±0.05	1.33±0.06	40.58±1.21	13.23±0.30	26.24±0.87	78.88±2.45	15.19±1.07	126.67±6.67	0.00
FOC	RSG 888	1st	2.63±0.04	0.67±0.24	2.13±0.01	44.06±1.25	14.76±0.78	16.31±1.68	91.36±4.89	12.80±0.25	33.33±6.67	0.00
	RSG 888	2nd	2.79±0.20	0.78±0.11	1.85±0.06	44.81±0.51	13.95±0.31	37.43±2.17	119.17±0.99	13.80±0.76	60.00±6.67	0.00

Treatments	Genotype	Week	Phenol (mg/g dw of GAE)	Proline (µg/g dry weight)	Protein (mg/g fw)	PAL (Units/g freshweight)	TSS (mg/g fw)	Catalase (Units/g fw)	LOX (Units/mg protein)	SOD (Units/mg protein)	DSI	RCP(%)
	RSG 888	3rd	2.87±0.25	0.95±0.04	1.69±0.01	48.50±3.21	13.20±0.39	44.50±0.44	124.61±5.45	14.96±0.63	76.67±11.54	0.00
FOC+AMF	ICCV 96029	1st	3.19±0.10	1.17±0.08	2.38±0.14	74.82±7.72	15.29±1.15	49.40±2.36	34.29±3.37	17.59±0.82	13.33±3.33	22.67±3.31
	ICCV 96029	2nd	3.20±0.16	1.39±0.21	2.66±0.13	76.86±2.16	16.68±0.41	50.95±0.95	44.81±2.38	20.12±0.83	26.67±6.67	26.67±2.90
	ICCV 96029	3rd	3.35±0.19	1.66±0.20	2.81±0.12	82.01±1.87	17.28±0.79	48.64±1.44	62.58±6.41	23.44±0.44	26.67±6.67	35.33±0.67
FOC+AMF	CSJ 513	1st	2.10±0.04	0.88±0.07	2.13±0.02	64.22±2.97	13.94±0.52	44.02±2.03	62.38±1.59	18.43±0.95	6.67±6.67	15.33±0.67
	CSJ 513	2nd	2.22±0.04	1.23±0.12	2.52±0.05	62.52±9.51	15.56±1.28	40.50±1.68	67.77±1.78	19.94±0.30	13.33±6.67	20.00±0
	CSJ 513	3rd	2.47±0.19	1.69±0.15	2.85±0.16	67.08±4.54	16.61±0.50	35.03±1.95	63.13±7.97	17.92±1.20	33.33±6.67	24.67±1.76
FOC+AMF	PUSA 547	1st	2.51±0.04	0.99±0.44	1.79±0.15	77.14±4.25	15.17±0.03	49.30±3.16	95.23±7.08	23.09±4.07	13.33±6.67	16.00±1.15
	PUSA 547	2nd	2.65±0.24	1.10±0.32	2.05±0.02	79.63±4.66	16.09±0.02	49.62±3.83	93.48±6.15	23.81±3.75	26.67±6.67	22.67±2.90
	PUSA 547	3rd	2.80±0.25	1.62±0.21	2.39±0.05	82.39±1.4	16.54±0.51	50.66±0.39	90.15±4.55	25.11±3.15	40.00±0	28.67±1.76
FOC+AMF	RSG 888	1st	2.70±0.11	0.83±0.21	2.02±0.06	66.01±1.33	13.26±0.92	46.89±0.88	84.63±6.41	20.53±1.31	6.67±6.67	16.00±6
	RSG 888	2nd	2.79±0.04	1.23±0.22	2.45±0.00	64.05±2.76	14.99±1.21	42.90±0.42	94.91±4.12	22.17±0.48	20.00±0	18.67±0.6
	RSG 888	3rd	3.87±0.08	1.55±0.12	2.56±0.05	68.57±3.98	15.33±0.63	48.94±3.11	105.41±10.16	25.02±0.57	26.67±3.33	22.00±1.15

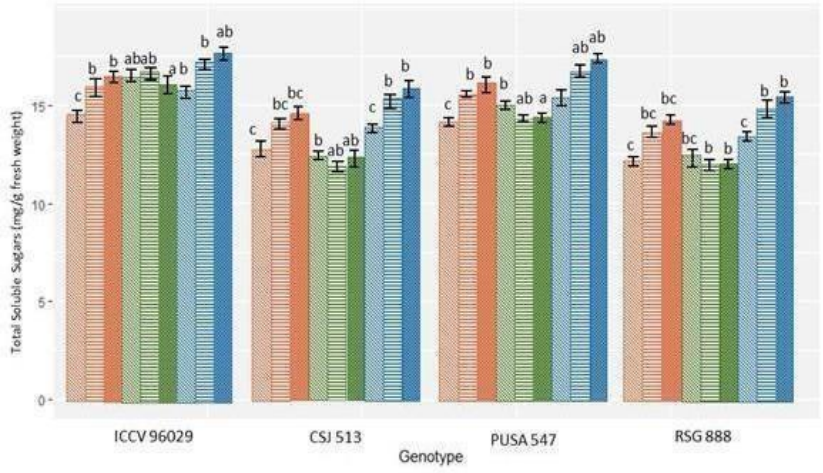
*PAL: Phenylalanine-ammonia lyase; TSS: Total Soluble Sugars LOX: Lipoxygenase; SOD: Superoxide Dismutase; GAE: Gallic Acid Equivalent; fw: fresh weight; CD: µmol conjugated diene produced; Catalase Units: µmol H_2O_2 decomposed/min; LOX units: µmol H_2O_2/min; PAL units: nmol cinnamic acid/min


Catalase activity (Unit/mg protein)
SOD activity (Unit/g protein)
ICCV 96029
CSJ 513
PUSA 547
RSG 888
Genotype
Treatments
Control
FOC treated
FOC+AMF trea
Weeks
Week1
Week2
Week3

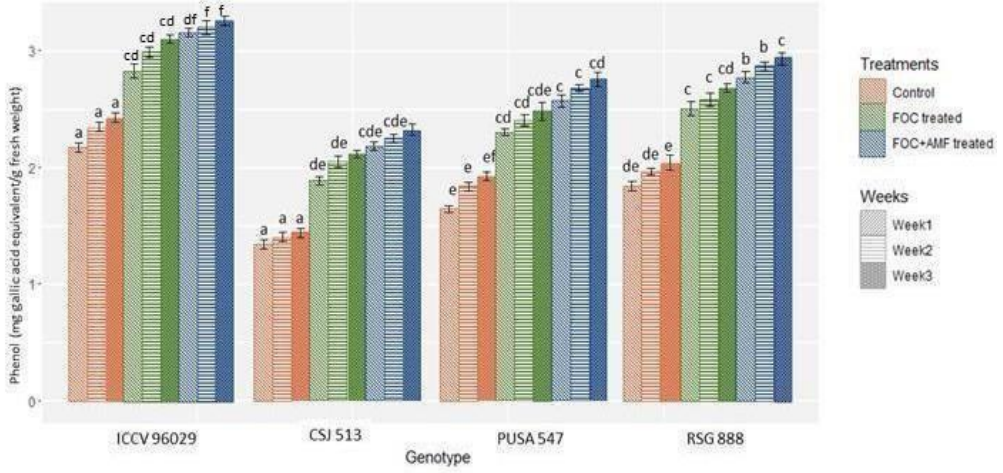
(a) **(b)**


LOX activity (nmol CD/min/g fresh weight)
PAL activity (nmol cinnamic acid/min.g fresh weight)
ICCV 96029
CSJ 513
PUSA 547
RSG 888
Genotype
Treatments
Control
FOC treated
FOC+AMF treated
Weeks
Week1
Week2
Week3

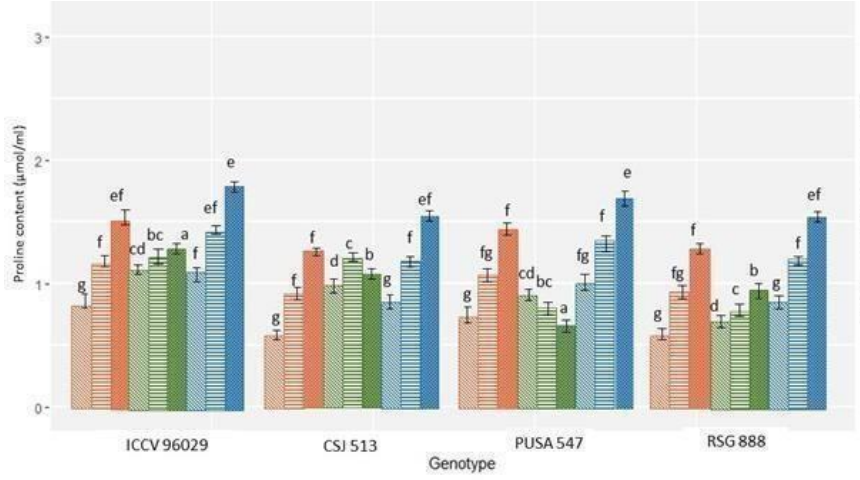
(c) **(d)**


Total Soluble Sugars (mg/g fresh weight)
ICCV 96029
CSJ 513
PUSA 547
RSG 888
Genotype

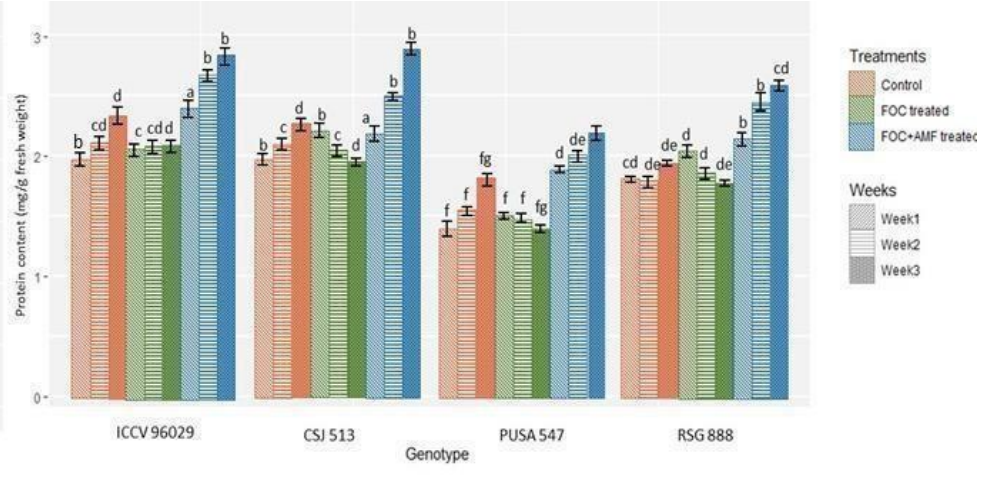
(e)


Phenol (mg gallic acid equivalent/g fresh weight)
ICCV 96029
CSJ 513
PUSA 547
RSG 888
Genotype
Treatments
Control
FOC treated
FOC+AMF treated
Weeks
Week1
Week2
Week3

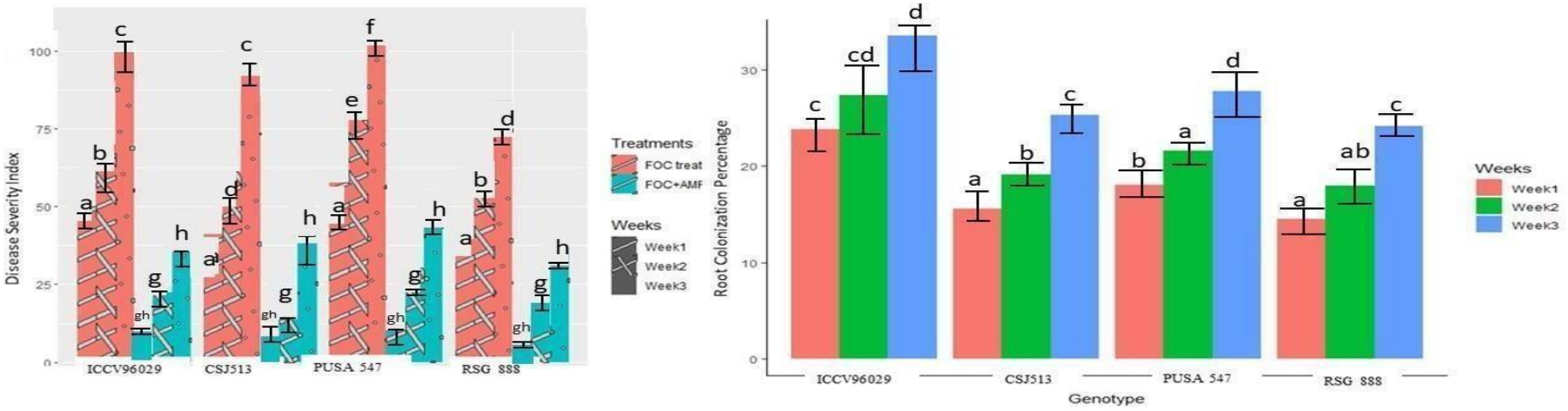
(f)


Proline content (µmol/ml)
ICCV 96029
CSJ 513
PUSA 547
RSG 888
Genotype

(g)


Protein content (mg/g fresh weight)
ICCV 96029
CSJ 513
PUSA 547
RSG 888
Genotype
Treatments
Control
FOC treated
FOC+AMF treated
Weeks
Week1
Week2
Week3

(h)



(i) **(j)**

Figure 4.14: Statistical representation of changes in biochemical parameters and Disease severity index of four susceptible chickpea genotypes under Control, FOC treated and FOC+AMF treated conditions observed for over three consecutive weeks

AMF colonisation showed a positive correlation with a number of metabolic markers. The FOC+AMF treated plants exhibited the high levels of lipoxygenase (LOX) activity, phenol content, and superoxide dismutase (SOD)activity, in contrast to the FOC-treated or control plants. This implies that the generation of this defense-related mechanism is stimulated by AMF colonisation, which help the plant withstand FOC infection. Regardless of the treatment, phenol content increased with time in all the selected genotypes. Thephenol content increased in FOC treated plants which further increased in the plants treated with FOC+AMF. The genotype ICCV 96029 showed the highestconcentration, indicating that this genotype may have a stronger defense system. Although, the phenolic compounds are known to provide structural integrity and support to the plants the rise in phenol content in FOC+AMF treated was not very significant as compared to the FOC treated plants. Similarstudies have been reported in literature where co-inoculation with *Fusarium* andAMF have resulted in variable responses of phenol content at periodic intervalsafter infection (da Trindade *et al*., 2019; Saallah *et al*., 2020). The proline content was found to be increasing at a very less significant rate for over three weeks in the FOC treated plants indicating very slow response to stress inducedby FOC. However, the addition of AMF and FOC both resulted in a much pronounced and significant increase in proline content indicating a quick response to ameliorate the stress caused by the pathogen. Furthermore, the genotype RSG 888 showed the significantly highest levels of LOX activities. Such increase in LOX activity on stress encounter has been observed in previousresearches also. It is found to be triggered by AMF addition however, steadily goes down with time. In our experiment, the same trend was seen in PUSA 547in which the LOX activity was triggered as a result of AMF addition but startedto fall down at third week of infection indicating the utilisation of LOX in synthesis of Jasmonic acid. LOX is a plant defense enzyme, and its activation upon AMF penetration might be a control mechanism to regulate fungal growthwithin the plant (Shah *et al*., 2024; da Trindade *et al*., 2019).

The SOD activity was although found to be increasing in the FOC treated plants; however, the increase was not significant over the course of three weeks.

This slow increase in the defense enzyme is an indicator of reduced defense mechanism in the susceptible genotypes. With the highest SOD activity in PUSA 547 in the treatment where AMF is added along with FOC, an increase in SOD activity was observed for over three weeks. As SOD also linked with the lower oxidative damage and increase in membrane stability; in the plants colonized with AMF, the AMF

inoculation thus showed a positive effect on disease control (Villani *et al*., 2021; Wang *et al*., 2020). In all the genotypes, FOC treatment increased catalase activity relative to controls. The FOC+AMF treatments resulted in a further increased catalase activity. This trend in catalaseactivity was observed over the course of three weeks. Catalase activity for PUSA 547 was highest overall across all treatments and time periods. This indicated that this genotype showed a stronger antioxidant defense system on AMF inoculation. These results regarding the catalase activity were congruent with the results reported previously in which the AMF inoculation caused an increase in the catalase activity (Fiorilli *et al*., 2018; Rahou *et al*., 2021). Moreover, phenylalanine ammonia lyase (PAL) activity increased across all thegenotypes. In our experiment, the highest activity on AMF inoculation was observed in PUSA 547 and RSG 888. The increase in this activity was non- significant in FOC treated plants however; it increased significantly for over three weeks in FOC+AMF treated plants. The PAL activity showed an initial increase from week 1 to week 2 and then these activity levels declined steadily.These observations indicated that the PAL activity was triggered as a response to the stress. However, the reduction in PAL activity at third week after coinoculation of FOC and AMF indicated a reduced need of phenolic compounds as a result of regulation of the stress conditions (da Trindade *et al*.,2019; Mirjani *et al*., 2023). This was concluded as the phenol content did not correlate with the PAL activity (da Trindade *et al*., 2019).

The levels of protein content decline on the inoculation of pathogen to the plants however, a notable increase in the protein content was observed in the FOC+AMF inoculated plants. The previous researches have also revealedsimilar results in which the susceptible genotypes showed a significant decline in protein content however, a rise in protein content is noticed on biocontrol inoculation (Farhana *et al*., 2022; Manghwar *et al*., 2020).

The plants treated with FOC had a consistently reduced concentration of total soluble sugar (TSS) than the control which is associated with wilting response due to lack of osmotic adjustment. In our study, the highest content of TSS wasobserved in ICCV 96029. A progressive yet less significant increase in soluble sugar content was observed in all FOC+AMF treated plants which continued for two consecutive weeks indicating the AMF induced accumulation of sugarsto avoid wilting. Whereas, in third week, this accumulation was less significant due to lower need for the osmotic adjustments. Another reason for a less significant change in the TSS content could be

increased root colonisation withAM fungus as it is well known that the AMF improves source-to-sink flow, redirecting sucrose from leaves to roots (Goddard *et al*., 2021).

The changes in disease severity index (DSI) were one of the most crucial findings. Although DSI rose in both the FOC and FOC+AMF treatments, it was noticeably greater in the plants treated with FOC. Notably, when co-immunizedwith FOC and AMF, PUSA 547 showed the largest reduction in disease severity (about 71.72%). This demonstrates how AMF colonisation, as opposed to FOCinfection alone, may successfully lessen the severity of the disease. Hence, the most promising genotype for further investigations on gene expression using transcriptome analysis was PUSA 547, which was determined by the observed reactions to the co-inoculation with FOC and AMF. Compared to FOC treatment alone, this genotype showed a significant reduction in disease severity(Duc and Post, 2018; Khanna *et al*., 2021; Spagnoletti *et al*., 2020). Furthermore,PUSA 547 had substantial phenol content, indicating the possibility of a potent inherent defense mechanism. Although more research is necessary to completely understand the underlying mechanisms, PUSA 547 is a useful option to investigate AMF-mediated resistance in chickpeas against FOC infection.

These biochemical changes demonstrated that by enhancing the activity of various antioxidants involved in the reduction of oxidative damage induced by ROS, AMF promotes the accumulation of secondary metabolites in the plants and helps in protection of host plants from pathogens, mitigating the harmful effects caused by the pathogen (da Trindade *et al*., 2019; Villani *et al*., 2021).

4.6.2. Structure Equation Model and Path analysis

The response of four chickpea genotypes to treatments with arbuscular mycorrhizal fungi (AMF) and the fungal pathogen *Fusarium oxysporum* f. sp. *Ciceri* (FOC) was revealed through a study of the structural equation model (SEM). The model looked into how the AMF root colonization percentage(RCP) and other biochemical parameters affected the disease severity index(DSI) (Figure 4.15).

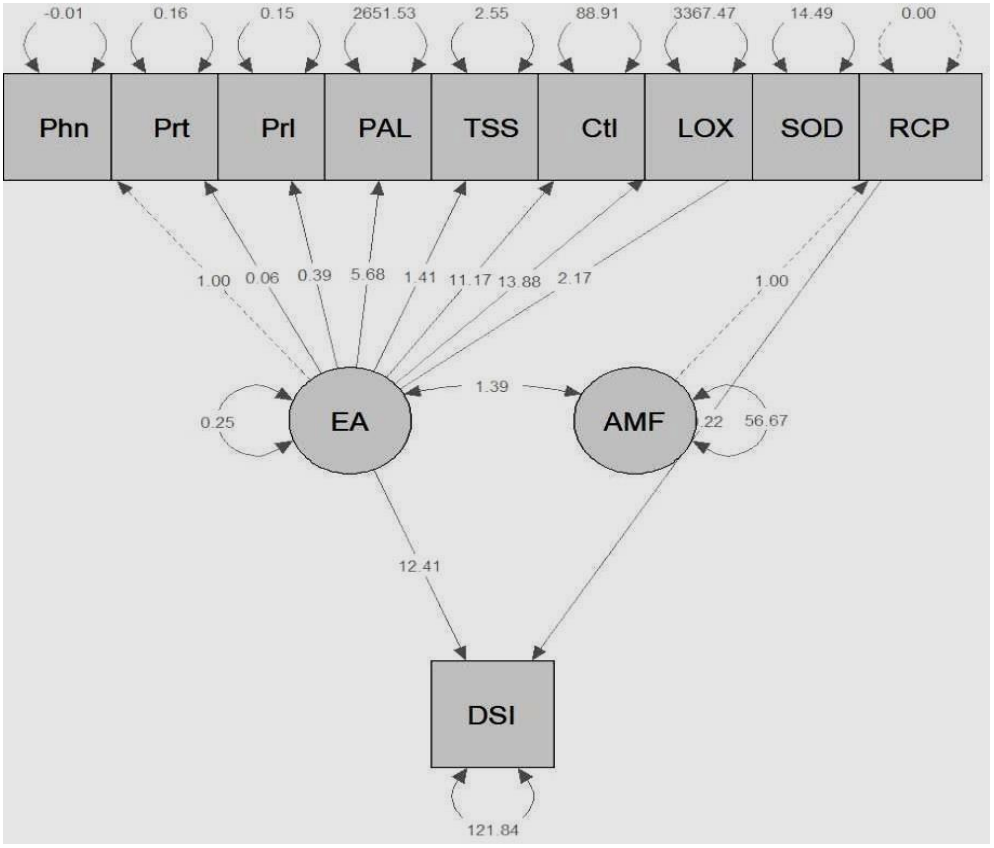


Figure 4.15 Structural Equation Model showing correlation between the Enzyme activity (EA) and AMF root colonization percentage (RCP) on the disease severity index (DSI) of the plants. The path coefficient numbers in between the arrows indicate the effect of relationship and P–values. The solid and dotted lines represent significant and non-significant relationship respectively.

Significant correlations were found between the activity of various important biochemical substances, such as protein, proline, total soluble sugars (TSS), lipoxygenase (LOX), catalase, superoxide dismutase (SOD), and phenylalanine ammonia lyase (PAL), and the percentage of AMF root colonisation. This implies that the accumulation of these defense related biochemicals was triggered by AMF colonisation, and they successfully contributed towards the plant's defense systems against FOC infection. The phenol concentration and DSI did not significantly correlate, according to the model. This suggests that, in the current experiment, phenol levels may not be the main factor driving the severity of the disease. Similar results have also been reported in experiments assessing the correlation between the AMF colonization and various biochemical parameters (Rasouli *et al*., 2023 and Thangaraj *et al*., 2022). Furthermore, a strong overall fit of the model to the data is indicated by the chi-square p-value of less than 0.05 with 34 degrees of freedom. This shows that the

observed data provided strong support for the proposed relationships between the variables. The experiments done by Tao *et al*., 2020 with inoculation of biocontrol fungi to control diseases showed similar results.

4.6.3. Transcriptome analysis

Our goal in this novel study was to elucidate the subtle variations in gene expression patterns between chickpea plants co-inoculated with arbuscularmycorrhizal fungus (AMF) and those inoculated only with *Fusarium oxysporum* f. sp. *Ciceris* (FOC). Using transcriptome analysis, we compared plants co- inoculated with AMF and those inoculated with FOC alone in order to find differentially expressed genes (DEGs) linked to defense systems in plants. Thisstudy helped to shed light on how AMF colonisation affects the molecular reactions of different genotypes of chickpeas during FOC infection. PUSA 547,the genotype that was selected for this study, was used in our attempt to understand the biological processes that underlie these differential inoculation circumstances.

4.6.4. Transcriptome quality and mapping statistics

Two biological replicates of FOC+AMF treated plants, FOC treated plants, and one of control plant were used for the transcriptome analysis of the various treatments. RNA sequencing of 5 samples using Illumina HiSeq2500 produced 224.76 million reads for inoculation and control treatments. The quality of RNAwas checked using Qubit, and Tapestation. The Control and FOC+AMF treatedplants had more RNA concentration than the FOC treated plants. The RNA integrity no. (RIN) was maximum (8.5) in the control and FOC+AMF treated samples and minimum in FOC treated ones (8.1). The QC results showed that all the treatments were admissible for further processing. The detailed description of the QC report is presented in Table 4.17 and the RNA QC on gel is shown in the Figure 4.16.

Table 4.17: The detailed QC summary of RNA extracted from differentially treated chickpea plants.

S.No.	Sample Name	RIN	Concentration	Volume(µl)	Total Yield(ng)	DV Value %	Result
1	T1 (Control)	8.5	274.40	20	5488	77.82	Pass
2	C2_1 (FOC+AMF treated)	8.5	235.20	20	4704	76.04	Pass
3	C2_2 (FOC+AMF treated)	8.3	104.61	20	2092	73.25	Pass
4	T2_1 (FOC treated)	8.5	90.05	20	1801	70.8	Pass
5	T2_2 (FOC treated)	8.1	79.18	20	1584	82.64	Pass

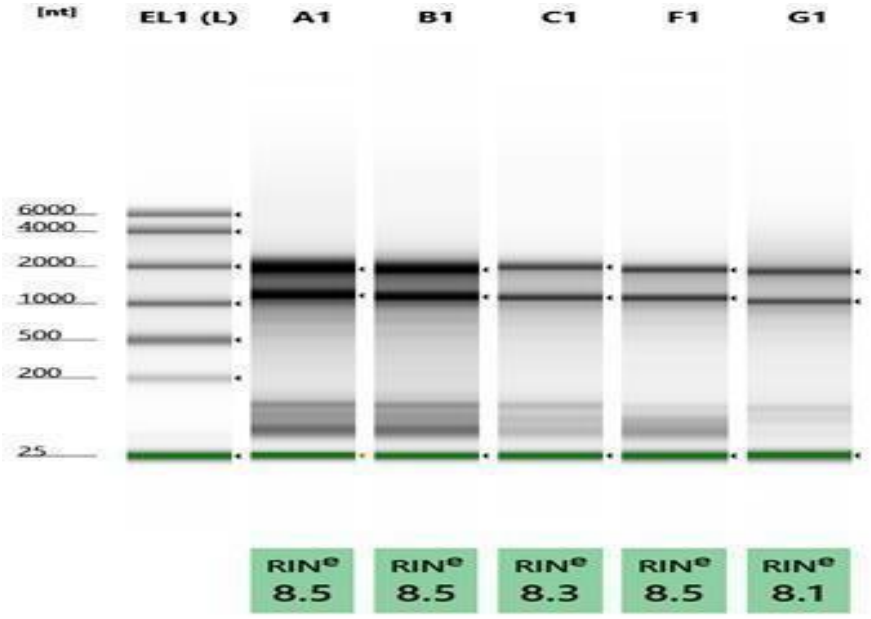


Figure 4.16. RNA quality check of the differentially treated plants using gel electrophoresis

4.6.5. Pre- Processing data

Utilising Adapter Removal, adapter-free, high-quality trimming at Q30 reads were achieved by eliminating the reads with adapter contamination and low basequality. A total of 217.1 million reads (89.74%) were subsequently mapped ontothe chickpea reference genome out of the 533.95 million (97.14%) good qualityreads that were collected overall. The summary of raw and pre-processed data is given in Figure 4.17, Table 4.17 and Table 4.18.

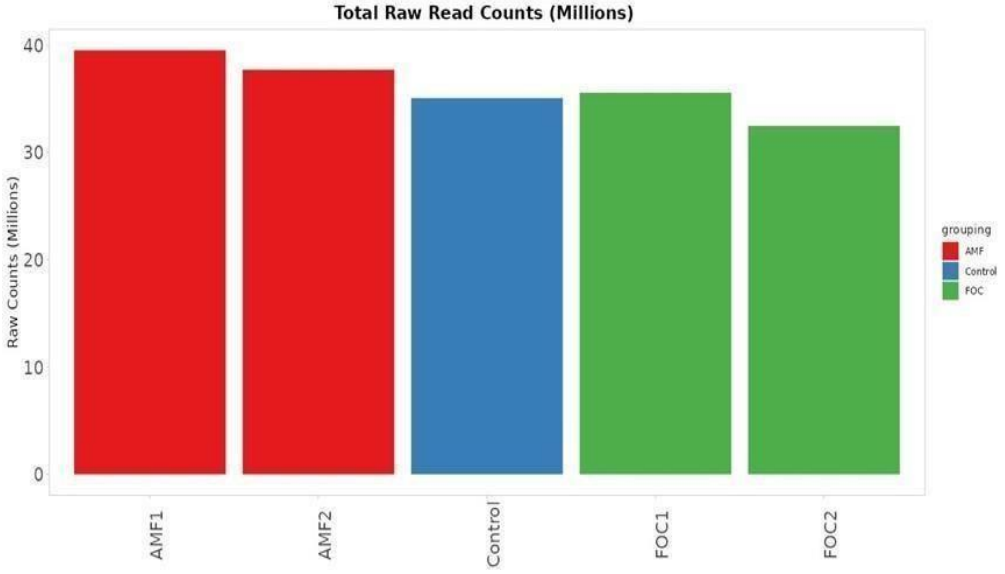


Figure 4.17: Graphical representation of the raw counts in FOC+AMF treated replications, FOC treated replications and Control plants after pre-processing.

Table 4.18: Alignment summary

Samples	Aligned Reads
C2-1 (FOC+AMF treated)	90.78%
C2-2 (FOC+AMF treated)	90.20%
T1 (Control)	96.85%
T2-1 (FOC treated)	84.93%
T2-2 (FOC treated)	84.59%

4.6.6. Differential analysis of transcriptome data

Over the course of the five samples, 30,344 genes were found in the overall expression estimation file. Following the stringent quality filters, 20,602 were deemed of good quality and processed for further analysis. Using the iDep 2.0 database, 12,986 of these genes were successfully converted to Ensembl gene IDs, while the remaining 7,616 genes were kept with their original IDs. Using EdgeR, expression analysis was carried out. Expression values were converted using the formula Log2 (CPM+c), where

'c' stands for a constant and CPM stands for counts per million. The results of pairwise significant differential expression features are presented in the following Table 4.19.

Table 4.19: The details of differential analysis of transcriptomics data of chickpea

Comparisons groups	Up	Down
FOCAMF-FOC	5027	2866
FOCAMF-Control	4292	1954
FOC-Control	2265	2373

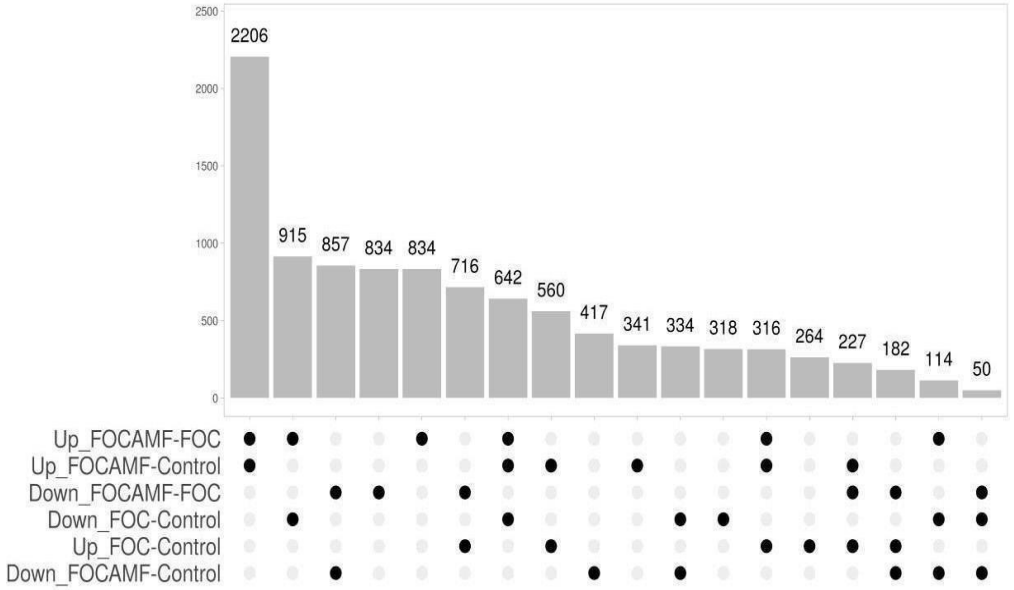


Figure 4.18: Upset plot showing an overview of differentially expressed genes (DEGs) in various combinations of FOC, Control and FOC+AMF treatments.

The volcano plots display the top 5 most significant genes under the comparisongroups FOC-Control, FOCAMF-Control and FOCAMF-FOC (Figure 4.18).

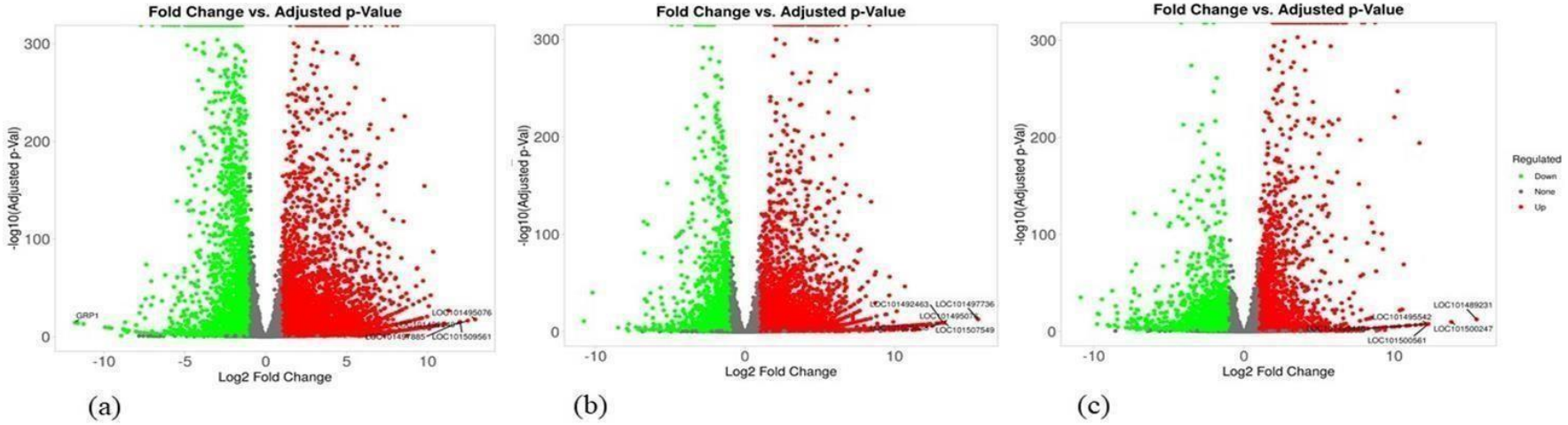


Figure 4.19: Volcano plot showing the most significant DEGs up and downregulated under the three different comparison groups. (a) FOCAMFFOC, (b) FOCAMF- Control and (c) FOC-Control

4.6.7. Principal Component Analysis

With PC 1 representing 74.69% variation and PC 2 representing 21.7% variation, the PCA unambiguously demonstrated differences between the control, FOC inoculation and FOC+AMF infected samples, showing a high expression variations among the treatments. The expression variation among the biologicalreplicates was fairly small (Table 4.20) (Figure 4.20).

Table 4.20: Summary of Principal Component Analysis

	PC1	PC2	PC3	PC4	PC5
FOCAMF1	-135.301	16.7141	-0.46782	-23.6626	4.19E-14
FOCAMF2	-135.277	16.1687	0.882826	23.68127	3.54E-14
Control	44.73942	-119.743	-0.46207	-0.07598	6.00E-14
FOC1	114.0468	42.42336	31.72493	-0.47624	5.39E-14
FOC2	111.7918	44.43681	-31.6779	0.533576	7.41E-14

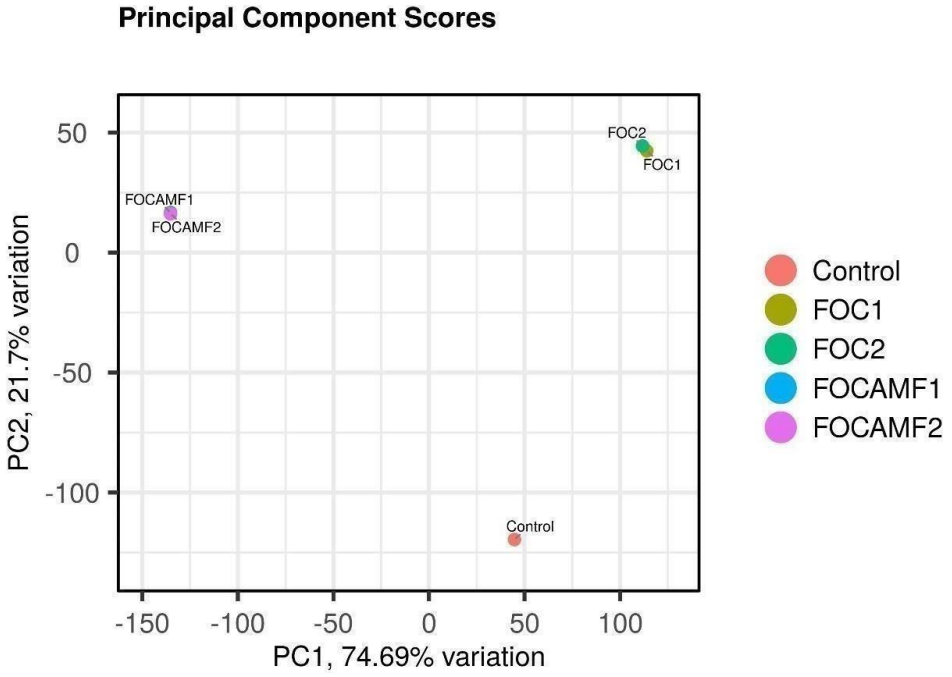


Figure 4.20: Principal Component Analysis of the response of chickpea plants under differential treatments

4.6.8. Functional classification of differentially expressed genes

Out of all significant DEGs, 4513 were given a gene ontology (GO) term which were assigned GO keywords were found for the differentially expressed genes (DEGs), which were distributed among biological processes (4261), molecularfunctions (5813), and cellular components (3749) (Figure 4.21).

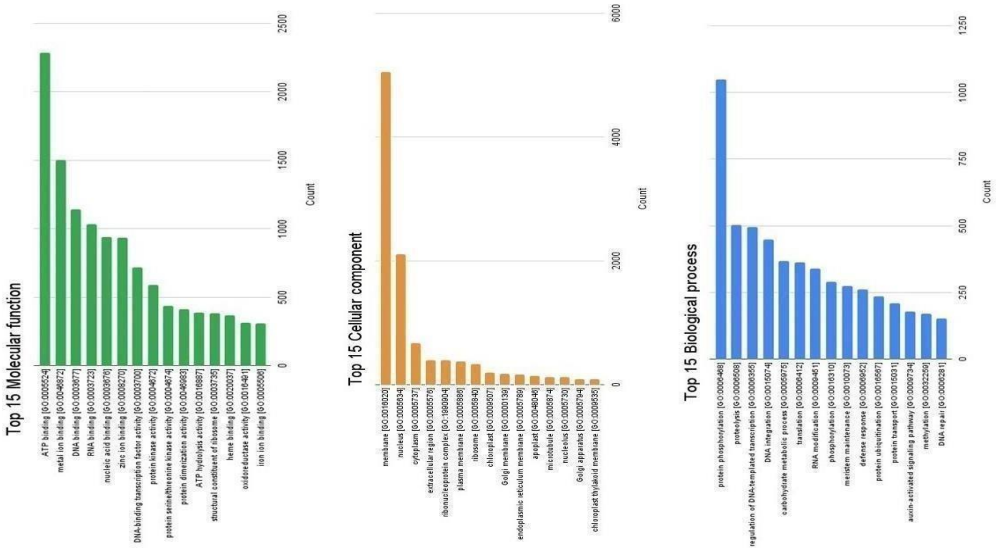


Figure 4.21: Plot of top 15 molecular functions, cellular components and biological processes available in annotation

4.6.9. Pathway analysis

The pathway analysis was done for the comparison group FOCAMFFOC using the parametric analysis of gene set enrichment (PGSEA). The pathway and process enrichment scores of each gene were examined for statistical significance in relation to each biological process, and the list of co-expressed genes was cross-referenced with the GO database. Moreover, pathways were used to group genes. The pathway analyses were conducted utilizing fold- change values derived from limma or DESeq2 for all genes. The significance cut-off for pathway analysis was set at 0.1, and the top 20 pathways were identified.

4.6.9.1. Biological processes

The significant pathways upregulated in the FOC+AMF and downregulated in FOC treated samples were polysaccharide metabolic process, polysaccharide biosynthetic process, hydrogen peroxide catabolic process, however the downregulated pathways were nuclear export, regulation of protein dephosphorylation etc. These pathways are linked to plant defense systems, implying that the presence of arbuscular mycorrhizal fungus (AMF) may increase the plant's ability to produce polysaccharides for cell wall strengthening and hydrogen peroxide for oxidative stress reduction in response to FOC infection. In contrast, FOC-treated samples showed downregulation in nuclear export and protein dephosphorylation pathways. The inhibition of these pathways may imply a weakened cellular function and regulatory systems in response to FOC infection alone, thereby increasing the plant's vulnerability to the pathogen. The

top 20 pathways involved in biological processes are presented in Figure 4.22.

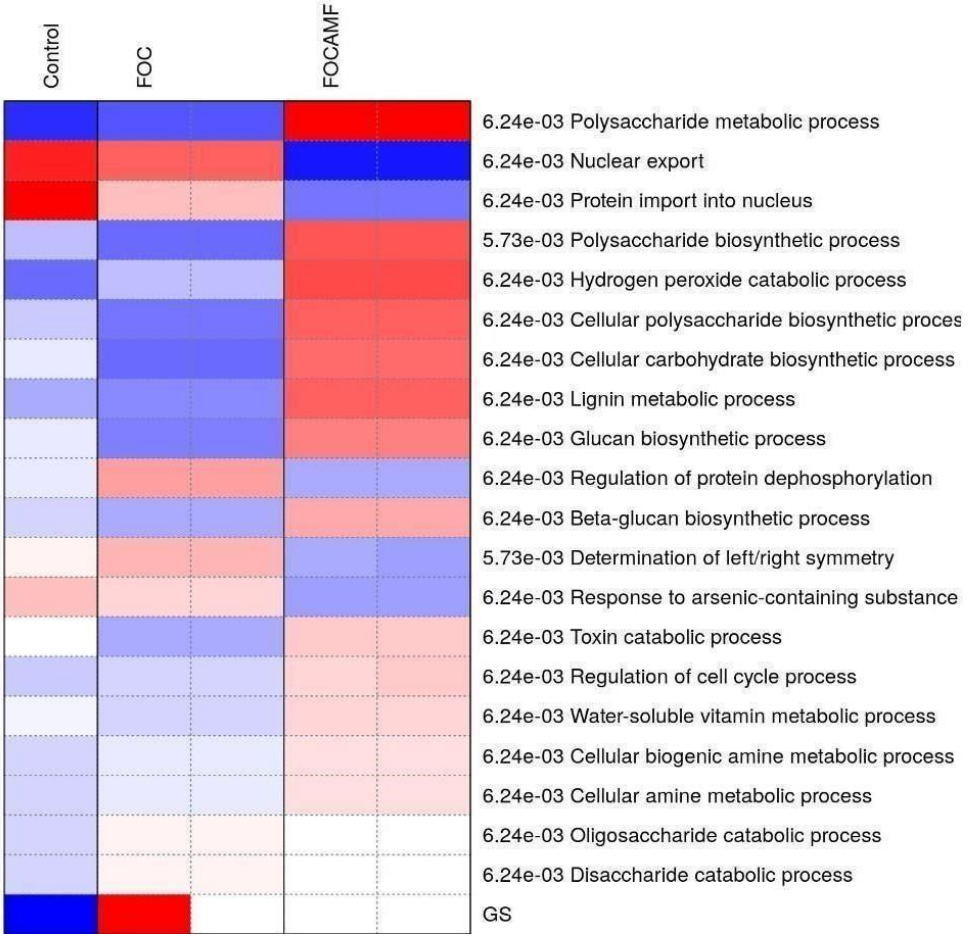


Figure 4.22: Heatmap showing the top 20 pathways associated with biological processes under all the treatments. Blue colour indicated downregulation and red indicates upregulation of pathways. GS: colour scale

4.6.9.2. Cellular components

The cellular component category's most upregulated pathways in FOC+AMF treated samples and downregulated in the FOC treated samples were cell wall, external encapsulating structure, intrinsic component of plasma membrane,origin recognition complex etc. However the downregulated components in FOC+AMF treated samples were transcription regulator complex, endosome membrane, histone methyltransferase complex etc. These alterations indicated that when AMF are present, structural integrity and defense mechanisms are enhanced in response to FOC infection. In contrast, in FOC-treated samples, downregulation of some of the important pathways may imply a reduced function of regulation and cellular signalling, perhaps making the plant more susceptible to FOC infection. The heatmap presented in Figure 4.23 gives a detailed description of the up and downregulation of the cellular components under all the

treatments.

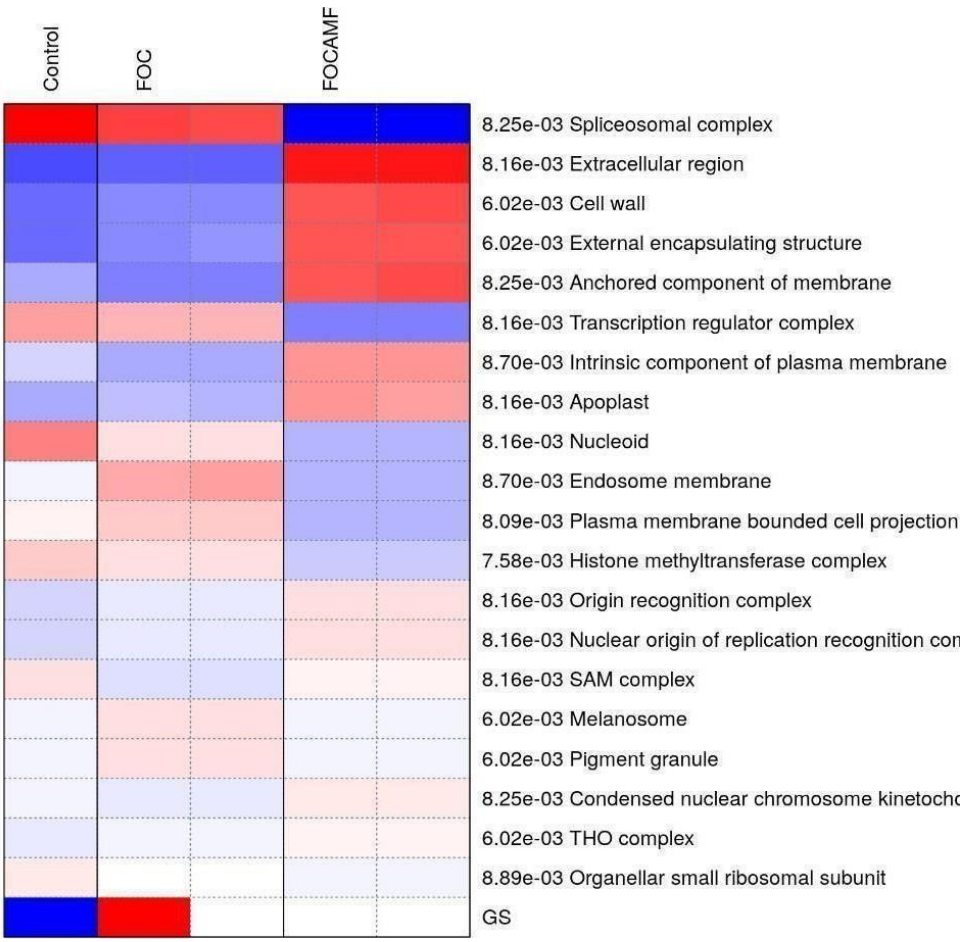


Figure 4.23: Heatmap showing the top 20 pathways associated with cellular components under all the treatments. Blue colour indicated downregulation and red indicates upregulation of pathways. GS: colour scale

4.6.9.3. Molecular function

The molecular function category was significantly enriched in pathways related to microtubule motor activity, DNA replication origin binding, polygalacturonase activity, peroxidase activity, palmitoyl hydrolase activity, however, suppression was observed in pathways linked to the molecular functions like exonuclease activity, transcription factor binding, sucrose transmembrane transporter activity etc., in the FOC+AMF treated samples.These functions play important roles in a variety of cellular processes, includingcytoskeletal dynamics, DNA replication, and defense responses, implying that AMF may improve the plant's ability to defend against FOC infection via manymolecular mechanisms (Figure 4.24).

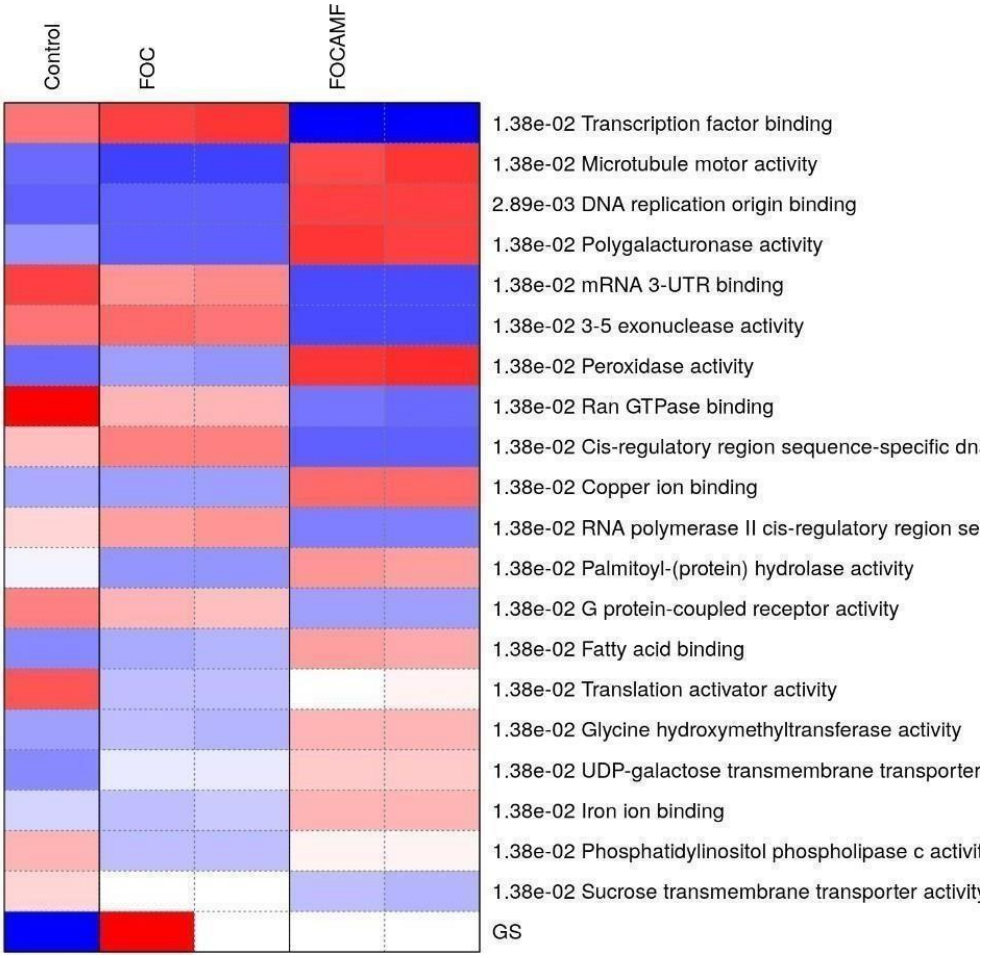


Figure 4.24: Heatmap showing the top 20 pathways associated with cellular components under all the treatments. Blue colour indicated downregulation and red indicates upregulation of pathways. GS: colour scale

4.6.10. Differentially expressed transcription factor families

Understanding the regulatory mechanisms behind plant responses to diverse environmental stresses depends significantly on the identification and characterisation of transcription factors (TFs). According to the transcriptome data 181 transcription factors (TFs) were found in the chickpea genome. 162 of the TFs were correctly classified into their respective families by using the PlantTFDB database to assign families to them. Interestingly, the distribution among various families showed distinct patterns of expression during stressful situations. The families bHLH (24 members), MYB (gene s35 members), AP2/ERF (46 members), WRKY (12 members), and NAC (9 members) were all well represented. Moreover, different numbers of members contributed to the total count for a number of other TF families, including PRE6, Heat Stress, Trihelix, GATA, TCP, BZIP, MADS, BEE-3, TGA, Homeobox, and Myblike. Nevertheless, a number of TFs—19 total—were not assigned to any families since the

PlantTFDB database did not contain those.

The transcriptional responses of chickpea to FOC and FOC+AMF treatments were shown to be significantly regulated by bHLH, MYB, AP2/ERF, WRKY, and NAC, among other well-represented TF families. These TF families have been the subject of in-depth research, and it is established that they play a role inboth biotic and abiotic stress responses. Plant defense mechanisms, including pathogen response, hormone signalling, and stress tolerance, are regulated by a variety of families, including bHLH (basic helix-loop-helix), MYB, AP2/ERF (APETALA2/ethylene-responsive factor), WRKY, and NAC (NAM, ATAF, and CUC). Additionally, other TF families, such as PRE6, Heat Stress, Trihelix,GATA, TCP, BZIP, MADS, BEE-3, TGA, Homeobox, and Myb-like, contributeto the transcriptional landscape of chickpea under stress.

Upregulated pathways in FOC+AMF treated plants include microtubule motor activity, DNA replication origin binding, and peroxidase activity, indicating improved cellular processes and defense responses (Garg *et al*., 2023; Volpe *et al*., 2023). In contrast, FOC-treated plants showed downregulation of pathways involved in fatty acid binding, and iron ion binding, indicating potential disturbances in lipid metabolism and nutrient uptake (Jiao *et al*., 2021). The biochemical data showing a positive connection between AMF colonisation anddefense-related gene expression are corroborated by the transcriptome analysis. The biochemical study reveals an increase in LOX and SOD activity, which is consistent with the upregulation of genes linked to defense mechanisms, including phenol content, lipoxygenase (LOX), and superoxide dismutase (SOD), in plants treated with FOC and AMF (da Trindade et al., 2019; Villani *et al*, 2021; Shah *et al*, 2024). The downregulation of pathways linked to fatty acid binding and iron ion binding in the transcriptome study is consistent with the decrease in catalase activity shown in plants treated with FOC+AMF in the biochemical investigation. This implies a coordinated response in which antioxidant enzyme activity is modulated by AMF colonisation to counteract FOC infection (CCS HAU, 2018 Jiao *et al*., 2021; Math *et al*., 2019).

Genes linked to stress response pathways found in transcriptome data are consistent with the rise in proline content identified in all genotypes in responseto stress in the biochemical analysis. A general stress response to FOC infection and AMF colonisation is shown by both analyses (Mirjani, 2023). Furthermore, the activation of cellular pathways associated with the cell wall, exterior encapsulating structures, and apoplast in AMF-treated plants emphasises the strengthening of physical barriers and

defense mechanisms against pathogen ingress (Puccio *et al*., 2023). In terms of biological pathways, AMF-treated plants showed upregulation in polysaccharide biosynthesis and hydrogen peroxide catabolism, indicating active structural component synthesisand ROS detoxification (Yadav *et al*., 2022; Pal *et al*., 2024). In contrast, FOC-treated plants showed downregulation of pathways related with ligandmetabolism and cellular glucose production, reflecting changes in energy metabolism and signalling activities under pathogen stress (Foucher *et al*., 2020).Most substantially, the disease severity index in FOC+AMF treated plants was lowered by 71.72 percent when compared to FOC-treated plants. This reductionhighlights AMF's possible protective effect against *Fusarium* wilt, emphasising its beneficial role in increasing plant resistance to infections (Khanna *et al*., 2022; Hashem *et al*., 2021). The transcriptome analysis revealed differential expression of genes linked to stress response pathways and protein metabolism,which is consistent with the reported increase in protein content in FOC+AMF treated plants and the observed fall in protein content upon FOC inoculation. Furthermore, modifications in sugar metabolism pathways found in the transcriptome data are reflected in the variations in total soluble sugar concentration seen in the biochemical study. (Manghwar *et al*., 2020; Formela- Luboińska *et al*., 2020; Farhana *et al*., 2022). The transcriptome analysis identified pathways linked to improved defense responses and ROS detoxification, which is consistent with the considerable decrease in disease severity index (DSI) observed in FOC+AMF treated plants, especially in genotype PUSA 547. As indicated by earlier research, this emphasises the possibility of AMF-mediated resistance against FOC infection. (Duc and Post, 2018; Hashem *et al*., 2021; Khanna *et al*., 2021; Spagnoletti *et al*., 2020).

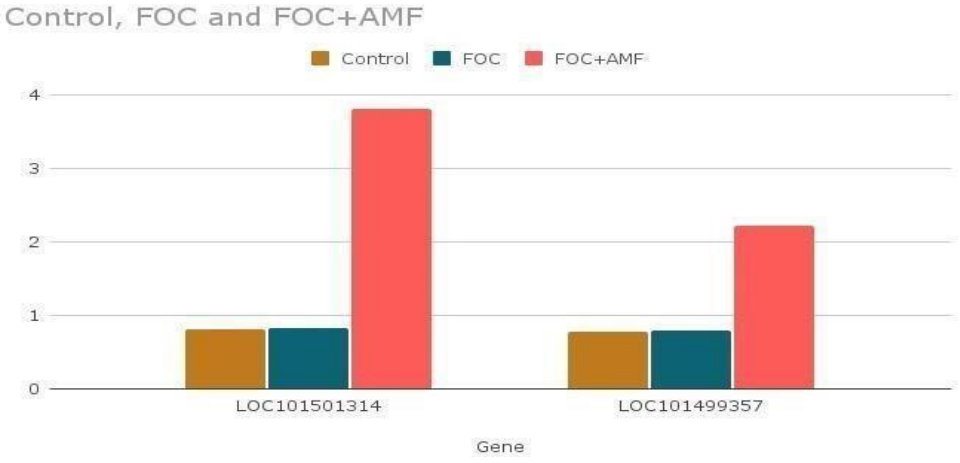
Overall, the transcriptome analysis confirms the biochemical results and illuminates the underlying processes of plant defense and stress adaptation by offering molecular insights into the biochemical responses seen in chickpea genotypes under FOC and FOC+AMF treatments.

4.6.11. Validation of gene expression

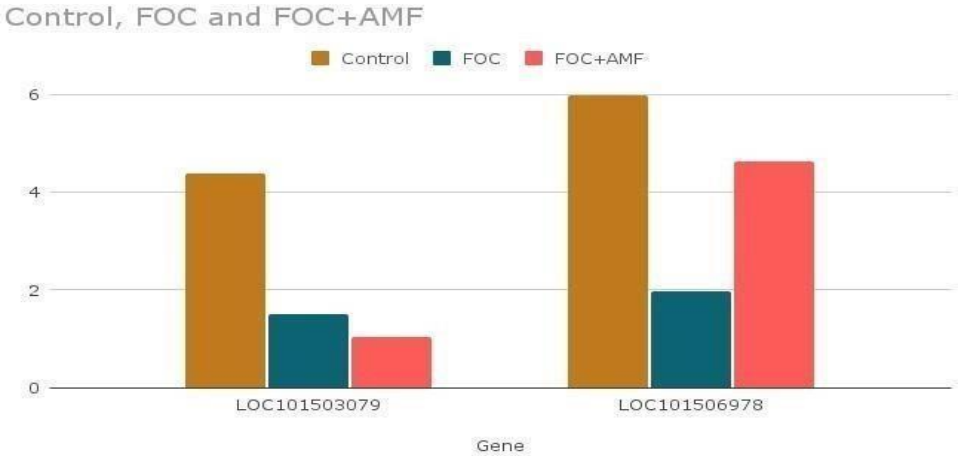
The highly significant differentially expressed genes which werespecific to the stress, were selected from all the data as shown in Table 4.21. The relative gene expression was determined by real-time PCR. Gene-specific primers designed to amplify the gene and the Actin gene were used in the RT PCR using SYBR Green chemistry. The relative gene expression was calculatedbased on Ct values using the comparative 2(-ΔΔCt) method.

Table 4.21: The list of genes selected for validation by RT PCR

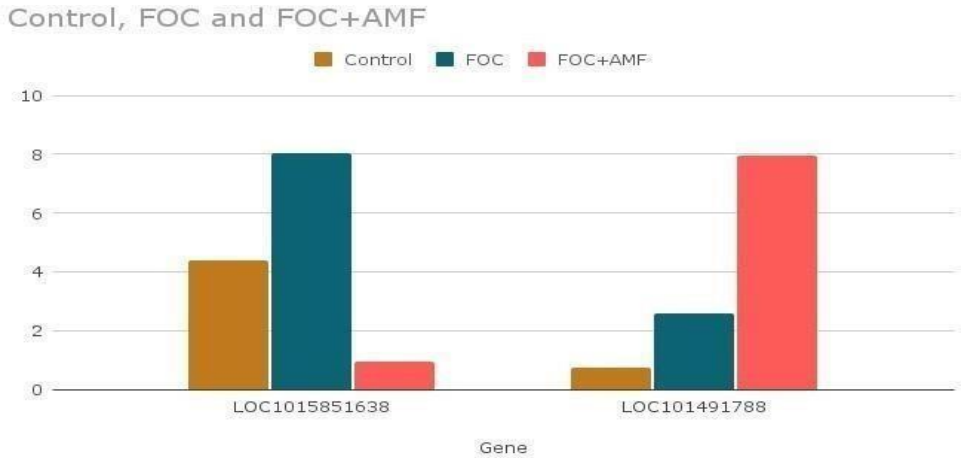
GeneID	NCBI description
LOC101499357	Peroxidase- 3
LOC101502158	ethylene-responsive transcription factor 9
LOC101491788	protein NRT1/ PTR FAMILY
LOC101505466	ATP phosphoribosyltransferase 2, chloroplastic-like
LOC101513079	potassium channel AKT2/3
LOC101515358	UDP-glucose: glycoprotein glucosyltransferase
LOC101506978	photosystem II 22 kDa protein, chloroplastic
LOC101507594	phenylalanine ammonia-lyase 1-like
LOC105851638	cytochrome P450 CYP736A12-like
LOC101501314	catalase activity



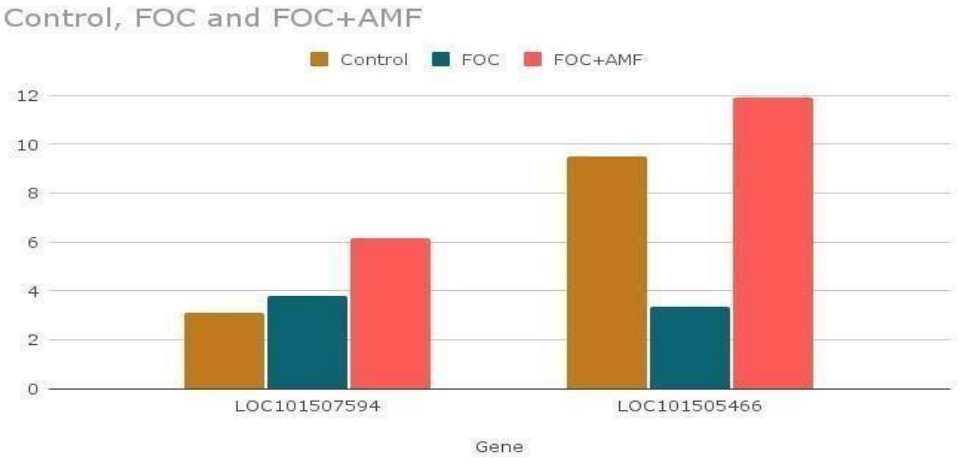
(a) **(b)**



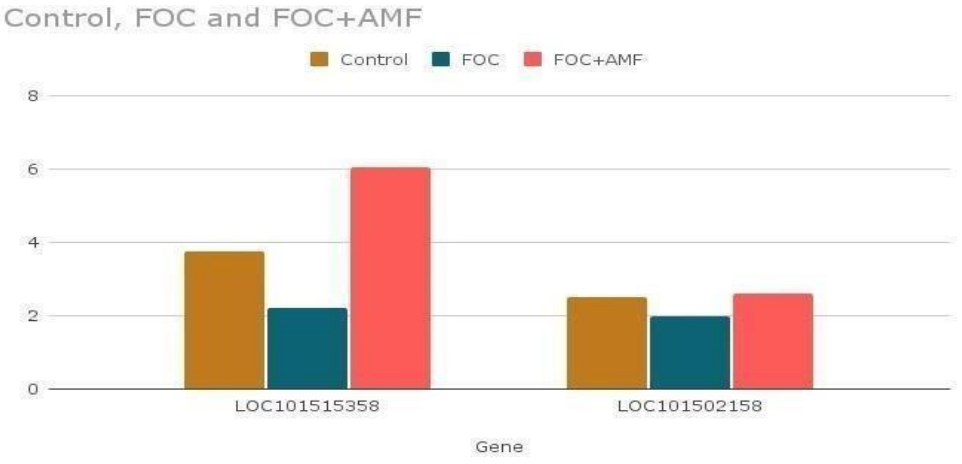
(c) **(d)**



(e) **(f)**



(g) **(h)**



(i) **(j)**

Fig. 4.25: Validation of gene expression using qRT-PCR

The highly significant differentially expressed genes unique to fungal stress were selected from the data , as shown in Figure 4.25. The primers used for selected gene validation are listed in Table 3.9. The relative gene expression was evaluated using real-time PCR.

Catalase activity, which is critical for alleviating oxidative stress, increased dramatically by thrice in FOC+AMF inoculation plants, compared to a moderate0.82-fold increase in FOC inoculated plants alone, highlighting catalase's criticalinvolvement in stress reduction. The defense-related gene peroxidase-3 was increased by 2.2 times in FOC+AMF-treated plants, indicating improved defense mechanisms. Similarly, AMF increased the expression of the ethylene- responsive transcription factor 2.5-fold. The NRT1/PTR family protein, whichis involved in nutrient transport, was elevated 2.5-fold in FOC-infected plants and even more than 9-fold in FOC+AMF treated plants. ATP phosphoribosyltransferase 2 activity, which is required for nucleotide biosynthesis, was highest in FOC+AMF treated plants, with an 11-fold increase, indicating higher metabolic activity. UDPglucose: glycoprotein glucotransferase,which is critical for protein glycosylation, was increased 6- fold in FOC+AMF treated plants versus 2.1fold in FOC treated plants. The PAL-1-like function, which is related with phenylpropanoid biosynthesis, increased 6.1-fold in FOC+AMF treated plants, the highest of the conditions.

Cytochrome P450, a detoxification enzyme, was highest in FOC-treated plants, decreased in control plants, and lowest in FOC+AMF-treated plants, indicating a shift in detoxification pathways. Photosystem II 22kDa protein was increased by 5.9 and 4.6-fold in normal and FOC+AMF treated plants, respectively, but downregulated in FOC-infected plants, indicating alterations in photosynthetic efficiency. Finally, the potassium channel AKT2/3, which is crucial for ion transport, was downregulated in FOC+AMF treated plants with only a 1.02-foldincrease, whereas it was increased by 4.3-fold in FOC-infected plants, indicatingdifferential ion transport regulation under stress circumstances. These RT-PCR results were consistent with the consistent with the results obtained from the results of transcriptome analysis.

Overall, the transcriptome analysis revealed that the key genes involved in plant defense, hormonal signaling, and symbiotic interactions were significantly upregulated in AMF-treated samples. These included **pathogenesis-related proteins** (e.g., *PR1*, *PR5*), which are crucial for activating early defense responses, and **glutathione S-transferases** (e.g., *GSTU19*), which play a major role in detoxification and alleviating oxidative stress. Enzymes such as **phenylalanine ammonia lyase** (*PAL1*) and **lipoxygenase** (*LOX2*) were also upregulated, indicating activation of the phenylpropanoid and jasmonic acid pathways. In addition, **ABC transporter genes** (e.g., *ABCG36*) facilitated the exchange of nutrients and defense compounds.

Transcription factors like **WRKY33**, **MYB1**, and **ERF1B** were notably expressed, suggesting a regulatory role in AMF-induced systemic resistance. These findings collectively highlight the involvement of AMF in priming defense signaling networks and enhancing host resilience against wilt stress.

SUMMARY AND CONCLUSION

The objective 1 of the study aimed to screen and evaluate chickpea genotypes against *Fusarium oxysporum f. sp. Ciceris* (FOC) using agronomical and molecular traits. Morphological characterization of FOC revealed typical traits consistent with the previous studies. Plant infection was confirmed through microscopic examination, and subsequent isolation of the pathogen confirmed its identity. Agronomical assessment under FOC infection revealed significant differences in wilt response among genotypes, with genotype and growth stage significantly influencing the response. The pre- and 45 DAS stages exhibited varying degrees of wilt severity across genotypes. Categorization of genotypes based on wilting percentage highlighted varying degrees of resistanceand susceptibility. Disease severity index, biomass, and yield varied significantlyamong genotypes, with ANOVA results indicating significant genotype and stage effects. Correlation analysis revealed strong relationships between wilt incidence, disease severity, and yield, emphasizing the impact of FOC infectionon chickpea productivity. Divergence analysis revealed genetic diversity of the genotypes and the cluster analysis grouped genotypes based on agronomical traits, revealing distinct resistance/ susceptibility profiles. Molecular analysis using STMS markers corroborated agronomical findings, segregating genotypes into clusters based on resistance/susceptibility. Overall, genotypes HC 1, GNG 2144, and C 235 were identified as highly resistant, while PUSA 547, ICCV 96029, CSJ 513, and RSG 888 were most susceptible to FOC race 3. These findings suggest that the identified resistant genotypes can be prioritized in breeding programs to develop cultivars with improved wilt resistance. Additionally, molecular markers associated with resistance may be utilized for marker-assisted selection in future research.

Objective 2 focused on screening and selecting suitable arbuscular mycorrhizal fungi (AMF) species against *Fusarium* wilt. Significant variations were observedin biochemical parameters across treatments, indicating potential benefits of AMF in enhancing plant resilience. Treatments significantly influenced biochemical responses, with AMF-treated plants exhibiting higher phenol content, enhanced protein synthesis, and antioxidant activity. Root colonization percentage was highest in plants treated with ProVam, correlating with reduced disease severity. Correlation analysis revealed strong associations between biochemical parameters and disease severity, highlighting the role of AMF in mitigating *Fusarium* infection. ProVam emerged as the most effective

treatment, enhancing plant defense mechanisms and forming beneficial mycorrhizal associations. Based on these results, ProVam containing *Glomus intraradices* can be recommended for field-level application to improve chickpea resistance against *Fusarium* wilt and promote plant health under pathogen stress.

In objective 3, the study investigated the mass production of AMF using Root Organ Culture (ROC) with a focus on the most promising treatment, ProVam. The morphological description of *Glomus intraradices* isolated from consortia of ProVam is provided, along with the inoculationof root segments with pre-germinated spores. The germination percentage, viability, root colonization percentage, root biomass, and spore density are evaluated over time. The results indicated a gradual increase in spore germination and viability over the observation period, with significant root colonization and biomass increase. Correlation analysis revealed positive associations between days after inoculation (DAI), spore density, root biomass, and root colonization percentage, indicating progressive establishment of AMF in roots. Additionally, AMF colonization correlates with increased levels of lipoxygenase (LOX) activity, phenol content, and superoxide dismutase (SOD) activity, suggesting enhanced defense mechanisms in plants. Structural equation modeling (SEM) reveals significant correlations between AMF root colonizationpercentage and various biochemical parameters, influencing the disease severityindex (DSI). These findings advocate the use of *in-vitro* produced AMF, particularly through ROC systems, as a scalable and efficient approach to formulate bio-inoculants for sustainable crop protection.

The objective 4 examined the susceptible chickpea genotype using transcriptome analysis which elucidated differential gene expression patterns between plants co-inoculated with AMF and those inoculated with *Fusarium oxysporum f. sp. Ciceris* (FOC) alone. The upregulation of pathways related to defense responses and ROS detoxification in AMF-treated plants suggests improved resistance against FOC infection. Functional classification of differentially expressed genes and pathway analysis further highlight the molecular mechanisms underlying AMF-mediated resistance, including enhanced polysaccharide biosynthesis, hydrogen peroxide catabolism, and activation of stress-responsive transcription factor families. Validation of gene expression through real-time PCR confirms the findings of the transcriptome analysis, providing molecular insights into the biochemical responses of chickpea genotypes under FOC and FOC+AMF treatments. Future studies can build upon these transcriptomic insights to identify key resistance genes and regulatory networks, which

may serve as targets for genetic engineering or genome editing to develop durable *Fusarium* wilt resistance.

Conclusions

This study comprehensively evaluated chickpea genotypes for resistance to *Fusarium* wilt, integrating agronomic, molecular, and mycorrhizal approaches. By unraveling the complex interplay between host, pathogen, and beneficial microbe, we identified resilient genotypes and elucidated the mechanisms underpinning their resistance. The efficacious application of arbuscular mycorrhizal fungi, particularly *Glomus intraradices* obtained from *in-vitro* source (ProVam), as a biocontrol agent was demonstrated, emphasizing the potential of sustainable, integrated disease management strategies. Our findings provide a foundation for breeding programs aimed at developing chickpea cultivars with enhanced *Fusarium* wilt tolerance while promoting agro-ecosystem health. These insights can be applied to formulate AMF-based bioformulations for field-level use, reduce dependence on chemical fungicides, and improve crop yields under pathogen pressure. Additionally, resistant genotypes identified in this study can be directly introduced into chickpea improvement programs or used as donor lines in marker-assisted selection for resistance traits.

Overall, this integration of biochemical, morphological, and transcriptomic approaches offers a comprehensive understanding of AMFmediated resistance in chickpeas, contributing to sustainable agricultural practices and improved crop resilience to fungal infections. Further research into these molecular pathways and gene expression validation could advance studies in plant- microbe interactions and crop protection strategies.

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Action Taken Report of last ETP

S. no.	Comments	Action taken	Page number
1.	The graphs for every parameter needs to be appropriately made with relevant X and Y axis units	The graphs have been edited appropriately with proper X and Y units and titles	74-78, 91-93
2.	Timelines needs to be appropriately mentioned in presentation.	Timeline has been added in the thesis and will be added inpresentation also	150
3.	Scholar should take care of units of measurement like for Total phenolic content.	The appropriate units of measurements have been added in the graphs as well as tables.	74-78, 91-93
4.	Scholar should also explore more biochemical and enzymatic parameters.	The results have been prepared according to the newly edited andRDC approved objectives and synopsis.	74-78, 91-93

Publication Details										
S. No	**Type of Paper**	**Name of the Journal/ Conference/ Book**	**Jounal indexing**	**Title of the Paper**	**Published Date**	**Volume & Issue Number**	**ISSN/ISBN Number**	**Impact Factor/ SJR**	**Type of paper (Research/ Review)**	**Whether this is thesis work or not (Yes/No)**
1	Journal Paper	Plant Science Today	Scopus	Screening of chickpea genotypes from different agroclimatic areas against *Fusarium oxysporum* f. sp. *Ciceris* (race 3) using morpological and molecular markers	Jun-23	-	ISSN 2348-1900	0.9	Research	Yes
2	Journal paper	Plant Cell Biotechnology and Molecular Biology		Methods of evaluation and screening of chickpea (*Cicer arietinum*) genotypes for wilt disease tolerance and potential usage of arbuscular mycorrhizal fungi	10-Jun-21	22(37&38):73-83; 2021	ISSN 0972-2025	SJR Q4	Review	Yes

				for biocontrol of wilt: A review						
3	Chapter	Springer		Green Manuring and its role in soil health management	May-20	Soil Biology (SOILBIOL,volume 59)	978-3-030-44364-1		Chapter	No
4	Chapter	Springer		Molecular taxonomy, Diversity and Potential applications of genus *Fusarium*	Jun-21	Fungal Biology	978-3-030-67561-5		Chapter	Yes
5	Conference	International Conference on Bioengineering and Biosciences (ICBB-2022)		Screening of chickpea genotypes from different agroclimatic areas against *Fusarium oxysporum* f. sp. *Ciceris* (race 3)	18-19 November 2022				Research	Yes
6	Conference	National conference on Sustainable Development and socio-economic upliftment of agrarian society		Effect of salt stress on Biological functionality of legumes and approaches to enhance tolerance	26-27 October 2023				Review	No

Timeline of Research Work

August 2019-July 2020	August-October, 2020	November, 2020-Feb 2021	March, 2021-September, 2021	October, 2021 -May, 2022	June, 2022-November, 2022	December, 2022 -May, 2023	June, 2023-December, 2023	January, 2024-July, 2024
Course workand synopsis approval	Paper writing							
		Preliminary screening						
			Paper writing and procuring pathogen culture from ITCC, IARI					
				Mass multiplication of pathogen and screening of chickpea genotypes, analysis				
					Child care leave			
						Mass multiplication of AMF and screening of genotypes for RNA-sequencing		
							RNA-sequencing	
								Data analysis and Thesis presubmission