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Research Article

Expression analysis and characterization of resistance (R) genes in contrasting Fusarium wilt resistant chickpea genotypes

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ABSTRACT

Fusarium wilt is one of the most notorious diseases of crop plants. This disease leads to huge crop losses in chickpea with substantial monetary value worldwide. The objective of the current study was to characterise *Fusarium oxysporum* f. sp. *cicris* (*Foc*), the causal agent of chickpea wilt, on morphological basis followed by the assessment of resistance of thirty chickpea germplasm accessions to *Foc* in a pot experiment. The second objective of this research was to study gene expression and characterisation of specific resistance (R) genes in different Fusarium wilt-resistant chickpea genotypes. The morphological identification and characterization of *Foc* was done by culturing the fungus potato dextrose agar (PDA) medium. Fusarium wild resistance was assessed by challenging 30 chickpea genotypes to artificial inoculum of *Foc* by using a pre-defined disease rating scale. Different resistance levels were showed by different chickpea genotypes ranging from highly resistant to highly susceptible. Two accessions demonstrated resistance, 15 were moderately resistant and the rest of the genotypes were moderately susceptible to susceptible. The *in-silico* expression analysis of R genes showed that two R genes, *LOC101499430* and *LOC101499568* were upregulated in chickpea roots, indicating their probable role in Fusarium wilt resistance. The genes were further assessed for their expression in response to Fusarium inoculation in contrasting Fusarium wilt resistant and susceptible chickpea genotypes. The resistant genotypes showed significantly higher expression levels of the selected R genes in comparison with susceptible genotypes. This study provides valuable insights into evaluating chickpea germplasm for Fusarium wilt resistance and identification of candidate resistance genes which could be employed for enhancement of Fusarium wilt resistance in chickpea.

Keywords: Resistance (R) genes; Fusarium wilt, Chickpea; Expression analysis.

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INTRODUCTION

Fusarium fungi pose significant challenge for chickpea cultivation, as exemplified by *F. oxysporum* f. sp. *cicris* (*Foc*) (Jalali and Chand, 1992). *Foc* is a fungus that proliferates readily, causing wilting in chickpea plants with great rapidity. It thrives in particular regions with low rainfall, where environmental conditions are conducive to disease development. *Foc* targets chickpea plants during the seedling and flowering stages. This leads to a reduction in the yield, which can be substantial (Ali *et al.*, 2018). Infected plants can lose up to 61% of their yield when infected during the seedling stage, or 43% during post-anthesis.

The mean annual yield loss due to *Foc* attack is estimated to range from 10% to 50% (Subhani *et al.*, 2012). Different studies indicate that Fusarium wilt is more prevalent in the Asian subcontinental region, the United States, Turkey, and Spain (Halila and Strange, 1996; Nene *et al.*, 1989). Symptoms of fusarium wilt in chickpeas typically manifest between 21 to 26 days post-sowing. In Pakistan, Fusarium wilt is the most prevalent fungal disease affecting chickpeas, with significant economic consequences (Ali *et al.*, 2018).

This disease has become a significant challenge for chickpea over the past four to five years due to the prolonged dry season in the country. Different approaches to disease management are therefore essential. Biotechnology and molecular marker methods are being used to support crop improvement initiatives by targeting genes responsible for plant disease resistance. These genes, found in plant genomes, confer immunity to pathogens. They work by producing R proteins. Plants have sophisticated R genes that can recognize specific pathogen effectors. This recognition can occur by direct binding or by detecting changes in a host protein caused by the effectors. (Jones and Dangl, 2006).

Resistance gene coding for NB-LRR proteins (nucleotide-binding/leucine-rich repeats domains) regulate a vast array of infection resistance reactions, frequently with the aim of preventing further pathogen development (Rasul *et al.*, 2019). There are typically several hundred different resistance genes in each plant genome that are critical to the control of different pathogens. These resistance (R) genes often show selectivity against pathogen strains. The hypothesis is that there is a coordinated mechanism between the plant's resistance gene and the pathogen's avirulence gene that aims to establish specificity in the resistance response. This implies a cooperative action involving receptors or ligands for both the Avr and R genes. (Numberger *et al.*, 2004).

An effector can manipulate the cell destination its host, triggering a NLR associated with either the intended goal or a diversion. Plant breeders frequently rely on R genes to confer beneficial resistance, although the efficacy of existing resistance in plants may vary depending on the pathogen or the resistance gene and the pathogen effector. The location of resistance genes can exert a significant influence on the pathogen, leading to either modification or elimination, in comparison to avirulence and effector genes. It is notable that few resistance genes demonstrate long-term stability, while certain R genes, especially those found in compact groupings of similar genes, possess the ability to swiftly develop novel pathogen targets within brief durations. (Friedman and Baker, 2007).

By keeping the above facts in mind, the present study was designed to characterize *Foc* based on morphological features and to assess the resistance levels of thirty chickpea germplasm accessions through *Foc* inoculation in a pot experiment, documenting disease severity responses ranging from highly resistant to highly susceptible and highlighting promising genotypes for further breeding efforts aimed at enhancing Fusarium wilt resistance in chickpea. The other main objective was to study the expression analysis and characterization of selected resistance (R) genes in contrasting Fusarium wilt resistant chickpea genotypes.

MATERIALS AND METHODS

Pathogen sampling, pathogen isolation, purification, and characterization

The infected roots of chickpea plants were gathered from the Pulses Research Institute at the Ayub Agriculture Research Institute (AARI) in Faisalabad. These samples were carefully stored in sealed plastic bags and transported to the laboratory for further analysis. Potato Dextrose Agar (PDA) was used for *Foc* isolation. For its preparation we mixed 20g Potato starch, 20g Dextrose and 15g Agar Agar in one-liter distilled water. By thoroughly mixing on hot plate, the PDA was autoclaved for 20 minutes at 121^0C and 15 psi pressure. After autoclave media was poured in sterile petri plates in sterile conditions and let it stay for solidification in biosafety cabinet. In the meantime, infected roots were cut into 1cm pieces, and 2% Sodium Hypochlorite solution was used for their surface sterilization by immersing them for 30 seconds. After sterilization roots were washed with distilled water thrice. Excess water was removed by blotting the samples with sterile filter paper. Subsequently, the samples were placed on PDA plates supplemented with antibiotics such as 25 mM streptomycin, with three to four root pieces per plate. The plates were then incubated for 3 days at 28°C in an incubator. Pathogen purification was carried out using the hyphal tip method, and identification was based on various criteria including colony pattern, plate color, spore shape, size, and structure and presence of micro and macroconidia.

Staining of *Foc* spores and mycelia

Foc spores were stained using the lactophenol blue staining method. To prepare the staining solution, 10 grams of phenol, 10 ml of glycerol, 10 ml of lactic acid and 0.02 grams of trypan blue were dissolved in 10 ml of distilled water. Fresh staining solution was prepared by combining lactophenol-trypan blue with 95% ethanol in a 1:2 ratio. The *Foc* spores and mycelia were boiled in water bath for 2 minutes at 95^0C in the staining solution and left to stand at room

temperature for 12 to 36 hours or until the desired intensity of staining had been achieved. The samples were then destained with a chloral hydrate solution (50 grams of chloral hydrate dissolved in 20 ml of distilled water) and photographed using a compound microscope according to Keogh *et al.* (1980).

Plant cultivation and screening of germplasm against Fusarium wilt

Thirty chickpea germplasm samples were obtained from the Department of Plant Breeding and Genetics (PBG) at the University of Agriculture Faisalabad. These germplasm accessions included 5009, Ch-7, 1021, 6022, 6009, 6016, 1056, Noor-2009, 6008, 1027, 6060, 6035, 214, 115, 1001, 6015, 220, 1009, 290, Wanhaar-2000, 2004, Pb-2008, 4112, 6013, 1032, 4056, 1017, Cs-30, 1821, and Cm-2008. The germplasm screening was done by growing and inoculating the plants in half liter plant pots. Chickpea seeds were planted in pots in a greenhouse. Each treatment involved sowing ten pots per genotype. Fusarium was cultured on PDA media. Spores were extracted, after the growth of two weeks by using the chilled water method through multiple (4) layers of sterile muslin cloth. The spores of *Fusarium oxysporum* f. sp. *ciceri* were quantified using a hemocytometer in the laboratory. The inoculum was adjusted to a concentration of 4.5×10^5 spores/ml in distilled water. This prepared inoculum was applied to the plants near their roots using a needleless syringe in a uniform manner across all plants.

Disease assessment and disease rating scale:

The progression of disease in chickpea plants was monitored and documented at different stages of growth to evaluate its impact. An already reported disease rating scale for the measurement of disease severity was adopted (Iqbal *et al.,* 2005). The detail of disease rating scale is given below.

1: Highly Resistant = <1% wilted plants
2: Resistant = 1-10% wilted plants
3: Moderately Resistant = 11-20% wilted plants
4: Susceptible = 21-50% wilted plants
5: Highly Susceptible = >51% wilted plants

***In-silico* gene expression analysis of R-genes from chickpea**

In-silico Gene Expression was done by retrieving the expression data of different R-genes from Chickpea Transcriptome Database (CTDB) (Verma *et al.*, 2015). The R-genes transcript IDs of chickpea were determined by using CTDB tBlastn Program. For gene expression the data was retrieved from database of different plant parts and the heat map was made by using Microsoft Excel. Different intensities of red and blue colors to different boxes were given by conditional formatting on the basis of expression values.

Expression analysis of selected R-genes in genotypes with contrasting resistance

RNA isolation and primer designing

Total RNA was isolated from roots of two susceptible (1009 and 6060) and two resistance genotypes (214 and 6009) by using using TRIzol reagent (Macedo and Ferreira 2014). Complementary DNA (cDNA) was synthesized through reverse transcription process by reverse transcriptase PCR from total RNA through Superscript III 1st Strand Synthesis Kit from Thermo Scientific according to the manufacturer's instructions. Two genes were selected from the *in-silico* expression analysis with the highest expression in the roots which were *LOC101499430* (with transcript ID on CTDB TC00496) and *LOC101499568* (with transcript ID on CTDB TC00633). Primers were design using amplifX at https://inp.univ-amu.fr/en/amplifx-manage-test-and-design-your-primers-for-pcr. The primers used for *LOC101499430* were Forward 5'-GGTGCCAAAGTCTTCAAACGTG- 3', and Reverse 5'-ACGCCTGCAATCGTCCATCTT-3'. Similarly, for *LOC101499568* RGA the primer pair was Forward 5'-GAGGGATGCATTTCCCTTACAAGC-3' and Reverse 5'-AGGCAAGGTCTCAATGTGAGTG-3'. The primer designing was done by using coding sequences of both genes.

Semi-quantitative reverse transcriptase PCR

For semi-quantitative reverse transcriptase PCR, the concentration of cDNA from different treatments was equalized after measurement of NanoDrop spectrophotometer (Thermo Scientific). After equalizing the cDNA, the gene specific primers mentioned in the previous section were used to quantify the expression of these R-genes from the DNA bands on the gel electrophoresis according to Ali *et al.* (2013).

RESULTS

Characterization of *Foc* on morphological basis

The morphological identification of *Foc* pathogen was done by culturing the pathogen on the PDA (Figure 1). The colony shape, color and texture as well as the shape and size of macrospores microspores showed it resemblance with *Foc* fungus reported earlier in the literature.

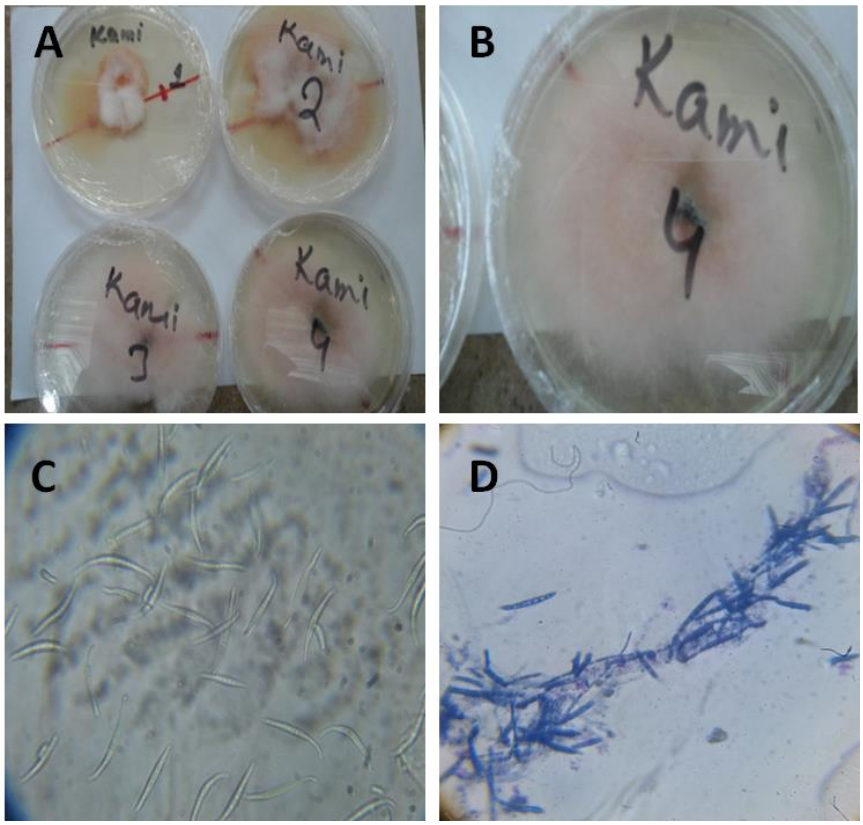

Figure 1. Purification and characterization of *Fusarium oxysporum* from wilted chickpea plants. A and B: Colony shape, color and texture, C: Macrospores of *Foc*, D: Macro and micro spores of *Foc* after Keogh staining.

Assessment of resistance level of chickpea germplasm in response to *Foc*

Thiry germplasm accessions of chickpea were subjected to *Foc* inoculum in the pot experiment. The results demonstrated differential response of different germplasm accessions. Only two genotypes displayed the resistance response while 15 genotypes showed moderate resistance response (Figure 2). The rest of the germplasm accession exhibited susceptible response with 6 showing highly susceptible behavior and 7 with moderate susceptibility. Two genotypes 6009 and 214 were found resistant, whereas 15 genotypes including 5009, Ch-7, 6022, 1056, 6008, 1027, 115, 1001, 220, Wanhaar-2000, Pb-2008, 4056, 1017, Cs-30, and Cm-2008 showed moderately resistance response to Fusarium wilt. On the other hand, 7 genotypes viz., 1021, 6016, Noor-2009, 6035, 290, 2004, and 6013 were moderately susceptible while 6 genotypes (6060, 6015, 1009, 4112, 1032, and 1821) were highly susceptible.

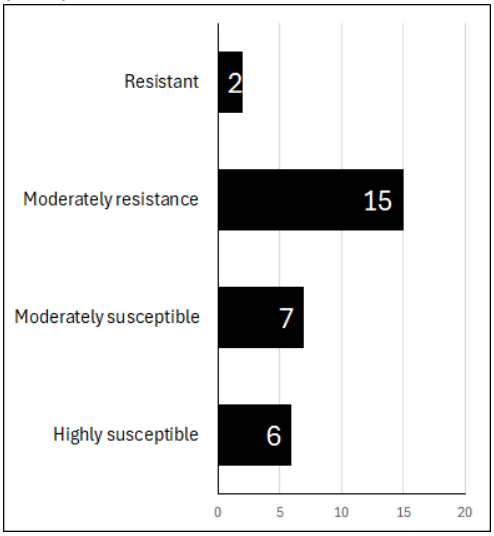

Figure 2. Graphical representation of germplasm resistance response to *Foc*

***In-silico* gene expression analysis of chickpea R-genes in different plant parts**

In-silico gene expression of 31 R-genes was performed by using the expression values of these transcripts from Chickpea Transcriptome Database (CTDB) (Figure 3). The results displayed that most of the R-genes were not expressed in different plant parts. However, two R-genes *LOC101499430* (with transcript ID on CTDB TC00496) and *LOC101499568* (with transcript ID on CTDB TC00633) were highly expressed in the roots of chickpea followed by transcripts TC00299 and TC05117 which were also expressed in the roots. Overall, most of the transcripts were expressed in roots as compared to the other plant parts. The two R-genes having very high state of expression were chosen for *in-vivo* gene expression studies in chickpea genotypes with contrasting resistance levels to Fusarium wilt.

Identifier	Shoot	Root	Mature leaf	Flower bud	Young pod
TC00011	76	94.4	15.5	13	3.7
TC00017	55.9	82.6	7.8	30.3	47.6
TC00021	42.5	0	7.8	43.2	29.3
TC00076	33.5	82.6	0	25.9	43.9
TC00085	4.5	0	0	4.3	0
TC00299	42.5	141.6	62	60.5	62.2
TC00316	60.4	82.6	15.5	38.9	33
TC00337	17.9	23.6	15.5	51.9	11
TC00496	46.9	200.5	54.2	47.6	22
TC00633	2.2	200.5	7.8	4.3	3.7
TC01261	15.7	35.4	23.2	17.3	3.7
TC01498	20.1	59	7.8	34.6	7.3
TC01626	4.5	35.4	7.8	13	14.6
TC01659	2.2	70.8	0	8.7	0
TC01875	2.2	0	0	17.3	3.7
TC02487	13.4	47.2	7.8	13	65.9
TC02522	2.2	70.8	7.8	0	3.7
TC02794	0	11.8	0	0	0
TC02937	13.4	35.4	0	8.7	7.3
TC03328	20.1	0	15.5	0	3.7
TC04064	11.2	23.6	0	17.3	0
TC04227	4.5	0	7.8	13	7.3
TC04229	17.9	47.2	31	4.3	3.7
TC04281	2.2	11.8	0	4.3	0
TC04688	0	0	0	4.3	3.7
TC05117	2.2	153.3	0	4.3	14.6
TC05440	2.2	0	0	8.7	0
TC07010	6.7	0	0	17.3	0
TC07558	8.9	47.2	15.5	13	0
TC13287	4.5	11.8	7.8	4.3	0
TC20721	2.2	0	0	0	3.7

Figure 3. Analyzing gene expression in chickpeas across various plant parts using computational approaches. Blocks highlighted in red indicate elevated expression levels, while blue blocks indicate low expression levels.

Expression analysis and characterization of R-genes in contrasting Fusarium wilt resistant chickpea genotypes

The *in-vivo* expression of two R-genes naming *LOC101499430* (with transcript ID on CTDB TC00496) and *LOC101499568* (with transcript ID on CTDB TC00633). For this purpose, total RNA was isolated from roots of two susceptible (1009 and 6060) and two resistance genotypes (214 and 6009) followed by the synthesis of cDNA as

discussed in the section of material and methods. The phenotypes of control and inoculated plants is given in the Figure 3. The difference between the resistant and susceptible genotypes is clearly demonstrated by the genotypes.
For measuring the expression of two R-genes in contrasting chickpea genotypes, PCR products of four samples were run on agarose gel to check visualize their expression in the contrasting genotypes (Figure 5). The RGA, *LOC101499430* showed very high expression in the resistant genotypes 214 and 6009 varieties and very minor expression in the control plants. Similarly, *LOC101499568* exhibited good expression in resistant cultivars. In contrast, there was no significant expression was observed in susceptible verities i.e., 1009, 6060.

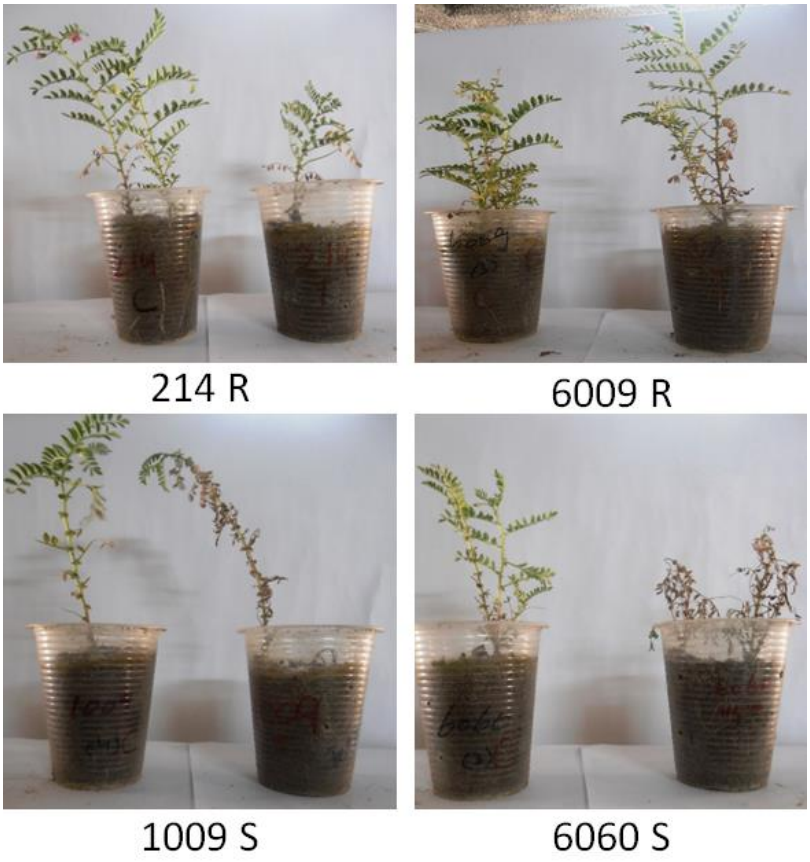

Figure 4. Symptoms of *Foc* on chickpea resistant and susceptible genotypes. In each figure left-side plant is control plant and right-side plant is inoculated with *Foc*.

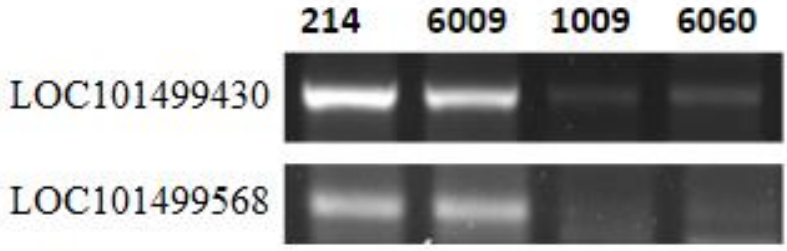

Figure 5. Semi-quantitative RT-PCR for measuring the expression of two R-genes in contrasting chickpea genotypes.

DISCUSSION

The aim of this study was to examine the enhanced chickpea varieties for their resistance to Fusarium wilt, alongside the utilization of novel methodologies to analyze the resistance genes within the chickpea genome. Additionally, the study involved in vivo expression analysis of specific R-genes. An assessment of chickpea germplasm was conducted to differentiate between responses indicating resistance and susceptibility. Understanding the characteristics of the associated pathogens is crucial for accurate evaluation (Jabbar *et al.*, 2020). For developing effective management

strategies and identification, it is essential to perform their characterization accurately. Which includes morphological examination of *Foc* (Younesi *et al.*, 2015), assessment of colony morphology (color, shape and texture) and microscopic characteristics.

During the evaluation of chickpea germplasm against *Foc*, significant diversity was observed in their resistance levels. The main theme of this study highlights the breeding efforts to gain resistance against Fusarium wilt. In results, only two cultivars were observed resistant while 15 genotypes showed moderate resistance. Other remaining genotypes showed susceptibility or moderate susceptibility. Chaudhry *et al.*, (2006) also evaluated different germplasms against *Foc*. Similarly, Chaudhry *et al.*, (2007) also screened 196 different germplasms to evaluate immune and highly resistant lines. Another research conducted by Babu and Ravikumar (2009) showed that, in resistant genotypes there was less inhabitation of pollen tubes as compared to the susceptible genotypes.

Chickpea Transcriptome Database (CTDB) was used to retrieve the transcriptomic data of chickpea and by suing computational approach distinct expression patterns of R-genes were observed (Verma *et al.*, 2015). Different R-gene transcripts showed variable expressions in different plant parts. However, R-genes within roots showed the highest expression. Hence, this result proves the hypothesis that roots play a key role in the interaction of soil-borne pathogens (*Foc*) and plants (Ali *et al.*, 2018). By observing roots expression, two R-genes i.e., LOC101499568 and LOC101499430 were selected to further investigate the expression under field conditions. Between susceptible and resistant genotypes their distinct differences were observed. The expression of both R-genes in resistant genotypes was elevated in contrast to susceptible genotypes, revealed the significant function in providing resistance to Fusarium wilt. A recent study Priyadarshini *et al.* (2023) demonstrated that, the expression profile of R-genes fluctuates over the time in both resistant and susceptible genotypes against *Foc*. The *Foc* resistant Quantitative Trait Loci (QTLs) and Lucine-rich repeats (commonly associated with wilt resistance) were also observed to aligned within the WRKY group.

CONCLUSIONS

The results concluded that there is diversity for resistance in the available germplasm of chickpea tested as on 2 genotypes showed resistance response to *Foc*. The *in-silico* analysis showed that the transcripts of R genes were mainly expressed in roots and very less expression was found in the other plant parts. The two R genes *LOC101499568* and *LOC101499430* was highly expression in the resistance line as compared to susceptible lines demonstrating some role in the resistance against *Foc*.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this research.

COMPETING OF INTEREST

The authors declare no competing interests.

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