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Molecular Characterization of Fusarium and Screening of Cowpea [*Vigna unguiculata* (L.) Walp.] Germplasms against Fusarium Wilt (*Fusarium oxysporum* f. sp. Tracheiphilum)

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ABSTRACT

Background: Fusarium wilt caused by fungal pathogen, *Fusarium oxysporum* is one of the soil-borne diseases that cause 30 to 100% yield reduction with high plant mortality and severe problem in cowpea production.

Methods: The *Fusarium* spp. isolate was obtained from infected cowpea plants and morphological and molecular characterization were done using universal primers. Similarity and phylogenetic evolutionary relationship analysis were performed with database. The fusarium wilt symptoms occurrence in each germplasm were scored using disease rating scale by artificial inoculation of 10% culture.

Result: Isolated *Fusarium* spp. was an anamorphic species with medium growth rate (5 mm/day), characterised by white cottony to pinkish colonies, aseptate microconidia (12.879 × 3.570 µm) with false head, septate macroconidia (32.409 × 5.297 µm) along with chlamydospores. While, 90-100% homology was observed between isolate (Accession no: MZ706473) and fusarium reference database. Phylogenetic evolutionary analysis showed that the isolate was closely related to *F. oxysporum* (OL679453.1) strain supported by 52% bootstrap. Artificial screening of 115 cowpea germplasm under pot culture, identified 18 resistant lines with 0% symptoms. The wilting intensity was maximum during flowering or pod stage than seedling stage in cowpea. The identified resistant cowpea varieties could be used as donor parent in future resistant breeding programmes.

Key words: Artificial screening, Cowpea, Disease scale, *Fusarium oxysporum*, Pathogenicity.

INTRODUCTION

Being short gestation, low input and resilience crop, cowpea in farming system becomes an innovative approach for climate change, soil degradation and nutrient depletion. Having originated in Africa, where the large genetic diversity of wild types occurs spreads into all continents, now mainly grown in many parts of Asia, Europe, USA, Central America and South America (Tetteh *et al*., 2020). Adaptability of the cowpea in all over is due to its high resilience to harsh conditions, especially hot and dry environments and poor soils (Boukar *et al*., 2018). Cowpea plays multifunctional traits, including food for human consumption as grains, tender leaves and pods; maintains soil-ecology balance through nitrogen fixation by facilitating symbiosis with nodulating bacteria; and provides fodder for livestock, it also helpful as a cover crop or an erosion-preventing crop; it aids in suppressing weeds; supports in the retention of moisture and contributes significant economic productivity and environmental sustainability (Omomowo and Babalola, 2021).

Fusarium wilt caused by fungal pathogen, *Fusarium oxysporum* f. sp. tracheiphilum (fot), is one of the soil-borne diseases that cause major threat in many of the cowpea growing area; substantially reduces the yield of 30 to 100% with high plant mortality and severe problem in cowpea production (Pottorff *et al*., 2014). The occurrence and epidemic spread of the pathogen depends on the soil factors like nutrient status, temperature (25-28°C), moisture and resistant varieties. The pathogen invades into the plant through roots and enters the vascular system; infected plants exhibit leaf chlorosis, necrosis, wilting, vascular discoloration and death of the plant with severe economic loss (Omoigui *et al*., 2018). Symptoms of fusarium wilt begins with sudden yellowing of foliage to defoliation; at the severe conditions, the lower portion and upper part of the tap root system together forms as swollen like structure and slowly became shredded and disintegrated (Senthilkumar, 2003). Wide range of host plants including family members of Leguminosae, Malvaceae and Solanaceae and the presence of dormant thick-walled asexual chlamydospore, which

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germinates by the induction of plant root exudates, in the soil crust are the major constraints to control the fusarium pathogen completely. Though it is seed and soil borne pathogen, is very difficult to manage using fungicide alone; and continues usage of fungicide for soil fumigation, mainly methyl bromide leads to environmental damage as well as occurrence of resistant strains of pathogen (Omoigui *et al*., 2018). So, the use of resistant varieties is one of the cost effective and environmentally safe method to control fusarium wilt in cowpea. Hence, the study aimed at identification and characterization of causal organism and screening of cowpea genotypes to identify resistant against fusarium wilt.

MATERIALS AND METHODS

Present study was conducted at Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2019-20, for molecular characterisation of pathogen causing fusarium wilt in cowpea and screening of cowpea varieties against the same for disease resistance breeding programme.

Isolation, identification and multiplication of *Fusarium oxysporum* spp.

The infected cowpea plants (*Vigna unguiculata* L.) were collected from field, stem and roots were washed in running water to remove adhered soil particles. The infected collar portion and root bits were cut into small pieces of 1-2 mm size and surface sterilized with 0.1% mercuric chloride ($HgCl_2$) followed by three times washes with sterile water. The 4-5 small bits were transferred aseptically into a petridish containing solidified Potato Dextrose Agar (PDA) amended with streptomycin sulphate and tetracycline hydrochloride to minimize chances of bacterial growth. Plates were incubated at 28°C and undergone periodic observation. From 3 days old culture plates, 5 mm disc of fungus was sub-cultured into fresh 15 ml of sterilized and solidified PDA, incubated for 3 days at 28±2°C. The mycelial growth, colony characters and spore specifications were recorded three days after inoculation (DAI) on daily basis. The pathogen was characterized according to the epitypification given by Lombard *et al*. (2019) under microscope. Isolated fungus was multiplied in sand maize medium, which was prepared by mixing 90 g of riverbed sand and 10 g of maize meal (9:1) with 20 ml of distilled water in 250 ml of Erlenmeyer flask; and sterilized the mix at 15 lb for 20 minutes. After cooling, total of 10 bits of fungal culture along with PDA was aseptically transferred into the flask and closed air tightly; incubated for 15 days at room temperature (28±2°C) for multiplication.

Genomic DNA extraction, PCR amplification and nucleotide sequencing

The genomic DNA of pure fungal isolates was extracted using NucleoSpin® Plant II Kit. The quality of the DNA isolated was checked using agarose gel (0.8%) electrophoresis. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). The ITS ribosomal DNA regions were amplified by PCR using universal primer pairs, ITS1-ITS4. PCR reaction mixture contained 200 ng fungal genomic DNA, 5 µl of 2× Phire Master Mix, 4 µl distilled water and 0.25 µl of each primer. Amplification was done in a Gene Amp-9700 thermal cycler (ABI, USA). Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA).

Sequence analysis

The sequences were trimmed, assembled and consensus sequences were obtained using BioEdit software. The obtained consensus sequences were deposited in NCBI database under accession number MZ706473. The obtained nucleotide sequence was searched through BLASTN at GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) to compare with ITS reference sequence available in the database. Multiple sequence alignment of ITS sequence with reference sequence from gene bank was performed using Clustal W available in BioEdit Software. Phylogenetic evolutionary relationship analysis was performed using Molecular Evolutionary Genetic Analysis software (MEGA 7) by Neighbor-joining method with 1000 bootstraps run.

Screening of cowpea genotypes against *Fusarium oxysporum* spp.

Seedling screening was conducted in completely randomised design with three replications along with control during 2019-20. A set of 115 cowpea genotypes consisting of germplasm from NBPGR, New Delhi, AVRDC, Taiwan, Dept. of Plant Breeding and Genetics, College of Agriculture, Vellayani and released varieties in Kerala Agricultural University (KAU) were collected for the study. The *Fusarium* isolate multiplied in sand maize medium (9:1) were incorporated to the sterilized soil at the rate of 10% (w/w). One week old cowpea seedling from each genotype was transplanted to the soil containing *Fusarium* and a control plant were also maintained and monitored regularly. The number of days to wilt, disease intensity, disease scoring and disease reaction were assessed visually and weekly, starting at 20 days post inoculation. The disease intensity (Chattopadhyay and Sen, 1996) was calculated by using the formula:

$$\text{Disease intensity} = \frac{\text{Sum total of the score}}{\text{Total number of the plant assessed}} \times \frac{100}{\text{Maximum grade}}$$

Each genotype sown in a replicated manner with control plants were scored on a 0-4 disease rating scale described by Senthilkumar (2004).

RESULTS AND DISCUSSION

The culture had medium growth rate (5 mm/day), when it was cultured aseptically in PDA media from infected stem (Fig 1). Maximum radial growth and growth parameters with abundant sporulation were noticed in *Fusarium* spp. grown on potato dextrose agar (Gupta *et al*., 2010). *Fusarium*

Supplementary Table 1: Comparison of morphological characters of the present isolate with *Fusarium oxysporum* spp. previously described.

Species	Shape of conidiospore	Average radial growth	Shape and size (µm) of		Shape and size (µm) of chlamydospores	Special features
			Microconidia	Macroconidia		
Present isolate*	Unbranched and branched monophialides	Medium growth rate of 5 mm/day	Aseptate, abundant, oval to ellipsoidal, cylindrical, straight, or curved form and 12.879 ×3.570 µm in size	3-5 septate, thin walled, fusoid, pointed ends, occasionally falcate with terminal cell, hooked, pedicellate basal cell 32.409×5.297 µm in size	Terminal and intercalary in position, globose, 7 µm diam	Aerial conidia forms aggregates of aseptate microconidia forms false head structure at the tip of phialides, smooth thin walled, aseptate
Fusarium oxysporum on	Unbranched and branched monophialides	3.0-4.0 mm/d at 24°c	No septate, oval-ellipsoidal, straight to curved 5.0~12 × 2.2~3.5 µm	Usually 3~5 septate, fusoid -subulate and pointed at both ends 27~46 × 3.0~4.5 µm	Globose to subglobose, formed intercalarily or terminally, 5-10 µm diam	Aerialconidia forming small false heads the tips of the phialides, hyaline, ellipsoidal to falcate, smooth- and thin-walled, 0-1-septate

Table 1: Number of days to taken wilt, disease score, intensity and reaction in 115 cowpea germplasms.

Name	No. of days taken to wilt	Disease score (0-4)	Disease intensity (%)	Disease reaction
TCR79	40	4.0	80.0	HS
TCR80	48	1.0	20.0	MR
TCR81	44	3.3	66.6	S
TCR83	44	1.3	26.6	MS
TCR84	44	1.0	20.0	MR
TCR85	48	1.3	26.6	MS
TCR86	53	1.0	20.0	MR
TCR87	53	0.0	0.00	R
TCR88	53	1.3	26.6	MS
TCR89	48	2.6	53.3	S
TCR101	53	2.0	40.0	MS
TCR104	48	2.6	53.3	S
TCR105	48	1.3	26.7	MS
TCR106	48	3.0	60.0	S
TCR107	53	1.3	26.7	MS
TCR108	53	1.6	33.3	MS
TCR109	53	1.6	33.3	MS
TCR110	53	2.0	40.0	MS
TCR111	53	3.0	60.0	S
TCR112	26	2.0	40.0	MS
TCR113	26	2.6	53.3	S
TCR115	26	1.0	20.0	MR
TCR116	26	1.0	20.0	MR
TCR117	26	3.0	60.0	S
TCR118	26	2.3	46.7	MS
TCR119	26	2.6	53.3	S
TCR122	26	3.0	60.0	S
TCR124	26	1.6	33.3	MS
TCR125	26	1.0	20.0	MR
TCR126	26	1.3	26.7	MS
Aryanadu local	31	3.0	60.0	S
Ayyanthole local	21	4.0	80.0	HS
Cherthala local I	21	2.6	53.3	S
Cherthala local II	40	3.0	60.0	S
Elamadu local II	21	0.0	0.00	R
Kadambarakonam local	15	4.0	80.0	HS
Kallicaud local	21	0.0	0.00	R
Kallicaud local II	40	2.6	53.3	S
Kanjikuzhi local	21	0.0	0.0	R
Kochi local	21	0.0	0.0	R
Kollam local I	21	1.3	26.7	MS
Kollamcode local	21	1.3	26.7	MS
Koovappally local	21	0.0	0.00	R
Kottayam thattathi local	21	0.0	0.00	R
Kumil local	21	3.0	60.0	S
Mavelikara local	21	0.0	0.00	R
Nelladu local III	21	3.6	73.3	S

Table 1: Continue...

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Nellanadu local	21	0.0	0.00	R
Nellanadu local II	37	2.6	53.3	S
Nenmara local II	21	0.6	13.3	MR
Nenmara local III	21	4.0	80.0	HS
Nenmara local IV	21	2.0	40.0	MS
Nenmara local V	21	2.6	53.3	S
Nenmara local VI	21	3.0	60.0	S
Omallur local I	21	2.6	53.3	S
Padavalam payar	37	2.6	53.3	S
Palakkad local I	21	2.0	40.0	MS
Palakkad local	31	3.6	73.3	S
Peyad local	21	1.0	20.0	MR
Puthenpeedikayil local	21	3.0	60.0	S
Vellavalli payar	29	1.0	20.0	MR
Vlathankara local I	29	0.6	13.3	MR
Vlathankara local II	29	1.0	20.0	MR
Wayanadu local I	29	0.0	0.00	R
Wayanadu local II	29	0.0	0.00	R
Muttathkonam local	29	0.0	0.00	R
Kottayam local I	29	1.0	20.0	MR
Kalliyoor local	29	1.3	26.6	MS
Aranmula local	32	1.0	20.0	MR
Kilimanur local	32	1.6	33.3	MS
Elamadu local I	32	2.6	53.3	S
Kulashekharam local I	32	0.0	0.00	R
Nilamel local	32	1.0	20.0	MR
Kottarakkara local	32	0.0	0.00	R
Anchal local II	32	1.0	20.0	MR
VI001920 A-R	44	4.0	80.0	HS
VI034386	44	0.0	0.00	R
VI034392	44	1.3	26.7	MS
VI036720	44	3.3	66.6	S
VI036721	44	4.0	80.0	HS
VI040951	44	1.0	20.0	MR
VI041083	44	1.3	26.6	MS
VI041680	44	1.0	20.0	MR
VI041703-A	44	3.0	60.0	S
VI046192	44	3.0	60.0	S
VI046645-B	44	3.6	73.3	S
VI046648-B	44	4.0	80.0	HS
VI046653	44	0.0	0.00	R
VI047617	44	1.3	26.6	MS
VI048020	44	1.0	20.0	MR
VI048484	36	2.6	53.3	S
VI048488	32	4.0	80.0	HS
VI048533	44	0.0	0.00	R
VI050893	44	3.3	66.6	S
VI050896	44	1.6	33.3	MS
VI055471	44	1.0	20.0	MR
VI057906-A	44	1.0	20.0	MR
VI057936-B	44	1.0	20.0	MR
VI061032	44	1.6	33.3	MS

Table 1: Continue...

Table 1: Continue...

VI061064	44	1.3	26.6	MS
VI061095	44	2.6	53.3	S
VI062338	44	2.0	40.0	MS
VI064556	44	0.0	0.00	R
VI050941	44	1.0	20.0	MR
VI061014	44	2.0	40.0	MS
Malika	34	1.000	20.00	MR
Sharika	34	1.000	20.00	MR
Bhagyalakshmi	34	2.667	53.33	S
Lola	34	1.333	26.67	MS
Vyjayanthi	34	1.000	20.00	MR
Vellayani Jyothika	34	1.667	33.33	MS
Githika	34	2.000	40.00	MS
Manjari	34	0.667	13.33	MR
Anaswara	34	1.333	26.67	MS
Kanakamony	34	4.000	80.00	HS

oxysporum spp. is an anamorphic species circumscribed by some morphological criteria, basically shape of macroconidia, structure of microconidiophore and formation and disposition of chlamydospores. The colonies of isolate were appressed to floccose in texture, white on the upper surface, reddish brown or faint pink on the lower side of the petridish (Fig 2). Some species of fusarium produces no colour, while others usually produce pale purple to dark magenta pigment on agar media (Janevska and Tudzynski, 2018), but there is no relationship between pathogenicity and pigments produced (Lombard *et al*., 2019). The conidiophores consisted of monophialides on aerial mycelium and produces asexual spores such as microconidia, macroconidia and chlamydospores, which lacks sexual reproduction. Microconidia were aseptate, abundant, oval to ellipsoidal, cylindrical, straight or curved form and 12.879×3.570 µm in size. Aggregates of aseptate microconidia forms false head structure was a characteristic feature of *Fusarium oxysporum* spp. Microconidia produced from intercalary phialides in false heads (Ohara and Tsuge, 2004). The macroconidia were 3-5 septate, thin walled, fusoid, pointed ends, occasionally falcate with terminal cell, hooked, pedicellate basal cell and 32.409×5.297 µm in size. Chlamydospores when present were terminal and intercalary in position (Fig 3). The comparison of isolate with already characterized fusarium pathogen by Lombard *et al*. (2019) was depicted (Supplementary Table 1).

The ITS region contains multiple tandem repeats of ribosomal RNA in the haploid genome, which are very useful in species identification, hence it has been considered as a standard marker in DNA bar-coding of fungal species (Janevska and Tudzynski, 2018). Based on the assembled sequences from both forward and reverse primers, the obtained ITS sequences containing ITS1-ITS4 deposited in the gene bank at NCBI (accession number MZ706473)

(Fig 4). Basic Local Alignment Search Tool (BLAST) analysis showed that the assembled sequence had high percentage of similarity index (99-100%) and query recovery (100%) with the *Fusarium oxysporum* reference sequences (Supplementary Table 2). Phylogenetic analysis revealed that the isolate is less distant and diverse from *Fusarium oxysporum*. The phylogenetic tree divided into clades and subclades, in which the isolate was related to potential *Fusarium oxysporum* (OL679453.1) strain supported by 52% bootstrap (Fig 5).



Fig 1: Basal swelling of fusarium infected cowpea plants.

Cowpea accessions were abundantly available with huge diversity in characters, which could be useful in the improvement programme and in developing varieties with specific traits (Nalawade *et al*., 2021). Screening of cowpea germplasm against fusarium wilt resistance are not done so far. The screening of 115 cowpea germplasm collected from different sources were resulted in 18 resistant (R) genotypes with 0% incidence of symptoms, these genotypes need to be further evaluated for the fusarium linked characters under sick plot at field level. Remaining 26 were moderately resistant (MR) with 0-25% incidence of symptoms, 31 were moderately susceptible (MS) with 26-50% incidence of symptoms, 31 were susceptible (S) with 51-75% incidence of symptoms and 9 genotypes were highly

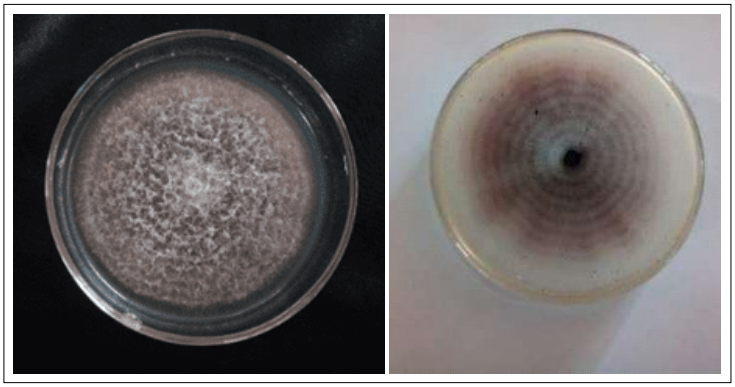


Fig 2: Colony characters of *Fusarium oxysporum* spp.

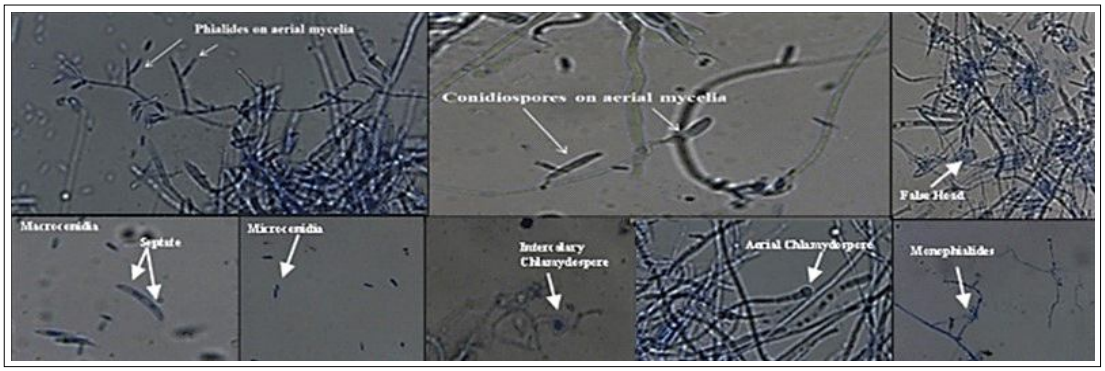


Fig 3: Spore characters of *Fusarium oxysporum* spp.

Supplementary Table 2: Sequence of isolate, homology and identity percentage.

Isolates/acc no	Gene size	Homology	Accession No	Query cover	Identity
MZ706473	754	*Fusarium oxysporum* isolate KUMBPJBT-70	OL679453.1	100%	100%

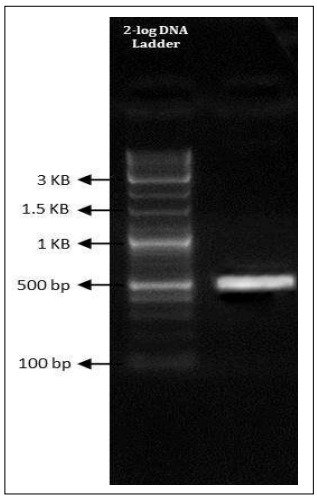


Fig 4: Gene amplification at 500kb with ITS1-ITS4 universal primer.

susceptible (HS) with >75% incidence of symptoms (Table 1) (Fig 6). Artificial screening of many crops against fusarium wilt reaction were done and scored accordingly in chickpea (Kumar *et al*., 2019), lentil (Roy *et al*., 2021). Single dominant gene with lack of maternal effect and linkage; controls the resistant reaction in cowpea against fusarium wilt (Omoigui *et al*., 2018). The dominant inheritance Fusarium wilt resistance were also observed in pigeon pea (Singh *et al*., 2018). So, the crop improvement for resistant breeding through the marker assisted selection become uncomplicated and easily recognizable; which can shorten the breeding cycle.

The wilting symptom began after 20 days up to 53 days of inoculation in all genotypes, which means the disease severity was more during flowering or reproductive stage than seedling stage; and incidence of disease varied greatly within the genotypes. Late wilt percentage was

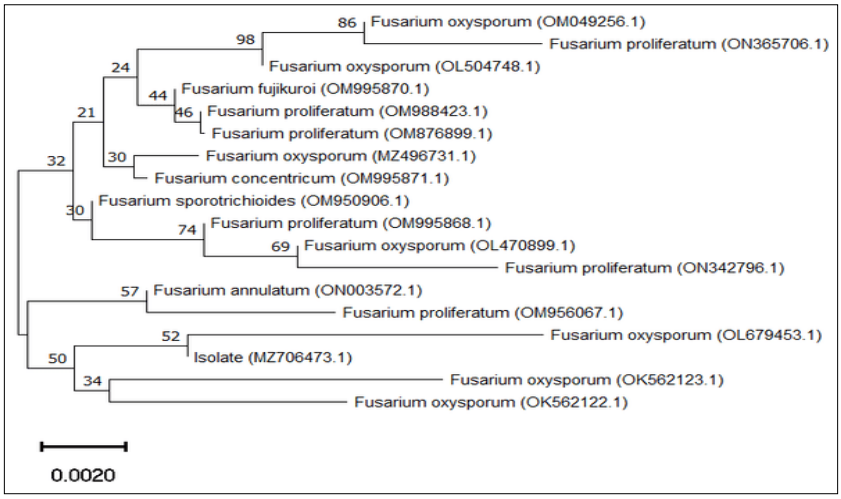


Fig 5: Phylogenetic analysis of isolate sequence with reference sequences from database.

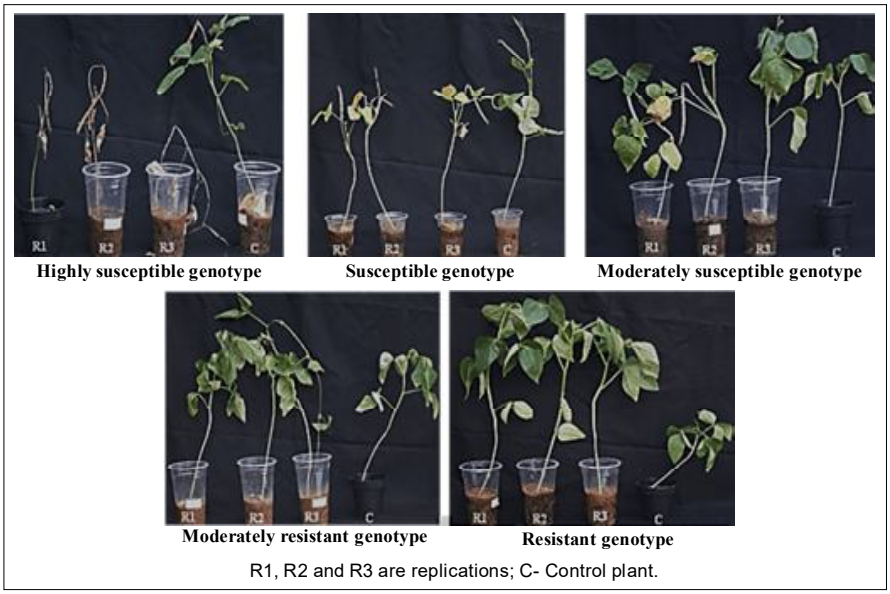


Fig 6: Different disease reaction of cowpea germplasms.

greater than early wilt percentage in all the genotypes of chickpea under pot culture screening than sick plot method (Yadav and Kumar, 2019). Disease intensity were ranged from 0-80%, with reaction of resistant and highly susceptible respectively. The eighteen cowpea genotypes identified as resistant against fusarium wilt could be used as donor parents for wilt resistance in resistant breeding programme.

CONCLUSION

Fusarium wilt in cowpea causes tremendous decline in the cowpea production, and the management cannot be done completely due to is soil-born nature. It causes yellowing, necrosis, vascular discoloration, basal swelling, and fully distraction of the plant. The pathogen produces asexual spores like aseptate microconidia, 3-5 septate macroconidia and thick walled, dormant chlamydospores, without sexual reproduction. The wilting symptom in cowpea seedlings under artificial screening, began after 20 days up to 53 days of inoculation in all genotypes, with more disease severity during flowering or reproductive stage than seedling stage; and incidence of disease varied greatly within the genotypes. The resistant cowpea varieties identified in the study might be used as donor parents for resistant breeding programme.

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Conflict of interest

The authors declare no conflicts of interest.

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