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MOLECULAR AND PHENOTYPIC EVALUATION OF PIGEONPEA GENOTYPES FOR RESISTANCE TO *FUSARIUM* WILT BY *IN VIVO, IN VITRO* AND MARKER ASSISTED SCREENING METHODS

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ABSTRACT

A comprehensive *in vivo* and *in vitro* assessment was conducted to identify resistant genotypes in pigeonpea against *Fusarium udum*. Further molecular validation was done using SSR markers. Sixty pigeonpea genotypes were screened *in vivo* in sick plot maintained at AICRP on Pigeonpea, ZARS, Kalaburagi. *In vitro* screening was done following root dip technique developed using the same 60 genotypes. Further these genotypes were subjected to screening using three SSR markers (ASSR 1, ASSR 23 and ASSR 363) specific to *Fusarium* wilt resistance in pigeonpea. In field screening out of 60 genotypes four genotypes (ICP X 140203-B-1, TDRG 272, ICP 8863, and GRG-811) were found resistant. 41 genotypes showed moderately resistant reaction and remaining 17 genotypes showed a susceptible reaction. When these genotypes were subjected to *in vitro* screening through root dip method, two genotypes (ICP X 140203-B-1 and ICP 8863) recorded resistant, 37 genotypes recorded as moderately resistant and remaining 23 genotypes showed susceptible reaction. To confirm the resistance and susceptibility of these genotypes at their genetic level, the genotypes screened using SSR markers. The markers amplified a total of six polymorphic alleles with an average polymorphic information content value of 0.48. Cluster analysis, done by UPGMA, grouped the 60 pigeonpea genotypes into two main clusters according to their *Fusarium* wilt reaction. Based on the Mann- whitney U test and simple regression analysis, the three markers were found to be significantly associated with *Fusarium* wilt resistance. The phenotypic variation explained by these markers was 24.08 per cent. The *in vitro* screening method developed offered low cost, short time and short space consuming platform for identification of resistance in pigeonpea against *Fusarium* wilt which otherwise take whole one season in field screening at sick plot. The correlation between marker presence and phenotypic resistance highlighted the robustness of SSR markers used in the study as tools for marker-assisted selection. These findings not only provide insights into the genetic basis of resistance and their stability but also offer a reliable framework for developing *Fusarium* wilt-resistant pigeonpea varieties. Combination of *in vivo*, *in vitro* and molecular techniques assessed in the study also offer rapid, assured and reliable methods for identification of wilt resistant cultivars in parallel to speed breeding programmes.

Key words : Speed breeding programmes, Phenotypic resistance, Pigeonpea genotypes, *Fusarium* wilt.

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millspaugh] is a major *Kharif* pulse crop of India. The crop is most suitable for intercropping as it is slow growing and does not compete with short duration annual crop. Green pigeonpea seeds are highly nutritious, contains high levels of proteins and significant amount of essential amino acids like lysine, methionine and tryptophan. Dry pigeonpea seeds contain protein (20–22%), carbohydrate (57.3%), fat (1.5%) and ash (8.1%). Its protein has two globulins, cajanin and concajanin accounting for 58% and 8%, respectively. The dried stalks are used as fuel, for making baskets and thatching material. Besides the ability of plant to fix atmospheric nitrogen makes pigeonpea an important

component of sustainable cropping system (Keerti *et al.*, 2022). Biotic stresses are regarded as the primary constraint to pigeonpea production in the Indian subcontinent, with *Fusarium* wilt being the most destructive disease, followed by Sterility mosaic disease, Macrophomina root rot, and Phytophthora blight. (Pande *et al.*, 2013). Pigeonpea wilt caused by a fungus *Fusarium udum* Butler severely hampers its productivity and causes yield losses upto 30 to 100 per cent (Dhar *et al.*, 2005). The disease being soil borne in nature, it is difficult to manage through fungicide alone. In addition to high cost of fungicides, continuous use of fungicides results in detrimental effect on environment and development of resistant strains of the pathogen. Therefore, adopting resistant cultivars is considered the most effective strategy and sustainable way to control the disease. The study of literature suggested that resistant sources have been identified in pigeonpea against *Fusarium* wilt (Singh *et al.*, 2016), but it must be a continuous procedure since after some years it's bound to breakdown since pigeonpea is often cross-pollinated crop and pathogen may overtake the resistance over time. Therefore, development of wilt resistant varieties in pigeonpea is a major challenge and needs to be addressed on priority basis. Along with traditional screening method, there is a need to develop more rapid, reliable and repeatable methods of identifying resistant cultivars in pigeonpea against the *Fusarium* wilt. The advancement of scientific methods has enabled use of various biotechnological tools in screening of host plants against particular diseases especially using molecular markers. These markers have the ability to indicate genetic variation among the genotypes and particular set of populations. The microsatellite or SSR markers are considered most useful due to high polymorphism, ability to detect multi-allelic variation and co-dominance nature enabling them for reproducible results (Rehman *et al.*, 2023). These are either genomic SSR markers or EST-SSR markers that have been developed and mapped for numerous plant species in order to evaluate genetic diversity and phylogenetic relationships for genetic resource utilisation and conservation. Therefore, SSR markers are considered neutral markers (Sagar *et al.*, 2023). Cultivated pigeonpea are known to have low polymorphism, hence SSR markers are ideal for studying the genetic diversity (Kimaro *et al.*, 2020). In the present investigation, the pigeonpea genotypes were initially screened in wilt sick plots where the genotypes showed resistant, moderately resistant or susceptible reaction, later *in vitro* screening was carried out for the same genotypes. These finding were further confirmed through SSR markers, which clearly differentiated these genotypes into either resistant or susceptible genotypes.

Materials and Methods

***In vivo* screening of pigeonpea genotypes in wilt sick plots :** Field screening of 60 pigeonpea genotypes and two checks along with susceptible and resistant check ICP 2376 and ICP 8863 was carried out in wilt sick plot maintained at ZARS, Kalaburagi. The entries were sown in two rows at spacing of 60×20cm in three replications. Other agronomical practices were followed as per package of practices. Observations for wilt incidence were recorded at seven-day intervals, starting from 30 days after sowing up to 180 days. Based on the observations taken the disease incidence was calculated and the genotypes were categorized as resistant, moderately resistant or susceptible by using the disease rating scale of AICRP on *Kharif* Pulses.

Wilt incidence (%)	Reaction
0.00-10.00	Resistant
10.10-30.00	Moderately resistant
>30.00	Susceptible

***In vitro* screening of pigeonpea genotypes through root dip method:** Sixty pigeonpea genotypes and two checks were screened through root dip method following the below procedure standardised. Seven day old pigeonpea seedlings were raised in sterile sand, removed from sand slowly without much disturbance to root system, root tips were trimmed slightly, dipped in *Fusarium udum* inoculums for 30 seconds having concentration of 6 x 10^5 (7 days old culture) and transplanted to pots consisting sterile soil. Observations for wilt incidence were recorded at 15 days post inoculation.

Screening of pigeonpea genotypes using SSR markers

Extraction of DNA : Young pigeonpea seedling leaf tissues were collected for all the 60 genotypes and two checks from 15 day old seedlings raised in pots. The genomic DNA of all the samples was extracted using the traditional CTAB (Cetyltrimethyl Ammonium Bromide) method. Young leaf samples (100 mg) from each genotype were collected, washed, and ground in 750 µl of preheated CTAB buffer. After incubation at 65 °C for 60 minutes with intermittent mixing, the mixture was centrifuged at 8000 rpm (4°C, 5 min), and the supernatant was collected. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed gently, and centrifuged at 14,000 rpm (4°C, 15 min) to remove cellular debris. The aqueous phase was

transferred to a fresh tube, mixed with chloroform: isoamyl alcohol (24:1), and centrifuged again. The resulting supernatant was combined with pre-chilled isopropanol and incubated overnight at -20 °C for DNA precipitation. The next day, DNA was pelleted by centrifugation, washed with 70% ethanol, air-dried, and dissolved in 40 µl of nuclease-free buffer. To remove RNA contamination, 10 µl of RNase A was added, followed by incubation at 60 °C before storing the sample at -20°C.

Primer	Forward sequence	Reverse sequence	Annealing Tm(^{0}C)	Reference
ASSR-1	GTCCGTTG AAAAACAA AGAG	CGTTTTA GGTTTCT TCTCTGC	55	Singh *et al.* (2013)
ASSR-23	CTTTCCCT TCTCTCTC AACAC	AAGCAG AAGCAGA AGCAGAG	55	
ASSR-143	AACCGAT GCTTTCTT CTACTAC	ACTCAAC GGTGCTA CTCATC	55	
ASSR-363	GGGAGA AGTATAAG GAGAAATG	TCACCCT TTGATAA TGTTCC	55	

PCR amplification: The DNA samples isolated were subjected to amplification by three SSR primers namely ASSR 1, ASSR 23 and ASSR 363 (Table 1). Polymerase chain reaction (PCR) reaction mixture (15 µl) consisted of 2µl of genomic DNA, 7 µl of Master mix, 1 µl reverse and forward primer each and 4 µl nuclease free water. DNA amplification was carried out in a thermal cycler (Veriti, Singapore) with PCR reaction cycles consisting an initial denaturing step at 94°C for 3 min followed by 35 cycles with a denaturing step at 94°C for 30 s, a primer annealing step at 55°C for 1 min and an extension step at 72°C for 1 min. After the last cycle, samples were kept at 72°C for 5 min for final extension (Table 1). The PCR products of each sample were subjected to electrophoresis on 5 per cent agarose gel along with a 100 bp DNA ladder (Thermo Fisher Scientific, USA). The gel electrophoresis was carried out for three hours at 60 V. After completion, the agarose gel was visualized in a gel document machine (UVITEC, U. K). Upon confirmation of the amplifications, the gel was photographed in a gel documentation unit and different amplicons obtained for each sample were measured for their band size in comparison with marker.

Table 1 : Details of PCR reaction steps.

Steps	Programme		No. of cycles
	Temp (°C)	Duration	
Initial denaturation	95	3 min	1
Denaturation	95	30sec	35 cycles
Annealing	55	1 min	
Extension	72	3 min	
Final extension	72	5 min	1

Results and Discussion

In vivo screening of pigeonpea genotypes in wilt sick plots

In the present study during the *Kharif* season of 2023-24, 60 pigeonpea (*Cajanus cajan*) genotypes were evaluated for resistance to *Fusarium* wilt (*Fusarium udum*) in sick plot. Diseased plants expressed symptoms starting from 30 days after sowing. The pathogen being soilborne in nature, infects the host plant by entering the vascular system through root tips or wounds, leading to progressive chlorosis of leaves and branches, wilting, and eventual root system collapse (Jain and Reddy, 1995). Although the fungus infects the plant during the early seedling stage, symptoms do not become visible until later in crop development (Reddy *et al.*, 1990; Hillocks *et al.*, 2000). The initial symptoms included loss of turgidity in leaves and interveinal clearing. Leaves exhibited slight chlorosis, sometimes turning bright yellow before wilting (Reddy *et al.*, 1990). A distinguishing characteristic of the disease partial wilting of the plant, resembling water deficiency despite sufficient soil moisture was noticed in affected plants. Lateral root infection responsible for partial wilting and tap root infection causing total wilt (Nene, 1980; Reddy *et al.*, 1993) both were visible. The most notable internal symptom with a distinct purple band extending upward from the base of the main stem was visible among the wilting plants. Infected xylem tissues developed black streaks, leading to brown or dark purple bands on the stem surface of partially wilted plants.

In this study, the 60 pigeonpea genotypes and two checks were categorized based on their mean percent disease incidence (PDI), which ranged from 7.47 per cent to 86.83 per cent. Four genotypes—ICP X 140203-B-1, TDRG 272, ICP 8863, and GRG-811 were identified as resistant, with ICP X 140203-B-1 showing the lowest disease incidence at 7.47 per cent, followed by TDRG 272 (8.2%), ICP 8863 (9.09%), and GRG-811 (9.75%). Additionally, 41 genotypes were classified as moderately resistant, with disease incidence between 10.01 per cent and 30 per cent. The remaining 17 genotypes were categorized as susceptible, with disease incidence exceeding 30 per cent. Among the susceptible genotypes, ICP 2376, used as a susceptible check, recorded the highest disease incidence at 89.30 per cent. The resistant check, ICP 8863, confirmed its resistance with a disease

Table 2 : Comparison of wilt incidence and disease reaction in pigeonpea genotypes screened against *F. udum* through different techniques.

S. no.	Genotypes	Screening methods			
		Field screening		Root dip	
		PDI (%)	Reaction	PDI (%)	Reaction
1	ICP x 140203- B1	7.47	R	6.67	R
2	GRG 811	9.75	R	15.00	MR
3	GRG 152	15.24	MR	13.34	MR
4	TS-3R	47.7	S	58.35	S
5	NAM 2217	19.69	MR	26.67	MR
6	TDRG 272	8.2	R	16.67	MR
7	CORG 9701	41.88	S	36.33	S
8	PHULE TUR	21.11	MR	18.35	MR
9	ICPL 87	25.86	MR	23.33	MR
10	ICP x 140196-B-1	36.11	S	41.67	S
11	NAM 2314	18.7	MR	21.76	MR
12	NAM 2284	21.93	MR	21.55	MR
13	NAM 314	22.37	MR	18.38	MR
14	NAM 2282	23.71	MR	23.39	MR
15	NAM-88	20.15	MR	28.73	MR
16	IC 73885	48.6	S	48.33	S
17	IC 73058	45.37	S	41.67	S
18	IC74013	20.76	MR	16.60	MR
19	IC 405218	34.68	MR	31.67	S
20	IC 73898	25.47	MR	26.67	MR
21	IC 73995	22.62	MR	23.33	MR
22	IC73975	18.45	MR	26.67	MR
23	IC 73952	24.17	MR	28.33	MR
24	WRG 93	18.26	MR	16.67	MR
25	PT -0012	34.62	MR	43.33	S
26	WRGE -150	18.99	MR	26.68	MR
27	CRG 18004	25.16	MR	16.67	MR
28	BDN -2019-29	17.41	MR	18.33	MR
29	ICAKTM 19424	48.55	S	43.33	S
30	MIRA	60.15	S	58.33	S
31	WGR 443	33.45	MR	31.67	S
32	SKNP 2122	25.98	MR	21.67	MR
33	PA 714	51.56	S	41.66	S
34	BAUPP 19 -11	43.42	S	51.66	S
35	NTL 1127	14.36	MR	26.65	MR
36	AL 2362	48.5	S	66.77	S
37	NUPPC -68	48.61	S	48.34	S
38	SKNP 2107	28.63	MR	23.33	MR
39	IPAE 22-1	49.72	S	48.33	S
40	PAU 881	69.3	S	43.35	S
41	NAAM 88	23.81	MR	21.67	MR
42	PT 11- 16	53.76	S	53.38	S
43	PA -6	34.76	MR	28.38	S
44	WRG 225	30.53	MR	42.67	S
45	LRG 489	20.05	MR	48.33	S
46	RKVP 1165	73.99	S	58.43	S
47	PT 12- 19 -2	16.78	MR	51.67	S
48	KRG 33	21	MR	28.67	MR
49	ICP x 140213- B-3	26.19	MR	28.33	MR
50	ICP x 140188-B-3	31.11	S	33.32	S
51	EC 843239	16.27	MR	18.34	MR
52	NAM 2329	27.2	MR	23.33	MR
53	ICP x 140217 B-1	14.58	MR	‘16.67	MR
54	NAM 2292	27.08	MR	28.33	MR
55	NAM 2151	23.05	MR	26.67	MR
56	NAM 2085	24.11	MR	26.66	MR
57	WRGExICP 15028	18.11	MR	18.33	MR
58	IC 73959	21.88	MR	23.33	MR
59	IC 73969	25.08	MR	25.00	MR
60	IC 73961	15.03	MR	18.33	MR
61	ICP 8863 (R.C)	9.09	R	3.33	R
62	ICP 2376 (S.C)	89.3	S	78.34	S

incidence of 9.09 per cent (Table 2, Fig. 1). Based on these observations, the genotypes were grouped into three categories: resistant, moderately resistant, and susceptible. Similar findings were reported by different scientists earlier from their findings. Bisht *et al*. (2022) reported six resistant genotypes and seven moderately resistant genotypes with a disease incidence ranging between 21 and 40 per cent. Ravikumara *et al*. (2022) identified significant variability in disease reactions among pigeonpea genotypes. Their study revealed 12 genotypes with resistant reactions (0–10.00% PDI), 14 with moderate resistance (11–30.00% PDI), 11 with moderately susceptible reactions (31–35.00% PDI) and 15 highly susceptible genotypes exhibiting over 50 per cent disease incidence. Shinde *et al*. (2021) screened 24 pigeonpea genotypes and identified five genotypes as resistant, with wilt incidences of less than 10 per cent. Additionally, four genotypes were categorized as susceptible with wilt percentages between 20.1 and 40 per cent, while the remaining genotypes exhibited moderate resistance. These reports are in concurrence with our findings of classifying pigeonpea genotypes in three main broad groups based on their resistance nature.

***In vitro* screening of pigeonpea genotypes through root dip method :** After confirming the age of

Table 3 : Response of *Fusarium* wilt associated SSR markers screened agaisnt 60 pigeonpea genotypes

S. no.	Genotypes	Approximate size of amplification product(bp)		
		ASSR 1	ASSR 23	ASSR 363
1	ICP x 140203- B1	100 (-)	130 (-)	180 (-)
2	GRG 811	100 (-)	130 (-)	180 (-)
3	GRG 152	100 (-)	130 (-)	180(-)
4	TS-3R	120 (+)	150 (+)	200 (+)
5	NAM 2217	100 (-)	130 (-)	180 (-)
6	TDRG 272	100 (-)	130 (-)	180 (-)
7	CORG 9701	120 (+)	150 (+)	200 (+)
8	PHULE TUR	100 (-)	130 (-)	180 (-)
9	ICPL 87	100 (-)	130 (-)	180 (-)
10	ICP x 140196-B-1	120 (+)	130 (+)	180 (+)
11	NAM 2314	100(-)	130 (-)	180 (-)
12	NAM 2284	100 (-)	130 (-)	180 (-)
13	NAM 314	100 (-)	130 (-)	180 (-)
14	NAM 2282	100 (-)	130 (-)	180 (-)
15	NAM-88	100 (-)	130 (-)	180 (-)
16	IC 73885	120 (+)	150 (+)	200 (+)
17	IC 73058	120 (+)	150(+)	100 (+)
18	IC74013	100 (-)	130 (-)	180 (-)
19	IC 405218	120 (+)	150 (+)	200 (+)
20	IC 73898	100(-)	130(-)	180(-)
21	IC 73995	100 (-)	130 (-)	180 (-)
22	IC73975	100 (-)	130 (-)	180 (-)
23	IC 73952	100 (-)	130 (-)	180 (-)
24	WRG 93	100 (-)	130 (-)	180 (-)
25	PT -0012	120 (+)	150 (+)	200 (+)
26	WRGE -150	100 (-)	130 (-)	180 (-)
27	CRG 18004	100 (-)	130 (-)	180 (-)
28	BDN -2019-29	100 (-)	130 (-)	180 (-)
29	ICAKTM 19424	120 (+)	150 (+)	200 (+)
30	MIRA	120 (+)	150 (+)	200 (+)
31	WGR 443	120 (+)	150 (+)	200 (+)
32	SKNP 2122	100 (-)	130 (-)	180 (-)
33	PA 714	120 (+)	150 (+)	200 (+)
34	BAUPP 19 -11	120 (+)	150 (+)	200 (+)
35	NTL 1127	100 (-)	130 (-)	180 (-)
36	AL 2362	120 (+)	150 (+)	200 (+)
37	NUPPC -6B	120 (+)	150 (+)	200 (+)
38	SKNP 2107	100 (-)	130 (-)	180 (-)
39	IPAE 22-1	120 (+)	150 (+)	200 (+)
40	PAU 881	100 (-)	130 (-)	180 (-)
41	NAAM 88	100 (-)	130(-)	180 (-)
42	PT 11- 16	120 (+)	150 (+)	200 (+)
43	PA -6	120 (+)	150 (+)	200 (+)
44	WRG 225	120 (+)	150 (+)	200 (+)
45	LRG 489	120 (+)	150 (+)	200 (+)
46	RKVP 1165	120 (+)	150 (+)	200 (+)
47	PT 12- 19 -2	120 (-)	150 (+)	200 (+)
48	KRG 33	100 (-)	130 (-)	180 (-)
49	ICP x 140213- B-3	100 (-)	130 (-)	180 (-)
50	ICP x 140188-B-3	120 (+)	150 (+)	200 (+)
51	EC 843239	100 (-)	130 (-)	180 (-)
52	NAM 2329	100 (-)	130 (-)	180 (-)
53	ICP 8863 (R.C)	100 (-)	130 (-)	180 (-)
54	ICP 2376 (S.C)	120(+)	150(+)	200(+)
55	ICP x 140217 B-1	-	-	-
56	NAM 2292	-	-	-
57	NAM 2151	-	-	-
58	NAM 2085	-	-	-
59	WRGE x ICP 15028	-	-	-
60	IC 73959	-	-	-
61	IC 73969	-	-	-
62	IC 73961			

Sign within parentheses indicates the presence (+)/absence (“) of a SSR band. ‘+’ indicates presence of a band specific to *Fusarium* wilt susceptible check, ICP 2376 and ‘–’ indicates presence of a band at different position than in ICP 2376

the seedlings and the concentration of *Fusarium* inoculum for *in vitro* screening of pigeonpea against *F. udum*, seven-day-old seedlings of 60 genotypes and two checks, raised separately in plastic cups filled with sterile sand, were screened. The seedlings were dipped in a seven-day-old *F. udum* culture broth at 75 per cent concentration for 1-2 min and incubated for seven days.

Among the 60 genotypes and two checks, only one, ICP × 140203-B1, was found to be resistant, with a disease incidence of 6.67 per cent. The resistant check, ICP 8863, also remained resistant, with no incidence of wilt (Fig 2). Moderate resistance was observed in 37 entries with disease incidence ranging from 10 per cent to 30 per cent, whereas 23 genotypes were found to be susceptible. The susceptible check ICP 2376 showed a 78.34 per cent wilt incidence (Fig. 3). Based on the percentage of disease incidence, these genotypes were grouped into the resistant, moderately resistant and susceptible categories (Table 2, Fig. 4).

These results support previous research highlighting the role of controlled inoculation and incubation in reliably assessing disease resistance. (Nene and Kannaiyan, 1982) performed the root dip method where the disease incidence of the genotypes varied from 5 per cent to 75

Table 4 : SSR Marker analysis of Pigeonpea genotypes.

S. no.	Marker	Observed band size	No. of alleles	Polymorphic alleles	Per cent polymorphism	PIC	MI
1	ASSR 1	100-120	2	2	100	0.48	48.9
2	ASSR23	130-150	2	2	100	0.48	48.9
3	ASSR363	180-200	2	2	100	0.48	48.9

Table 5 : Association of SSR markers with *Fusarium* wilt resistance based on Mann-Whitney U and simple marker analysis.

S. no.	Markers	Mann-Whitney U		Single marker analysis
		W- value	P value	R^2
1	ASSR-1	646	0.003	24.08
2	ASSR-23	646	0.003	24.08
3	ASSR-363	646	0.003	24.08

per cent, while the resistant check (ICP 8863) remained unaffected. Haware and Nene (1994) were the first to use the root dip technique and observed more than 90 per cent wilt incidence within 20-26 days in genotypes ICP 2376, ICP 8518 and ICP 6997. Most recently Kshirsagar, 2023 screened 15 genotypes of pigeonpea against *Fusarium udum* using an inoculum concentration 6×10^5. Resistant pigeonpea germplasms exhibited a disease incidence between 0 per cent and 20 per cent, while moderately resistant germplasms showed a disease incidence ranging from 40 per cent to 50 per cent. Susceptible germplasms had a disease incidence between 75 per cent and 95 per cent.

Molecular screening using SSR markers : In this study, three SSR markers—ASSR-1, ASSR-23 and ASSR-363—were used to assess genetic variability and confirm resistance-associated alleles in 60 pigeonpea genotypes which were screened *in vivo* and *in vitro*. These markers produced six polymorphic alleles, with two per primer. ASSR-1 amplified ~120 bp in the susceptible check and ~100 bp in the resistant check (Fig. 5), ASSR-23 produced ~150 bp in the susceptible and ~130 bp in the resistant check (Fig. 6), while ASSR-363 amplified ~200 bp in the susceptible and ~180 bp in the resistant check (Fig. 7). Among the 60 genotypes, 29 matched the amplicon size of resistant check, 25 matched with the amplicon size of susceptible check, and eight showed no amplification, likely due to a lack of polymorphism in target regions (Fig. 8). These findings align with Singh *et al.* (2016), where ASSR-1 amplified 120 bp in susceptible genotypes and 100 bp in resistant genotypes, ASSR-23 produced 150 bp in susceptible genotypes and 135 bp in resistant genotypes and ASSR-363 yielded 200 bp in susceptible genotypes and 170 bp in resistant genotypes (Table 3).

The Polymorphic Information Content (PIC) value for SSR markers ASSR-1, ASSR-23 and ASSR-363 was 0.48, indicating polymorphism. This suggests sufficient genetic variation to differentiate pigeonpea genotypes based on resistant and susceptible reaction. The Marker Index (MI) was 48.9, reflecting a high ability to distinguish genetic variation (Table 4). Singh *et al.* (2013) also reported two polymorphic alleles per marker, with PIC values of 0.49 for ASSR-1, 0.42 for ASSR-23, and 0.76 for ASSR-363.

Mann Whitney U test for pigeonpea genotypes for *Fusarium* wilt resistance using SSR markers: SSR marker data was analyzed using the nonparametric Mann-Whitney U test to assess their association with *Fusarium* wilt resistance in pigeonpea. This test effectively handles small sample sizes and evaluates distribution differences between groups. The analysis yielded a consistent W value of 646 for all three markers, indicating uniform trends. A p-value of 0.003 for each marker, which is below the significance threshold ($p < 0.05$), confirmed statistically significant differences between groups (Table 4). Singh *et al.* (2013) also found a significant association of six SSR markers, including ASSR-1, ASSR-23, and ASSR-363, with *Fusarium* wilt resistance using K-W ANOVA on 36 pigeonpea genotypes.

Single marker Analysis (SMA) of pigeonpea genotypes for *Fusarium* wilt resistance: Single marker analysis was performed using genotypic data (presence or absence) and phenotypic data (disease scores) to identify the association of SSR markers with *Fusarium* wilt resistance. This data was then subjected to linear model regression analysis. All markers exhibited significant R^2 values, with each marker explaining phenotypic variation of 24.08 per cent (Table 5). The results indicated that all 3 SSR markers were linked to wilt resistance in pigeonpea genotypes. Among the markers linked to *Fusarium* wilt disease resistance, those accounting for more than 20 per cent of the explained phenotypic variation (R^2%) of the trait were considered important. With this we could conclude that the markers used in the study are associated with FW disease and can act as

Table 6 : Comparative analysis of SSR markers reaction with different screening techniques employed for screening against *F. udum* causing wilt in pigeonpea.

S. no.	Genotypes	Screening methods		
		Field screening	Root dip	Molecular screening
1	ICP x 140203- B1	R	R	R
2	GRG 811	R	MR	R
3	GRG 152	MR	MR	R
4	TS-3R	S	S	S
5	NAM 2217	MR	MR	R
6	TDRG 272	R	MR	R
7	CORG 9701	S	S	S
8	PHULE TUR	MR	MR	R
9	ICPL 87	MR	MR	R
10	ICP x 140196-B-1	S	S	S
11	NAM 2314	MR	MR	R
12	NAM 2284	MR	MR	R
13	NAM 314	MR	MR	R
14	NAM 2282	MR	MR	R
15	NAM-88	MR	MR	R
16	IC 73885	S	S	S
17	IC 73058	S	S	S
18	IC74013	MR	MR	R
19	IC 405218	MR	S	S
20	IC 73898	MR	MR	R
21	IC 73995	MR	MR	R
22	IC73975	MR	MR	R
23	IC 73952	MR	MR	R
24	WRG 93	MR	MR	R
25	PT -0012	MR	S	S
26	WRGE -150	MR	MR	R
27	CRG 18004	MR	MR	R
28	BDN -2019-29	MR	MR	R
29	ICAKTM 19424	S	S	S
30	MIRA	S	S	S
31	WGR 443	MR	S	S
32	SKNP 2122	MR	MR	R
33	PA 714	S	S	S
34	BAUPP 19 -11	S	S	S
35	NTL 1127	MR	MR	R
36	AL 2362	S	S	S
37	NUPPC -68	S	S	S
38	SKNP 2107	MR	MR	R
39	IPAE 22-1	S	S	S
40	PAU 881	S	S	R
41	NAAM 88	MR	MR	R
42	PT 11- 16	S	S	S
43	PA -6	MR	S	S
44	WRG 225	MR	S	S
45	LRG 489	MR	S	S
46	RKVP 1165	S	S	S
47	PT 12- 19 -2	MR	S	S
48	KRG 33	MR	MR	R
49	ICP x 140213- B-3	MR	MR	R
50	ICP x 140188-B-3	S	S	S
51	EC 843239	MR	MR	R
52	NAM 2329	MR	MR	R
53	ICP 8863 (R.C)	R	R	R
54	ICP 2376 (S.C)	S	S	S
55	ICP x 140217 B-1	MR	MR	Unamplified
56	NAM 2292	MR	MR	Unamplified
57	NAM 2151	MR	MR	Unamplified
58	NAM 2085	MR	MR	Unamplified
59	WRGE x ICP 15028	MR	MR	Unamplified
60	IC 73959	MR	MR	Unamplified
61	IC 73969	MR	MR	Unamplified
62	IC 73961	MR	MR	Unamplified

indicators for resistance genes.

According to Singh *et al.* (2013), the six SSR marker ASSR-1, ASSR-23, ASSR-148, ASSR-229, ASSR-363 and ASSR-363 were substantially linked to resistance to *Fusarium* wilt. Of these, ASSR-363 explained the greatest amount of phenotypic variation brought on by FW resistance, accounting for 56.4 per cent of the variation, while the other markers explained phenotypic variation ranging from 23.7 to 56.4 per cent.

Cluster analysis of pigeonpea genotypes for *Fusarium* wilt resistance using SSR markers: Genetic similarity values were used from 60 pigeonpea genotypes and 3 SSR markers to create a dendrogram based on resistance and susceptibility to the *Fusarium* wilt reaction. The dendrogram was constructed using Jaccard's similarity coefficient and UPGMA, classifying genotypes into two main clusters using the R software (Fig. 9). Cluster A with 31 resistant genotype and Cluster B with 23 susceptible genotypes. The Jaccard's similarity coefficient was ranging from 0.0 to 0.8. Prajapati *et al.* (2014) identified two clusters with the highest and lowest similarity matrix values at 0.90 and 0.52, respectively. Similarly, Bisht *et al.* (2022) identified three different clusters based on wilt resistant genotypes.

Correlation between screening methods and SSR marker analysis for *Fusarium* wilt resistance in pigeonpea: Genotypic analysis of pigeonpea for *Fusarium* wilt resistance was conducted through field screening, *in vitro* screening, and molecular screening

Susceptible check- ICP 2376

Resistant check – ICP 8863

Field view of screening of pigeonpea genotypes in sickplot against *Fusarium* wilt

Fig. 1 : *In vivo* screening of pigeonpea genotypes in wilt sickplots against *Fusarium* wilt.

Fig. 2 : Response of ICP 2376 seedlings against 75 per cent *F.udum* inoculum oncentration.

Fig. 3 : Response of ICP 2376 seedlings against 75 per cent *F.udum* inoculum concentration.

using SSR markers (ASSR-1, ASSR-23, and ASSR-363). Field and *in vitro* screening classified genotypes as resistant (R), moderately resistant (MR), or susceptible (S), while SSR markers confirmed molecular resistance or susceptibility. In molecular screening three genotypes (ICP x 140203-B1, GRG 811, and TDRG 272) amplified at 100 bp, 130 bp, and 180 bp with markers ASSR 1, ASSR 23 and ASSR 363, respectively. These amplicon sizes are similar to ICP 8863 (Resistant check), hence confirming resistance. Among the three genotypes GRG 811 and TDRG 272 showed resistant reaction in the field but moderately resistant reaction through root dip method. This could be because under *in vitro* conditions all the environmental factors are eliminated and the pathogen are in direct contact with the host. 21 genotypes that were susceptible in field and *in vitro* screening matched the amplicon size of susceptible check ICP 2376 confirming its susceptibility. Of 33 moderately resistant genotypes, 26 matched the amplicon size of resistant check ICP 8863, indicating genotypic resistance, which may be because molecular screening detects specific resistance-associated alleles at the genetic level, which may not always translate directly to field performance due to gene expression regulation or interactions with other genetic and environmental factors, while seven genotypes matched the amplicon size of susceptible check, suggesting genotypic susceptibility. Their moderate field resistance may result from environmental factors influencing disease severity. PAU 881 was susceptible in field and *in vitro* screening but resistant in molecular screening, indicating genetic resistance that may not be fully expressed under field and *in vitro* screening conditions. While PAU 881 has potential for resistance

Response of GRG 152 seedlings against *F. udum*

Response of GRG 152 seedlings against *F.udum*

Response of ICPx140203-B1 seedlings against *F.udum*

Response of TS- 3R seedlings against *F.udum*

Fig. 4 : *In vitro* screening of pigeonpea genotypes through root dip method.

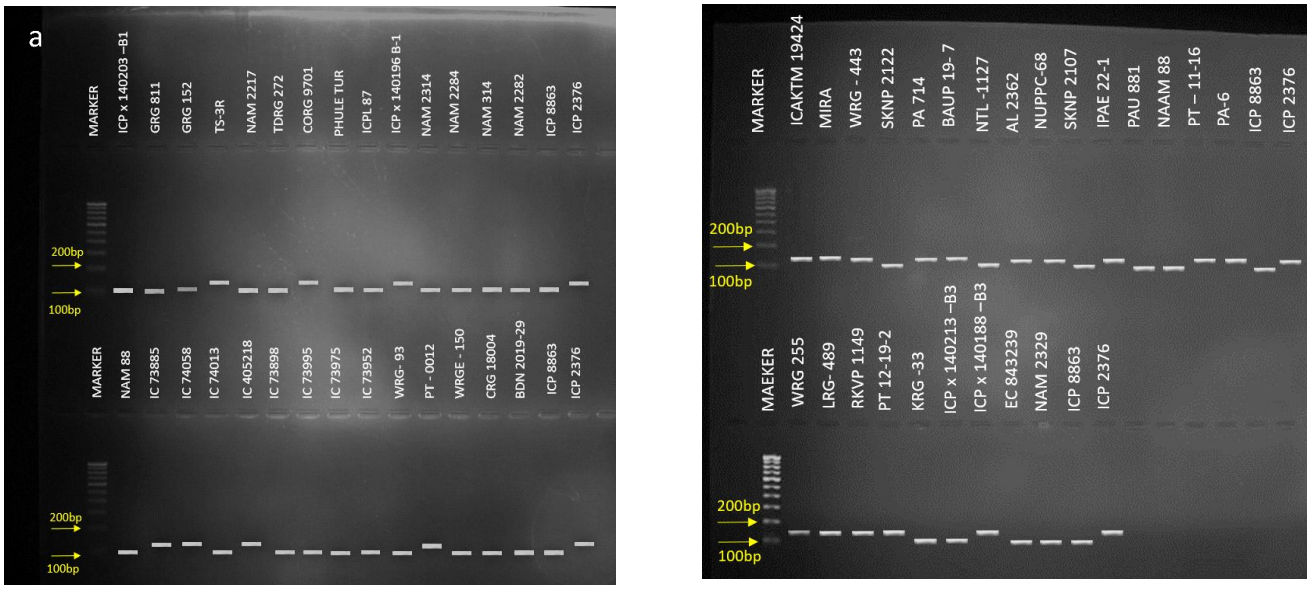

Fig. 5 : Amplification of pigeonpea genotypes by ASSR-1 marker indicating resistance and susceptibility of genotypes against *Fusarium* wilt.

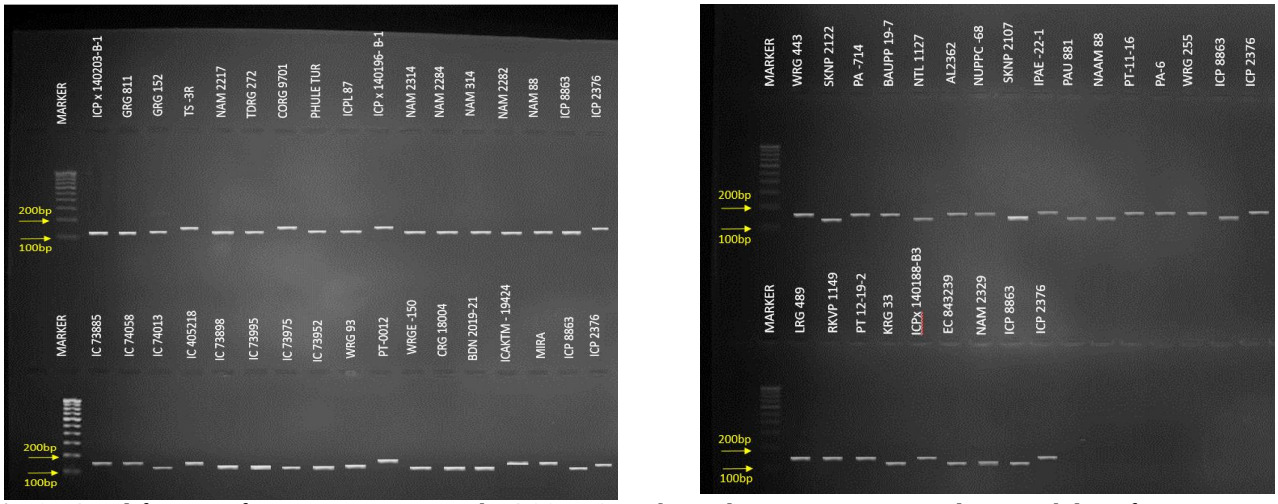

Fig. 6 : Amplification of pigeonpea genotypes by ASSR-23 marker indicating resistance and susceptibility of genotypes against *Fusarium* wilt.

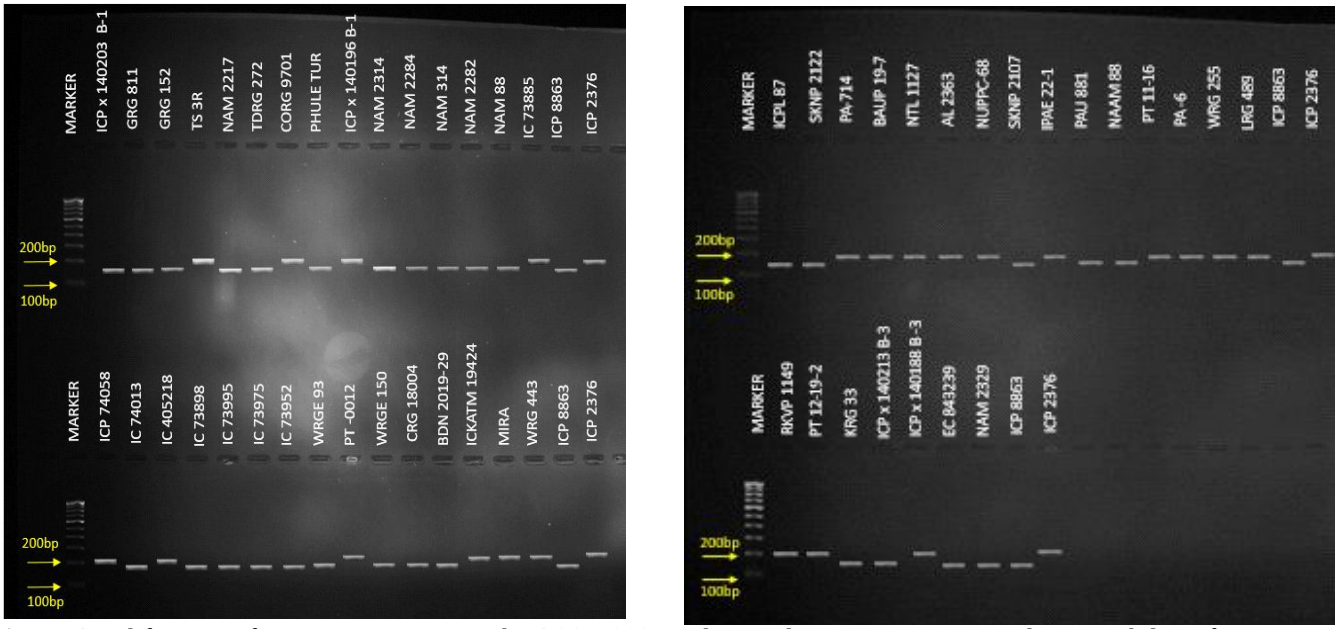

Fig. 7 : Amplification of pigeonpea genotypes by ASSR 363 markers indicating resistance and susceptibility of genotypes against *Fusarium* wilt.

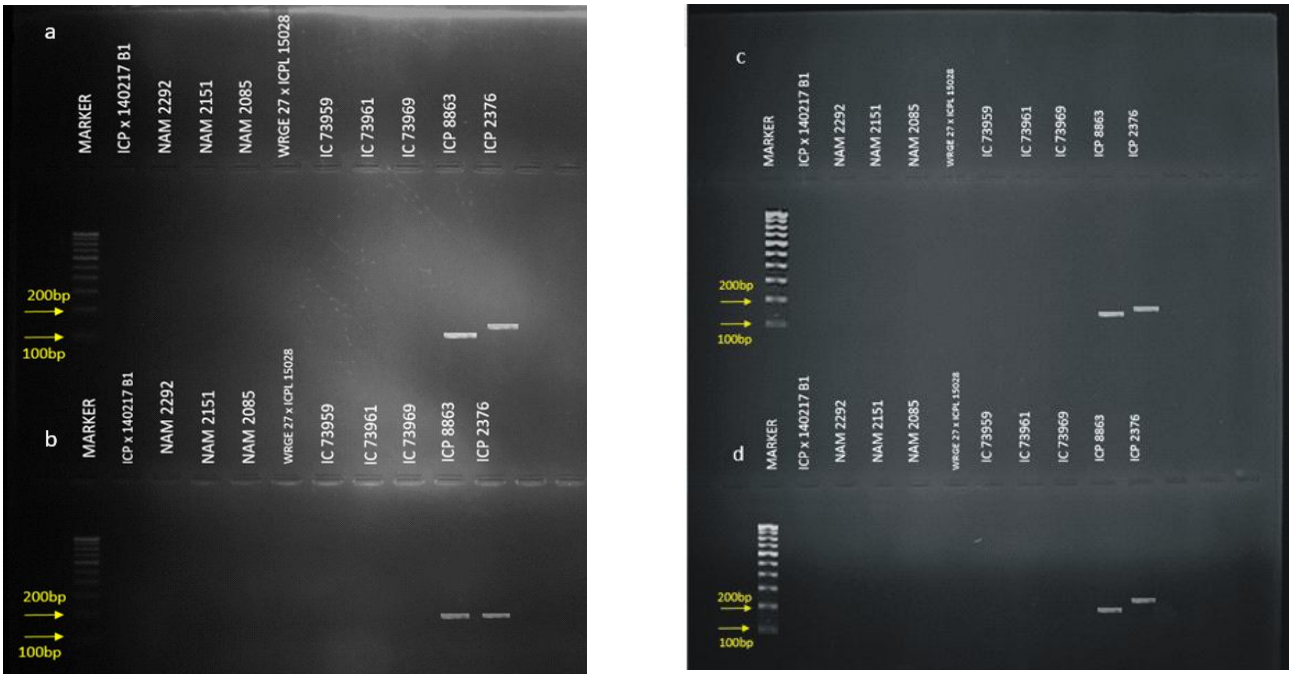

Fig. 8 : Pigeonpea genotypes that remained unamplified with the SSR markers a) ASSR-1 b) ASSR-143 c) ASSR -23 d) ASSR-363.

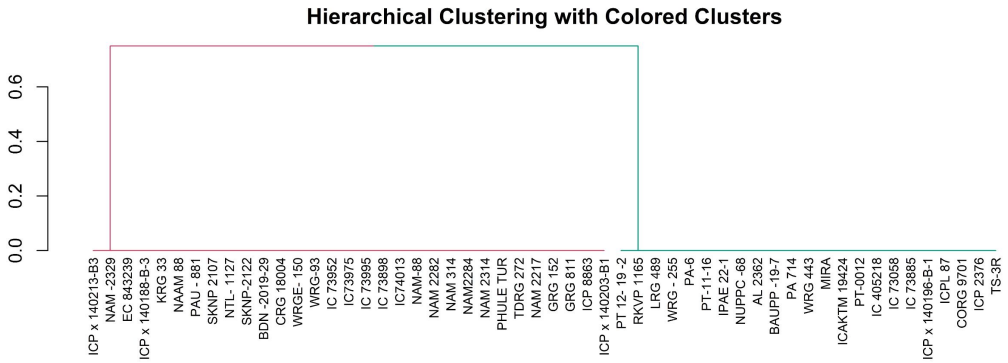

Fig. 9 : Dendrogram of pigeonpea genotypes based on their response to SSR markers generated by UPGMA cluster analysi.

breeding, its inconsistent field performance limits its immediate use. (Table 6). Overall, SSR markers effectively confirm resistance and are valuable for *Fusarium* wilt resistance breeding in pigeonpea.

Conclusion

The molecular characterization of pigeonpea genotypes using SSR markers successfully validated resistance against *Fusarium* wilt by identifying specific alleles linked to resistance traits. This validation highlighted the potential of SSR markers for accurately predicting resistance traits and enabled the differentiation of resistant and susceptible genotypes with high precision. These findings provide a scientific basis for utilizing SSR markers in marker-assisted selection to enhance the efficiency of breeding programs. By enabling the rapid and precise screening of large pigeonpea germplasm collections, SSR markers accelerate the identification and selection of resistant genotypes. The insights gained from this molecular approach contribute to understanding the genetic mechanisms underlying *Fusarium* wilt resistance. These results accelerate and hasten future research and breeding efforts aimed at developing *Fusarium* wilt-resistant pigeonpea varieties. The inability of primes to amplify few genotypes gives scope for research and development of more specific and reliable markers.

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