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Genetic Diversity Studies and Screening for Fusarium Wilt (*Fusarium udum* Butler) Resistance in Wild Pigeonpea Accessions, *Cajanus scarabaeoides* (L.) Thouars

NR Nagaraja*[1], MB Arun Kumar[2], CT Manjunath Prasad[2], Rajkumar[3], Parimal Sinha[2] and Jaagrati Jain[2]
[1]*ICAR-Central Plantation Crops Research Institute, Regional Station, Vittal-574243, Karnataka, India*
[2]*ICAR-Indian Agricultural Research Institute, New Delhi-110012, India*
[3]*ICAR-Central Plantation Crops Research Institute, Kasaragod-671124, Kerala, India*

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Wild relatives play an important role in the genetic improvement of cultivated crops. *Cajanus scarabaeoides* (L.) Thouars is one of the important wild species, which possess several desirable traits. Investigations on 67 accessions of *C. scarabaeoides* and 3 cultivated varieties for total soluble seed protein profiles using Sodium Dodecyl Sulphate-Poly Acryl amide Gel Electrophoresis resolved the protein bands ranged from 4-11. Based on the presence or absence of bands, similarity values were calculated and dendrogram was constructed using Unweighted Pair Group Method with Arithmetic Mean analysis. The dendrogram formed two main clusters. The first main cluster consisted of three cultivated varieties and 55 accessions of *C. scarabaeoides* and the second main cluster comprised of remaining 12 accessions of *C. scarabaeoides.* Similarity percentage ranged from 38.5 to 100. ICP15728 is the lone genotype which is distinctly different from remaining genotypes formed separate sub-cluster in the second main cluster with 38.5% similarity. Thirty eight accessions of *C. scarabaeoides* were screened against Fusarium wilt under glass house conditions. Of the 38 accessions screened seven accessions (ICP12707, ICP15689, ICP15692, ICP15732, ICP15744, ICP15748 and ICP15754) showed resistance to Fusarium wilt. Therefore, these accessions could be utilized in breeding programmes to increase the levels of resistance to Fusarium wilt in the pigeonpea cultivars, after further testing.

Key Words: *Cajanus scarabaeoides* accessions, Fusarium wilt, Genetic variability, Resistance screening, Wild species

Introduction

Pigeonpea is an important grain legume crop grown in the tropics and subtropics. The Indian subcontinent, eastern Africa and central America are the world's three main pigeonpea producing regions (Mallikarjuna *et al*., 2011) with Indian subcontinent contributing 75-80% to world's production (Sharma *et al*., 2012).

Pigeonpea is attacked by a range of biotic and abiotic factors, which are major constraints to increase productivity of pigeonpea (Mallikarjuna *et al*., 2011). Among the biotic factors, more than 60 pathogens including fungi, bacteria, viruses, mycoplasma, and nematodes can infect pigeonpea. Of these, Fusarium wilt and Phytophthora blight are widespread across regions (Reddy *et al*., 2012).

Fusarium wilt caused by soil-borne fungus *Fusarium udum* Butler is an important fungal disease prevalent in the pigeonpea growing areas it is more severe in Indian subcontinent (Singh *et al*., 2013). In Indian subcontinent the crop losses ranged from 16 - 47% (Prasad *et al*., 2003) and wilt incidence is believed to have increased significantly over the time (Gwata *et al*., 2006). The annual losses due to wilt have been estimated at US$71 million in India and US$5 million in eastern Africa (Reddy *et al*., 2012).

Although it has been suggested that wilt incidence can be reduced by various practices, host plant resistance would be the most effective, economical and environmental friendly management practice for disease control. There are only few sources of resistance reported for Fusarium wilt (Prasanthi *et al*., 2009; Karimi *et al*., 2010). But the wild relatives of crops are important sources of resistance to biotic and abiotic constraints (Mallikarjuna *et al*., 2007). Pigeonpea offers a rich source of variability in the form of wild relatives, which could be utilized for disease resistance, good agronomic traits, enhancing nutritional quality, identification and diversification of cytoplasmic base of cytoplasmic male sterility (CMS) system etc. (Singh *et al*., 2013).

**Author for Correspondence: Email: naga_gene@yahoo.com*

Cajanus scarabaeoides, C. acutifolious, C. sericeus and *C. albicans* are some of the wild *Cajanus* species with resistance to pigeonpea pod borer *Helicoverpa armigera* (Sujana *et al.*, 2008). Hence, assessment of genetic variability present in accessions of *C. scarabaeoides* is important for better utilization of germplasm. Successful attempts were made by researchers to study the genetic diversity present in cultivated and wild species which includes seed protein analysis (Ladizinsky and Hamel, 1980; Jha and Ohri, 1996), which are accession specific help in detecting diversity among the wild and cultivated species.

The protein profiling of germplasm and genetic markers have been widely and effectively used to determine the taxonomic and evolutionary aspects of several crops (Murphy *et al*., 1990; Khan, 1990; Das and Mukarjee, 1995; Ghafoor *et al*., 2002). Knowledge of genetic variability present in crop plants is important to plan the breeding programmes. Therefore, this study was designed to assess the genetic variability and also to screen the accessions of *C. scarabaeoides* to identify the Fusarium wilt resistance which could be utilized in pigeonpea disease resistance breeding programmes.

Materials and Methods

Plant Material

The experimental material for genetic diversity study comprised of seeds of 67 accessions of *C. scarabaeoides* collected from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana (Table 1) and three cultivated varieties of pigeonpea *viz.,* Pusa 33, Pusa 855 and Pusa 991. Thirty eight accessions of *C. scarabaeoides*, three cultivated varieties (Pusa 33, Pusa 855 and Pusa 991) and one susceptible variety-Bahar (as a check) was used to screen against Fusarium wilt resistance (Table 2).

Preparation of Protein Samples

The total soluble proteins were analyzed following the procedure described by Laemmli (1970). Five seeds of each genotype were ground to powder and defatted using defatting solvent mixture. Defatting was performed for 9 h with at least three solvent changes and later the ground material was air dried by leaving it open at room temperature overnight. Protein extraction was carried out by homogenizing finely ground cotyledon meal in 25mM Tris-glycine buffer (pH 8.3) overnight. Clear supernatant was decanted after centrifuging it for

Table 1. Details of *C. scarabaeoides* accessions used for total soluble proteins studies

ICP No.	Alternative accession identifier	Origin
12707		India
15683	ICPW 082	India
15684	ICPW 083	India
15685	ICPW 084	India
15687	ICPW 086	India
15688	ICPW 087	India
15689	ICPW 088	India
15690	ICPW 089	India
15691	ICPW 090	India
15692	ICPW 091	India
15693	ICPW 091	India
15694	ICPW 093	Sri Lanka
15695	ICPW 094	Sri Lanka
15696	ICPW 095	Myanmar
15697	ICPW 096	India
15699	ICPW 098	India
15700	ICPW 099	India
15702	ICPW 101;JM 4880	India
15703	ICPW 101;JM 4893	India
15704	ICPW 103;JM 4989	India
15705	ICPW 104;JM 5012	India
15706	ICPW 105;JM 5040	India
15707	ICPW 106; JM 5054	India
15708	ICPW 107; JM 5056	India
15709	ICPW 108;JM 5076	India
15710	ICPW 109	India
15711	ICPW 110	India
15712	ICPW 111	India
15713	ICPW 112	India
15716	ICPW 115	India
15717	ICPW 116	India
15718	ICPW 117	India
15721	ICPW 120	Philippines
15722	ICPW121	India
15724	ICPW 123;RPSP 458	India
15725	ICPW 124;RPSP 827	
15726	ICPW 125	India
15727	ICPW 126	India
15728	ICPW 127	India
15730	ICPW 129	India
15731	ICPW 130; Jangalapalli	India
15732	ICPW 131	India
15733	ICPW 132; ARKS 12347	India
15734	ICPW 133; EC 121206;NT2510	Australia
15735	ICPW 134;EC 121207;NT 2495	Australia
15736	ICPW 135; EC 122342	Fiji
15737	ICPW 136;EC 122344	Fiji
15738	ICPW 137; IW 3339	India
15739	ICPW138;IW 4353	India
15740	ICPW 139;IW 3463	India
15742	ICPW141;IW 2458.	Australia
15744	ICPW143;IW 2496	Australia
15745	ICPW 144; NT2510	Australia
15746	ICPW 145	India
15747	ICPW 146	India
15748	ICPW 147	India
15749	ICPW 148	India
15750	ICPW 149	India
15751	ICPW 150	India
15752	ICPW 151	India
15753	ICPW 152	India
15754	ICPW 153	India
15755	ICPW 154	India
15756	ICPW 155	India
15757	ICPW 156	India
15758	ICPW 157	India
15759	ICPW 158	India

10 min. at 10,000 rpm and 0.2 ml of the Tris-glycine extract was taken and diluted with equal amount of the working sample buffer in each genotype. The mixture was boiled for 10 min., cooled and used for electrophoresis fractionation.

Gel Electrophoresis

The gel of 1mm thickness was prepared by using resolving gel and stacking gel. While preparing the gel all the reagents of resolving gel were mixed well and poured between the plates of the cassette. Care was taken to avoid air bubbles to be trapped in the gel solution and 3/4th of the cassette was filled with resolving gel and it was allowed to polymerize. After the resolving gel gets polymerized the reagents of the stacking gel were mixed thoroughly and poured carefully above it and a comb was inserted immediately without trapping any bubble and gel was allowed to polymerize.

Once the gel was polymerized 5µl of the protein sample was loaded in each well using a syringe. The cassette was fitted into the electrophoresis tank and also the lower and upper tanks were filled with electrode buffer. The electrophoresis apparatus was connected to the power pack by fitting the electrodes into sockets of the identical colour. Voltage and power were turned to maximum. Current was adjusted to 1.5 m Amp per well till the tracking dye reached the bottom of the gel.

Gel Fixing and Staining

The gel was carefully removed from the plates after the run and immersed in 150ml of fixing solution (*i.e.* 15% Tri-Chloro Acetic acid-TCA%) overnight. The gel was then rinsed with distilled water and mixture of 15ml of 2% comassie blue and 100ml of 15% TCA was added inside the tray containing the gel. Staining was done till the bands developed well. Later the stain was tipped off and the gel was destained in distilled water till the background was clear. Later the gels were washed and photographed.

Evaluation and Documentation

The electrophorograms were prepared measuring the distance of each band from the point of loading. The relative mobility (Rm) of each band was calculated as ratio of distance traveled by the band to the distance traveled by tracking dye and the bands are numbered on the basis of increasing Rm values.

Calculation of Similarity Indices

Similarity index values were calculated based on proportion of common fragments between two lanes by using the following formula:

$$F = \frac{2Mxy}{Mx+My}$$

Where, F is the similarity index, Mx is the number of bands in accession x, My is the number of bands in accession y and Mxy is the number of bands common to both x and y. Fx100 gives the percent similarity between two accessions, thus F=1.0 would mean that the patterns in the two accessions are identical. Phylogenetic tree indicating the relationship among the genotypes was obtained based on simple matching coefficients using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Numerical Taxonomy System (NTSYS-pc) analysis (Rohalf 1993).

Fusarium udum Spores

A virulent isolate of *F. udum* (spore concentration 10^{6}spores/g of soil) was collected from Division of Plant Pathology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi.

Raising of Seedlings for Fusarium wilt Resistance Screening

Seeds of *C. scarabaeoides*, cultivated varieties and susceptible variety of pigeonpea were grown in plastic seedling trays containing mixture of autoclaved riverbed sand, virulent isolate of *F. udum* and antibiotic. Seedlings were raised by watering with sterilized water.

Observations for Wilt Incidence

Observations were recorded regularly 15 Days after inoculation (DAI) to 45 DAI. The Percentage wilt incidence (PWI) was calculated as ratio of number of infected to the total number of plants and the genotypes were categorized using a disease rating scale of 1-9 (Nene *et al.*, 1981) as 1: resistant (no symptoms/ 0% incidence); 3: moderately resistant (10% or less incidence); 5: tolerant (11-20% incidence); 7: moderately susceptible (21-50% incidence); and 9: susceptible (51% or more incidence).

Results and Discussion

Genetic Diversity Studies

As a step towards biochemical characterization, 67 accessions of *C. scarabaeoides* along with three cultivated varieties were analyzed for total seed proteins using Sodium Dodecyl Sulphate-Poly Acryl amide Gel Electrophoresis (SDS-PAGE). The electrophoretic profile of total soluble proteins revealed characteristic banding pattern for all the 67 accessions and three cultivated

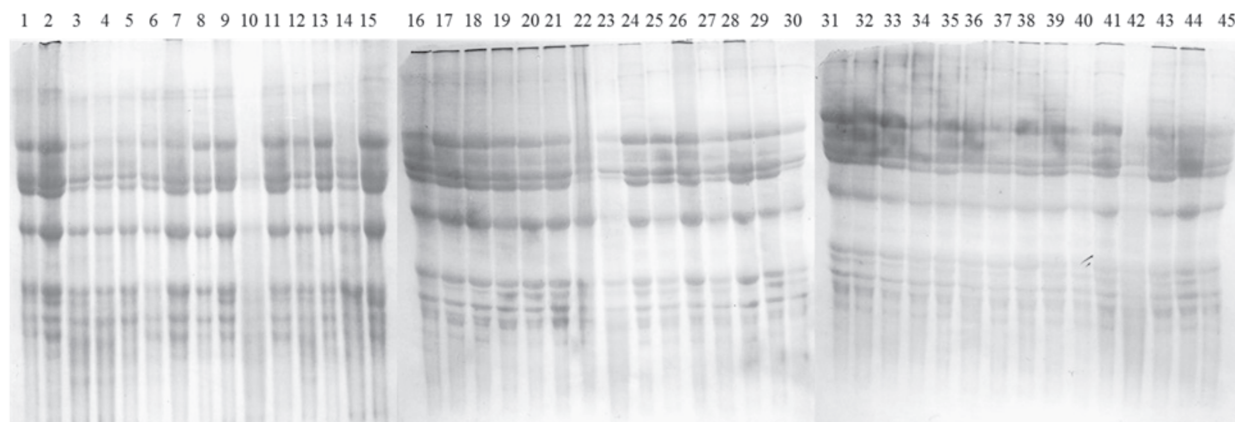

Fig. 1a. Electrophoregrams of accessions of *C. scarabaeoides* for total soluble proteins

Lanes 1-15; ICP15683, ICP15685, ICP15689, ICP15690, ICP15691, ICP15692, ICP15693, ICP15695, ICP15697, ICP15702, ICP15703, ICP15705, ICP15706, ICP15710, ICP15711. Lanes 16-30; ICP15737, ICP15740, ICP15742, ICP15744, ICP15747, ICP15748, ICP15749, ICP15750, ICP15751, ICP15752, ICP15753, ICP15754, ICP15755, ICP15756, ICP15757. Lanes 31-45; ICP15758, ICP15759, ICP15684, ICP15687, ICP15688, ICP15694, ICP15696, ICP15699, ICP15700, ICP15704, ICP15709, ICP15722, ICP15724, ICP15739, ICP15745

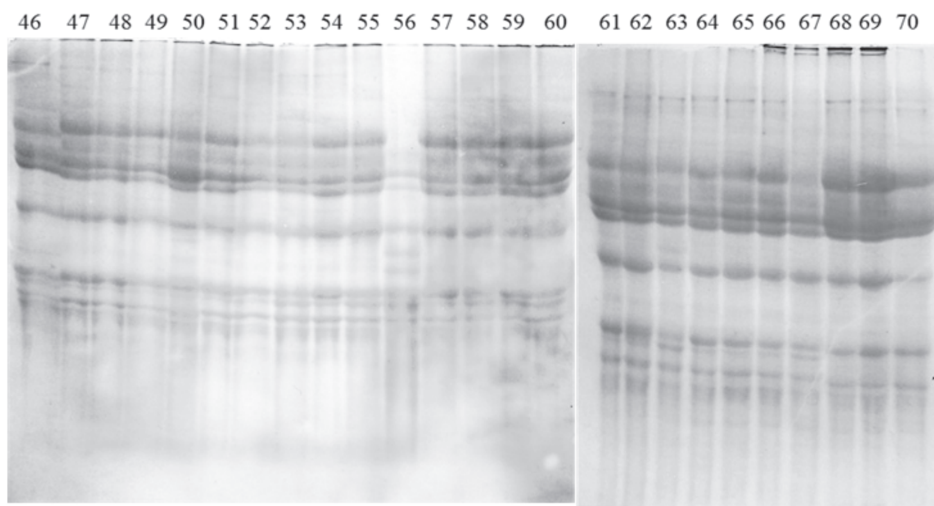

Fig. 1b. Electrophoregrams of accessions of *C. scarabaeoides* and cultivated varieties for total soluble proteins

Lanes 46-70; ICP12707, ICP15712, ICP15713, ICP15716, ICP15717, ICP15718, ICP15721, ICP15725, ICP15726, ICP15727, ICP15728, ICP15731, ICP15732, ICP15733, ICP15735, ICP15746, ICP15707, ICP15708, ICP15730, ICP15734, ICP15736, ICP15738, Pusa-33, Pusa-855, Pusa-991

varieties (Fig. 1a & 1b). The number of bands resolved ranged from 4, in case of ICP15710 to 11 (ICP15747, ICP15748, ICP15751, ICP15752, ICP15758, ICP15699, ICP15700, ICP15724, ICP15739). The band number *viz*., 4 (Rm0.34), 6 (Rm0.46), and 9 (Rm0.62) were common to all the accessions/ genotypes.

Based on the presence or absence of bands, similarity values were calculated for all pair wise comparisons made among 67 accessions of *C. scarabaeoides* and three cultivated varieties using Nei and Lis (1979) equation. The similarity values in percentage ranged from 38.5 to 100. Based on similarity matching coefficients, a dendrogram (phylogenetic tree) was constructed using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) analysis (Fig. 2). The dendrogram formed two main clusters for 67 accessions of *C. scarabaeoides* and three cultivated varieties. The first main cluster consisted of three cultivated varieties

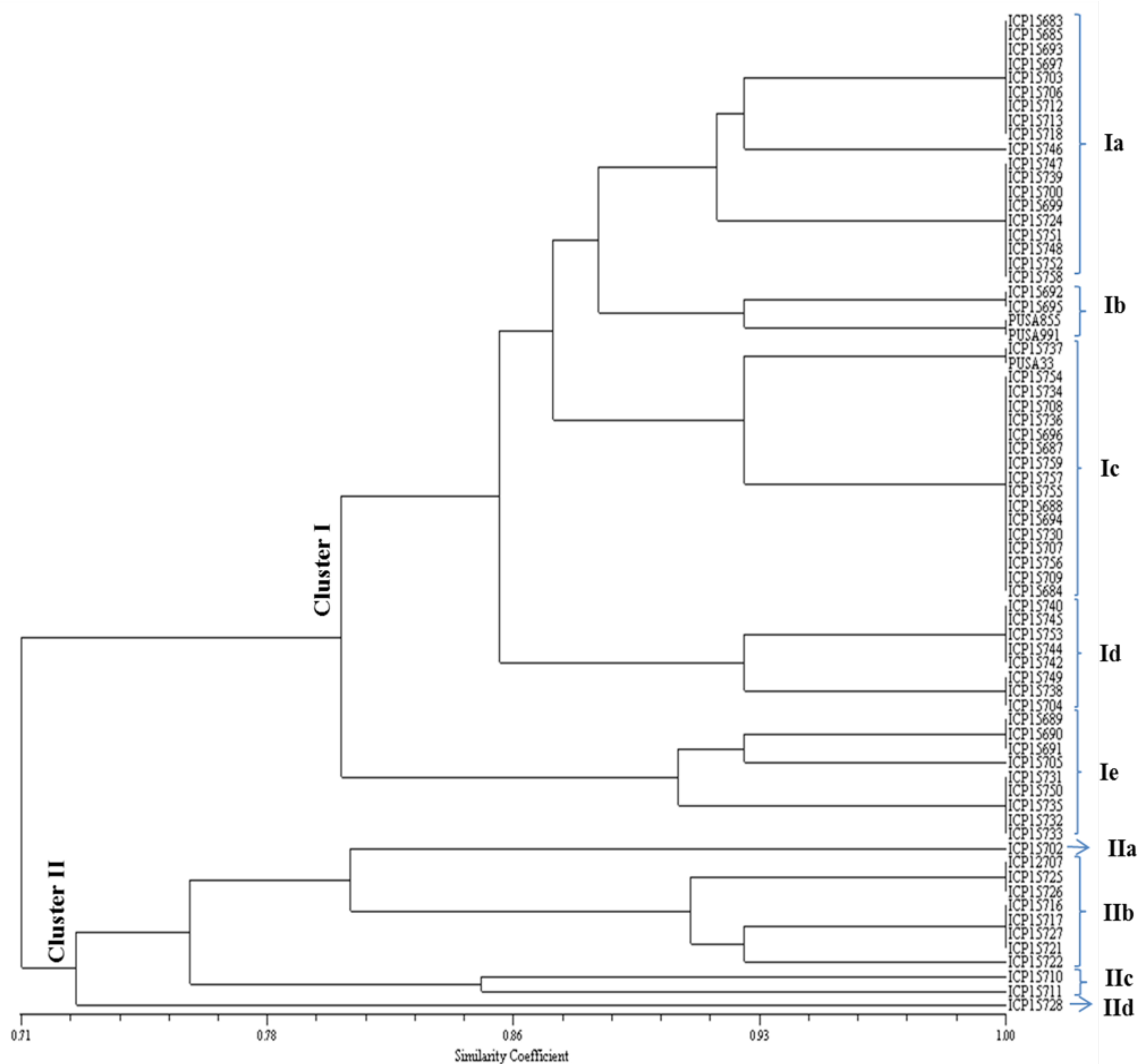

Fig. 2. Dendrogram based on total soluble proteins showing relationship of 67 accessions of *C. scarabaeoides* and three cultivated varieties of pigeonpea

and 55 accessions of *C. scarabaeoides* and the second main cluster comprised of remaining 12 accessions of *C. scarabaeoides.*

The first main cluster was further divided into five sub clusters viz., Ia (consisted of 19 accessions: ICP15683, ICP15685, ICP15693, ICP15697, ICP15703, ICP15706, ICP15712, ICP15713, ICP15718, ICP15746, ICP15747, ICP15739, ICP15700, ICP15699, ICP15724, ICP15751, ICP15748, ICP15752, ICP15758), Ib (two accessions and two cultivated varieties: ICP15692, ICP15695, Pusa-855, Pusa-991), IC (17 accessions & one cultivated variety: ICP15737, ICP15754, ICP15734, ICP15708, ICP15736, ICP15696, ICP15687, ICP15759, ICP15757, ICP15755, ICP15688, ICP15694, ICP15730, ICP15707, ICP15756, ICP15709, ICP15684, Pusa-33), Id (eight accessions: ICP15740, ICP15745, ICP15753, ICP15744, ICP15742, ICP15749, ICP15738, ICP15704), Ie (nine accessions: ICP15689, ICP15690, ICP15691, ICP15705, ICP15731, ICP15750, ICP15735, ICP15732, ICP15733). The second main cluster was also divided in to four sub-clusters *viz*., sub cluster IIa comprised of only one accession *i.e* ICP15702, sub cluster IIb

consisted of eight accessions *viz*., ICP12707, ICP15725, ICP15726, ICP15716, ICP15717, ICP15727, ICP15721, ICP15722, sub-cluster IIc consisted of two accessions *viz*., ICP15710, ICP15711 and sub cluster IId consisted of only one accession i.e ICP15728 which is diverse from all other genotypes.

Fusarium wilt Resistance Screening

C. scarabaeoides, a wild species of Indian origin, that has many desirable characters (Upadhyaya 2006), has multiple disease resistance (Kulkarni *et al*., 2003; Upadhyaya, 2006), is cross compatible with cultivated pigeonpea and inter-specific gene transfer is possible through conventional hybridization (Mallikarjuna *et al*., 2007). Therefore, in the present study 38 accessions of *C. scarabaeoides* were screened for Fusarium wilt resistance. The percent disease incidence ranged from 0-100% among the accessions of *C. scarabaeoides* (Table 2). Out of 38 accessions screened, seven accessions *viz*., ICP12707, ICP15689, ICP15692, ICP15732, ICP15744, ICP15748 and ICP15754 exhibited resistance with 0% disease incidence, and rest of the accessions showed moderately susceptible to susceptible reaction with percent disease incidence ranging from 22.22 to 100%. Three cultivated varieties (Pusa 33, Pusa 855 and Pusa 991) produced moderately susceptible to susceptible reaction with disease incidence ranging from 44.44 to 77.77% and the susceptible check (Bahar) showed 100% wilt incidence.

Out of seven accessions which exhibited resistant reaction with 0% of disease incidence, six accessions were placed in the first main cluster of the dendrogram (accession ICP15748 was placed at sub-cluster Ia, accession ICP15692 at sub-cluster Ib, accession ICP15754 at sub-cluster Ic, ICP15744 at sub-cluster Id and ICP15689, ICP15732 at sub-cluster Ie) and one accession ICP12707 at the sub-cluster IIb of the second main cluster. ICP15692 accession displayed resistance to Fusarium wilt in this study was also found to be resistant to both the mild and severe strains of *Pigeonpea sterility mosaic virus* in the earlier studies of Kulakarni *et al*. (2003).

A considerable amount of variation was observed based on SDS-PAGE that indicated the utilization of seed protein markers for germplasm classification in pigeonpea. SDS-PAGE of total soluble seed proteins was able to characterize and identify all the studied genotypes and can be employed effectively for identification of pigeonpea genotypes. This technique was employed by Drzewiecki (1990) in pea, Mudzana *et al*. (1995) in faba bean, Goyal and Sharma (2003) in cluster bean and these scientists successfully showed protein electrophoresis as powerful tool to identify the crop plants. For any successful crop improvement programmes characterization of variability present in wild relatives/ species is important. To assess the genetic variability present in crops and their wild relatives/ species total soluble proteins as biochemical markers can be employed.

Many studies were done previously on screening for resistance to Fusarium wilt and other diseases to identify the resistance source. To mention a few, Nene and Kannaiyan (1982) conducted experiment on field screening of more than 11,000 entries of pigeonpea and found 33 lines to be resistant to *F. udum*. Saxena *et al*. (1990) evaluated 33 accessions of *Atylosia scarabaeoides* at ICRISAT and detected accessions resistant to Fusarium wilt, Phytophthora blight, sterility mosaic and cyst nematode (*Heterodera* sp.). Prasanthi *et al*. (2009) screened 88 lines along with ICPL87119 and ICPL8863 resistant checks for Fusarium wilt under field conditions and artificial inoculation and identified 14 lines having 0-20% plant mortality. Jaagrati Jain (2006) tested 35 genotypes for multiple disease and insect resistance and identified five genotypes with multiple disease resistance to Fusarium wilt and sterility mosaic. Kumar *et al*. (2005) evaluated 115 wild *Cajanus* accessions belonging to six species viz. *C. albicans, C. platycarpus, C. cajanifolius, C. lineatus, C. scarabaeoides* and *C. sericeus* under greenhouse conditions in endemic locations of each isolate through mite-mediated virus inoculation against three *Pigeonpea sterility mosaic virus* isolates prevailing in peninsular India and found 15 accessions (ICP 15614, 15615, 15626, 15684, 15688, 15700, 15701, 15725, 15734, 15736, 15737, 15740, 15924, 15925 and 15926) showing resistance to all three isolates.

Seven accessions of *C. scarabaeoides viz.,* ICP12707, ICP15689, ICP15692, ICP15732, ICP15744, ICP15748, and ICP15754 showed resistance to Fusarium wilt caused by *F. udum* in this investigation could be utilized as a source of resistance in breeding programmes to increase the levels of resistance to Fusarium wilt in the pigeonpea cultivars after further testing. This will be of great help to pigeonpea breeders to develop resistance breeding programmes.

Table 2. Percent wilt incidence in the accessions of *C. scarabaeoides* and cultivated varieties of pigeonpea

Genotypes	Alternative accession identifier	Origin	Percent wilt incidence (PWI)
ICP12707		India	0
ICP15683	ICPW 082	India	25
ICP15685	ICPW 084	India	25
ICP15689	ICPW 088	India	0
ICP15691	ICPW 090	India	33.33
ICP15692	ICPW 091	India	0
ICP15693	ICPW 091	India	33.33
ICP15695	ICPW 094	Srilanka	71.42
ICP15697	ICPW 096	India	80
ICP15702	ICPW 101; JM 4880	India	100
ICP15703	ICPW 101; JM 4893	India	100
ICP15706	ICPW 105; JM 5040	India	100
ICP15710	ICPW 109	India	25
ICP15711	ICPW 110	India	100
ICP15712	ICPW 111	India	22.22
ICP15713	ICPW 112	India	100
ICP15716	ICPW 115	India	100
ICP15717	ICPW 116	India	75
ICP15718	ICPW 117	India	66.66
ICP15721	ICPW 120	Philippines	100
ICP15725	ICPW 124; RPSP 827	India	100
ICP15726	ICPW 125	India	100
ICP15728	ICPW 127	India	50
ICP15731	ICPW 130; jangalapalli	India	60
ICP15732	ICPW 131	India	0
ICP15733	ICPW 132; ARKS 12347	India	100
ICP15737	ICPW 136; EC 122344	Fiji	100
ICP15740	ICPW 139; IW 3463	India	100
ICP15742	ICPW141; IW 2458.	Australia	42.85
ICP15744	ICPW143; IW 2496	Australia	0
ICP15747	ICPW 146	India	80
ICP15748	ICPW 147	India	0
ICP15749	ICPW 148	India	33.33
ICP15751	ICPW 150	India	100
ICP15752	ICPW 151	India	100
ICP15753	ICPW 152	India	50
ICP15754	ICPW 153	India	0
ICP15755	ICPW 154	India	100
Pusa 33			50
Pusa 855			44.44
Pusa 991			77.77
Bahar			100

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