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Exploring New Sources of Viral Resistance in Cowpea Germplasm and Its Elaboration through Multivariate Analysis

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ABSTRACT

Background: Cowpea is a major food legume rich in protein but its production has been dwindling by several factors including viral infection due to various virus strains in all agro-ecological zones.

Methods: Sixty eight cowpea genotypes were screened against qualitative traits (leaf shape, seed surface, twinning tendency, anthocynin pigment, plant type, fodder type and cream color) and four seed borne viruses *viz.* cucumber mosaic virus (CMV), cowpea aphid borne mosaic virus (CABMV), black eye cowpea mosaic virus (BICMV) and bean common mosaic virus (BCMV) under both *in situ* and laboratory conditions using DAC-ELISA.

Result: Based on *in situ* screening, 16 genotypes were found resistant to all the four viruses, whereas for ELISA, 13 genotypes (27005, 27041, 27075, 27141, 27145, 27146, 27147, 27158, 27160, 27167, 27172, IT85F-1380 and IT86D-719) were found resistant to all. Twelve clusters were obtained from UPGMA based on disease severity. Genotype 27008 (Pakistan) was present in cluster VI and was susceptible to all antisera CMV, CABMV, BICMV and BCMV. Whereas 13 genotypes were present in cluster VIII which were found resistant to all the four antisera applied. Therefore, 13 genotypes suggested for safe use in any breeding program at developing resistant cultivars. First two factors obtained through PCA with eigen-values >1 contributed > 80 per cent variability. Twelve distinct groups were observed and these were in coordination with cluster analysis.

Key words: Cluster analysis, CMV, Cowpea, Seed borne viruses.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp] is an important food legume crop of tropics and sub-tropics. People across Sub-Saharan Africa and South East Asia are depending on this nutritionally rich legume that has ability of nitrogen fixation, drought and heat tolerant (Carvalho *et al.,* 2017; Boahen, 2017; Haruna and Usman, 2013). According to FAO 5.8 million tons of dry cowpea is produced annually from 11 million hectares planted all over the world (Xiong *et al*., 2016). In Pakistan, it is cultivated on marginal lands whereas available data is on all underutilized crops and not specifically documented for cowpeas.

More than 20 viral diseases contributing to low yield in cowpea. CABMV and BICMV are predominant amongst seed borne viruses (Bashir *et al*., 2002; Orawu *et al*., 2012). Economically these viruses can cause yield losses exceeding 87% and usually transmitted through aphids (Shoyinka *et al*., 1997). Most destructive in the world are being members of the genus potyvirus belonging to the family potyviridae, that cause widespread disease in cowpea (Rybicki and Pietersen, 1999). The captivating characteristics of potyviral diseases are the appearance of mosaic, vein clearing, mottling, deformation and stunting of plants.

To combat such viral diseases host plant resistance is considered to be the most economical and environment friendly. Heritable forms of resistance have been found in certain cultivars (Fraser, 1992). The use of resistant (R) varieties is cost effective for farmers, but considerable time and cost may be involved in developing varieties with appropriate levels of resistance as reported by Byoung-Cheorl *et al*. (2005). Previous reports indicated that several sources of genetic resistance to viruses in cowpea have been identified (Salem *et al*., 2010). The concerted efforts have transferred R genes into popular cowpea landraces to boost production for cowpea growers in many parts of the world including Pakistan (Arshad *et al*., 1998). The current study revealed systematic screening and identification of resistant genotypes to develop breeding material and new source of commercially available varieties. Sixty-eight genotypes were screened against four viruses using ELISA for identification of resistant genotypes after field observations. Multivariate analysis was employed to explore nature of seed borne viruses interaction and serology of viruses affect crop. Testing focused whether both techniques

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phylogenetic analysis and scattered diagram supported the same results under in situ and viral serological testing.

MATERIALS AND METHODS

Sixty-eight genotypes were acquired from PGRI, NARC, Islamabad, Pakistan. Collections representing Pakistan were 62, while 4 were obtained from International Institute of Tropical Agriculture (IITA) and one from China (Iqbal *et al.,* 2003). These genotypes were grown under field conditions at PGRI, NARC, Islamabad (33°44′ N and 73°08′ E, 540 m) in an augmented design with three replications. One local check was repeated after every 10 rows. One row of 4 meters length was planted for each genotype with row to row and plant to plant spacing as 75 cm and 20 cm, respectively. Recommended cultural practices were followed throughout the crop season (Iqbal *et al.,* 2017).

Qualitative traits

Field data on diverse qualitative traits (leaf shape, seed surface, twinning tendency, anthocynin pigment, plant type, fodder type and cream color) were recorded. Data were taken of individual plant and then their mean values were compared and analyzed using summary statistics with the help of MS Excel Windows 2010.

Serology and Direct antigen coating-enzyme linked immunosorbent assay (DAC-ELISA)

Leaves were collected from ten plants of each genotype from the field to screen against four viral antisera to cowpea seed borne virus *viz*., CMV, CABMV, BlCMV and BCMV for DAC-ELISA. As majority of the virus antisera are produced in rabbit, therefore, the rabbit immunoglobulin attached to antigens are probed with enzyme labeled anti-rabbit immunoglobulin produced in goat. In place of immuno-globulins, the use of Protein-A conjugate is equally good as that of anti-rabbit conjugate. The fresh and healthy samples were collected at 50% flowering stage for each genotype in polyethylene bags and stored at 4°C. ELISA buffer was used to ground leaves before loading in the ELISA plates. Antigens were prepared in 0.02 M carbonate buffer (pH 9.6) and used for coating ELISA plates followed by the addition of crude antiserum (not purified IgG as in case of DAS-ELISA) with its optimum concentration. 200 µL extract of each sample was loaded in each well of ELISA plate. Two wells each for healthy and positive control were used for each virus. The loaded plates were incubated at 37°C for 2 hours, then washed with washing buffer three times at interval of one minute and dried on paper towel (Bashir *et al.,* 2000).

Cross absorption of crude antiserum was made with healthy plant extract in conjugate buffer (antibody buffer) to have a 1:100 dilution, then filtering through cheese cloth. Two hundred µl cross-absorbed solution was added into each well of ELISA plate and incubated the plate at 37°C for 1 hr prior to adding it to micro titer plate. The solution of alkaline phosphatase (ALP) conjugate (Sigma-3687) was prepared in conjugate solution (pH 7.4) at a dilution of 1:1000 for all the viruses to be screened against specific antiserum. Two hundred µl of this solution was added in each well and incubated at 37°C for 2 hr. The substrate solution was prepared by dissolving P-Nitrophenyl Phosphate (PNPP) tablets in substrate buffer (pH 9.8). One tablet (5 mg) was used for 30 ml buffer. Substrate solution (200 µl) was added into each well and ELISA reading was taken using BIORAD Multiscan (Model Atto 550 nm Yamato Scientific Co. Ltd, Japan) 30 minutes after adding substrate buffer. Reading of the healthy wells was considered as positive. The data on disease rating were recorded as 0 (resistant), where no reaction and 1 (susceptible), where reaction was observed. Although, variation in reaction intensity was observed but even weak reaction (even slightly higher than control that was considered at ELISA reading < 0.200 and the values greater than this were considered as susceptible). Genetic linkage for disease reaction was constructed on the basis of Unweighted Pair Group Mean Averages (UPGMA) and scattered diagram using computer software STATISTICA version 6.0 and SPSS version 10.01 for windows.

RESULTS AND DISCUSSION

Cowpea genetic diversity existed in Pakistan comprising 39 genotypes from Punjab, 21 from KPK, 1 each from Baluchistan and Gilgit Baltistan. These areas are known as centers of biodiversity (Iqbal *et al.,* 2017). Qualitative traits presented in (Table 1) showed wide range of variability present in these traits. Leaf shape (LS) was divided into four descriptors. Forty two genotypes were ovate, 12 were lanceolate narrow and 14 were lanceolate broad whereas no line was observed as rhombic. Plants with ovate leaf shape were found to be best for moisture absorption and synthesis of food proteins while lanceolate broad and narrow leaf shape is also a distinguishing trait in cowpea which could have the ability to resist drought, this trait was prominent in exotic germplasm obtained from IITA, Nigeria and hence the plants with these characters could be utilized for breeding and hybridization of local and exotic cowpea in rainfed conditions. All plants were found to be glabrous *i.e*. without hairs (100%) which help in harvest and trample. Erect plant type was recorded in 49 lines was dominant trait as compared to prostrate in (13), pronounced (2) and spreading (4). Erect plant type is preferred for fodder use and during humid conditions get more light while prostrate type is preferred for planting under rainfed conditions as it facilitates moisture uptake and its efficient conservation. On the other hand genetic variation was recorded low in twinning tendency and anthocynin pigment. Further evaluation and exploitation of these traits with other economic traits may help to improve this trait (Al-Saady *et al*., 2018). Thirty two genotypes were identified and selected as fodder type, rest 36 were non-fodder type. Fodder type can be utilized as dual purpose (Iqbal *et al.,* 2021). Cream color in 29 lines was prominent which is commercially valued and needs to be multiplied.

Resistant sources are reported for one or two viruses but no investigation is available on multiple resistances

(Bashir *et al.,* 2002). ELISA (Clark and Adams, 1977) generally detects concentration of virus lower than 0.01 µg/ml as compared to immunodiffusion test (1 µg/ml), micro precipitin test (0.5 µg/ml), electron microscopy (0.1 µg/ml) and infectivity assay (0.05 µg/ml) (Van Dan Heuvel and Peters, 1989; Bar-Joseph and Garnsay, 1981).

As six antisera (CMV, CABMV, BICMV, CSMV, BCMV and SBMV) were used for detection, identification of cowpea seed borne viruses, two antisera (CSMV and SBMV) were not properly functioned as they gave high back ground reaction. Results presented in Table 1 generated by DAC-ELISA revealed that genotypes of diverse origin (27005, 27041, 27075, 27141, 27145, 27146, 27147, 27158, 27160, 27167, 27172, IT85F-1380 and IT86D-719) were found highly resistant to four viral antisera (CMV, CABMV, BICMV and BCMV). Resistant genotypes may serve as resistant source for developing new varieties. Only one genotype 27008 of local origin was found highly susceptible to all the viral antisera. Genotype number F0214 obtained from China was found resistant to CMV although susceptible to other three viruses. Seven genotypes (27001, 27006, 27012, 27025, 27027, 27052 and 27064) were found only resistant to black eye cowpea mosaic virus but susceptible to other three antisera. Twenty seven genotypes were observed resistant to cowpea mosaic virus, whereas susceptible to other three antisera. As a whole twelve different disease reactions of resistant and susceptible to different antisera were recorded through DAC-ELISA (RRRR, SSSS, SSRS, RSSS, SRRS, RSSR, RRSS, SSRR, SRRR, RRSR, RSRR and RRRS).

Previously CMV was not detected in cowpea germplasm collected from Pakistan (Bashir and Hampton, 1996). Genotypes *viz.,* 27008, 27001, 27006, 27012, 27025, 27027, 27052, 27064, 27003, 27009, 27011, 27017, 27018, 27022

Table 1: Cowpea germplasm based on different disease reactions of the 68 genotypes to four virus antisera (CMV, CABMV, BICMV and BCMV).

Disease reaction	Antisera	Numbers	Genotypes
RRRR	Resistant to all four antisera CMV, CABMV, BICMV and BCMV	13	27005 Rawalpindi, 27041 MuzafferGarh, 27075 Lahore, 27141 Muzaffer Garh, 27145 Gujranwala, 27146 Gujranwala, 27147 Gujranwala, 27158 Faisalabad, 27160 Okara, 27167 Sheikhupura, 27172 Chitral, IT85F-1380 Nigeria, IT86D-719 Nigeria.
SSSS	Susceptible to all antisera CMV, CABMV, BICMV, BCMV	1	27008 Rawalpindi.
SSRS	Susceptible to CMV, CABMV, BCMV but resistant to BICMV	7	27001 Attock, 27006 Rawalpindi, 27012 Rawalpindi, 27025 Jehlum, 27027 Gujrat, 27052 Chakwal, 27064 Islamabad.
RSSS	Resistant to CMV but susceptible to CABMV, BICMV, BCMV	27	27076 Lahore, 27077 Lahore, 27078 Kasur, 27081 Kasur, 27088 Okara, 27098 Chitral, 27099 Sheikhupura, 27102 Gilgit, F0214 China, 27106 Layyah, 27107 Bahawalpur, 27110 Kasur, 27115 Mansehra, 27118 Swat, 27120 Mansehra, 27121Dir, 27122 Dir, 27123 Dir, 27124 Dir, 27125 Chitral, 27126 Chitral, 27127 Chitral, 27140 Dir, 27149 Dir,27150 Dir, 27170 Dir, 27171 Chitral.
SRRS	Resistant to CABMV, BICMV but susceptible to CMV and BCMV	2	27003 Attock, 27009 Chakwal.
RSSR	Resistant to CMV and BCMV but susceptible to CABMV and BICMV	7	27083 Gujranwala, 27099 Sheikhupura, 27097 Chitral, 27105 Khushab, 27108 Okara, 27109 Faisalabad, IT95K-222-10 Nigeria.
RRSS	Resistant to CMV and CABMV but susceptible to BICMV and BCMV	3	27093 Haripur, 27100 Chakwal, 27119 Mansehra.
SSRR	Susceptible to CMV and CABMV but resistant to BICMV and BCMV	4	27011 Rawalpindi, 27017 Rawalpindi, 27018 Rawalpindi, 27022 Jehlum.
SRRR	Susceptible to CMV but resistant to CABMV, BICMV, BCMV	1	27144 Lahore.
RRSR	Resistant to CMV, CABMV and BCMV but susceptible to BICMV	1	27142 Multan.
RSRR	Resistant to CMV, BICMV and BCMV but susceptible to CABMV	1	IT95U-286 Nigeria.
RRRS	Resistant to CMV, CABMV and BICMV but susceptible to BCMV	1	27151 Dir.

and 27144, all were observed susceptible to CMV. The CMV symptoms were more severe in plants infected with mixed infection of BICMV than with single infection. This may be due to synergistic effect of two viruses (Pio-Ribeiro *et al.*, 1978). These lines may serve as resistant sources for breeding program provided their resistance is confirmed by artificial virus inoculation. There are several control strategies for viral diseases including the use of virus free seed or vegetative propagules, prevention of infection by breaks in cropping or control of weed hosts, prevention of transmission by vectors and breeding for resistance (Fraser, 1992).

Two types of viral symptoms were observed under field condition. Some plants showed bright yellowing with mosaic pattern and such types of symptoms were more common. Second type of symptoms appeared in the form of molting, vein banding and vein clearing, mosaic and yellowing. Among 16 genotypes which were observed resistant under field conditions due to no visible virus like symptoms, either single or mixed infection of two to three viruses gave reaction in three genotypes (27052, 27099 and F0214) by ELISA and these were considered as susceptible. The genotypes resistant to antisera were CMV (53), CABMV (21), BICMV (28) and BCMV (22), whereas, for susceptible ones were for CMV (15), CABMV (47), BICMV (40) and BCMV (46). Under field conditions, the viral disease increased with the passage of time and was attributed to favorable climatic factors, abundance of inoculum and vector for viruses or due to synergistic effect of two or more viruses. Overall viral disease incidence ranged from 0-66% based on ELISA results. Thirteen genotypes (27005, 27041, 27075, 27141, 27145, 27146, 27147, 27158, 27160, 27167, 27172, IT85F-1380 and IT86D-719) were resistant to all the four viral diseases (Fig 1).

In response to disease severity, twelve clusters were obtained from unpaired group mean averages (UPGMA). Cluster I, III, VI, VII, IX consisted of one genotype, cluster II (3), cluster IV (7), cluster V (27), cluster VIII (13), cluster X (4), Cluster XI (2) and cluster XII counted 7 genotypes in each case. Genotype 27008 originated from Pakistan was present in cluster VI and was susceptible to all antisera CMV, CABMV, BICMV and BCMV, whereas 13 genotypes were present in cluster VIII which were found resistant to all the four antisera applied. Out of these two were from IITA, Nigeria and 11 others were from Pakistan. The identified genotypes screened against seed borne viruses can be safely used in any breeding program. Clusters were diverse in nature and so were their responses to different antisera. This may prove their worth in future breeding programs or in combination with resistant, susceptible and highly susceptible lines. Cluster diagram indicated that resistant genotypes were placed in separate cluster, whereas susceptible to CMV, CABMV, BICMV and BCMV were grouped in different clusters. Genotypes resistant under field condition were from all the three sources, *i.e.*, China, Nigeria and Pakistan. PCA revealed first two factors with eigen-values greater than unity contributed more than 80 per cent variability among 68 genotypes for four viral diseases, hence a scattered diagram was constructed (Fig 2). Twelve distinct groups were observed and these were in coordination with cluster analysis. The genotypes resistant to all the four viruses were grouped in the upper right box along with other three genotypes which were resistant to three different viruses. On the contrary, the genotypes susceptible to all the viruses were in the lower left box.

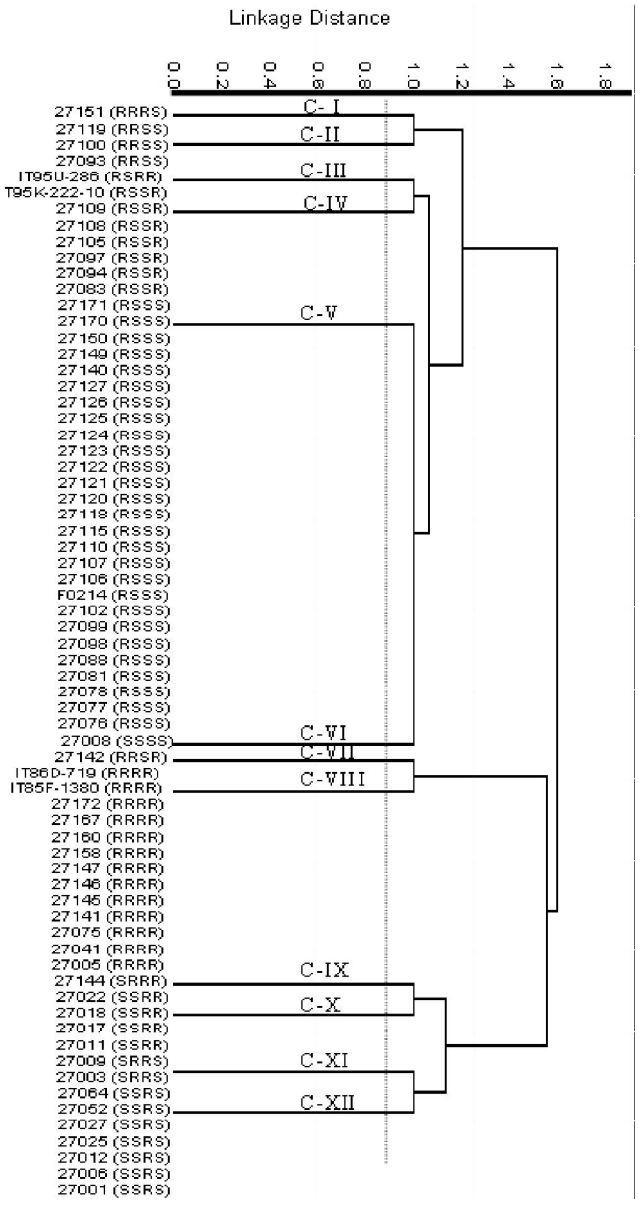

Fig 1: Cluster tree for 68 genotypes of cowpea based on four viral diseases. The order of viral antisera used was as CMV CABMV BICMV and BCMV. The symbol "R" stands for resistant and "S" for susceptible to particular virus.

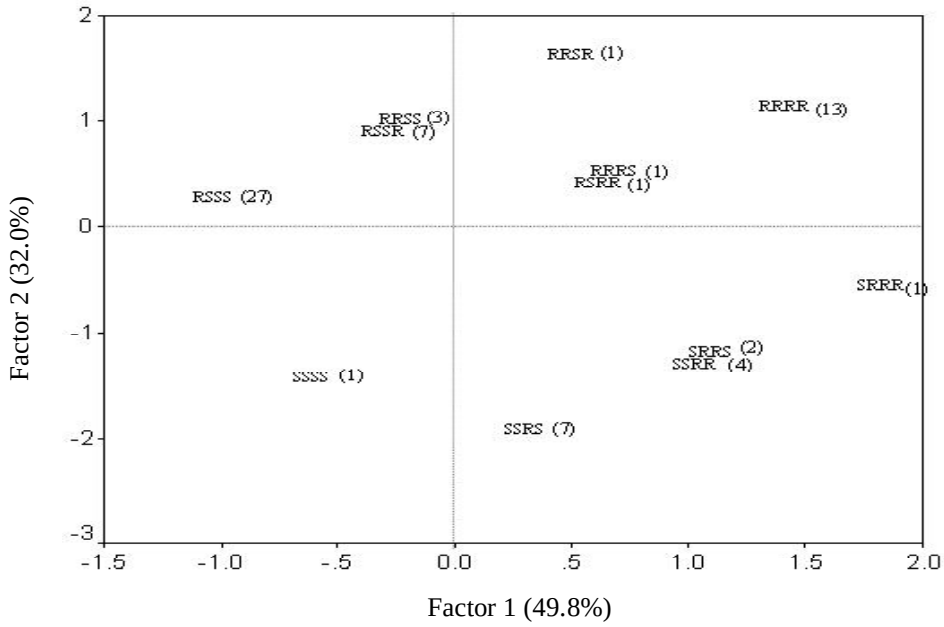

Fig 2: Scatter diagram for 12 clusters based on four viral antisera in cowpea. The order of viral antisera is; CMV, CABMV, BICMV and BCMV. The symbols "R" stands for resistant and "S" for susceptible to particular virus.

CONCLUSION

Thirteen genotypes (27005, 27041, 27075, 27141, 27145, 27146, 27147, 27158, 27160, 27167, 27172, IT85F-1380 and IT86D-719) were found resistant to all above viral diseases therefore, suggested for breeding programs aiming development of resistant cultivars with better yield potential.

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