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INTER-RELATIONSHIP BETWEEN SDS-PAGE MARKERS AND AGRONOMIC TRAITS IN CHICKPEA (*CICER ARIETINUM* L.)

A. GHAFOOR, F.N. GULBAAZ[1], M. AFZAL, M. ASHRAF[1] AND M. ARSHAD[2]

Institute for Agro Biotechnology and Genetic Resources (IABGR), National Agricultural Research Centre (NARC), Islamabad, Pakistan.
Corresponding author: Email: ghafoor59@hotmail.com

Abstract

Sixty accessions collected from major chickpea growing areas of the Punjab along with two check varieties were investigated for principal components analysis based on agronomic characters and seed proteins. Seed proteins were analyzed through slab type SDS-PAGE using 11.25% Polyacrylamide gel and 6 µl of sample quantity. First three principal components with eigenvalues more than 1 contributed 88.58% of the variability amongst 62 genotypes evaluated for 11 quantitative traits, whereas PC_4 to PC_{11} were less than unity. All the characters under study contributed genetic variance positively towards PC_1 except days to flowering where it was negative. Eight characters (branches, pods per plant, pods per branch, seeds per pod, 100-seed weight, biological yield, grain yield and harvest index) exhibited maximum effect on PC_1 and seven characters were positive for PC_2. Most of the accessions grouped on the basis of their origin for agronomic characters. Cluster analysis showed that accessions from same origins were grouped separately that might be due to frequent exchange of germplasm by the breeders or transport of grain to different markets from where the seed of various origins is disseminated through out the country. Out of 14 SDS-PAGE markers, 8 were polymorphic and gel was divided into three regions. The accessions with similar banding patterns were suggested to confirm by 2-D electrophoresis. SDS-PAGE alone did not exhibit high level of intra-specific variation, therefore, diverse accessions are suggested to be acquired from various sources. Agronomic characters were observed more reliable than protein peptides, but simultaneous study for both agronomical and biochemical analysis (protein and DNA) is suggested. Principal component and cluster analyses proved their validity to investigate inter-relationship among accessions for agronomic characters and SDS-PAGE markers, although agronomic evaluation exhibited more reliability.

Introduction

Agronomic, morphological and physiological traits are generally used to characterize the varieties, however such data may not provide an accurate estimation of genetic diversity because of environmental influence or due to the presence of multiple alleles or genes. Moreover, field-testing and evaluation of plant materials is often laborious and time consuming. Considering these difficulties, bio-chemical markers received more attention in recent years from the crop geneticists for assessment of genetic variability (Akhtar, 2001; Rabbani *et al.*, 2001). Among biochemical techniques, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is most widely used due to its validity and simplicity for describing genetic structure of crop germplasm. SDS-PAGE is a practical reliable method because seed storage proteins are largely independent of environmental fluctuation (Gepts, 1989; Murphy *et al.*, 1990; Ghafoor *et al.*, 2002). Use of seed protein electrophoresis has been able to detect qualitative and quantitative differences among cultivars in various crop species (Cooke, 1984; Gardiner & Forde, 1988). Both before and during electrophoresis, the proteins are continuously exposed to

[1]Department of Biological Sciences, Quaid-I-Azam University, Islamabad, Pakistan.
[2]Pulses Programme, National Agricultural Research Centre (NARC), Islamabad, Pakistan.

detergent SDS (Sodium Dodecyl Sulphate), a common cleaning agent found in toothpaste; its chemical formula is $CH_3 (CH_2)_{11} SO_4^- Na^+$. Approximately one molecule of detergent binds to each amino acid i.e., 1.4g of SDS binds to each gram of protein. At neutral pH, the detergent is negatively charged. SDS molecules repel one another, which forces the proteins with bound detergent into rod like shapes endowed with similar mass to charge ratios. The present study was conducted to study genetic diversity on the basis of agronomic traits and total seed protein to access their inter-relationship with special emphasis to geographic origin in chickpea.

Materials and Methods

Sixty accessions along with two check varieties (Punjab 91 and Paidar) were planted during November 2000 and harvested during April-May, 2001. Table 1 presents the status of chickpea germplasm collected from 5 districts of chickpea growing area of the Punjab. Two rows of 2 meter length for each plant progeny were planted with 75 cm and 10 cm inter and intra-row spacing, respectively. Recommended cultural practices were followed throughout the crop season to get healthy crop. Pesticides and fungicides were sprayed to save the crop from the infestation of pests and disease. Agronomic characters were recorded following IPGRI descriptors for chickpea (Anon., 1985). Days to flowering were recorded when 50% plants started flowering and days to maturity were recorded at 90% maturity when pods turned brown/black. Other quantitative data, plant height (cm), number of branches, number of pods, grain yield (g) and biomass (g) were recorded on ten competitive plants sampled randomly and then averaged to per plant basis. Seeds per pod were recorded on ten pods sampled at random within each accession/genotype. Pods per branch were calculated and expressed as pods per unit branch, whereas seed weight was recorded after counting 100 seeds by seed counter and weighed in grams. Harvest index was determined as economic yield expressed in percentage over total biomass.

Table 1. Collecting area of 62 genotypes of chickpea evaluated for agronomic characters and SDS-PAGE markers.

District	Total
Bahawalnagar	11
Bhakkar	17
Khushab	12
Layyah	18
Mianwali	2
Checks	2

For the extraction of proteins, single seed was ground to fine powder with mortar and pestle. Sample buffer (400 µl) was added to 0.01 g of seed flour as extraction liquid and mixed thoroughly in Eppendorf tube with a small glass rod. The extraction buffer contained the following final concentrations: 0.5 M Tris-HCl (pH 6.8), 2.5% SDS, 10% glycerol and 5% 2-mercaptoethanol. Bromophenol Blue (BPB) was added to the sample buffer as tracking dye to watch the movement of protein in the gel. Seed proteins were analyzed through slab type SDS-PAGE using 11.25% Polyacrylamide gel on ten samples for each accession. In order to check the reproducibility of the method two separate gels were run under similar electrophoretic conditions. The molecular weights of the dissociated polypeptides were determined by using molecular weight protein standards "MW-SDS-70

kit" from Sigma Chemical Company, USA. The SDS-PAGE of total seed protein was carried out in the discontinuous buffer system according to the method of Laemmli (1970). SDS-PAGE revealed that 11.25% acrylamide gel concentration, 6 µl of sample gave the best resolution as suggested by Iqbal (2001).

For quantitative traits, data were analyzed for principal component and cluster analyses with the help of computer software SPSS Version 10.01 and STATISTICA 6.0 for Windows 98. For SDS-PAGE, after staining and destaining the gels, depending upon the presence or absence of polypeptide bands, similarity index was calculated for all possible pairs of protein types. To avoid taxonomic weighing, the intensity of bands was not taken into consideration, only the presence of the bands was taken as indicative. Presence and absence of the bands were entered in a binary data matrix. Based on results of electrophoretic band spectra, accessions were plotted for first two PCs on the basis of geographic distribution.

Results

Agronomic traits

Table 2 presents basic statistics for 11 quantitative characters of 60 accessions along with 2 checks. High variance was observed for all the characters under study except seeds per pod. First three principal components with eigenvalues more than 1 contributed 88.58% of the variability amongst 62 genotypes evaluated for 11 quantitative traits (Table 3). Other components (PC_4 to PC_{11}) were less than unity hence could not prove their importance. Principal component 1 exhibited 60.39% of the total variation, PC_2 16.45% and PC_3 11.75% of the total variation. Characters that contributed more positively to PC_1 were, branches (0.917), pods per plant (0.953), pods per branch (0.657), seeds per pod (0.919), 100-seed weight (0.968), biological yield (0.957), grain yield (0.963) and harvest index (0.909), whereas days to flowering contributed least to first component. Days to flowering (0.964) and maturity (0.689) contributed maximum genetic variance to PC_2 and plant height was assessed significant for PC_3. Days to maturity were contributed by all the factor but high effects were observed for PC_2.

Table 2. Range, means, SE and variance for 11 quantitative traits in 62 genotypes of chickpea evaluated during 1997.

	Mean±SE	σ	σ^2	Minimum	Maximum
Days to flowering	140.03±0.61	4.80	23.05	124.00	155.00
Days to maturity	170.48±0.58	4.60	21.20	163.00	185.00
Plant height (cm)	51.00±0.74	5.83	33.98	37.60	66.90
Number of branches	12.31±0.46	3.60	12.99	5.80	22.60
Pods per plant	39.66±3.04	23.93	572.44	7.50	122.70
Pods per branch	3.70±0.30	2.37	5.62	0.68	13.10
Seeds per pod	1.13±0.03	0.22	0.05	0.47	1.81
100-seed weight (g)	15.78±0.60	4.72	22.31	10.88	27.49
Biological yield (g)	29.18±1.06	8.34	69.48	14.14	50.65
Grain yield (g)	7.19±0.44	3.47	12.05	1.74	18.35
Harvest index (%)	25.21±1.27	10.03	100.68	7.15	56.52

Table 3. Principal Components (PCs) for 11 quantitative characters in 62 genotypes of chickpea.

	PC_1	PC_2	PC_3
Eigen value	6.64	1.81	1.29
Proportion of σ^2	60.39	16.45	11.75
Commulative σ^2	60.39	76.83	88.58
	Eigen factor		
Days to flowering	-0.082	0.964	0.087
Days to maturity	0.667	0.689	0.627
Plant height	0.316	-0.415	0.814
Branches per plant	0.917	0.209	-0.261
Pods per plant	0.953	0.162	-0.179
Pods per branches	0.657	0.120	0.179
Seeds per pod	0.919	-0.234	0.120
100-seed weight (g)	0.968	0.048	-0.057
Biological yield per plant (g)	0.957	0.128	-0.139
Grain yield per plant (g)	0.963	-0.116	0.117
Harvest index (%)	0.909	-0.245	0.217

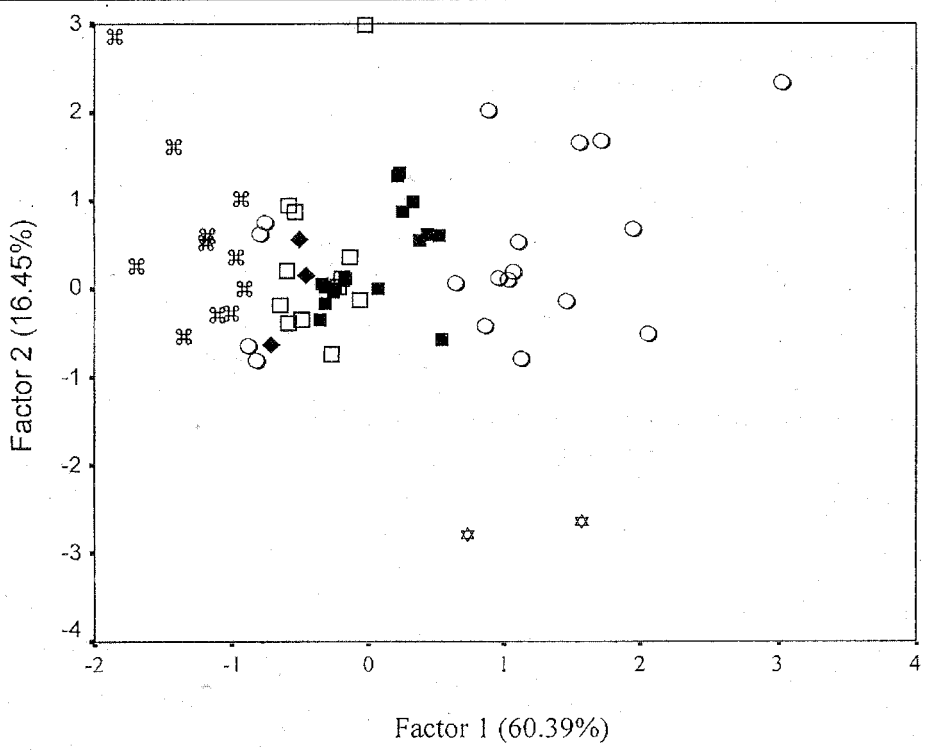

Fig. 1. Scattered diagram based on quantitative traits for first and second factors in chickpea. The marks represent as ○- District Layyah, ■- District Bhakkar, □- Districh Khushab, ◆- District Mianwali, ⌘- Bahawalnagar and ✡- approved varieties

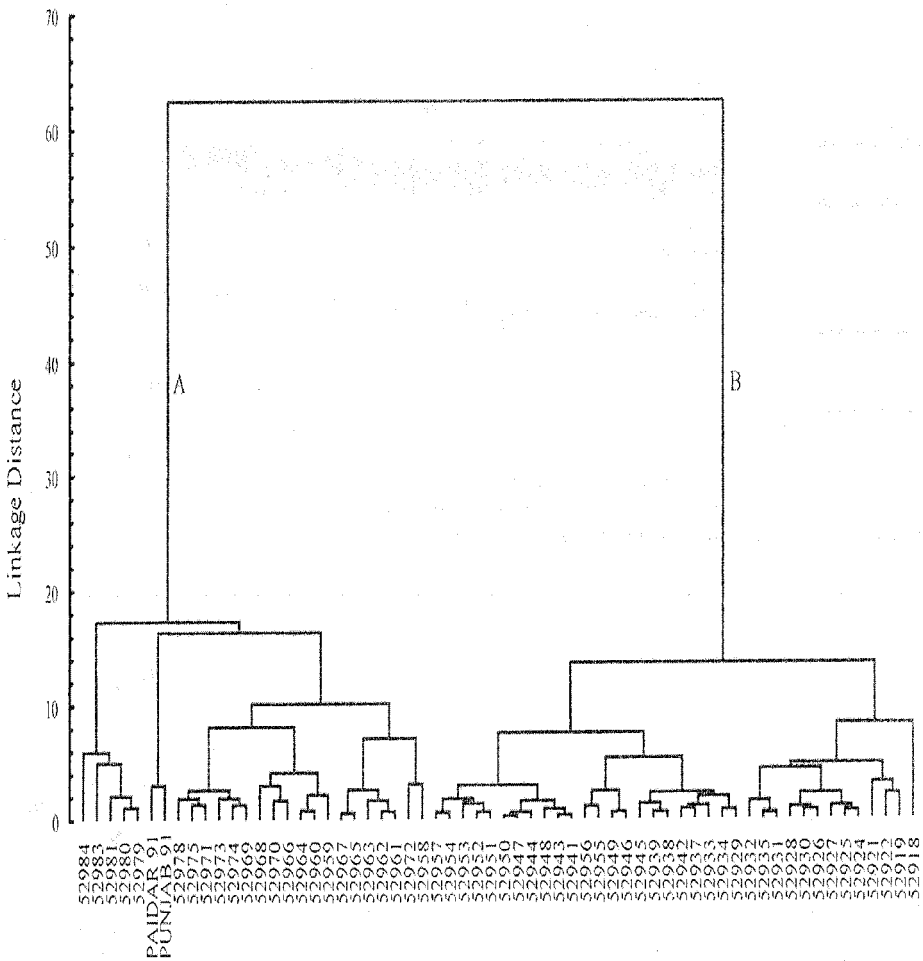

Fig. 2. Cluster diagram of 60 chickpea accessions along with 2 checks based on quantitative characters.

All the characters under study contributed genetic variance positively towards PC_1 except days to flowering where it was negative. In more detail, the first PC which explained 60.39% of the variance is positively associated with all the characters except one. Eight important yield contributing characters exhibited more positively, whereas days to flowering contributed least. This means that the populations with high PC_1 values are high yielding and formed by medium maturing plants characterized by high seed weight and harvest index. Seven characters contributed positively for PC_2 where days to maturity was observed with highest values for PC_2. It is evident that ten important plant characters contributed more positively to first 2 principal components and hence these could be established important for the material under investigation.

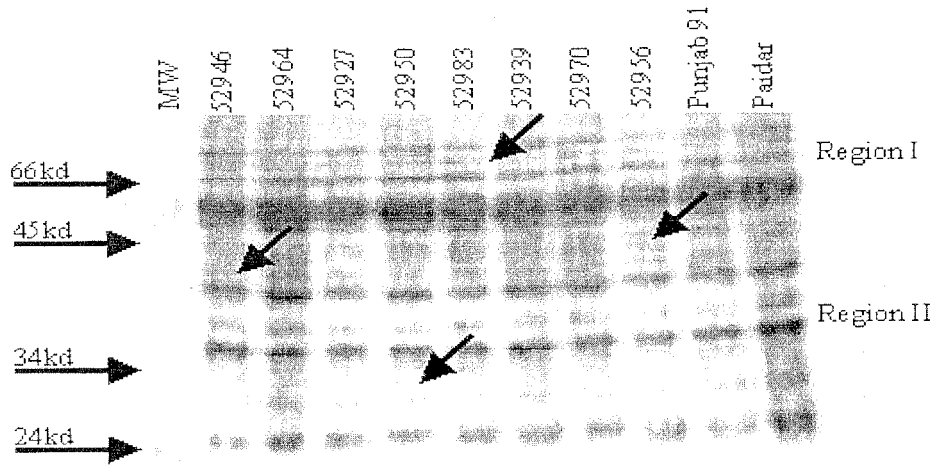

Fig. 3. Total seed protein pattern in chickpea accessions obtained by slab type gel electrophoresis using SDS-PAGE. The marker SDS-70 from Sigma Chemicals was used as standard. The arrows indicate variation among various accessions involved in the evaluation.

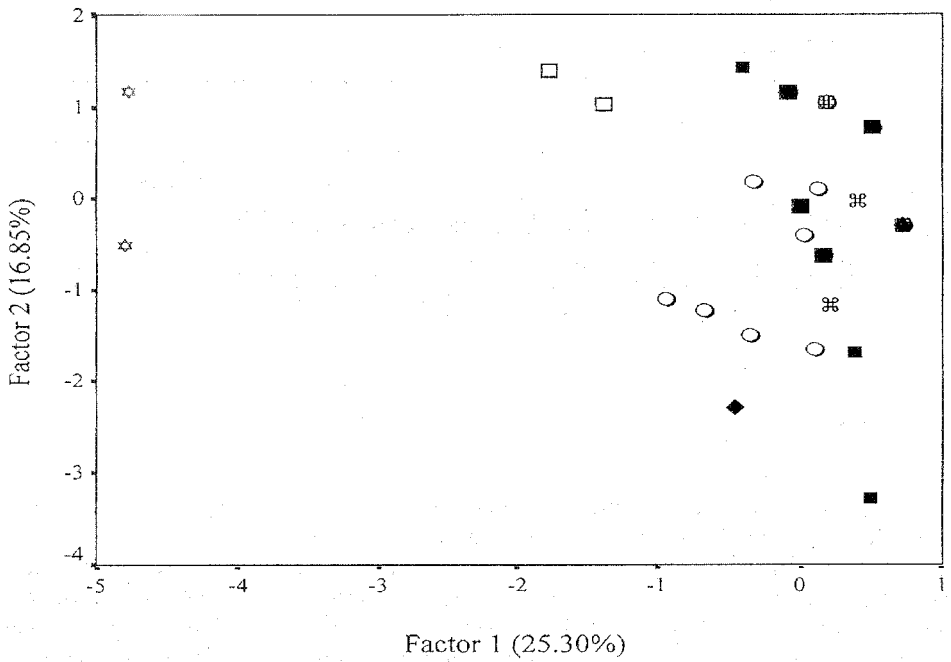

Fig.4. Scattered diagram based on SDS-PAGE markers for first and second factors in chickpea. The marks represent as ○- District Layyah, ■- District Bhakkar, □- Districh Khushab, ◆- District Mianwali, ⌘- Bahawalnagar and ✡- approved varieties

The accessions were plotted on the basis of geographic origin and source of seed collection, hence these were investigated as the genetic diversity was related to geographic origin or not. The PC_1 and 2 revealed one group in the left upper half, one in the right upper half, one in between of these two groups and one consisting checks was observed in the lower half of the graph (Fig. 1). Most of the accessions were grouped on the basis of their origin for quantitative characters.

Cluster diagram based on Euclidean dissimilarity using Ward's method revealed 2 major groups and if it is observed critically, six clusters were observed (Fig. 2). The group A consisted of 4 and group B consisted of 2 clusters. Both the checks were grouped together in the cluster II. Cluster I consisted of five accessions, all of these were collected from the district Layyah. Cluster III comprised of 12 accessions and out of these eight (52978, 52975, 52971, 52973, 52974, 52969, 52968, 52970) were collected from Layyah, where other four accessions (52966, 52964, 52960, 52959) originated from the district Bhakkar. Both of these districts are adjoining and well known due to chickpea cultivation. Cluster IV consisted seven accessions, 52967, 52965, 52963, 52962 and 52961 were collected from Bhakkar, 52972 from Layyah and 52958 originated from Khushab. Twenty three accessions were grouped together in cluster V and out of these eleven (52957, 52954, 52953, 52943, 52941, 52956, 52955, 52939, 52938, 52942, 52937) were collected from the district Khushab and it is important to note that in total twelve accessions were collected from Khushab and out of these 11 were grouped together in this cluster. Seven accessions viz., 52952, 52951, 52950, 52947, 52948, 52949 and 52946 which were collected from Khushab were grouped in this cluster. Both the accessions originated from the district Mianwali were also in this cluster. Two accessions (52933 and 52934) of this cluster were collected from Layyah and one (52929) originated from Bahawalnagar. This cluster consisted a mix population of accessions collected from various sites. Cluster VI consisted of thirteen accessions and out of these two (52932 and 52931) were collected from Layyah, one (52935) from Bhakkar and all others (52928, 52930, 52926, 52927, 52925, 52924, 52921, 52922, 52919, 52918) were from Bahawalnagar.

Seed Proteins

Forty-one accessions were homozygous on the basis of SDS-PAGE whereas others were heterozygous hence single seed descents could be isolated from these heterogeneous lines to establish pure-lines for future breeding programme. In total, 14 protein bands were recorded ranging from the Molecular Weight (MW) of 24 to 66 kd. Many protein subunits of lower MW were also observed but due to inconstancy in reproducibility they were not recorded. Occasionally, variation was also observed in the density or sharpness of a few bands but this variation was not taken into consideration.

Out of 14 protein subunits, 8 were polymorphic and 6 were monomorphic. On the basis of banding pattern, gel was divided into three regions (Fig. 3). Region I had 4 bands of more than 66 kd MW of which 3 were polymorphic. Region II ranged from 24 to 66 kd having 10 protein peptides, out of which 5 were polymorphic. In this region, the protein bands were observed with high degree of variation in quantitative term. The quantitative intensity of bands were not recorded at present although these may provide some information specific to chickpea. Weak protein bands were observed in the region III of lower molecular weight, hence not recorded due to inconsistency in presence. The

accessions were plotted on the basis SDS-PAGE markers to assess relationship to their origin (Fig. 4). Many accessions overlap each other due to similarity on the basis of SDS-PAGE markers. Two approved varieties were separated in the left upper part of the graph whereas few accessions were marked separately in other cases. Two accessions from District Khushab and four from District Layyah were clearly separated whereas others were mixed together or overlapped to each other.

The dendrogram based on SDS-PAGE markers for dissimilarity matrix using UPGMA showed a division into 5 clusters (Fig. 5). Clusters I consisted two approved varieties, cluster II consisted one accession (52946), cluster III comprised of four accessions viz., 52984, 52935, 52934, 52931, whereas cluster IV consisted 52932 accession. All the other accessions were grouped together to constitute one cluster at 1.5 linkage distance. For most accessions and protein subunits, no clear observation was recorded which could facilitate selection on the basis of SDS-PAGE for improving agronomic traits in chickpea from the material under investigation.

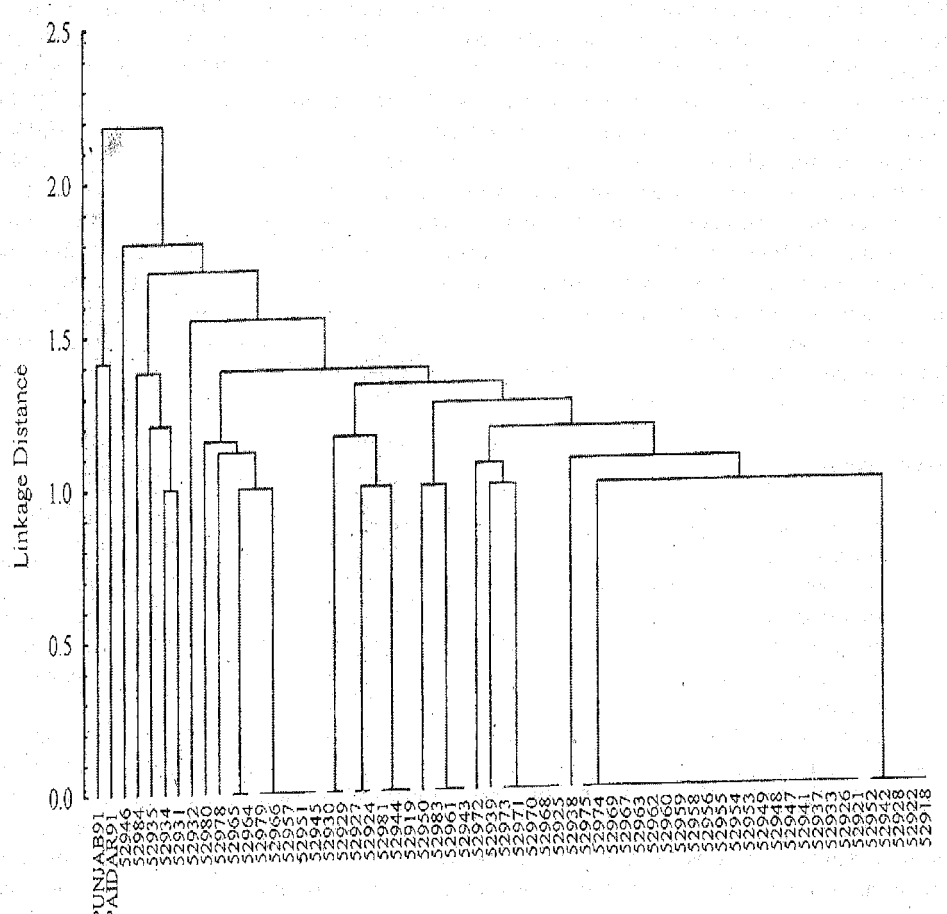

Fig. 5. Cluster diagram of 60 chickpea along with 2 checks based on SDS-PAGE makers.

Discussion

Germplasm evaluation must be considered the first step in any plant breeding programme and it is commonly based on a simultaneous examination of a large number of populations for several characters of both agronomic and physiological interest (Pezzotti *et al.*, 1994; Rabbani *et al.,* 1998; Ghafoor *et al.*, 2001a). Only PC_1 exhibited more than half of variability, hence considered cumulative of other components. The first component is strongly associated with high yield potential and yield contribution traits, thus more related to reproductive phase, whereas second component is associated with days to flowering and days to contributing 17.2% of the total variance, hence the populations in this component are more likely related to vegetative traits. The population with high PC_2 values are characterized by late flowering and maturity. The populations in this component were associated negatively with plant height, seeds per pod, grain yield and harvest index which revealed that the accessions in the population failed in appropriate partitioning of economic yield which ultimately reduced harvest index.

According to Perry & McIntosh (1991), differentiation according to geographical regions of origin is useful in substantiating the postulated regions of diversity or gene centres. The rare alleles, each only occurring in one or two apparently random populations can be considered to be mutants, migration or the results of other coincidental events (Van Hintum & Elings, 1991). Alleles common in the restricted areas occur mostly in the areas of high genetic diversity. This could indicate that migration of genetic material from one place to new regions, followed by some degree of contamination by mixture or out crossing with other landraces (Pecetti *et al.*, 1996).

Low intra-specific variation was observed among chickpea accessions and similar results had already been reported by Thakare *et al.*, (1987) and Mehrani (2002) in legumes who observed low intra-specific variation within one species in their study. SDS-PAGE showed that the method provided a tool for reliable germplasm discrimination based on genetic differences in seed storage protein comparison in chickpea. The accessions with similar banding patterns may be duplicated in the germplasm, but these are suggested to be confirmed by the use of 2-D electrophoresis focusing as suggested by earlier researchers (Celis & Bravo, 1984; Beckstrom-Sternberg, 1989; Higginbotham *et al.*, 1991). In the present studies intra-specific variation was limited and it was observed that SDS-PAGE alone did not exhibit high level of intra-specific variation, therefore, diverse accessions based on SDS-PAGE are suggested to be acquired from various sources, preferably from centre of diversity to build a broad based gene-pool with maximum variability. Further, for better management of genebank, a precise comprehensive knowledge of agricultural and biochemical data (protein and DNA) is essential to eliminate duplicates from germplasm collections.

SDS-PAGE in local chickpea germplasm did not reflect any clue either for agronomic preference and/or geographic distribution. For most accessions and protein subunits, no clear observation was recorded which could facilitate selection on the basis of SDS-PAGE for improving agronomic traits in chickpea from the material under investigation. PCA provide a good evaluation of landraces by identifying those that should be further evaluated at the genetic level (Rouamba *et al.,* 1996). Additional applications of this technique will certainly be found as its use becomes more widespread in fields of biological sciences, where it has been used extensively for more than two decades. Dasgupta & Das, (1984, 1985) considered multivariate analysis best for

choosing parents for hybridization. Suggestion has been made for selecting suitable stable diverse parents so as to streamline a crossing programme for increased grain yield in chickpea. Such studies would allow more efficient enhancement and use of genetic resources with a view to introduce desirable characteristics from landraces into improved cultivars. Kresovich & McFerson (1992) considered genetic diversity important in assessment of PGR management. Ahmad *et al.*, (1997) reported that first two canonical components contributed 85% of the variation between lentil genotypes. It was observed that PCA on the basis of quantitative characters was more distinct and exhibited more breeding value. Kumar & Arora (1992) observed in chickpea that the varieties with narrow genetic base were affected more by seasonal variation than those with broader genetic base, particularly under rainfed condition. Under such circumstances, availability of genetically diverse genotypes for hybridization programme becomes imperative. Dias *et al.*, (1993) and Amurrio *et al.*, (1995) reported no association between morphological characters and geographic origin. Analysis based on morphological characters was more reliable than on the basis of protein peptides that indicated more breeding value in chickpea, but simultaneous study for both agronomical and biochemical analysis (protein and DNA) is suggested.

Cluster analysis showed that many accessions from same origins were grouped separately which may be because of frequent exchange of germplasm by the breeders or transport of grain to different markets from where the seed of various origins is disseminated through out the country. According to Smith *et al.*, (1995), linkage cluster and PCA are useful for preservation and utilization of germplasm. Though accessions grouped together with greater morphological similarity, the cluster did not necessarily include all the accessions/genotypes from the same or nearby sites. The grouping pattern of landraces reflected association with geographic origin which is in contradiction to those presented by Amurrio *et al.*, (1995). Further Gupta *et al.*, (1991), Dias *et al.*, (1993) and Rabbani *et al.*, (1998) also reported no association between morphological characters and geographic origin.

In the present study, multivariate approach proved to be a useful tool in that it produced five clusters on the basis of provincial distribution much more differentiated if compared to the initial subdivision according to geographic sites of chickpea. The study confirmed the existence of a wealth of phenotypic divergence in the local chickpea germplasm. Further collecting missions to main chickpea growing areas with greater diversity could concentrate efforts on sampling as many geographically and ecologically distinct areas as possible, rather than collecting extensively from fields close to motorable roads within individual province (Pecetti *et al.*, 1996; Ghafoor *et al.*, 2001b). Laghetti *et al.*, (1998) suggested collecting expedition to the areas of where genetic erosion takes place in cowpea along with the areas where existing genetic diversity has not been yet gathered (Padulosi, 1993). SDS-PAGE was not very effective for studying intra-specific genetic diversity in cultivated chickpea alone rather wild *Cicer* could be included. Further, biochemical markers are suggested to enhance by adding DNA markers for studying diversity related to germplasm collections. Multivariate analyses proved its validity to establish genetic diversity and these statistics on the basis of quantitative characters revealed more reliability than SDS-PAGE markers.

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