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RELATIONSHIP BETWEEN SDS-PAGE MARKERS AND *ASCOCHYTA* BLIGHT IN CHICKPEA

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Abstract

The study was conducted to determine the relationship of chickpea genotypes towards blight disease reaction, *in vitro* growth of *A. rabiei* on sap extracted from chickpea and seed protein pattern by SDS-PAGE. Seed proteins were analyzed through slab type SDS-PAGE using 11.25% Polyacrylamide gel and 6 µl of sample quantity. Most of the genotypes grouped on the basis of disease reaction and *in-vitro* fungus growth but no association between disease and SDS-PAGE was observed. Out of 12 SDS-PAGE markers, 6 were polymorphic and gel was divided into three regions. The genotypes with similar banding patterns were suggested to test by 2-D electrophoresis and DNA markers for genetic diversity. Cluster analysis revealed mixed grouping of susceptible and tolerant genotypes that indicated no response for classifying chickpea for disease reaction on the basis of SDS-PAGE. A low level of genetic diversity was observed among 57 genotypes although those originated from diverse sources. As SDS-PAGE alone did not exhibit high level of variation rather disease rating was more reliable than protein peptides, but simultaneous study for both aspects (disease and biochemical analysis) is suggested. Due to less effectiveness of SDS-PAGE in resolving intra-specific genetic diversity in cultivated chickpea and disease reaction, wild *Cicer* spp., are suggested to be included. Further, biochemical markers are suggested to enhance by adding DNA markers (RAPD, RFLP, AFLP) in relation with *Ascochyta* blight for further evaluation and screening that will help in marker assisted selection (MAS).

Introduction

Chickpea is an important winter season food legume of Pakistan that is well adapted to marginal areas with low inputs. Its production in the country is, however, limited mainly due to occurrence of a foliar disease known as chickpea blight caused by *Ascochyta rabiei* (Pass) Lab. In case of blight epidemics, the yield losses caused by this disease have been estimated to the level of 48-70% (Malik & Bashir 1984; Nene, 1980) or more. Development of blight resistant varieties is the most effective and practical solution to this problem. For genetic improvement in chickpea against blight, it is important to use different sources of resistance available in the world collection of germplasm (Gurdip *et al*., 1991; Singh & Reddy 1991; Haware *et al*., 1995).

Ascochyta blight rating is a difficult task for a number of reasons: resistance is expressed on a quantitative scale; expression of resistant genes could be strongly influenced by the environment; unpredictable weather and a highly variable population of pathogen react differently with different genotypes (Melchinger, 1990). Under such conditions the efficiency of identifying resistant genotypes are suggested to combine with molecular markers for marker assisted selection. Considering difficulties in field evaluation and screening, bio-chemical markers received more attention in recent years from the crop geneticists for assessment of genetic variability (Akhtar, 2001, Ghafoor *et al*., 2002). Among biochemical techniques, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is widely used due to its validity and simplicity for

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describing genetic structure of crop germplasm. SDS-PAGE is practically a reliable method because seed storage proteins are largely independent of environmental fluctuation (Gepts, 1989; Murphy *et al*., 1990). The present study was conducted to investigate genetic diversity and disease rating along with total seed protein using SDS-PAGE to access their inter-relationship with emphasis to *Ascochyta* blight reaction.

Materials and Methods

Ranking of chickpea genotypes for disease resistance

Fifty-seven chickpea genotypes obtained from national and international sources were tested under artificial inoculation conditions against Ascochyta blight. Out of these, 23 genotypes were kabuli types (white seeded) and the others were desi types (brown seeded). Among all, 24 were approved varieties, whereas others were advanced lines obtained from International Centre for Agricultural Research in dry Areas (ICARDA) and national research centers (Table 1). The seeds of each genotype were surface sterilized with Clorox solution (0.1% available chlorine) for 2 minutes and were sown in disposable pots (7.5 x 15 cm) filled with sterilized soil and sand mixture (2:1) without additional nutrients. Five chickpea seedlings were maintained in each pot.

A blight susceptible variety C-727 was kept as control for comparison. Plants were grown in a greenhouse at 20±2 ^{0}C under natural light for 15 days before inoculation. Pots were watered from the top prior to inoculation. Two week old seedlings were inoculated by spraying aqueous spore suspension having an approximate concentration of 5 x 10^5 spores/ml. The inoculum was prepared from 15 days old culture of *A. rabiei* multiplied on chickpea grains according to the procedure described by Ilyas & Khan (1986). Inoculated seedlings were incubated under muslin cloth chamber for 7 days with 80% humidity. Disease observations were taken when the susceptible check lines were completely killed and disease rating were recorded on 1-9 disease rating scale (Singh *et al*., 1981).

Effect of chickpea plant extract on the growth of *Ascochyta rabiei*

Five gram shoot tips of each chickpea genotypes grown under healthy conditions were ground in 100 ml distilled water. After two hours, the water extract was drained off to get a clear solution. This extract was kept under UV light for 24 hours for sterilization. It was used to study the colony growth of *A. rabiei*. Ten ml water extract (as prepared above) was mixed with sterilized dextrose water agar medium (2%) and poured in (90 mm diameter sterilized Petri dishes. This was stirred with a glass rod while still hot under aseptic conditions. On solidification of the medium, it was inoculated with the actively growing culture of the fungus with the help of a cork borer, and was kept at 20±2^{0}C in an incubator for growth. Dextrose agar medium without plant extract served as control. The experiment was run in triplicate. Observations on colony growth were recorded 15 days after incubation.

Biochemical studies

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%

Table 1. Chickpea genotypes included for their biochemical aspects associated with blight resistance

S. No	Cultivars	Source	Seed type	Disease scale (1-9)	Disease reaction	Radial growth (cm)
1	Dasht	NARC	D	3.3	R	4.6
2	Parbat	NARC	D	4.0	T	5.1
3	C-727	AARI	D	9.0	S	4.9
4	C44	AARI	D	8.0	S	4.7
5	Punjab-91	AARI	D	8.0	S	4.7
6	Piadar	AARI	D	9.0	S	4.8
7	Noor-91	AARI	K	7.6	S	4.4
8	Bittle-98	AARI	D	7.0	S	4.6
9	Balkasar	BARI	D	3.0	R	4.7
10	Wanhar	BARI	D	4.7	T	4.8
11	CM-2000	NIAB	K	7.0	S	5.0
12	CM-98	NIAB	D	7.0	S	5.3
13	CM-88	NIAB	D	7.3	S	5.5
14	CM-72	NIAB	D	7.7	S	5.0
15	NIFA-88	NIFA	D	4.7	T	4.8
16	DG-92	RRI	K	8.7	S	5.3
17	DG-89	RRI	D	8.3	S	5.3
18	ILC 202	ICARDA	K	7.4	S	5.2
19	Pb-1	AARI	K	9.0	S	4.7
20	ILC-482	ICARDA	K	5.0	T	5.5
21	ILC-1929	ICARDA	K	5.3	T	5.3
22	ILC-3279	ICARDA	K	6.0	S	4.5
23	ILC-72	ICARDA	K	4.7	T	3.9
24	ILC-194	ICARDA	K	6.3	S	4.5
25	ILC195	ICARDA	K	4.3	T	4.6
26	ILC-200	ICARDA	K	3.7	R	4.6
27	ILC-201	ICARDA	K	6.3	S	4.4
28	AUG-424	UAF	D	9.0	S	4.6
29	NIFA-95	NIFA	D	6.7	S	4.4
30	C-235	AARI	D	9.0	S	4.6
31	Karak-1	ARS	D	7.0	S	4.5
32	V88194K	AARI	K	8.7	S	4.4
33	AAR-1	AARI	K	7.7	S	4.3
34	FLIP96-60C	ICARDA	K	7.0	S	4.3
35	FLIP97-17C	ICARDA	K	6.0	S	3.5
36	FLIP97-192C	ICARDA	K	5.0	T	4.1
37	FLIP97-179C	ICARDA	K	5.0	T	4.3
38	CH41/91	NIAB	K	7.7	S	4.0
39	NCS-2001	NARC	K	8.3	S	4.5
40	FLIP95-68C	ICARDA	K	5.7	T	5.1
41	NCS-950183	NARC	D	5.3	T	4.3
42	NCS-95004	NARC	D	5.3	T	4.6
43	NCS-95010	NARC	D	4.7	T	4.9
44	NCS-950212	NARC	D	4.3	T	4.5
45	92080	AARI	D	6.0	S	4.9
46	97047	AARI	D	4.7	T	4.6
47	90280	AARI	D	7.0	S	4.5
48	96052	AARI	D	7.7	S	4.5
49	96051	AARI	D	8.3	S	4.1
50	PBC-2000	AARI	D	6.7	S	5.2
51	93A082	AZRI	D	6.0	S	4.1
52	92A043	AZRI	D	5.0	T	4.2
53	CM72XILC3279	NARC	D	5.0	T	4.4
54	DC-1	RRI	D	4.0	T	3.5
55	CH40/89	NIAB	D	5.3	T	4.3
56	CM738/92	NIAB	D	8.0	S	3.5
57	CM2325/96	NIAB	D	8.0	S	3.4
	EMS			0.623		0.150
	LSD			1.276		0.482
	COV			12.15		8.56

NARC- National Agricultural Research Centre, Islamabad, AARI- Ayub Agricultural Research Institute, Faisalabad, BARI- Barani Agricultural Research Institute, Chakwal, NIAB- Nuclear Institute for Agriculture and Biology, Faisalabad, NIFA- Nuclear Institute for Food and Agriculture, Peshawar, RRI- Rice Research Institute, Dokri, Sindh, ICARDA- International Centre for Agricultural Research in Dry Areas, Allepo, Syria, UAF- University of Agriculture, Faisalabad, ARS- Agricultural Research Station, Karak, AZRI- Arid Zone Research Institute, Bhakhar

K- Kabuli (white seeded), D- Desi (brown seeded)

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. 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).

The data were analyzed for cluster analysis with the help of computer software STATISTICA Version 5.0 for Windows 98. For SDS-PAGE, after staining and distaining 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 and analyzed for cluster analysis (Sneath & Sokal, 1973).

Results

Disease response of chickpea genotypes

Of the 57 chickpea genotypes evaluated for the sources of resistance against *A. rabiei*, none of the line was found completely free from disease (Table 1). Among the kabuli types, 6 genotypes (ILC 482, ILC 72, ILC 195, ILC 200, FLIP 97-192C, FLIP 97-179C) obtained from ICARDA were tolerant to blight. None of local Kabuli type was tolerant. In case of desi types, 11 genotypes (Dasht, Parbat, Balkasar, Wanhar, NIFA 88, NCS 5010, NCS 950212, 97047, 92A043, CM 72/ILC 3279, DC 1) were tolerant and all of these have been developed by the local breeders (Table 2).

Table 2. Grouping of chickpea genotypes according to the reaction against blight.

Tolerant	
Desi type	Dasht, Parbat, Balkasar, Wanhar, NIFA-88, NCS-95010, NCS-950212, 97047, 92A043, CM72/ILC3279, DC-1
Kabuli type	ILC-482, ILC-72, ILC-195, ILC-200, FLIP97-192C, FLIP97-179C
Susceptible	
Desi type	C-44, Punjab-91, Piadar, Bittle-98, CM-98, CM-88, CM-72, DG-89, AUG-424, NIFA-95, C-235, Karah-1, NCS-950183, NCS-95004, 92080, 90280, 96052, 96051, PBC-2000, 93A082, CH40/89, CM738/92, CM2325/96
Kabuli type	Noor-91, CM-2000, DG-92, ILC-202, Pb-1, ILC-1929, ILC-3279, ILC-194, ICC-201, V88194K, AAR-1, FLIP96-60C, FLIP97-17C, CH41/91, NCS-2001, FLIP95-68C, ILC-263

Effect of chickpea plant extract on the growth of *Ascochyta rabiei*

No relationship was observed between disease reaction of 57 genotypes and *In vitro* growth of *A. rabiei* grown on sap extract from the same genotypes. Fungus growth was fast in sap from kabuli types, but no clear indication was observed that might help in

determining the resistance mechanism during *in-vitro* investigation (Table 1). Similar response was observed in case of susceptible genotypes. However, in case of some susceptible genotypes, fungus growth showed an increase. But on the other hand, in case of some susceptible genotypes, the growth was minimum as compared with others. It is interesting to note that the genotypes CM 72/ILC 3279 (F_9 generation) was tolerant although both of the parents were susceptible to disease. This might be due to additive genes controlling resistance mechanism present at various loci.

Seed proteins

On the basis of SDS-PAGE, 12 protein bands were observed with the Molecular Weight (MW) of 24 to 66 Kda. Many protein subunits of lower MW were also observed but due to inconsistency 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 in consideration. Out of 12 protein subunits, 6 were polymorphic and 6 were monomorphic. On the basis of banding pattern, gel was divided into three regions (Fig. 1).

Region I had bands of more than 66 Kda MW of which 2 were polymorphic. Region II ranged from 24 to 66 Kda having 8 protein peptides, out of which 4 were polymorphic. In this region, the protein bands were observed with high degree of variation in quantitative term. The quantitative intensity of bands was 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. On the basis of disease rating and radial growth, three clusters were observed (Fig. 2).

Cluster I consisted 14 genotypes, cluster II comprised 21 and cluster III of 22 genotypes. Out of 17 tolerant genotypes, 10 were grouped in the cluster III, 6 in cluster II and one in cluster I. The genotypes were plotted on the basis of SDS-PAGE and if cut at 1.5 linkages distance 4 clusters were observed (Fig. 3). Many genotypes overlap each other due to similarity on the basis of SDS-PAGE markers. Cluster I consisted three genotypes (AUG 424, C 235, NCS 2001), whereas cluster II consisted two genotypes (CM 2000, CM72/ILC 3279) and both of these were tolerant genotypes. One genotype (CH 41/91) was in cluster III and all the other 51 genotypes were in cluster IV. Within this cluster, this cluster comprised of mixed genotypes of susceptible and tolerant nature both kabuli and desi types that indicated no relationship between disease reaction and SDS-PAGE. A low level of genetic diversity was observed among 57 genotypes although these originated from diverse sources that might indicate exploitation of a portion of genetic diversity for chickpea improvement.

Discussion

Out of 11 tolerant genotypes 5 were approved varieties and others were advanced lines that indicated the visualization of breeding against chickpea blight by the national researchers. The tolerant genotypes are supposed to be the best sources for developing resistant cultivars by gene pyramiding as suggested by Horn (2001). There was no relationship in clustering on the basis of seed type, desi or Kabuli both for disease rating and SDS-PAGE. Low level of variation was observed for SDS-PAGE among chickpea genotypes included in the present study and similar results had already been reported by

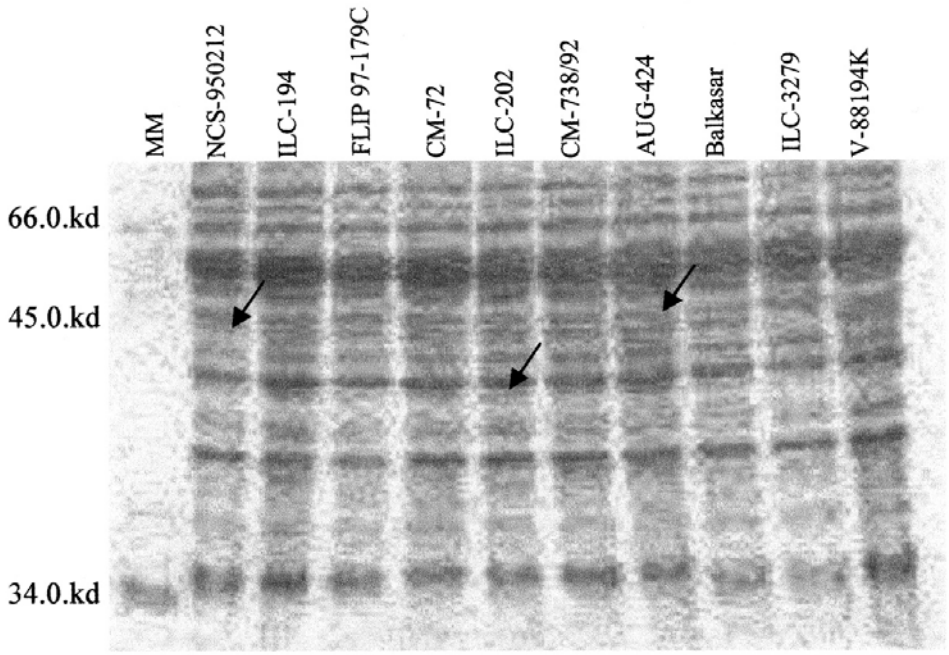

Fig. 1. Variation in seed proteins of chickpea genotypes. The molecular marker used in the gel was SDS-70 Kit. The arrows indicate variation in different regions.

Thakare *et al*., (1987), Iqbal, (2001), Mehrani, (2002) and Ghafoor *et al*., (2002) in legumes who observed low intra-specific variation within one species in their studies. SDS-PAGE showed that the method provided a tool for reliable germplasm discrimination based on genetic differences in seed storage protein comparison in chickpea, but no relationship among disease, seed type and protein peptides was observed. The genotypes with similar banding patterns may be duplicated, but these are suggested to be confirmed by the use of other biochemical markers including 2-D electrophoresis and DNA markers (Beckstrom-Sternberg, 1989; Higginbotham *et al*., 1991).

In the present study intra-specific variation was limited and it was observed that SDS-PAGE alone did not exhibit high level of intra-specific variation, therefore, diverse germplasm based on SDS-PAGE is suggested to be acquired from various sources, preferably from centre of diversity to build a broad based gene pool with maximum variability. Further, there was no relationship observed among three parameters i.e., *in vitro* growth of fungus, disease rating and SDS-PAGE for seed proteins, therefore for comprehensive knowledge of agricultural, biochemical data and *Ascochyta* blight reaction, there is a need to enhance the level of biochemical markers (protein and DNA). The SDS-PAGE in 57 chickpea genotypes did not reflect any clue either for fungus growth, disease reaction, seed type (desi or kabuli) and origin. For most genotypes and protein subunits, no clear observation was recorded which could facilitate selection on the basis of SDS-PAGE for improving disease resistance in chickpea from the material under investigation. Analysis based on disease rating and radial growth was more reliable

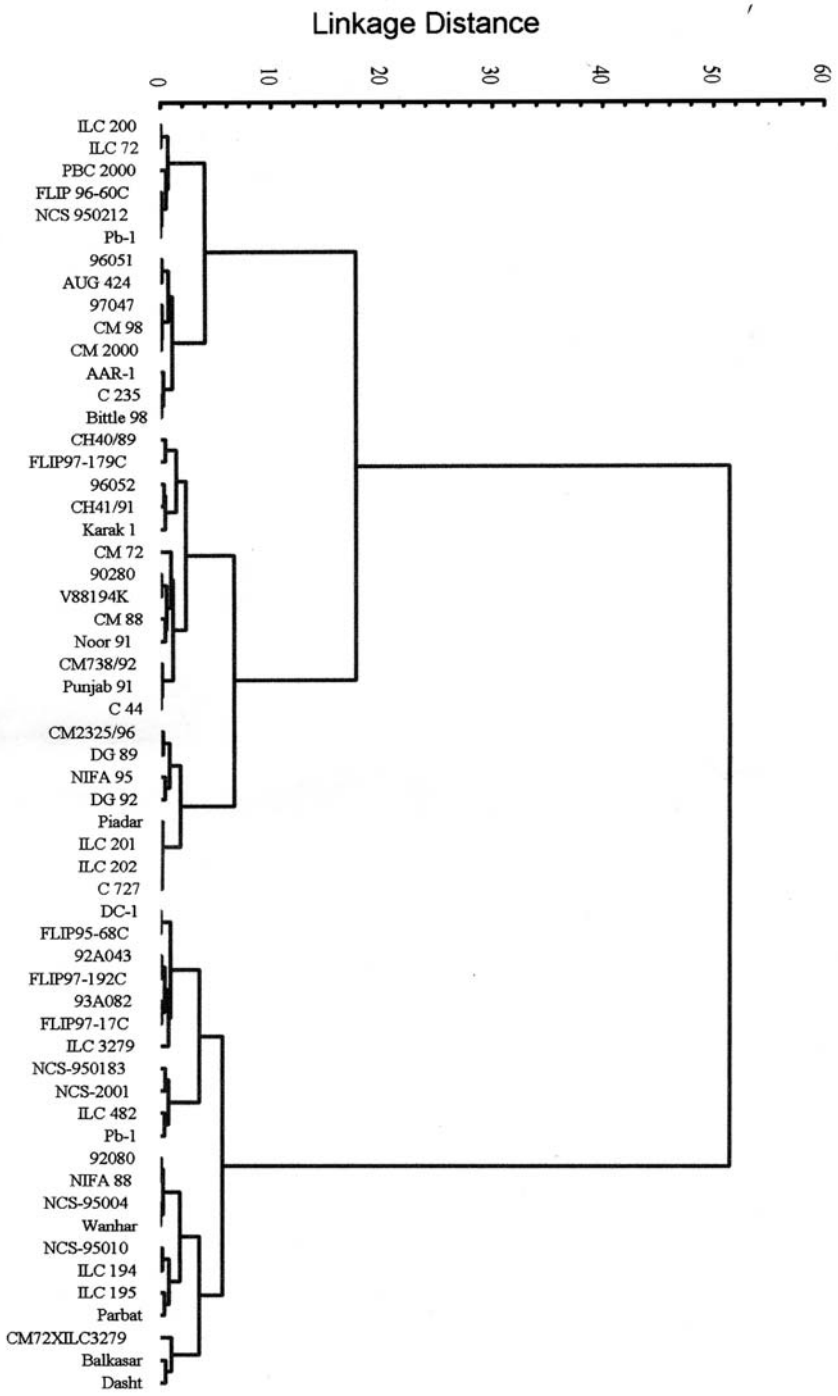

Fig. 2. Phenogram based on disease rating and radial growth in 57 genotypes of chickpea.

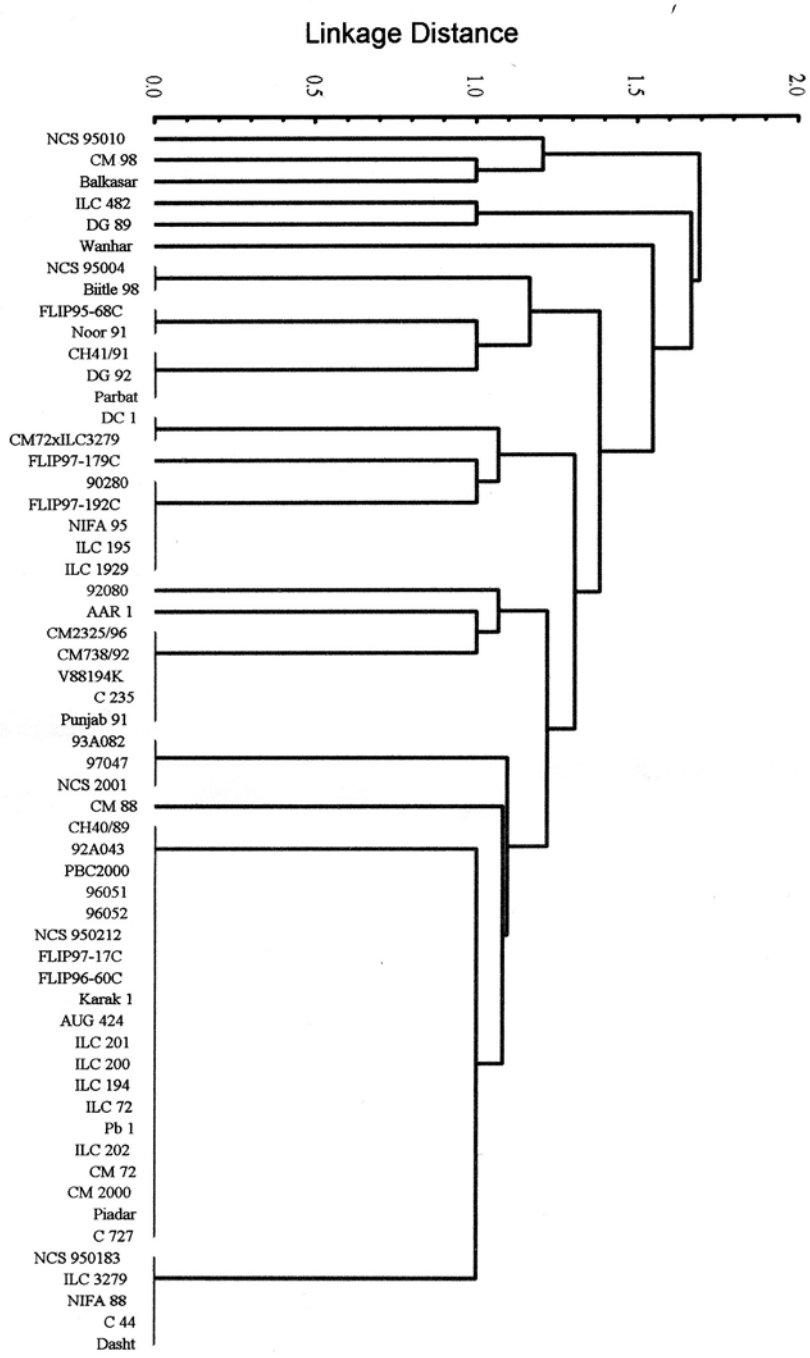

Fig. 3. Dendrogram based on six protein markers in 57 genotypes of chickpea.

than on the basis of protein peptides that indicated the use of enhanced biochemical markers. This situation indicated the independence of seed protein from disease reaction or complexity of genetics of this disease although DNA markers have been reported for *Ascochyta* blight in chickpea (Horn, 2001). SDS-PAGE was not very effective for studying intra-specific genetic diversity in cultivated chickpea and disease status alone rather wild *Cicer* could be included. Further, biochemical markers are suggested to enhance by adding DNA markers in relation to *Ascochyta* blight should be included for further evaluation and screening that will help in marker assistant breeding.

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