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Biochemical and cytological analysis of five cultivars of *Cicer* (chickpea)

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During the present study, protein estimation and protein profiling of five cultivars of *Cicer* namely Dhawal, ujjwal, Shubhra, DCP-92-3 and IPC-04-20 have been carried out. The protein content on per gram fresh weight basis was found highest in the Dhawal measuring 35.2± 3.83 mg followed by DCP-92-3 and lowest was found in the IPC-04-20 cultivar. The protein profiling of seed proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) revealed a high polymorphism between the five cultivars. The total number of polypeptide bands recorded was 36, the 4 bands were monomorphic and the rest32 were polymorphic with an average polymorphism of 88.88%. The Jacards similarity ranged from 0.25000 to 0.56000. The similarity index calculated was highest between Ujjwal and IPC-04-20 (37.5%) where as lowest was calculated between Shubhra and IPC-04-20 (22.22%) variety. The unweighted pair group method with arithmetic mean (UPGMA) clustering method revealed two major clusters in the dendrogram that is, cluster 1 and 2, comprising two varieties each. The Shubhra occupies a distinct place as depicted in the dendrogram. Moreover, cytological studies of the five cultivars by calculating the mitotic index were carried out. The mitotic index varied between 4.82 to 10.83% and was found highest in DCP-92-3 while as minimum value was observed in Ujjwal.

Key words: SDS-PAGE, UPGMA dendrogram, mitotic index, chickpea.

INTRODUCTION

The genus *Cicer* include 33 perennial, eight annual, one unspecified wild species as well as the cultivated ones (Van der Maesen, 1987). Chickpea is the second most important cool season pulse crop in the world and is grown in at least 33 countries including central and west Asia, South Europe, Ethiopia, North Africa, North and South America and Australia (Ladizinsky and Adler, 1976; Singh and Ocampo, 1997). It is native to South Europe and is the most important pulse crop of India, commonly grown in Uttar Pradesh, Panjab, Maharashtra, Rajasthan, Bihar and Madhya Pradesh accounting for more than 90% of the total area under it. India is the largest producer of chickpea, accounting for 66% of the world production (FAO, 2004). The average annual yield world wide (0.78 ton/ha) is considered to be somewhat lower than its potential yield (Singh et al., 1994; Sudupak et al., 2002). In India, gram is sown as Rabi crop at the end of the rainy season. Sowing takes place from September to November, and harvesting from February to April. Genotyping of different species is necessary for characterization of different accessions of crop gemplasm, testing varietal purity and registration of newly

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Abbreviations: UPGMA, Unweighted pair group method with arithmetic mean; **SDS-PAGE;** sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Table 1. Five experimental accessions of *Cicer*.

Accession name	Sample name	Source	Seed size
Dhawal	S1	IIPR, Kanpur	Extra bold
Ujjwal	S2	Same	Bold
Shubhra	S3	Same	Bold
DCP-92-3	S4	Same	Small
IPC-04-20	S5	Same	Small

developed cultivars (Chowdhury et al., 2002). Among numerous techniques available for assessing the genetic variability and relatedness, seed storage protein analysis represents a valid alternative to varietal identification (Manella et al., 1999). Seed storage protein profiling based on SDS-PAGE can be employed for various purposes, such as characterization of germplasm (Javid et al., 2004; Iqbal et al., 2005), varietal identfication, biosystematic analysis, determination of phylogenetic relationship between different species (Sammour, 1991; Isemura et al., 2001; Ghafoor et al., 2002). It is a useful tool for studying genetic diversity via sodium dodecyl sulphate polyacrylamide gel electrophoreseis (SDS-PAGE) (Sadia et al., 2009).

Seed storage protein markers are highly polymorphic and environmental influence on their electrophoretic pattern is limited (Gepts et al., 1986; Sadia et al., 2009). Genetic diversity of seed storage proteins has been reported for lima bean (Lioi et al., 1999), *Phaseolus vulgare* (Ferreira et al., 2000) and chickpea (Ghafoor et al., 2003). Phylogenetic relationship among *Cicer* species based on SDS-PAGE data has suggested that *Cicer reticulatum* is the wild progenitor of cultivated chickpea (Ahmad and Slinkard, 1992).

MATERIALS AND METHODS

The germplasm of five accessions of *Cicer* were obtained from Indian Institute of Pulse Research, Kanpur (U.P.) India. During the present study, five accessions were used for biochemical and cytological analysis. The details of five accessions are given in Table 1.

Protein estimation

Cicer being one of the highly proteinaceous crops was subjected to protein estimation in order to know the total protein content. The protein content was measured by using the method of Bradford et al. (1976) and was calculated by using the formula:

$$\text{Protein estimation} = \frac{12 \times \text{O. D.} \times \text{D. F.}}{0.28} \times 1000 \times \text{F. W.}$$

Where, O. D. is Optical Density, D. F. is dilution factor and F. W. is fresh weight.

Protein extraction

For total seed protein extraction from individual seed samples, 1 g seed of each accession was taken and ground into fine powder using pestle and mortar. 500 ml of protein extraction buffer was added to 0.01 g of seed flour and vortexed thoroughly to homogenize. The homogenate samples were centrifuged at 6,000 rpm for 10 min at room temperature. The extracted crude proteins were recovered as clear supernatant and were transferred to a new 1.5 ml eppendorf tubes and stored at 4°C until they were run on the polyacrylamide gel.

Protein profiling

Protein profiling of extracted samples was carried out through SDS-PAGE using 12% polyacrylamide gel. Electrophoresis was carried out at 75 V for 3 h. A protein marker was loaded as standard along with the samples with equal quantities of protein (4 ml) into each well of the gel. The gels were then fixed in solution (10% acetic acid and 40% ethanol) for 15 min with constant shaking and then stained with 0.2% (W/V) Commassie brilliant blue R 250 overnight on an electric shaker using Double Shaker Mixer Model DH-10. Destaining was carried out for a couple of hours followed by gel preservation, scanning and photography.

Protein imaging and data analysis

Gel photographing and documentation were carried out. With regard to variation in protein banding pattern, electrophorogram of each accession was scored for the presence or absence of bands and used to construct a dendrogram by the unweighted pair group mean and arithmetic average method (UPGMA).

Mitotic index

During the present study, the mitotic index was examined. Root tips of each accession were harvested at about 6 a.m., fixed in 3:1 ratio of ethyl alcohol and glacial acetic acid solution for about 6 h and then preserved in 70% alcohol for analysis. The mitotic index was calculated by using the formula:

$$\text{Mitotic index} = \frac{\text{Number of cells in division phase}}{\text{Total number of cells studied}} \times 100$$

RESULTS

Protein estimation

Cicer being a proteinacious crop was subjected to protein estimation. The protein content was found highest in Dhawal (35.2 mg/g F. W.) followed by DCP-92-3 (34.3 mg/g F. W.), Ujjwal (32.9 mg/g F. W.) and Shubhra (31.0

Table 2. Protein content of five accessions of *Cicer.*

Accession name	Protein content (mean ± standard error)
Dhawal	35.2 ± 3.83
Ujjwal	32.9 ± 3.16
Shubhra	31.0 ± 2.33
DCP-92-3	34.3 ± 3.83
IPC-04-20	28.7 ± 1.5

mg/g F. W.); while as the lowest protein content was observed in IPC-04-20 (28.7 mg/g F. W.) (Table 2). The variation might be due to different geographical regions.

Protein profiling

The SDS-PAGE of seed proteins of five genotypes was carried out to investigate the genetic diversity at molecular level. Seed storage protein profiling showed distinct polymorphism in electrophoretic banding patterns and led to detection of 36 polypeptide bands in total. Out of 36 bands, only four were monomorphic and the rest 32 were polymorphic. The average polymorphism was 88.88%. Similar results were also reported by Nisar et al. (2007) in Chickpea. Polymorphism was evident in all seed proteins of diverse molecular weights among all accessions but major diversity was found in low molecular weight region. The molecular weights of peptides ranged from 99 to 40 KD with the presence or absence of particular band. The maximum protein bands generated in case of Dhawal (21) and the minimum (16) in Shubhra. The protein band for highest molecular weight (that is, 99 KD) was generated in all the five accessions while that of lowest molecular weight (that is, 40 KD) was generated in IPC-04-20 (Table 3 and Figure 2). The similarity index calculated was found highest between Ujjwal and IPC-04-20 (that is, 37.5%), and between Dhawal and DCP-92-3 (30.76%) while as minimum similarity was found between Shubhra and IPC-04-20 (22.22%) (Table 4).

The data obtained from SDS-PAGE analysis was used for construction of dendrogram using unweighted pair group mean and arithmetic average (UPGMA) procedure is presented in Figure 1. Five accessions were grouped in two clusters. Cluster I and cluster II, comprising of two accessions each. The cluster analysis revealed that Ujjwal and IPC-04-20 are very close to each other. Accession Shubhra occupies a distinct place as revealed in the dendrogram.

Cytological study

The cytological study was done to find out the mitotic index. The mitotic index varied from 4.82 to 10.83% (Table 5). It was found highest in DCP-92-3 (10.83%) and in Dhawal (8.98%) which revealed that these accessions have highest power of division, while as the minimum mitotic index was found in Ujjwal (4.82%) and Shubhra (7%) which indicates that these have low rate of division. Similarity index of different protein bands was calculated by the formula:

$$\text{S. I.} = \frac{\text{Total number of similar bands}}{\text{Total number of bands}} \times 100$$

Jaccard's similarity index

Jaccard's similarity index is given in Table 6.

DISCUSSION

Previously morphological and cytological assay procedures were used to estimate existing genetic variability in the crops of commercial importance including legumes (Islam and Shepherd, 1991). These essay procedures though were successful in many cases but were not considered suitable for large scale screening mainly because of limited number of markers and time consuming for the essay procedures. Most recently, protein markers (especially seed storage proteins) are being used for better and more reliable estimation of genetic distances among species/-lines/populations (Weber et al., 2005). Comparison of seed storage proteins have been found to provide no biological basis for separating closely related small and large seeded lentils (Ladizinsky, 1979). Same is the case with chickpea genotypes evaluated in the present study as no difference in seed storage proteins of bold and small seeded genotypes was observed. The protein profiling of seed proteins using SDS-PAGE revealed a high polymorphism between the five cultivars. The total number of polypeptide bands recorded was 36, the 4 bands were monomorphic and the rest 32 were polymorphic with an average polymorphism of 88.88%. The Jacards similarity ranged from 0.25000 to 0.56000. The similarity index calculated was highest between Ujjwal and IPC-04-20 (37.5%); whereas lowest was

Table 3. Presence and absence of bands of different molecular weights in different samples.

Band No.	R. F. value	M. W. K. D.	S1	S2	S3	S4	S5
1	0.01	99	+	+	+	+	+
2	0.02	98	+	+	+	+	+
3	0.03	97	+	+	+	-	+
4	0.05	95	+	-	-	+	+
5	0.06	94	+	+	+	-	+
6	0.07	93	+	+	-	+	+
7	0.08	92	+	-	+	-	-
8	0.10	90	-	+	-	+	+
9	0.11	89	+	-	+	+	-
10	0.12	88	+	-	-	-	-
11	0.13	87	-	+	-	-	+
12	0.15	85	+	+	+	+	+
13	0.16	84	+	+	+	-	-
14	0.17	83	-	-	+	+	-
15	0.18	82	+	+	-	+	-
16	0.20	80	+	-	+	+	-
17	0.21	79	+	+	-	-	-
18	0.22	78	-	-	+	-	-
19	0.23	77	-	+	-	+	+
20	0.25	75	+	+	-	-	+
21	0.27	73	-	+	+	+	+
22	0.30	70	+	-	-	+	-
23	0.31	69	-	+	+	-	+
24	0.33	67	-	-	-	+	-
25	0.35	65	+	+	+	+	+
26	0.36	64	-	+	+	-	-
27	0.37	63	-	+	-	+	-
28	0.38	62	+	-	-	-	+
29	0.41	59	+	-	-	-	-
30	0.42	58	+	-	-	+	-
31	0.45	55	-	-	-	-	+
32	0.46	54	-	-	-	+	-
33	0.48	52	+	+	+	-	+
34	0.50	50	-	+	-	-	-
35	0.52	48	-	-	-	-	+
36	0.60	40	-	-	-	-	+

The symbols (+) and (-) indicate the presence and absence of a band, respectively.

calculated between Shubhra and IPC-04-20 (22.22%) variety. The UPGMA clustering method revealed two major clusters in the dendrogram that is, clusters 1 and 2, comprising two varieties each.

The Shubhra occupies a distinct place as depicted in the dendrogram. Similar results were also reported by Nisar et al. (2007) in Chickpea. The results of present studies are further strengthened by previous finding of Ghafoor et al. (2003), Yasmin et al. (2010), Asghar et al. (2003), Ferreira et al. (2000) and Dasgupta and Singh (2003) who reported high genetic diversity in various legume species using protein profiling.

Conclusion

Genetic diversity is important for improving any crop species. An understanding of the magnitude and patterns of genetic diversity in crop plants has important implications in breeding programs and for conservation of

Table 4. Similarity index of five accessions of *Cicer* using SDS-PAGE.

Sample	Name of samples				
	S1 (%)	S2 (%)	S3 (%)	S4 (%)	S5 (%)
S1	100				
S2	29.16	100			
S3	27.02	27.77	100		
S4	30.76	26.31	23.52	100	
S5	24.39	37.5	22.22	26.31	100

Where, S1 is Dhawal, S2 is Ujjwal, S3 is Shubhra, S4 is DCP-92-2 and S5 is IPC-04-20.

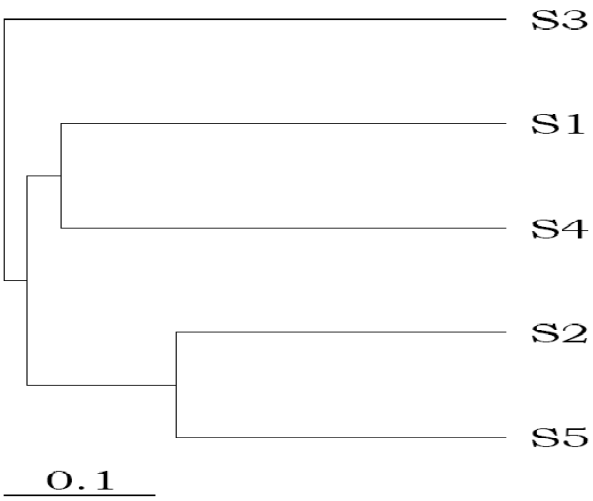

Figure 1. UPGMA dendrogram depicting phylogenetic relationships among five *Cicer* accessions.

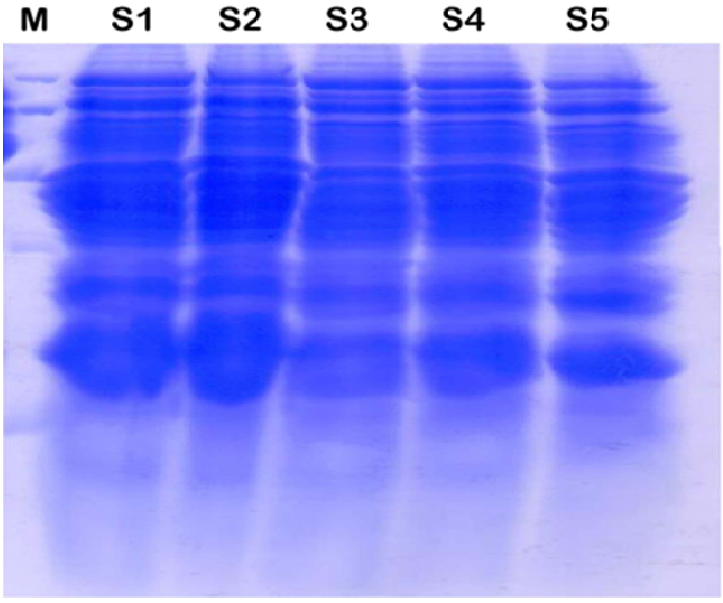

Figure 2. Protein profile of five accessions of *Cicer* produced using SDS-PAGE.

Table 5. Mitotic index of five *Cicer* accessions.

Accessions name	Mitotic index (%)
Dhawal	8.98
Ujjwal	4.82
Shubhra	7
DCP-92-3	10.83
IPC-04-20	7.63

Table 6. Jaccard's similarity index of five *Cicer* accessions.

	S1	S2	S3	S4	S5
S1					
S2	0.41379				
S3	0.37037	0.38462			
S4	0.40741	0.77037	0.32000		
S5	0.33333	0.56000	0.25000	0.33333	

genetic resources. From the result of the study, it is clear that higher amount of genetic diversity of seed storage proteins is present in chickpea genotypes which can be utilized in breeding programs aimed at increasing level of genetic diversity which ultimately will be useful for the development of new improved genotypes of chickpea. Evaluation of genetic diversity and identification of chickpea accessions by SDS-PAGE is easy and early approach and it is also useful for molecular weight analysis of chickpea seed storage proteins. It is concluded from the result that Shubhra and IPC-04-20 are genetically dissimilar; hence, it is recommended that these two accessions should be used for future breeding programs.

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