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GENETIC DIVERSITY ANALYSIS IN FIVE ACCESSIONS OF *TRIGONELLA* USING CYTOLOGICAL STUDY, PROTEIN ESTIMATION AND SDS-PAGE

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ABSTRACT

Genetic variation of cultivars is very interesting in reducing genetic vulnerability as well as stabilizing production. In this regard, a study was undertaken to evaluate the genetic diversity among five accessions of *Trigonella* viz., IC-143851 (A1), IC-144225 (A2), IC-332236 (A3), IC-371755 (A4) and IC-433589 (A5). For the first assay, mitotic index and protein estimation were evaluated. A1 accession had the highest mitotic index (15.40%), while A4 accession had the lowest (6.83%). Highest and lowest protein contents were observed in case of A3 (31.0±1.16) and A5 (23.2±0.80) accessions, respectively. Total seed storage protein profiles were examined using SDS-PAGE. The proteins were resolved in 27 bands with 24 polymorphic peptides. The similarity coefficient calculated on the basis of presence and absence of bands ranged from 0.23-0.55. Following the UPGMA algorithm of similarity coefficients, the accessions could be clustered into two similarity groups. Cluster 1 consisted of two accessions (A1 and A3) while the cluster 2 grouped rest of the accessions (A2, A4 and A5). Clustering based on seed storage protein profiles provides information about the phylogenetic relationship of accessions as all the accessions have at least one or more unique seed storage protein marker that can separate them from one another.

Keywords: Genetic Diversity ,SDS PAGE,Mitotic index,Trigonella Protein profile.Phylogenetic

INTRODUCTION

The family Fabaceae, or bean family, is the second big family of flowering plants in the world with 650 genera and18, 000 species (Rakhee *et al.*, 2004). It includes many crops useful for food, forage, fiber, wood and ornamental purposes. Few members of the family such as chickpea, soybean, fababean, fenugreek, lentil, pea etc. are consumed as grain legumes. The grain legumes are plants used as food in the form of unripe pods, mature seeds or immature dry seeds, directly or indirectly (Rachie and Roberts, 1974).

The genus *Trigonella* is one of the largest genera of the tribe Trifoliatae in the family Fabaceae and sub-family Papilionaceae (Balodi and Rao, 1991), represented by about 110 species in the world (Sechmen *et al.*, 1998). Among *Trigonella* species, *Trigonella foenum graecum* (commonly known as fenugreek) is a flowering annual, with autogamous white flowers occasionally visited by insects. Indigenous to countries on the eastern shores of Mediterranean, fenugreek is widely

cultivated in India, Egypt, Ethiopia, and Morocco and occasionally in England (Polhil and Raven, 1981). Fenugreek is extensively grown in the tropical and subtropical regions of India during winter season for its seeds, tender shoots and fresh leaves. The current productivity of fenugreek is 1245 kg/ha. The value added products of fenugreek such as fenugreek seeds, fenugreek powder and oleoresins are exported to Europe, North America, South Africa and some Asian countries (Malhotra and Vahishtha, 2008). Traditionally it is consumed as fresh vegetable and as a spice to add flavor to the Indian cuisines. Fenugreek is gaining importance due to its rare medicinal properties (Sharma *et al.*, 1990). According to *Ayurveda*, fenugreek is herbal drug that is bitter or pungent in taste. It is effective against anorexia and is a gastric stimulant (Rajagopalan, 2001). Diosgenin, a steroidal saponin present in its seeds has been shown to induce apoptosis in a variety of tumor cells (Shishodia and Aggarwal, 2006). Aggarwal and Shishodia (2006) cloned and characterized a small cystine -rich peptide which has antifungal properties in nature (Olli *et al.*, 2007; Olli and Kirti, 2006). Fenugreek seed powder has been shown to demonstrate antidiabetic effect by stabilizing glucose homeostasis and carbohydrate metabolism in type-1 diabetes (Preet *et al.*, 2006).

Across the world only known and well-defined cultivars are grown in specific areas. Gene banks also harbor scanty germplasm collection of *Trigonella* species (Hymowitz, 1990). The neglected and the underuse status of these locally important crops indicates a risk of disappearance of important plant material developed over thousands of years. One of the important factors restricting the development of better varieties and their large-scale production is that very little information is available about the genetic diversity, inter- and intra-specific variability and genetic relationship among these species. Therefore, attempts to analyze possible untapped genetic diversity becomes extremely essential for breeding and crop improvement.

Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) is used for its validity and simplicity to describe genetic structure of crop germplasm, but its implication has been limited mainly to cereals because of less polymorphism in most of the legumes (Ghafoor *et al.*, 2002). Seed protein analysis by SDS-PAGE is a tool to understand the genetic diversity at protein level among the genotypes. It is less expensive, reproducible, reliable and efficient method. Seed protein markers are widely used for the identification of varieties (Cooke 1984) of agricultural and horticultural crops. The validity and simplicity of seed protein profiling has been well documented (Cooke 1988). High stable and reliable seed protein profiling make it a powerful tool in elucidating the taxonomic and evolutionary problems of cultivars (Ladizinsky *et al.*, 1979; Das and Mukarjee, 1995). Researchers have used seed protein profiling by SDS-PAGE as genetic marker system since many years. The use of genetic and seed protein marker can be used to select elite accessions collected from different agro-climatic regions for crop improvement programs. Comparative studies on the proteomic data in leguminous species has been reported (Yasmin *et al.*, 2010). The present study was initiated to study the genetic diversity based on seed protein profiling across the selected accessions.

MATERIALS AND METHODS

Seeds of five accessions of *Trigonella* viz., IC 143851, IC 144225, IC 332236, IC

371755 and IC 433589, designated in the experiment as A1, A2, A3, A4 and A5, respectively, were collected from National Bureau of Plant Genetic Resource (NBPGR), New Delhi, to study the genetic variation based on cytological study, protein estimation and protein profiling. Protein extraction and protein estimation were conducted at Indian Grassland and Fodder Research Institute (IGFRI), Jhansi (U.P.), India.

Mitotic index:

Mitotic index was determined from the root tips of the experimental species. Root tips were harvested and fixed in 3:1 ratio of alcohol and glacial acetic acid. After six hours of fixation, the root tips were transferred to 70% alcohol for preservation. Mitotic index was then calculated by using the formula:

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

Protein estimation

Protein content was measured by using the method of Bradford *et al.,* (1976). The protein concentration was determined by using Bovine Serum Albumin as a standard and the total protein content was estimated by using the following formula:

$$\textbf{Protein content} = \frac{12 \times O.D \times Df}{0.28} \times 1000 \times \text{plant weight}$$

Protein profiling

Extraction of proteins

Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was used to characterize the protein profiles of the accessions by using 12.25% (w/v) separating gel and 4.5% (w/v) staking gel as developed by Laemmli (1970). 1.0 g seeds from each accession were ground to fine powder with mortar and pestle for the extraction of proteins. 400µl of protein extraction buffer was added to 0.01g seed flour and vortexed thoroughly to homogenize. For purification the homogenates were centrifuged at 15000 rpm for 10 minutes at room temperature. The extracted crude proteins were recovered as clear supernatant, which were transferred into1.5 ml centrifuge tubes and stored at 2^0C until electrophoresis.

Electrophoresis (SDS- PAGE)

The elecrophoretic procedure was carried out using slab type SDS-PAGE (AE 6530 M, Japan) with 12.25% polyacrylamide gel. A resolving gel (3.0 M Tris HCl, pH 8.0, 0.4% SDS) and a 4.5% stacking gel (0.4 M Tris HCl, pH 7.0, 0.4% SDS) was prepared and polymerized chemically by the addition of 17 ml of N, N, N, N tetra methylene diamine and 10% ammonium persulphate. Electrode buffer solution was poured into the bottom pool of the apparatus. Gel plates were placed in the apparatus carefully so as to prevent bubble formation at the bottom of gel plates. Then electrode buffer (0.025 M Tris, 0.12 M Glycine, 0.12% SDS) was added to the top pool of the apparatus. 15µl of the supernatant was loaded with the help of a micropipette into each well of the gel. The apparatus was connected with uninterrupted electric supply (100 V) until

the bromophenol blue reached the bottom of the gel. Gels were then stained for one hour with the staining solution containing 0.2% (w/v) Comassie Brilliant Blue R 250, dissolved in 10% (v/v) acetic acid, 40 % (v/v) methanol and water in the ratio of 10:40:50. Gels were destained in a solution of methanol, acetic acid and water in the ratio of 30:10:60, respectively. After destaining, gels were photographed using gel documentation systems.

Gel analysis

The gels were analyzed directly and data recorded on the basis of presence and absence of protein bands. Similarity index was calculated for all possible pairs of proteins. Presence and absence of bands were entered in a binary data matrix. Based on result of electrophoresis band spectra Jaccard's (1979), a dendrogram was constructed by the unweighed pair group method with arithmetic mean (UPGMA). All calculations were carried out using NTSYS-pc software version 2.2 (Rohlf, 2004).

RESULTS AND DISCUSSION

Evaluation of germplasm is essential to ensure its efficient and effective use. A significant variation was observed in case of the mitotic index (Table 1) of different accessions of *Trigonella* during the present investigation. Highest percentage of mitotic index (15.40%) was observed in case of A1, followed by A5 and A2. In case of A3 a mitotic index of (7.56 %) was observed. The lowest among all the accessions (6.83%) was found in case of A4. The accessions with higher mitotic index revealed that these accessions might have highest power of division. Different workers from time to time studied cytological characters among different species and revealed different rates of genetic variation at intra- and inter-specific level (Al-Wadi and Gamal, 2007; Bhat and Kudesia, 2011). Martin *et al.* (2011) found that some endemic species of *Trigonella* in Turkey have higher mitotic index by virtue of the fact that they are widespread while other endemic species are restricted to some localities only because of their lower mitotic index.

Total protein content in the seeds of *Trigonella* varied from 23.2-31.0 mg/g with a mean value of (27.04 mg/g.). The lowest protein content was found in A5 accession and the highest protein content was recorded in case of A3. A narrow range of variation was observed in rest of the accessions (Table 1). Our results are in consistency with the findings of Makaii *et al.* (2004) who revealed 30-32% protein in case of *Trigonella*. Sammour *et al.* (2007) also revealed genetic diversity on the basis of protein estimation in *Latharus sativa* and found the same result. The difference observed in case of the protein content may be attributed to the environmental factors such as geographical area, season of collection, elevation and annual temperature, precipitation and soil fertility (Vargas *et al.*, 2000) and thus expression of different genes. More studies are needed to determine the effects of the environment on the amount of the total seed proteins in seeds of plants.

Electrophoresis of proteins is a powerful tool for population genetics (Parker *et al.*, 1998). The most commonly used proteins are seed storage proteins, which are known to be polymorphic with respect to size, charge, or both these parameters (Cooke, 1984; Martinez *et al.*, 1997). Germplasm characterization based on morphological traits is not up to the mark and requires confirmation at molecular or at least at protein level. Electrophoresis of proteins is a powerful tool for detection of the genetic diversity and the SDS-PAGE of seed

protein is particularly considered a reliable technology because seed storage proteins are highly independent of environmental fluctuations (Iqbal *et al.*, 2005; Javid *et al.*, 2004). Genetic diversity of *Trigonella* germplasm elucidated through SDS-PAGE of proteins from seeds revealed distinct electrophoretic patterns. Twenty seven bands ranging from 50.0 to 97.0 kDa were recognized among five accessions, three were monomorphic (Table 2, Figure 1). The genotypes showed considerable variation in protein band number ranging from 12 to 17. Out of 27 peptide bands 24 bands were polymorphic with 88.88% polymorphism. Our finding reveals that considerable intra-specific variation was available in the analyzed accessions. The variation in the major bands was present in case of A1 and A5, where as the accessions showed variations for the minor bands. Band number 5 (93kDa), 7 (89 kDa) and 27 (50 KDa) were common in all the accessions. Polypeptide band number 8 (87 kDa, A1), 12 (78 kDa, A1) 18 (69 kDa, A2), 21 (65 kDa, A4), 23 (59 kDa, A4), 24 (58 kDa, A3) and 26 (56 kDa, A5) were accession specific. Band number 20 (67 kDa) was absent only in case of A1. Species specific bands may be exploited for hybrid identification in breeding experiments (Maity *et al.*, 2009).

The cluster analysis performed using UPGMA revealed two distinct clusters as evident from dendrogram (Figure 2) constructed from Jaccards similarity matrix. Cluster 1 consisted of two accessions (A3 and A1) while the cluster 2 grouped rest of the accessions (A2, A4 and A5). Clustering based on seed storage protein profiles provides information about the phylogenetic relationship of genotypes as all the genotypes have at least one or more unique seed storage protein marker that can separate them from one another and also from other *Trigonella* genotypes. The results are in consistence with Hameed *et al.* (2009).

The SDS-PAGE results revealed that the total amount of polymorphism accounted for principal component was 88.88% which revealed a considerable genetic diversity among the studied accessions. Our results are in consistence with that of Landizinsky (1979), who found genetic diversity among three species of fenugreek based on seed protein profiles. The variability within the investigated accessions agrees with previous biochemical studies (Chowdhury and Slinkard, 2000; Tadesse and Bekele, 2001, 2004). Various reports on the same line are present from the previous investigations. Sammour *et al*. (2007) used SDS-PAGE technique in *Latharus sativa* and found 72.72% polymorphism in case of seed proteins .The electrophoretic analysis of seed proteins in the *Trigonella* accessions revealed a considerable intra-specific variation. This observation is consistent with the electrophoretic data of *Latharus sativa* (Przybylska *et al*., 1998). Marked protein polymorphism may be explained by the presence of out crossing in this self-pollinated species as has been augmented by Chowdhury and Slinkard (1997) in case of *Latharus sativa.* Kaumar and Tata (2010) found 85.57 % polymorphism in case of chilli peppers. Present protein profiles of the selected experimental accessions of *Trigonella* revealed that accessions A4 and A2 are very close to each other at molecular level. These accessions almost possess same similarity index of about (0.50), (Table 3).

Seed protein patterns can be used as a promising tool for distinguishing cultivars of particular crop species (Jha and Ohri, 1996; Mennella *et al*., 1999). However, only few studies indicated that cultivar identification was not possible with the

SDS-PAGE method (Ahmad and Slinkard, 1992; De Vries, 1996). The SDS-PAGE is considered to be a practical and reliable method for species identification (Gepts, 1989).

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Table 1. Mitotic index and protein content of the five experimental *Trigonella* accessions.

Parameters studied	*Trigonella* Accessions				
	A1	A2	A3	A4	A5
Mitotic index (%)	15.40	14.46	7.56	6.83	14.66
Protein content (mg g^{-1} FW)	27.3±0.99	25.5±0.91	31.0±1.16	28.2±1.02	23.2±0.80

Table 2. Molecular weight of polypeptide bands of the five experimental *Trigonella* accessions.

Band Number	Rf Value	Molecular Weight (KDa)	*Trigonella* Accessions				
			A1	A2	A3	A4	A5
1	0.03	97	–	+	–	+	+
2	0.04	96	+	–	+	–	–
3	0.05	95	+	–	–	+	+
4	0.06	94	+	+	+	–	–
5	0.07	93	+	+	+	+	+
6	0.10	90	+	+	+	–	+
7	0.11	89	+	+	+	+	+
8	0.13	87	(+)	–	–	–	–
9	0.14	86	–	+	–	–	+
10	0.15	85	–	+	–	+	+
11	0.16	84	+	–	–	–	+
12	0.22	78	(+)	–	–	–	–
13	0.25	75	–	+	–	+	+
14	0.26	74	–	+	+	+	–
15	0.27	73	+	–	+	+	+
16	0.28	72	+	–	+	–	–
17	0.30	70	–	–	+	+	+
18	0.31	69	–	(+)	–	–	–
19	0.32	68	–	–	+	+	+
20	0.33	67	–	+	+	+	+
21	0.35	65	–	–	–	(+)	–
22	0.38	62	–	–	+	–	+
23	0.41	59	–	–	–	(+)	–
24	0.42	58	–	–	(+)	–	–
25	0.43	57	–	+	–	–	+
26	0.44	56	–	–	–	–	(+)
27	0.50	50	+	+	+	+	+
Total number of bands			12	13	14	14	17

(+) indicates species specific

Table 3. Jaccard's binary similarity coefficients of the five experimental *Trigonella* accessions.

Accession No.	A1	A2	A3	A4	A5
TI	1				
T2	0.25000	1			
T3	0.44444	0.35000	1		
T4	0.23810	0.42105	0.40000	1	
T5	0.31818	0.50000	0.40909	0.55000	1

Figure 1. Electrophoretic seed protein profiles of the five experimental *Trigonella* accessions.

M T1 T2 T3 T4 T5

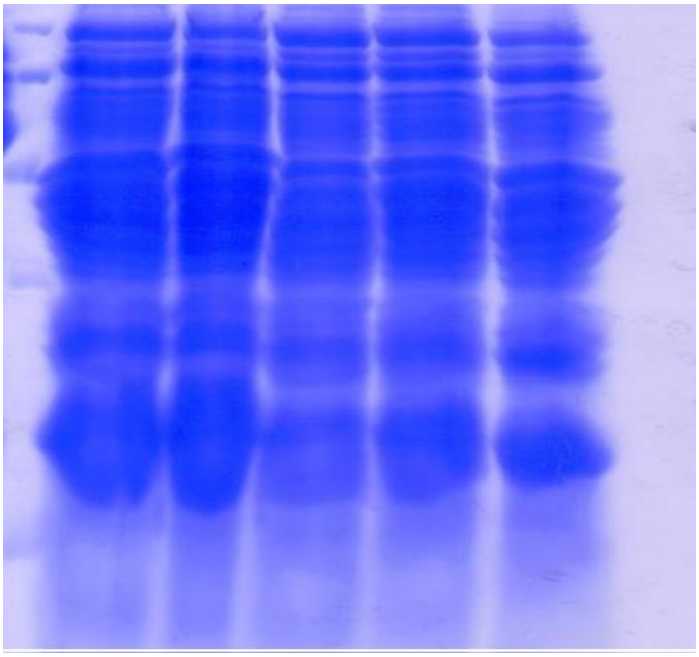
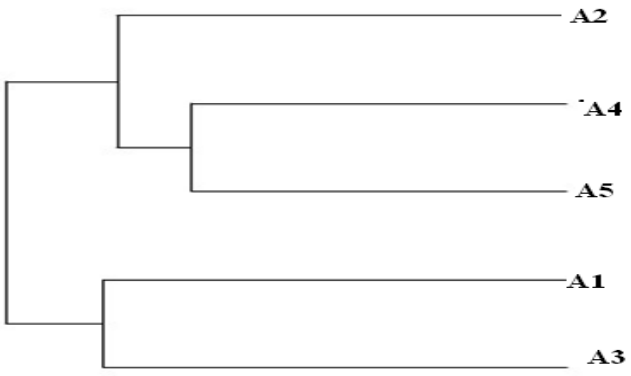


Figure 2. UPGMA dendrogram showing relationship among the five experimental *Trigonella* accessions based on genetic distance of SDS-PAGE.



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