IDENTIFICATION OF SEED STORAGE PROTEIN POLYMORPHISM IN *AZADIRACHTA INDICA* ECOTYPES USING SDS-PAGE

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Abstract : The present investigation was carried out during 2013-14 with twenty Neem (*Azdirachta indica*) ecotypes for protein profiling of seed protein through SDS-PAGE. The profiles of neem ecotypes were studied by extracting the total seed proteins from 20 ecotypes and performed SDS-Polyacrylamide gel electrophoresis. The total seed protein concentration ranged from 10.2-15.4 mg/ml at 660nm and on the basis of banding patterns through SDS-PAGE, indicated that the number of bands found in ecotypes ranged from 08 to 15. The bands on the gel were divided into 2 categories, major and minor bands. The major bands recorded in B1, B2, B3, B5, R2, G1, G2, G3, G4, G5 and Z1 ecotype, less bands recorded in R4, R5, Z3 and Z4 compared to the other varieties while there is no clear band in the B4, R1, R3, Z2 and Z5 ecotype. The total seed protein variation were also analyzed using Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) and resultant cluster analysis based on the data of protein profiling, classified twenty ecotypes into two major groups. Finally the study concluded that, the protein variability analysis clearly showed that there was low heterogeneity because most of the varieties were in the same cluster with respect to seed storage protein.

Keywords : *Azadirachta indica*, seed storage protein, protein profile, cluster analysis, SDS PAGE

I. INTRODUCTION

The neem has now been universally accepted as a "wonder tree" due to its multitude of uses (Dhaliwal et al., 2004). There are an estimated 25 million trees growing all over India (Rembold, 1996), of which 5.5% are found in Karnataka and it is in the third place next to Uttar Pradesh (55.7%) and Tamilnadu (17.8%) occupying the first two places respectively. An understanding of the extent and organization of genetic diversity of this species could be useful for both its genetic improvement and conservation (Kundu 1999a). Provenance studies have shown high levels of variability in neem with respect to survival, growth, morphological and physiological characters(Rajawatetal.1994;Kundu and Tigerstedt 1997, 1999; Kundu et al. 1998).

Several biochemical markers such as proteins and isozymes have served as an important tool to detect genetic relationships in plants (Mukhlesur *et al*., 2004). Protein polymorphism serves as genetic markers as they are direct products of active genes and are quite polymorphic and generally heritable (Gepts, 1990). The polymorphism observed in the protein profiles reflects the changes in the active part of the genome. Although protein polymorphism can be analyzed through a variety of techniques, polyacrylamide gel electrophoresis (PAGE) is generally favored technique for rapid analysis (Fergusan and Grabe, 1986; Smith and Smith, 1986; Raymond *et al*., 1991) due to its validity and simplicity for describing genetic variations (Ahmed and Slinkard, 1992). This technique has been used effectively to decipher genetic diversity among/ between genotypes in different plant species (Cook, 1984; Mukherjee and Datta, 2008). The present study was undertaken with an objective to evaluate the seed protein variation among the neem accessions collected from four ecological regions.

II. MATERIAL AND METHODS

2.1Plant material

Twenty seed samples of *Azadirachta indica* were collected from four districts Bidar, Gulbarga, Raichur of Hyderabad- Karnataka region and Zaheerabad, Telangana. For each accession 100-200 seeds were collected from five different regions from each district. A detailed description of the neem accessions used in the present study is given in Table 1.

2.2Total protein extraction

200 mg of neem seed was weighed and ground with the addition of 1.5 ml of extraction buffer and transferred into a 2 ml of microfuge tube. The sample was homogenized in extraction buffer (100 mM Tris HCl pH 6.8, 20 mM EDTA, 80% glycerol, 10% SDS and 0.1% 2-mercaptoethanol). The homogenate were centrifuged at 10,000 rpm for 30 min, the supernatant were collected as a crude protein sample and stored at -20^{0}C.

2.3Determination of protein concentration

Protein concentrations of 20 genotypes of neem seeds were determined according to Lowry *et al*., (1951). Bovine serum albumin was used as the standard protein. 1 ml of sample was mixed with 5 ml 0.01% Copper sulphate, 0.02% Sodium tartrate and 2% Calcium carbonate in 0.1 M NaOH. The mixture was left for 10 minutes and then mixed with Folin Ciocalteau's phenol reagent diluted 1:2 in distilled water. After incubation of 30 min at room temperature, absorbance at 660 nm was measured with a spectrophotometer. Distilled water (1ml) was used as a reference to prepare assay.

2.4SDS-PAGE Analysis

The molecular weight of the protein was determined by using SDS-PAGE (Genei, Bangalore, India). SDS-PAGE was carried out on a vertical slab gel according to the method of Laemmli, (1970). Protein samples were mixed with an equal volume of sample buffer containing 1% SDS (w/v), 5% β-mercaptoethanol (w/v), 0.003% bromophenol blue (w/v), and 10% glycerol (w/v), in 0.063 M Tris HCl pH 6.8 were boiled at 100^0C for 3 min in eppendorff tubes. 12% separating gel (pH 8.8) was poured in electrophoresis plates and allowed it to solidify. 5% stacking gel (pH 6.8) was poured on separating gel and the comb was inserted and allowed it to solidify. The sample was electrophoresed along with markers at 50V for 3 h with 0.025 M Tris HCl (pH 8.3) electrode buffer containing 0.192 M glycine and 0.1% SDS. After electrophoresis the gel was processed by CBB staining to observe the prominent bands.

2.5Data Analysis

Gels were scored for the presence (1) and absence (0) of every protein band. These binary data were analyzed using the software "PAST". A genetic dissimilarity matrix was calculated according to Jaccard's similarity index (JSI) and cluster analysis was done by the UPGMA method using the variance algorithm in PhyElp 1.4 software.

Table 1: Ecotypes under study for Protein profiling

Accession lane no.	Code	Ecotypes
1	B1	Bidar, Karnataka
2	B2	Bidar, Karnataka
3	B3	Bidar, Karnataka
4	B4	Bidar, Karnataka
5	B5	Bidar, Karnataka
6	R1	Raichur, Karnataka
7	R2	Raichur, Karnataka
8	G1	Gulbarga, Karnataka
9	G2	Gulbarga, Karnataka
10	G3	Gulbarga, Karnataka
11	G4	Gulbarga, Karnataka
12	G5	Gulbarga, Karnataka
13	Z1	Zaheerabad, Telangana
14	R3	Raichur, Karnataka
15	R4	Raichur, Karnataka
16	R5	Raichur, Karnataka
17	Z2	Zaheerabad, Telangana
18	Z3	Zaheerabad, Telangana
19	Z4	Zaheerabad, Telangana
20	Z5	Zaheerabad, Telangana

III. RESULTS

3.1Total Seed Protein

The aromatic amino acid of neem protein reacts with the Folin Ciocalteau reagent (Lowry's reagent) and forms a complex which is blue purple color complex (Singh, *et al.*, 2005). In Lowry's method the neem protein extract showed the absorbance at 660 nm. The concentration of protein ranged from 10.2-15.4 mg/ml.

3.2SDS PAGE Analysis

In this study, crude proteins of 20 different neem ecotypes were separated by SDS-PAGE electrophoresis for characterization and evaluation of genetic diversity among the given set of varieties. The molecular weight marker of 10 to 205 KDa was used for this purpose. Electrophoregram showing proteins banding pattern of different neem ecotypes are presented in Fig. 1. The result recorded from the figure shows that the total number of bands varies from 08 to 15 in different varieties. The bands on the gel were divided into 2 categories, category first comprises of major bands, second involve minor bands based on the glance (sharpness) of the bands. The major bands, particularly from the ecotypes B1, B2, B3, B5, R2, G1, G2, G3, G4, G5 and Z1 were common among most of the varieties but the other lane shows a variation. The neem ecotype R4, R5, Z3 and Z4 were showing a less number of protein bands as compared to the other varieties while there is no clear band in the ecotype B4, R1, R3, Z2 and Z5.

3.3Cluster analysis by SDS-PAGE

Cluster analysis was carried out on the results of SDS PAGE using the windows offline software "PAST" in order to investigate genetic variation among the given neem varieties. The result of cluster analysis is given in the dendrogram (Fig. 2) on the bases of linkage distance by the procedure of "unweighted pair group method with arithmetic means" (UPGMA). Cluster analysis, sorting the neem ecotypes into 2 major groups (lineages) at linkage distance 1.2 and which are further distributed into 7 clusters at linkage distance 0.8. The first group is composed of 2 clusters and there are 5 clusters in the second group. Among the lineage first, clusters 0.0 and 0.4 and 4 each were containing two varieties R5-Z2 and Z3-Z4 respectively. Similarly, among the second lineage, clusters 4, 5 and 7 each has three accessions (B5, G2 & G5), four accession (R2, G1, G3 & Z1) and six accessions (B2, B3, B4, R1, R3, & R4) respectively. And linkage distance cluster 3 and 6 consist of single accession G4 and B1 respectively.

3.4Similarity & distance indices

The data obtained from SDS PAGE was scored on the presence (1) and absence (0) of the bands and entered into a binary data matrix. Based on the results of electrophoretic band spectra, similarity index was calculated for all possible pairs of electrophoregram. The similarity matrix, thus generated was converted to a dissimilarity matrix and used to construct the dendrogram by the unweighted

pair group average method (UPGMA). The data were analyzed using PAST software. Jaccard coefficient dissimilarity ranged between 0.00 and 1.00 (Table 2). The lowest similarity was exhibited between B1 and R5, B1 and Z2 whereas the highest similarity was observed between B2, B3, B4, R1 and Z5; B2, B3, B4 and R3, R4; R2, G1 and G3; R3 and Z5; R4 and Z5 accessions.

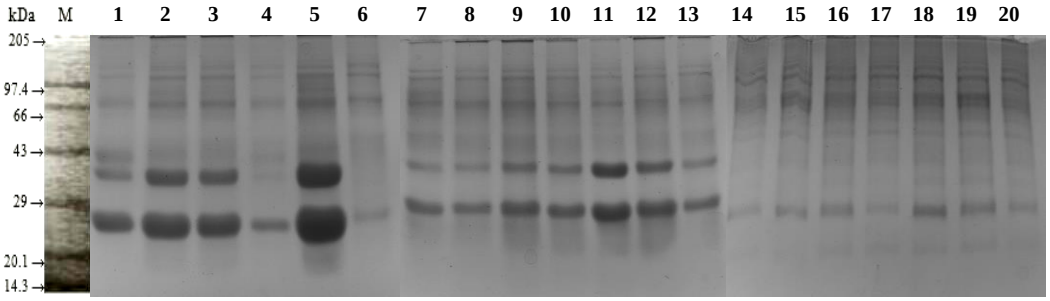

Fig 1: Banding pattern of total seed protein of neem ecotypes

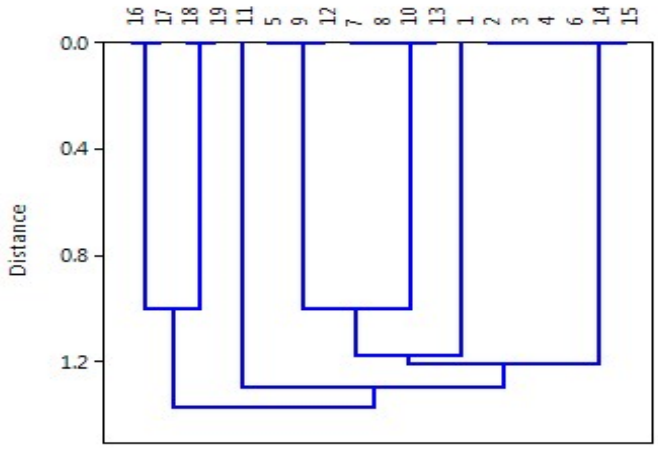

Fig 2: Dendrogram generated by PAST Software

Table 2: Similarity distance matrix based on Jaccard coefficient of *Azadirachta indica* biotypes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1	0.6	0.6	0.6	0.6	0.6	0.8	0.8	0.6	0.8	0.6	0.6	0.8	0.6	0.6	0.33	0.33	0.5	0.5	0.6
2	0.6	1	1	1	0.6	1	0.8	0.8	0.6	0.8	0.6	0.6	0.8	1	1	0.6	0.6	0.8	0.8	1
3	0.6	1	1	1	0.6	1	0.8	0.8	0.6	0.8	0.6	0.6	0.8	1	1	0.6	0.6	0.8	0.8	1
4	0.6	1	1	1	0.6	1	0.8	0.8	0.6	0.8	0.6	0.6	0.8	1	1	0.6	0.6	0.8	0.8	1
5	0.6	0.6	0.6	0.6	1	0.6	0.83	0.83	1	0.83	0.6	1	0.83	0.6	0.6	0.6	0.6	0.83	0.83	0.6
6	0.6	1	1	1	0.6	1	0.8	0.8	0.6	0.8	0.6	0.6	0.8	1	1	0.6	0.6	0.8	0.8	1
7	0.8	0.8	0.8	0.8	0.83	0.8	1	1	0.83	1	0.8	0.83	1	0.8	0.8	0.5	0.5	0.6	0.6	0.8
8	0.8	0.8	0.8	0.8	0.83	0.8	1	1	0.83	1	0.8	0.83	1	0.8	0.8	0.5	0.5	0.6	0.6	0.8
9	0.6	0.6	0.6	0.6	1	0.6	0.83	0.83	1	0.83	0.6	1	0.83	0.6	0.6	0.6	0.6	0.83	0.83	0.6
10	0.8	0.8	0.8	0.8	0.83	0.8	1	1	0.83	1	0.8	0.83	1	0.8	0.8	0.5	0.5	0.6	0.6	0.8
11	0.6	0.6	0.6	0.6	0.6	0.6	0.8	0.8	0.6	0.8	1	0.6	0.8	0.6	0.6	0.6	0.6	0.5	0.5	0.6
12	0.6	0.6	0.6	0.6	1	0.6	0.83	0.83	1	0.83	0.6	1	0.83	0.6	0.6	0.6	0.6	0.83	0.83	0.6
13	0.8	0.8	0.8	0.8	0.83	0.8	1	1	0.83	1	0.8	0.83	1	0.8	0.8	0.5	0.5	0.6	0.6	0.8
14	0.6	1	1	1	0.6	1	0.8	0.8	0.6	0.8	0.6	0.6	0.8	1	1	0.6	0.6	0.8	0.8	1
15	0.6	1	1	1	0.6	1	0.8	0.8	0.6	0.8	0.6	0.6	0.8	1	1	0.6	0.6	0.8	0.8	1
16	0.33	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.6	0.5	0.6	0.6	0.5	0.6	0.6	1	1	0.8	0.8	0.6
17	0.33	0.6	0.6	0.6	0.6	0.6	0.5	0.5	0.6	0.5	0.6	0.6	0.5	0.6	0.6	1	1	0.8	0.8	0.6
18	0.5	0.8	0.8	0.8	0.83	0.8	0.6	0.6	0.83	0.6	0.5	0.83	0.6	0.8	0.8	0.8	0.8	1	1	0.8
19	0.5	0.8	0.8	0.8	0.83	0.8	0.6	0.6	0.83	0.6	0.5	0.83	0.6	0.8	0.8	0.8	0.8	1	1	0.8
20	0.6	1	1	1	0.6	1	0.8	0.8	0.6	0.8	0.6	0.6	0.8	1	1	0.6	0.6	0.8	0.8	1

IV. DISCUSSION

The results from SDS-PAGE analysis of neem protein indicate a differential banding pattern for different neem ecotypes, but the overall degree of variation is relatively low. The molecular weight marker of 10 to 205 KDa was used for this purpose. Electrophoregram showing proteins banding pattern of different neem ecotypes are presented in Fig. 1. The result recorded from the figures shows that, the total number of bands varies from 8 to 15 in different varieties. The bands on the gel were divided into 2 categories, category first comprises of major bands, second involve minor bands based on the glance (sharpness) of the bands. The major bands, particularly from the ecotypes B1, B2, B3, B5, R2, G1, G2, G3, G4, G5 and Z1 were common among most of the

varieties but the other lane shows a variation. The neem ecotype R4, R5, Z3 and Z4 were showing a less number of protein bands as compared to the other varieties while there is no clear band in the ecotype B4, R1, R3, Z2 and Z5. The diversity in high molecular weight protein subunits is the result of gene silencing in some varieties encoding these proteins (Lawrence and shepherd, 1980). The major bands having a similar banding pattern in some ecotypes, but minor bands show variations. There is also a difference in the density of common major bands.

Dendrogram was calculated from the Jaccard similarity coefficient and un-weighted pair group method with averages constructed by the software PAST. Cluster analysis (Fig. 2), sorting the neem ecotypes into 2 major groups (lineages) at linkage distance 1.2 and which are further distributed into 7 clusters at linkage distance 0.8. The first group is composed of 2 clusters and there are 5 clusters in the second group. Among the lineage first, clusters 0.0 and 0.4 and 4 each contain two ecotypes R5-Z2 and Z3-Z4 respectively. Similarly, among the second lineage, clusters 4, 5 and 7 each has three ecotypes B5 and G2-G5, four ecotypes R2, G1-G3 and Z1 and six ecotypes B2-B3-B4 and R1-R3-R4 respectively. And linkage distance cluster 3 & 6 consist of a single ecotype from G4 and B1 respectively. The data obtained from SDS PAGE was scored on the presence (1) and absence (0) of the bands and entered into a binary data matrix. Based on the results of electrophoretic band spectra, similarity index was calculated for all possible pairs of electrophoregram. The similarity matrix, thus generated was converted to a dissimilarity matrix and used to construct the dendrogram by the unweighted pair group average method (UPGMA). The data were analyzed using PAST software. Jaccard coefficient dissimilarity ranged between 0.00 and 1.00 (Table 2). The lowest similarity was exhibited between B1 and R5, B1 and Z2 whereas the highest similarity was observed between B2, B3, B4, R1 and Z5; B2, B3, B4 and R3, R4; R2, G1 and G3; R3 and Z5; R4 and Z5 accessions. The dendrogram as a whole, represent the low heterogeneity because most of the varieties were in the same cluster. Fufa *et al*., (2005) reported that the genetic diversity estimates based on seed storage protein were lowest because they were the major determinants of end use quality, which is a highly selected trait. However, this helps to classify varieties in different groups. From the result of this study it is concluded that evaluation of genetic diversity and identification of neem varieties by SDS-PAGE is easy and early approach and it is also useful for molecular weight analysis of neem seed storage proteins.

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