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INTER- AND INTRA-SPECIFIC GENETIC DIVERSITY ANALYSIS IN THIRTY GENOTYPES OF *WITHANIA SOMNIFERA* (ASHWAGANDHA) USING PROTEIN PROFILE PATTERN

SUNITA KHATAK[*], OM PRAKASH YADAV[a] and RAMENDER KAITH

Department of Biotechnology Engineering, University Institute of Engineering and Technology
Kurukshetra University, KURUKSHETRA – 136119 (Haryana) INDIA
[a]Medicinal and Aromatic Plants Section, C. C. S. H. A. U., HISAR – 125001 (Haryana) INDIA

ABSTRACT

Thirty genotypes of *Withania somnifera* (L) Dunal also known as Ashwagandha were collected from three different states of India and diversity analysis was carried out using electrophoregrams using gradient gels (9-15%) from four to five week old leaves. A total of 11 bands were generated having Rm (Relative mobility) value ranging from 0.05 to 0.65 and of M. wt. in range of < 6.5 kDa to 45 kDa. The electrophoregram obtained using SDS-PAGE were used to estimate genetic similarity matrix, which ranged from 0.18 to 1.0 indicating high variability. The average similarity index value was 0.569. The dendrogram constructed from SDS-PAGE revealed one major cluster comprising all genotypes at a similarity coefficient of 0.55, which further bifurcates into two distinct clusters. Distinct clustering of genotypes of particular location was not observed although protein profile as general was considered as biochemical fingerprints of particular genotypes. Unique bands were observed using protein profile analysis distinguishing particular genotypes from rest. A unique band of Rm value 0.56 and 0.65 of molecular weight less than 6.5 kDa were found to be present in all the genotypes from Mandsaur district (M.P.) and Haryana along with two genotypes WS-223 and WS-224 from Udaipur (Rajasthan), while rest of the genotypes lack this specific band. Genotype WS-124 was clearly distinguished showing presence of a single band with Rm value (0.21) of molecular weight 22.3 kDa. The genotypes collected from different locations did not form well defined distinct clusters and were interspersed with each other.

Key words: SDS-PAGE, TEMED, APS, Polymorphism, Cluster analysis, Electrophoregrams.

INTRODUCTION

There is burgeoning need for the promotion of medicinal plants in India, firstly because they are re-emerging as a health tonic due to the mounting costs of prescription

[*]Author for correspondence; E-mail: sunkhatak@rediffmail.com; Fax: 01744-238967

drugs in the maintenance of personal health and secondly, these are providing livelihood to a significant number of people in the rural as well as urban communities. Moreover due to over exploitation of medicinal herbs from the natural resources, some of the species are threatened of being extinct from ecosystem. Last but not the least in the international market, the opportunities are emerging day by day for trade of medicinal herbs obtained from medicinal plants to fetch foreign exchange for country. India has probably the oldest, richest and most diverse cultural traditions in use of medicinal plants. Out of a large number of medicinal plants known in present scenario, *Withania somnifera* (L.) Dunal commonly known as Ashwagandha or Asgandh finds extensive use as a medicinal herb in the traditional system of medicine as a rasayana & medhya rasayana and its use in Ayurvedic & Unani extends back over 3000 to 4000 years[1]. It is also known as winter cherry and Indian ginseng belongs to *Solanaceae* family is an important small woody shrub or herb that grows upto 30 to 50 cm in height (maximum of 150 cm), usually clothed with whitish stellate hairs; leaves are ovate, entire and thin up to 10 cm long. It is known as adaptogens or vitalizers, which cause adaptive reactions to disease and appear to produce a state of non-specific increased resistance (SNIR) to adverse effects of physical, chemical, and biological agents[2]. Roots and leaves extract of *W. somnifera* indicates that it possesses many qualities such as anti-inflammatory[3], antitumour[4], antistress, antioxidant effect, immunomodulatory properties and neural system effect[5].

It is an ingredient in many formulations prescribed for a variety of musculoskeletal conditions e.g., arthritis, rheumatism[6] and as a general tonic to increase energy, improve overall health, longevity, and prevent disease in athletes, the elderly and during pregnancy. Clinical trials and animal research support the use of Ashwagandha for anxiety, cognitive and neurological disorders, inflammation and Parkinson's disease[7,8]. Ashwagandha has been used successfully in Ayurvedic medicine for centuries and therefore, proper identification of genotype is important for protection of both; the public health and industry. Various chromatographic techniques and marker compounds have been used to standardize botanical preparations but chemical complexity and variable sources are the limitations associated with the identification of genotypes. Polypeptide or protein profiling is a useful genetic marker system with functional/expressed gene diversity and has been applied to several lines of research including gene mapping, gene regulation, development genetics, evolution, and cultivar identification[9]. The gel electrophoresis methods, which are relatively easy to perform, fast and inexpensive have been widely used since late 70s. However, these suffer from several limitations such as their limited numbers, environmental dependence, limited variability generation and temporal & spatial expressions. The analysis of proteins using SDS polyacrylamide gels electrophoresis (SDS-PAGE) is one of the most simple, reliable and reproducible separation technique for protein analysis. Several researchers have

confirmed the usefulness of different SDS-PAGE procedures in plant taxonomic, evolutionary and genetic relationship studies[10].

EXPERIMENTAL

Material and methods

Plant material

Seeds of thirty selected genotypes of *W. somnifera* (L.) Dunal (Table 1) were procured from Medicinal Aromatic & Under Utilized Plant (MA & UUP) Section, Department of Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar, Haryana. Seeds were sown in randomized block design during *kharif* season. Three week old fresh leaves were taken as plant material for protein extraction.

Table 1: A brief description of *W. somnifera* genotypes used during present investigation

S. No.	Genotypes	Source
1	WS-124	Udaipur, Rajasthan
2	WS-201	-do-
3	WS-202	-do-
4	WS-204	-do-
5	WS-205	-do-
6	WS-206	-do-
7	WS-210	-do-
8	WS-213	-do-
9	WS-218	-do-
10	WS-220	-do-
11	WS-223	-do-
12	WS-224	-do-
13	WS-226	-do-
14	WS-90-100	Mandsaur (M.P.)
15	WS-90-103	-do-

Cont…

S. No.	Genotypes	Source
16	WS-90-104	-do-
17	WS-90-105	Mandsaur (M.P.)
18	WS-90-117	-do-
19	WS-90-125	-do-
20	WS-90-126	-do-
21	WS-90-129	-do-
22	WS-134 (C)	-do-
23	WS-90-135	-do-
24	WS-90-136	-do-
25	WS-20 (C)	-do-
26	Adinath	Neemuch (M.P.)
27	Local	Hisar, Research Farm Area
28	HWS-04-1	Haryana
29	HWS-04-2	-do-
30	HWS-04-3	-do-

Protein extraction/Sample preparation

SDS-PAGE was carried out on 9-15% gradient gels (Table 2) using discontinuous buffer system of Laemmli[11] and vertical slab gel electrophoresis apparatus. The leaves of each of the thirty genotypes of Ashwagandha were collected after 4-5 weeks of germination. These were oven dried at 70ºC for 48 hrs. The leaves were ground manually using pestle and mortar. Leaf powder (300 mg) was depigmented using solvent mixture (Chloroform: Methanol: Acetone in 1:1:1) thrice. The supernatant was discarded and pellet was air dried overnight. The dried material was mixed with 0.7 mL of working 1X sample buffer (0.125 M Tris HCl, 4% SDS, 20% glycerol, 0.05% bromophenol blue, 0.3 M β-mercaptoethanol, pH-6.8) and left overnight at room temperature. The samples were heated for 10 min in water bath at 60ºC and then centrifuged at 14,000 rpm at 10ºC for 20 min. The clear supernatant was used for electrophoresis. The samples were boiled at 100ºC for 3-4 min before loading into the wells. An aliquot containing 50 µg of sample protein was loaded in each well.

Table 2: Composition of polyacrylamide (9-15% gradient) gels

S. No.	Stock Solution	Stacking gel (mL)	Resolving gel	
			9%	15%
1.	Acrylamide-bisacrylamide solution (30%)	1.33 mL	6.0 mL	10.0 mL
2.	4xStacking gel buffer (1.0 m Tris-HCl, pH 6.8)	1.25 mL	-	-
3.	4xresolving gel buffer (1.5 M Tris-HCl, pH = 8.8)	-	5.0 mL	5.0 mL
4.	(10%) SDS	0.100 mL	0.2 mL	0.2 mL
5.	(1%) APS	0.100 mL	0.150 mL	0.150 mL
6.	TEMED	0.010 mL	0.015 mL	0.015 mL
7.	SdH_2O	7.210 mL	8.635 mL	4.635 mL
8.	Total volume	10 mL	20 mL	20 mL

Protein quantification

Lowry method[12] was used to determine the quantity of extracted proteins spectrophotometrically taking Bovine Serum Albumin as standard protein at 630 nm wavelength.

SDS-Page gel electrophoresis

A monomer solution for the appropriate resolving gel was prepared by combining all reagents as mentioned in composition of gels (Table 2) except ammonium persulphate (1%) and TEMED. Then APS and TEMED were gently mixed into monomer solution and the mixture was poured between the gel plates placed in gel casting assembly with the help of a gradient maker. Two different concentrations of resolving gel solution 9% and 15% (1.5 M Tris-HCl buffer, pH 8.8) were prepared and poured in two chambers of gradient maker. First of all 15% gel solution was poured and after that gel concentration continuously decreased till it reached 9%. It was immediately overlayered with distilled water. Polymerization was achieved approximately in 45 min at room temperature. Stacking solution (1.0 M Tris HCl buffer, pH 6.8) was prepared and poured on the top of resolving gel. A well forming comb inserted between the gel plates avoiding trapping of bubbles immediately. Stacking gel was allowed to polymerize at room temperature for 30-45 min. Electrophoresis apparatus was

assembled and upper and lower reservoirs were filled with electrode buffer (SDS-Tris glycine, pH 8.3). The comb was removed from the stacking gel and wells were washed 2-3 times with electrode buffer. Electrophoresis was carried out at room temperature at a constant current of 15 mA till the samples were in the stacking gel and at 25 mA, once the samples entered into the resolving gel. The run was continued till the tracking dye reached 1 cm above the bottom edge of the gel.

Gel staining and destaining

The gels were stained using Coomassie brilliant blue R-250 overnight and destaining was carried out at room temperature. After completion of electrophoresis staining and background destaining relative mobilities (R_m values) were calculated (Table 3) for each of the marker proteins and resolved proteins by following formula.

$$R_m \text{ value} = \frac{\text{Migration distance of protein band (mm)}}{\text{Migration distance of tracking dye (mm)}} \quad \ldots(1)$$

Relative mobility is the ratio between the distance traveled by the protein and the tracking dye in the resolved gel. The gels were photographed by using white light transilluminator of Alpha imager EC (Alpha Innotech, India).

SDS-Page data analysis

Data, fragment sizes were estimated from gel by comparison with molecular weight marker (Bangalore Genei, India) and were scored as discrete variables using '1' to indicate presence and '0' to indicate absence of a band. Banding pattern was scored simply by visual observations, where faint bands were neglected and only clear and distinct bands were scored. This 0/1 matrix was used to calculate the genetic similarity to estimate all pair-wise differences in the amplification product for all genotypes. The genetic associations between varieties were evaluated by calculating the Jaccard similarity coefficient (Table 4). The scores were entered into a database program (Microsoft Excel) and compiled in a binary matrix for phenetic analysis using NTSYS-PC (Numerical Taxonomy and Multivariate Analysis) system. Jaccard's similarity coefficient was calculated using Simqual subprogramme of NTSYS-PC and a dendrogram was constructed to study relatedness among 30 Ashwangandha genotypes produced by means of unweighted pair group method with arithematic average (UPGMA) analysis. Two dimensional and three dimensional principal component analysis was estimated by eigen value calculated using NTSYS-PC software

RESULTS AND DISCUSSION

The SDS-PAGE is very simple and a reliable technique for the analysis of genetic diversity on the basis of their protein profile[13]. The protein SDS-PAGE data showed that the Jaccard's similarity coefficient ranged from 72.2% to 100% (Table 3). Lowest Jaccard's similarity value represents maximum diversity. SDS PAGE on gradient gels (9-15%) was used to study the protein profile from leaves in 30 genotypes of Ashwagandha. In total, 11 bands were observed having Rm value (Relative mobility) ranging from 0.05 to 0.65 (Table 3). A maximum of 11 bands were observed in genotype WS-134 (C) from Mandsaur district (Madhya Pradesh) and a minimum of one band was observed in genotype WS-124 procured from Udaipur (Rajasthan). The 0/1 binary matrix data of protein banding pattern from different genotypes was used to generate similarity matrices among 30 genotypes of (*W. somnifera*) using 'simqual' subprogramme of software NTSYS-pC. The similarity indices for different genotypes ranged from 0.18 to 1.00 showing high variability at protein level. However, some bands with specific Rm values characteristics of particular genotypes were identified successfully. Maximum similarity was observed 100 percent between genotypes WS-206, and WS-210, genotype WS-220 and WS-223 from Udaipur (Rajasthan) along with between genotypes WS-90-100, and WS-90-103, WS-224 and WS-90-100 and WS-90-103 and WS-224 from Mandsaur district (Madhya Pradesh) including genotype Adinath (M.P.) and HWS-04-1 from Haryana.

A minimum similarity 18 per cent was observed between genotypes WS-201 and WS-224, (Raj.) between genotype WS-201 and WS-90-100 and between WS-134 (C) (MP) and WS-223 (Rajasthan) between genotype WS-205 (Rajasthan) and WS-134 (C) (M.P.) between genotypes WS-220 (Raj.) and WS-134 (C) and between genotypes WS-223 and WS-134 (C). Banding pattern obtained using SDS- PAGE was not good enough for identification of all genotypes of a particular location to be clustered together. Instead the genotypes of Udaipur, Rajasthan revealed 100 per cent similarity with the genotypes of same locations as well as that procured from Mandsaur district of Madhya Pradesh. The average similarity index value was 0.569.SDS-PAGE and it was used to study banding pattern in 30 genotypes of Ashwagandha using leaf protein, which revealed a total of 11 bands with Rm value in the range of 0.05 to 0.65 and molecular weight ranged from 6.5 to 45 KDa. The band with a Rm value (0.05) with a molecular weight of 45 KDa was found to be present in 26 genotypes out of 30 except WS-124, WS-201, WS-220, WS-223. While band with Rm value (0.1) was found to be present in only 10 genotypes with a molecular weight 37 KDa, which comprised of genotypes from different locations viz. WS-206, WS-210, WS-213, WS-226, WS-224 from Udaipur (Rajasthan), WS-90-100, WS-90-103, Ws-90-126, WS-134 (C) alongwith WS-90-136, from Mandsaur district (M.P.) Band with Rm value (0.15) with

molecular weight 29 KDa was located in 16 genotypes, which includes genoytpes WS-204, WS-213, WS-223, WS-224, WS-226, from Udaipur (Rajasthan), and genotype WS90-100, WS-90-103, WS-90-104, WS-90-105, WS-90-117, WS-90-126, WS-90-129, WS134 (C), WS-90-136, from Mandsaur (M.P.), and 2 genotypes HWS-04-2, and HWS-04-3 from Haryana. Band with Rm value (0.18) was found to be present in only 4 genotypes, WS-202, WS-204, from Udaipur (Rajasthan) WS-90-105 and WS-134 (C) from Mandsaur district (M.P.), which was specific to these genotypes with a molecular weight of 25.6 KDa. Band with Rm value (0.21) of molecular weight 22.3 KDa was found to be present in 17 genotypes, which includes, WS-134 (C), WS-90-126, WS-90-125, WS-90-117, WS-90-105, WS-90-103 and WS-90-100 from Mandsaur district (M.P.) while 10 genotypes from a total of 13 genotypes from Udaipur (Rajasthan) except WS- 205, WS-213 and WS-201. Band with a Rm value (0.27) of molecular weight (20.1) KDa was present in 15 genotypes including eight genotypes from Udaipur (Rajasthan), and seven genotypes from Mandsaur district (M.P.). A total of 13 genotypes revealed the presence of band with Rm value (0.30) of molecular weight 17 KDa including four genotypes from Udaipur (Rajasthan) and eight genotypes from Mandsaur district (M.P.) along with one genotype HWS-04-2 from Haryana.

Ten genotypes showed the presence of band with Rm value (0.36) of molecular weight 14.3 KDa, which comprised of two genotypes from Haryana, Three genotypes from Mandsaur district (M.P.) and five genotypes from Udaipur (Rajasthan). Band with Rm value 0.42 with molecular weight of 6.5 KDa was found to be present in 25 genotypes out of 30 except WS-90-135, WS-90-129, WS-90-104, WS-124 and WS-201. Band with Rm value 0.56 and 0.65 of molecular weight less than 6.5 KDa were found to be present in all the genotypes from Mandsaur district (M.P.) and Haryana along with two genotypes WS-223 and WS-224 from Udaipur (Rajasthan), while rest of the genotypes lack this specific band. A minimum of one band with Rm value 0.21 of molecular weight 22.3 KDa clearly demarked genotype WS-124. Asghar *et al.*[14] conducted a similar genetic diversity study in twenty-nine accessions of chickpea (*Cicer arietinum*) germplasm and obtained dendrogram by the UPGMA analysis, which classified all accessions into five clusters. Similarly, Bhat and Kudesia[15] reported the genetic similarity among five species of family Solanaceae and divided in two main clusters. The results of PCA analysis based on SDS-PAGE largely corresponded with the result of the cluster analysis (Fig. 1). The protein patterns obtained from our study can serve as a vital input to the conventional method of varietal identification that relies solely on morphological character.

Cluster tree analysis

A dendrogram was constructed using similarity matrix data obtained from 30 genotypes of Ashwagandha revealing relationship on leaf protein pattern basis using Jaccard

similarity coefficient of NTSYS-pC software. All the 30 genotypes of ashwagnadha were grouped together into one major cluster at a similarity coefficient of 0.55 (Fig. 1). Major cluster comprised of all the 30 genotypes and was further seperated into 2 major clusters-cluster I and cluster II at a similarity coefficient of 0.59 and 0.68, respectively. The cluster I at a similarity coefficient of 0.59 was further diversified, outgroping genotypes HWS-04-2 (Haryana) from that of 6 genotypes from Mandsaur (M.P.) that were WS-90-126, WS-90-129, WS-134 (C), WS-90-136, WS-20 (C), WS-90-135, along with 2 genotypes from Haryana, which were HWS-04-2 and HWS-04-03 and genotype WS-224 and WS-226 of Udaipur (Rajasthan) at a similarity coefficient of 0.63. The cluster I at a similarity coefficient of 0.63 was further diverged into two subcluster, subcluster I and subcluster II at a similarity coefficient of 0.66 and 0.71, respectively. The subcluster I (0.66) outgrouped the genotypes WS-223 and WS-224 from Udaipur (Rajasthan) from rest of the genotypes of this subcluster.

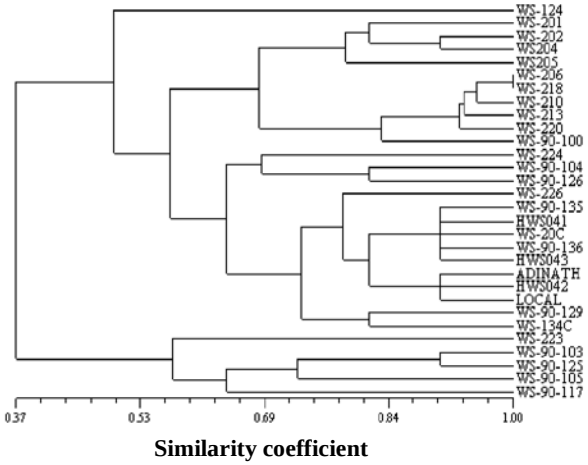

Fig. 1: Dendrogram depicting the interrelationship among thirty genotypes of Ashwagandha

This subcluster I is delineated into one group comprising genotypes WS-90-126, WS-90-129 and WS-90-136 together at similarity coefficient of 0.66 all procured from Mandsaur districts (M.P.). The subcluster II at a coefficient of 0.71 was further diversified into two subgroups, subgroup III and subgroup IV at a similarity coefficient of 0.78 and 0.82, respectively. The subgroup III at a coefficient of 0.78 separated the genotypes WS-226, WS-90-117 from that of WS-90-125 and WS-134 (C) at a similarity coefficient of 0.89. Similarly, subgroup IV of subcluster II further outgrouped the genotypes HWS-04-I and HWS-04-3 of Haryana at a similarity coefficient of 0.82 while grouped the genotypes WS-90-135 and WS-20 (C) from Mandsaur (M.P.) at a similarity coefficient of 0.89. As compared cluster II at a coefficient of 0.68 divided into two subgroups, subgroup V and

subgroups VI at a similarity coefficient of 0.68 and 0.79. Subgroup V (0.68) outgrouped the genotypes WS-90-104, WS-90-105 from Mandsaur (M.P.) and local (Haryana) altogether from ten genotypes, which includes WS-124, WS-201, WS-202, WS-204, WS-205, WS-206, WS-210, WS-213, WS-218 and WS-220 all from Udaipur (Rajasthan) along with one genotypes Adinath from Neemuch tehsil of Madhya Pradesh and 2 genotypes WS-90-100 and WS-90-103 from Mandsaur district M.P.

The subgroup V (0.79) was further diverged into 2 subsubgroups I, II, Subsubgroup I at a similarity coefficient 0.80 grouped the 5 genotypes WS-202, WS-205, WS-220, WS-90-100, WS-90-104 from that of 7 genotypes WS-124, WS-204, WS-201, WS-210, WS-213, WS-218 and Adinath. Genotypes WS-124, WS-204 WS-210 showing hundred percent similarity were further separated out from genotypes WS-201, WS-206, WS-213, WS-218 and Adinath at coefficient of 0.89. Genotype WS-220 from Udaipur (Rajasthan) was out grouped at a coefficient of 0.83 from WS-202 and WS-205 all from Udaipur Rajasthan. Similarly genotypes WS-90-100 and WS-90-103 were outgrouped at a coefficient of 0.79. Thus the protein pattern revealed high level of polymorphism among genotypes as revealed by similarity matrix also.

PAGE methods are now well established as an effective means of analyzing leaf proteins and subsequently identifying crop cultivars and to inter relate them. Their uses have been reviewed by several researchers in various medicinal plants of *Solanaceae* family. Characterization of different cultivars by studying protein polymorphism has been reported by several workers[16-20]. These protein markers can further be validated by PCR based molecular markers. Inamdar *et al*.[21] reported electrophoretic technique to be a versatile tool to study genetic relationship among different genotypes of sorghum. The protein banding pattern clearly differentiated the nine cultivars on the basis of soluble seed proteins. Morphologically similar genotypes of potato (*S. tuberosum*) were differentiated on the basis of electrophoregram obtained using tuber protein, although most of the genotypes were identical in their banding pattern as reported by Mishra *et al*.[22] Electrophoretic banding pattern obtained by SDS-PAGE technique was studied in 30 groundnut genotypes[23], where protein banding pattern was specific for each genotype. Some genotypes revealed unique bands, which help in identifying them individually, Mishra *et al*.[24] observed 12 bands using SDS-PAGE in different cotton cultivars. Five bands with Rm value 0.2352, 0.2942, 0.3882, 0.5294 and 0.5882 were reported to be common in all cultivars, while one band (Rm value 0.3529) could discriminate the *arboreum* and *hirsutum* cultivars using seed storage protein. Zecevic *et al.*[25] conducted similar experiment to study phylogenetic relationship using protein profile pattern on the basis of SDS-PAGE electrophoresis of eight genotypes of pepper (*C. annuum*). The dendrogram revealed distinctness of genotypes within different varieties and within same variety. However, the clustering of genotypes was in accordance

to that observed on basis of morphological parameters. Mann et al.[26] studied genetic diversity in chickpea (*Cicer arietinum*) using seed protein separation by using SDS-PAGE. A total of 39 bands were observed ranging from (Rm value 0.11 to 0.95). A minimum of 14 bands and a maximum of 34 bands were observed. The dendrogram constructed separated the 30 genotypes into 2 broad clusters at dissimilarity coefficient of 51%.

Conclusively the medicinal plants are rich in secondary metabolites that interfere with electrophoretic separation of proteins. Therefore, we developed an optimized method for extraction of proteins from leaf of Ashwagandha. The SDS-PAGE is one of the simple and reliable markers for genetic diversity analysis and cultivar identification, which can be useful to determine the correct starting material for plant breeding. SDS-PAGE using leaf protein revealed an 11 banded electrophoregram with Rm value ranging from 0.05 to 0.65 and mol. wt. in range of 6.5 kDa to 45 kDa. The SDS-PAGE data was used to compute similarity matrix, which ranged from 0.18 to 1.00 and thus, corroborate with that obtained using DNA markers. WS223, WS-90-100 and WS 90-102 genotypes of *W. somnifera* although from different locations showed 100% similarity in their protein profile.

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