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A Taxonomic Analysis of Albumin Seed Storage Proteins in Eight Tunisian Pomegranate (*Punica granatum* L.) Ecotypes

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Abstract: In order to improve the understanding of the taxonomy of some Tunisian pomegranate ecotypes, albumin fraction from pomegranate seed proteins has been analyzed by means of SDS-PAGE (Sodium dodecylsulphate polyacrylamide gel electrophoresis). The albumin (32% from total storage protein) represents three groups. The first group ran from 116 to 58 kDa, the second group ran from 46 to 33 kDa and the third groups of four subunits ran from 23 to 15 kDa. We conducted two methods to analyze Albumin gel; gel analysis (Gelpro software Ver. 3.1) and Image processing (Image J software Ver. 1.38). Tow dendrograms were obtained with rooted trees from drawgram drawing program. In most cases first and second clusters were in agreement. They practically lead to same cluster group, in exception of supplementary cluster obtained in image processing method with Jebali cultivar.

Key words: *Punica granatum* L., albumin, seed storage proteins, SDS-PAGE, image processing, ImageJ

INTRODUCTION

The pomegranate, a temperate climate species that requires high temperatures to mature properly, is one of the oldest known edible fruits. This fruit has been successfully adapted to the Mediterranean climate.

Diversity of pomegranate germplasm in Tunisia based on fruit characteristics were done primarily based on morphological characteristics by Mars and Marrakchi (1999). However, is not sufficient to identify pomegranate cultivars because the differences among them are often subtle and misleading. More robust genotypic traits free from strong environmental influence are required for proper identification and estimation of genetic diversity among cultivars. To date, only one study has been done using AFLP markers to reveal diversity among some Tunisian pomegranate (Jbir *et al*., 2007). The use of molecular markers such as seed proteins fingerprints would supply complementary useful information in pomegranate ecotypes taxonomy. The aim of this study is to characterize some Tunisian pomegranate based on albumin polypeptide patterns.

Seed storage proteins were initially classified according to their solubility into albumins (water soluble), globulins (saline soluble), prolamins (alcohol soluble) and glutelins (residue) by Osborne (1924). In this study we are interested in albumin fraction that forms about 32% from seed storage protein.

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MATERIALS AND METHODS

Plant Material

Eight accessions, representing five denominations, were included in the present study (Table 1). They are represented by adult trees maintained in the same collection at Gabès in South Tunisia by Mars and Marrakchi (1998). From each ecotype, 10 pomegranates were randomly picked. After extracting the seeds by hand, we are concerned in the contents of woody portion from seeds.

Albumins Extraction from Pomegranate Seeds

In order to extract all classes of storage proteins from pomegranate, we developed a fractionation protocol of the various categories of proteins while basing on their difference in solubility as described previously by Nasri and Triki (2007). Defeating pomegranate seeds was performed with Soxhlet method and extracted with distilled water (pH = 6.8), (0.1 g mL^{-1}) by two stirring steps of 20 min at 4°C and then centrifuged at 12,000 rpm for 20 min at 4°C. Supernatant was freeze-dried as Albumin fraction. Thereafter the protein concentration from each sample was determined by the method of Bradford (1976).

Protein Determination Using Bradford Assay

The protein content of each sample was quantified using the standard Bradford assay. Standard solutions of reagent grade BSA (Equitech-Bio, Inc., Kerriville, TX) were prepared containing 0-400 μg protein. Samples were covered with parafilm mixed and incubated for 5 min before absorbance measurement at 595 nm in spectrophotometer (Anthelie Advanced, Microbeam, S.A). All standard and unknown samples possessed the same solution matrix. The protein content of each sample was determined by fitting a least squares regression curve of the quantity of standard protein concentration vs. absorbance.

Electrophoresis for Protein Separation

Electrophoretic analyses were performed by SDS-PAGE according to method of Laemmli (1970). In this method, we use the same protocol that was reported by Nasri and Triki (2007). Samples were heated at 100°C for 5 min and 30 μL loaded onto SDS 12% PAGE Gels. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma) in glacial acetic acid-ethanol-water (10:40:50) overnight and destained with glacial acetic acid-water solution unless the background was clear.

Statistical and Bioinformatics Analyses

Two methods are selected to analyze Albumin gel: In first method we used gelpro software to score the present (1) or absent (0) of polymorphic bands in individual lanes. A phylogenetic tree from albumin gel was constructed by the maximum parsimony method using the PHYLIP program (Phylogeny Inference Package) (http://evolution.genetics.washington.edu/phylip.html). A similarity was estimated with the Jaccard's coefficient. The matrix of generated similarities was analyzed by the Unweighted Pairgroup Method with Arithmetic Average (UPGMA), using the SAHN clustering module.

Table 1: Origin and main characteristics of studied pomegranate accessions

Ecotype	Code	Origin	Main fruits characteristics
Beldi 1	BL1	Gafsa	Medium fruits and less colored juice
Chelfi 3	CH3	Testour	Medium fruits sweet juice and thin skin, soft seeded
Gabsi 3	GB3	Zerkine 2	Large and juicy fruits with thin skin
Jebali 3	JB3	El-Alia	Medium fruits with colored juice
Mezzi 1	MZ1	Tozeur	Acid medium fruits with colored juice
Rafrafi 5	RF5	Zerkine 2	Less colored fruits, hard seeded
Tounsi 5	TN5	Tozeur	Colored and abundant juice, soft seeded
Zehri 5	ZH5	Testour	Small and colored fruits with sweet taste

Image processing of albumin electrophoregram is performed by ImageJ software Ver. 1.38 (http://rsb.info.nih.gov/ij/). ImageJ is a public domain Java image processing program which appear to be a useful and excellent program for scientists in image processing by Daniel *et al*. (2005) and Shimoji *et al*. (2006). After converting image from albumin gel ImageJ allow us to quantify band intensity.

RESULTS AND DISCUSSION

The examination of Table 2 shows that the pomegranate seeds are rich in albumin. It represents about 52 mg g^{-1} of Dry-Weight (DW). The migration patterns of albumin subunits in pomegranate seeds are shown in Fig. 1. Three groups are identified. The first group ran from 116 to 58 kDa, the second group ran from 46 to 33 kDa and the third groups of four subunits ran from 23 to 15 kDa.

Automatic segmentation of protein bands from albumin gel produced by hybridizing techniques could add some useful details. Large intensity variation is accorded to bands within each lane in the image. Analysis is performed by ImageJ software. A number of pre-processing steps should be applied on the image prior to the automatic segmentation. First a homomorphic filter is applied to enhance the

Table 2: Contents of seeds storage proteins in Albumin from Tunisian ecotypes of pomegranate

Ecotype	Albumin (mg g^{-1} DW)
Beldi	52.67±5.25
Chelfi	47.16±0.53
Gabsi	54.27±4.33
Jebali	54.34±5.32
Mezzi	53.88±3.34
Rafrafi	62.23±4.12
Tounsi	45.43±1.90
Zehri	62.45±3.39

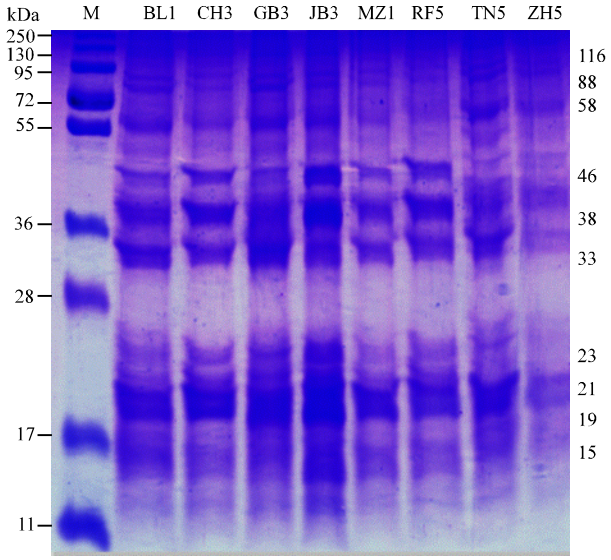

Fig. 1: Albumin polypeptide patterns obtained by SDS-electrophoresis from seeds of pomegranate ecotypes. M: Molecular-weight marker

Table 3: Quantification of pixel density of albumin contents in Tunisian pomegranate ecotypes

	Beldi	Chelfi	Gabsi	Jebali	Mezzi	Rafrafi	Tounsi	Zehri
Band 1	130±24.1	121±52	142±26.5	140±18.5	120±19.5	115±20	135±16.8	132±26
Band 2	95±6.9	60±4.7	105±16.1	102±16.7	62±7.4	72±12.9	103±20.3	97±13.4
Band 3	94±4.4	82±22	102±16	106±17.3	81±9.6	78±4.4	106±16.6	101±6
Band 4	322±45	302±31	306±27.2	306±39	102±4.5	112±9	225±52	212±11.3
Band 5	280±25.7	360±61.5	266±10	360±39	224±38	277±20	102±20.3	96±10.3
Band 6	462±84.1	401±78.1	390±11.7	540±48.1	286±56.3	317±34.2	206±18	161±13.1
Band 7	87±5	106±15.7	112±81.1	134±34.7	101±13.6	62±97	87±13.4	46±3.2
Band 8	460±50.8	442±34	407±18.2	721±64	366±29	396±7.6	390±7.6	167±24
Band 9	180±19.11	167±31.15	183±23	206±10.4	161±25.9	154±13	167±26.9	147±12.4
Band 10	46±4.6	64±1.6	65±3.2	97±14.3	22±1.7	21±2.5	25±4	21±1.3

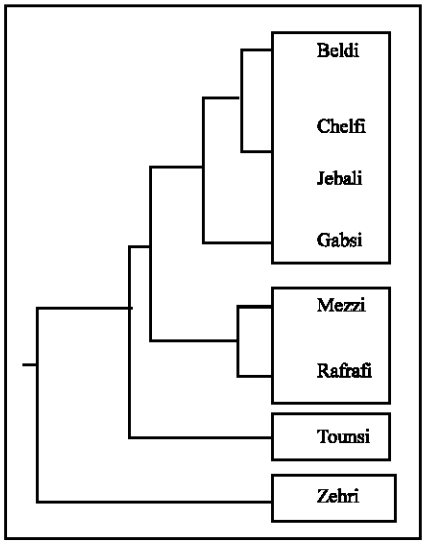

Fig. 2: UPGMA dendrogram of 8 Tunisian pomegranate ecotypes based on Albumin SDS-page profile according to band score (1, 0)

image followed by an edge preserving noise filtering processing to remove the noise in the image. Then a background normalization operation is applied to the resulting image. After these preprocessing operations, each lane in the image is detected and presented by a one-dimensional intensity profile (Table 3).

Ecotypes similarity was estimated with the Jaccard's coefficient (Table 3). The matrix of generated similarities was analyzed by the Unweighted Pairgroup Method with Arithmetic Average (UPGMA), According to Neighbor-Joining distance method and parsimony methods from Phylip package. We obtain rooted trees from drawgram drawing program in both first and second method which are presented, respectively in Fig. 2 and 3. In exception of last cluster obtained in second method with Jebali cultivar (Fig. 3) in most cases first and second clusters were in agreement. They practically lead to same cluster group. Group 1 (Beldi, Chelfi and Gabsi), group 2 (Mezzi and Rafrafi) group 3 (Tounsi) and last group formed by Zehri cultivar. Mainly the clusters were not in agreement with Morphometric characters studied by Mars in Mars and Marrakchi (1999). As a comparison, Mars reported also considerable phenotypic diversity among Tunisian pomegranate accessions. Significant differences in Tunisian pomegranate germplasm were due, mainly, to recombination combined with sexual and vegetative propagation, for long time and uncontrolled spread of plant material.

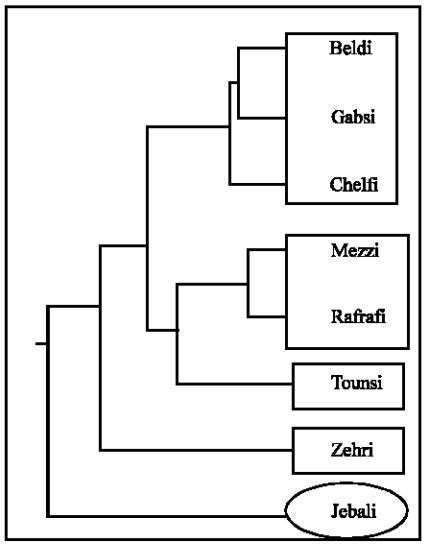

Fig. 3: UPGMA dendrogram of 8 Tunisian pomegranate ecotypes based on Albumin SDS-page profile according to pixel density

Divergence in added Jebali cluster obtained by image processing method can be accorded to the different principles and bases of classification of the two methods. In other part image noise from the Jebali line JB3 maybe in cause of this divergence. To perform a perfect evaluation additional steps are required like background normalization of resulting image. After these pre-processing operations, each lane in the image is detected and presented by a one dimensional intensity profile. An algorithm must be applied on the valleys in the resulting intensity profile for each lane in order to identify the corresponding valley as noise or a protein band (Zhan *et al*., 2005).

CONCLUSION

The studied pomegranate ecotypes are not introduced before in a Molecular characterization such us the molecular study performed by Jbir *et al*. (2007). Some of them are implicated in the morphological analyses performed by Mars and Marrakchi (1999), since we are using adult trees from the same collection.

As a comparison, to the morphological characterisation in Mars and Marrakchi (1999) results, considerable dissimilarities are observed among Tunisian pomegranate accessions. The derived UPGMA dendrogram proved that cultivars are generally clustered independently from their geographical origin Chelfi 3 and Zehri 5 from Testour, Gabsi 3 and Rafrafi 5 from Zerkine 2 and the some result with Mezzi1 and Tounsi 5 from Tozeur.

In exception of last cluster obtained in second method with Jebali cultivar, classical and image processing methods appear to be in agreement. Without image noise in gel profile, they practically lead to same cluster group. In the opposite case the classic method of taxonomy seem to be preferred for gel analysis by many scientists.

Development of new bioinformatic tools for Image processing and algorithmic modeling will support the second method and minimize error due to noise in gel profile. More and more publications are accorded to DNA and protein fingerprinting with image processing. A minimal skill of bioinformatics methods seems to be crucial to normalize gel images for taxonomic analyses.

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