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Preliminary Authentication of Some *Acacia* L. Species in Taif Highlands

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ALTHOUGH their wide distribution in arid lands and their many uses that include fodder and fuel besides the environmental values of soil stabilization, *Acacia* species are vulnerable to elimination in Saudi Arabia. In this study, seed morphology and patterns of their coat surface sculpture as revealed by scanning electron microscopy besides both seed proteins and seven isozymes profiles were employed for the discrimination and authentication the vulnerable Saudi Arabian *Acacia* collected from the western region of the kingdom. The scanning electron microscopic study displayed diversity in shape, dimensions, color, central aerole features and coat topography of seeds among different species to be characteristic for each species. Seed protein and isozyme profiles showed high variability among studied species. The UPGMA phenogram and genetic similarity analysis based on combination of seed morphology, protein and isozyme patterns confirmed the extensive genetic diversity existed in *Acacia* species.

Keywords: *Acacia*, SDS-PAGE, Isozyme, Seed surface, SEM.

The genus *Acacia* is considered one of the most important tree and shrub group in the sub-family Mimosoideae of Saudi Arabia. Ten species, two subspecies and four varieties of *Acacia* were recorded in Saudi Arabia (Collenette, 1999).Most species are centered in the western region, and they are little represented in Eastern and Northern parts of Saudi Arabia in different types of soils (Collonette, 1999, Chaudhary and Al-Jawaid, 1999). Most of *Acacia* species are important sources of browse fuel and pole timber; some are important commercial sources of gum and tannin. Some can be effectively utilized for shade, shelter, live fences, soil stabilization as well as street trees and ornamentals (Wickens, 1995).

Since 2009, Hegazy *et al.* (2009) mentioned that about 35% of the species that constitute the standing vegetation are vulnerable to elimination in Saudi Arabia because they are not represented in the seed bank. Therefore, proper identification is urgently needed for the preservation of economic species growing in extreme arid regions. Traditionally, subjective methods based on the morphological features such as shape, color, texture, and odor are used for the discrimination of herbal medicines. However, these methods are difficult to apply accurately for discrimination and authentication (Joshi *et al.*, 2004 and

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Zhang *et al*., 2007). Because of their validity and simplicity; biochemical protein markers (SDS-PAGE and isozymes) are still efficient tools used to address the interspecific and intraspecific diversity and are considered to be more accurate than those of traditional methods for authentication and discrimination among *Acacia* species (Casiva *et al*., 2002; Shukor *et al*., 2006 and Karakish *et al*., 2013).

Seed morphology has been shown to provide useful characteristics for the identification and classification of wide variety of plant taxa (Buss *et al*., 2001; Zhang *et al.*, 2005 and Gontchaova *et al*., 2009). In addition to gross morphology of seeds, sculpturing details of outer seed coat under the SEM are quite variable between different species and has been well recognized as a reliable approach for assessing phenetic relationship and identification of species or the other taxa (Koul *et al.*, 2000; Yoshizaki, 2003 and Javadi & Yamaguchi, 2004). AL-Gohary and Mohamed (2007) studied the surface sculpture of 11 Egyptian *Acacia* species and construct an identification key.

No previous work has been made on the Saudi Arabian *Acacia* species by using these techniques together, so the present work aimed at discrimination and authentication the vulnerable Saudi Arabian *Acacia* collected from the western region of the kingdom by using the scanning electron microscopy (SEM), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isozyme techniques.

Material and Methods

Fresh materials of nine species, one subspecies and two varieties of *Acacia* L. (*A. origena, A. asak, A. tortilis, A. johnwoodii, A. gerrardii var. gerrardii, A. gerrardii var. najdensis, A. etbaica, A. etbaica ssp. uncinata, A. laeta, A. seyal, A. raddiana* and *A. nubica*) were collected from the western region of Saudi Arabia. The collected materials were identified according to Collenette (1999) and Chaudhary (2001).

For scanning electron microscopic investigation, seeds were dehydrated in an acetone series, critical point dried using carbon dioxide and, together with dry seeds, were mounted directly on stubs using double-side adhesive tape, and sputter-coated with gold. Observations were made in a JEOL-JSM-639OLA auto scan SEM. The morphological characters of seeds; size, shape, color, surface texture, funicle position and four central areole features; shape, length of arms, size and color, have been described. Terminology of seed-coat surface sculpturing basically follows Stearn (1992) and Font Quer (1993). Seed multistate characters were transformed to two-state characters in coding (Sneath & Sokal, 1973 and Crisci & Lópezarmengol, 1983).

SDS-PAGE was performed in 12% acrylamide slab gels following the system of Laemmli (1970). Protein extraction was conducted by mixing 0.5 g of the seeds of each generation with an equal weight of pure, clean, sterile fine sand.

The seeds were then ground to fine powder using a mortar and pestle and were homogenized with 1.5 M Tris-HCl buffer, pH 8.8 in clean Eppendorf tube and where left in refrigerator overnight (Badr, 1995). Samples were centrifuged at 1000 rpm for 10 min. For electrophoresis, 10 µl of clear supernatant was mixed with 10µl of loading dye and 10 µl of mixture was loaded in the gel for each sample. After the run completed, the gel was stained by commassie blue R-250 for 6 hr, destained using mixture of methanol:glacial acetic acid:distilled water (3:1:6) and photographed.

The examined isozymes were: α-and β-esterases (α-and β-est), acid phosphatase (Acp), alcohol dehydrogenase (Adh), aldehyde oxidase (Alo), malate dehydrogenase (Mdh) and peroxidase (Px). For their extraction, three mature seeds of each sample were soaked in water for seven days. Then the seeds were homogenized in 1 ml extraction buffer (1 M Tris-HCl, pH 8.8) using a mortar and pestle; centrifuged at 10000 rpm for 10 min; the supernatant was kept at –20ºC until use. For isozymes separation, 10% (w/v) native-polyacrylamide gel electrophoresis method was used (Stegemann *et al*., 1985). For electrophoresis, 40 µl of extract was mixed with 20µl of treatment buffer and 40 µl of this mixture was applied to the well. For gels staining, protocols of Scandalios (1964) were used for α and β-Est.; Wendel & Weeden (1989) for both Ao and Acp; Weeden & Wendel (1990) for Adh; Jonathan & Wendell (1990) for Mdh & Heldt (1997) for Px. After run finished, gels were washed two or three times with tap water; fixed in ethanol: 20% glacial acetic acid (9:11 v/v) for 24 hr and photographed.

The produced clear well defined bands by using either the SDS-PAGE or isozymes techniques are used to estimate levels of polymorphism by dividing the polymorphic bands by the total number of scored bands. Differences in bands intensity among profiles of the different samples were not considered. Data generated by SEM, SDS-PAGE and isozyme techniques were used to compile a binary matrix for cluster analysis. The presence or absence of each seed, protein and isozyme character was treated as a binary character in preparation the data matrix (coded 1 and 0, respectively). Genetic similarity among species was calculated according to Dice similarity coefficient (Dice, 1945) and used to construct a dendrogram using unweighted pair group method with arithmetic average (UPGMA) by using SPSS-11 program (SPSS, 2011).

Results

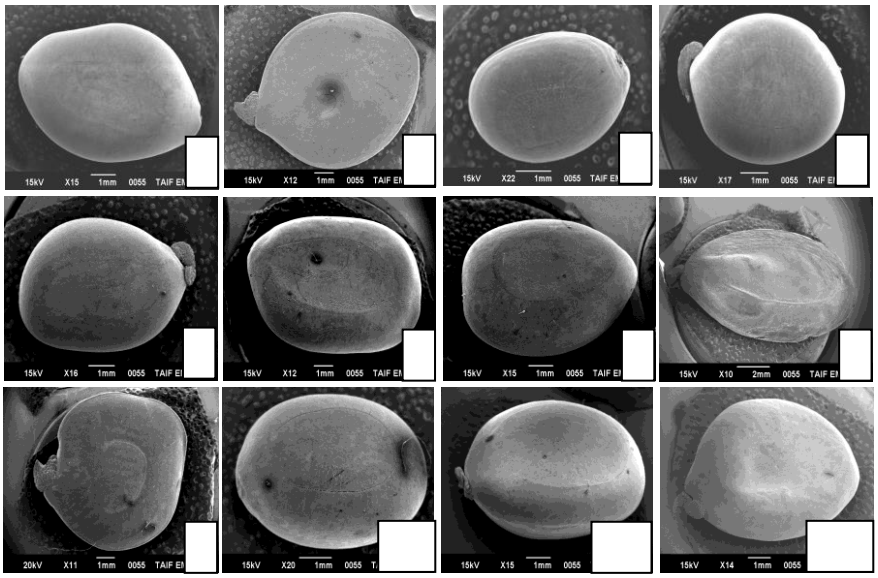
The SEM technique in this study was concentrated on description of seed morphology and seed surface sculpture for *Acacia* seeds as illustrated in Table 1 and Fig. 1. The morphological characters of seeds detected distinctive variations among different species. Funicle position was sub-terminal in all species except that of *Acacia laeta.* SEM investigation of seed coat sculpturing exhibited four distinct types of surface patterns namely; reticulate, granular, rugose and polygonal-discoid. Central aerole characters were distinguishable in all investigated species. Closed central aerole discriminated *A. origena, A. johnwoodii, A. gerrardii var. najdensis, A. etbaica* and *A. nubica* from other

species. The level of the central aerole varied among flat, concave and convex. Open central aerole with straight equal arms distinguished only *A. asak, A. etbaica ssp. uncinata* and *A. laeta*. The ratio between the central aerole area and seed surface area was 50% in all species, except that of *A. asak* and *A. laeta* was 10% and 25% respectively. The central aerole area was light in *A. asak, A. gerrardii var. najdensis, A. etbaica* and *A. laeta* and dark in other species.

TABLE 1. Macromorphological characters of seeds of *Acacia* species using SEM.

No.	Species	Size L x W mm	Shape	Color	Texture	Funicle	Central aerole			
							Shape	Length of arms	Size	Color
1	*A. origena*	4x6	Rhombic with pointed apex	Light brown	Reticulate tuberculate	Sub-terminal	closed concave	-	1/2 of seed area	Dark
2	*A. asak*	5x7	Obovate compressed	Olive-brown	polygonal-discoid	Sub-terminal	Open convex	equal arms	1/10 of seed area	Light
3	*A. tortilis*	3x4	Elliptic with pointed apex	Reddish brown	granular	Sub-terminal	open flat	not equal arms	1/2 of seed area	Dark
4	*A johnwoodii*	4x6	Elliptic with pointed apex	Light brown	Reticulate tuberculate	Sub-terminal	closed concave	-	1/2 of seed area	Dark
5	*A. gerrardii var. gerrardii*	4x6	Elliptic with round apex	Light brown	Reticulate tuberculate	Sub-terminal	open concave	not equal arms	1/2 of seed area	Dark
6	*A. gerrardii var. najdensis*	5x7	Rhombic	Dark green	Reticulate tuberculate	Sub-terminal	closed concave	-	1/2 of seed area	Light
7	*A. etbaica*	5x7	Elliptic with pointed apex	Light brown	Undulate-reticulate	Sub-terminal	closed flat	-	1/2 of seed area	Light
8	*A. etbaica ssp. uncinata*	6x10	Elongated compressed with pointed apex	Dark brown	Reticulate	Sub-terminal	open convex	equal arms	1/2 of seed area	Dark
9	*A. laeta*	8 x 8	Globose Compressed	Olive-brown	polygonal-discoid	Terminal	open convex	equal arms	1/4 of seed area	Light
10	*A. seyal*	4 x 5	Elliptic with round apex	Olive-brown	Rugose	Sub-terminal	open convex	not equal arms	1/2 of seed area	Dark
11	*A. raddiana*	5x8	oblong with pointed apex	Dark brown	Reticulate	Sub-terminal	open concave	not equal arms	1/2 of seed area	Dark
12	*A. nubica*	4x5	Elliptic with round apex	Light brown	Reticulate	Sub-terminal	closed concave	-	1/2 of seed area	Dark

(A) Seed Morphology



Seed surface sculpture

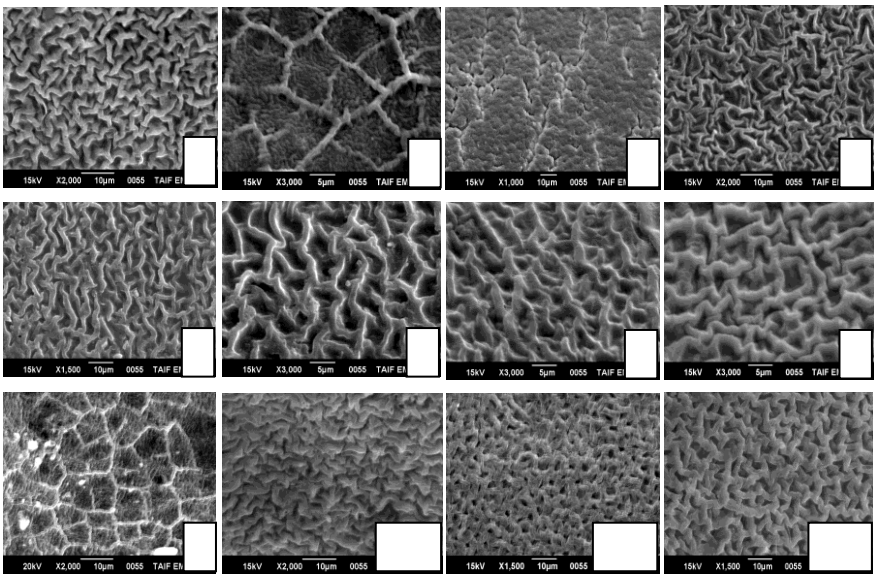


Fig. 1. SEM microphotographs of seed Morphology (A) and seed surface sculpture (B) of *Acacia* species. 1:*A. origena,* 2:*A. asak,* 3:*A. tortilis,* 4:*A johnwoodii,* 5:*A. gerrardii var. gerrardii,* 6:*A. gerrardii var. najdensis,* 7:*A. etbaica,* 8:*A. etbaica ssp. uncinata,* 9:*A. laeta,* 10:*A. seyal,* 11:*A. raddiana* and 12:*A. nubica.*

The produced SDS-PGE of seed protein profiles of the studied *Acacia* taxa are shown in Fig. 2. A total number of 62 detectable seed protein bands (subunits) were recorded. Molecular weight (Mw) of the storage protein subunits are ranged from 7.87 to 131.85 kDa. The profile revealed 12 common bands in all species and 50 non-shared bands including 8 unique bands, with polymorphism percentage 80.6%. Four unique bands that were detected at Mw 82.67, 80.29, 36.85 and 9.46 kDa, characterized *A. etbaica.* On the other hand, three unique bands with Mw 31.66, 19.73 and 13.78 kDa distigushed *A. raddiana,* and only one unique band was detected in *A. origena* with Mw 71.66 kDa. Band at Mw 62.84 kDa discriminated *A. etbaica and A. etbaica ssp. uncinata* from other species. *A. gerrardii var. gerrardii* and *A. gerrardii var. najdensis* had the same protein banding pattern.

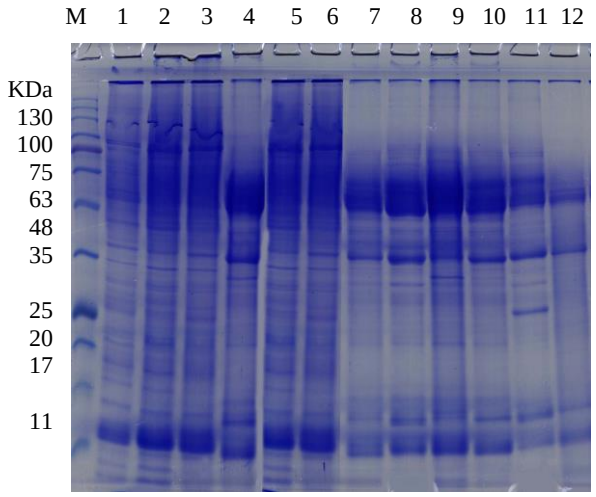


Fig. 2. The produced seed protein profile of *Acacia* species using SDS-PAGE technique. M: Standard protein marker, kDa: kilo Dalton. 1:*A. origena,* 2:*A. asak,* 3:*A. tortilis,* 4:*A johnwoodii,* 5:*A. gerrardii var. gerrardii,* 6:*A. gerrardii var. najdensis,* 7:*A. etbaica,* 8:*A. etbaica ssp. uncinata,* 9:*A. laeta,* 10:*A. seyal,* 11:*A. raddiana* and 12:*A. nubica.*

The produced zymograms of the used seven isozyme systems yielded 24 loci in *Acacia* samples as shown in Fig. 3. The highest number of loci (7) was recorded in α-esterase pattern; while Aldehyde oxidase and malate dehydrogenase patterns scored the least (2). The zymograms were determined after examining one population far each species. All examined isozymes exhibited distinctive variability among *Acacia* species. Acid phosphatase pattern recorded 3 loci through 4 bands. Six species, *i.e. A.asak, A. johnwoodii, A. gerrardii var. gerrardii, A. gerrardii var. najdensis, A. etbaica* and *A. etbaica*

ssp.uncinata had the same banding pattern (one band), as did *A. radiana* and *A. nubica* (two bands). Each of *A. origena* and *A. laeta* was distinguished by unique band. As Acid phosphatase pattern, alcohol dehydrogenase revealed that *A. gerrardii var. gerrardii, A. gerrardii var. najdensis* and *A. etbaica* had the same banding pattern (one band), as did *A. radiana* and *A. nubica* (two bands). *A.asak, A. johnwoodii* and *A. etbaica ssp.uncinata* had distinctive patterns. All species in aldehyde oxidase pattern recorded distinctive patterns except *A. gerrardii var. najdensis* and *A. etbaica* had the same banding pattern (two bands). Each of *A. johnwoodii, A. gerrardii var. gerrardii* and *A. nubica* characterized with one unique band. Malate dehydrogenase produced two groups, the first included *A.asak, A. laeta* and *A. radiana* and the second grouped *A. gerrardii var. gerrardii* and *A. nubica* had the same banding patterns (one band). Peroxidase pattern recorded 3 loci. *A. gerrardii var. najdensis, A. laeta* and *A. radiana* had the same banding patterns (one band), while *A. origena, A.asak, A. johnwoodii* and *A. nubica* distinctive patterns. On the other hand, esterases showed most variations of the seven enzymes tested. α-esterase pattern showed 12 distinctive patterns for *Acacia* species. *A.asak* detected the highest number of bands (11), from them two bands were unique. The other 4 characteristic unique bands were scored in *A.tortilis, A. etbaica ssp.uncinata, A. laeta* and *A. radiana.* β- esterase banding pattern recorded 4 loci through 10 bands. *A.asak* also recorded the highest number of bands (7), from them three bands were unique. Two characteristic unique bands were scored in *A. johnwoodii* and *A. radiana. Species A. gerrardii var. gerrardii, A. gerrardii var. najdensis, A. etbaica* and *A. etbaica ssp.uncinata* recorded the same banding pattern (one band).

Genetic similarity was calculated from the dice similarity index value for *Acacia* species based on combination of SEM, SDS-PAGE and isozymes data sets (Table 2). The maximum genetic similarity was 0.838 between *A. gerrardii var. gerrardii* and *A. gerrardii var. najdensis,* while the lowest genetic similarity of 0.375 was between *A. asak* and *A. nubica*. The phylogenetic relationships among *Acacia* species were analyzed by UPGMA method and presented in a dendrogram (Fig. 4). This revealed that, the samples are grouped into two main clusters (I and II). The first (I) comprised two subclusters (Ia and Ib). Subcluster Ia included *A. tortilis* and subcluster comprised *A. origena* with *A. gerrardii var. gerrardii* and *A. gerrardii var. najdensis*. Subcluster Ib grouped *A. asak* with *A. laeta*. The second (II) also comprised two subclusters (IIc and IId). Subcluster IIc included *A. radiana* and *A. nubica*, while subcluster IId comprised *A. johnwoodii* and subcluster grouped *A. etbaica* with *A. etbaica ssp. uncinata*, and *A. seyal.*

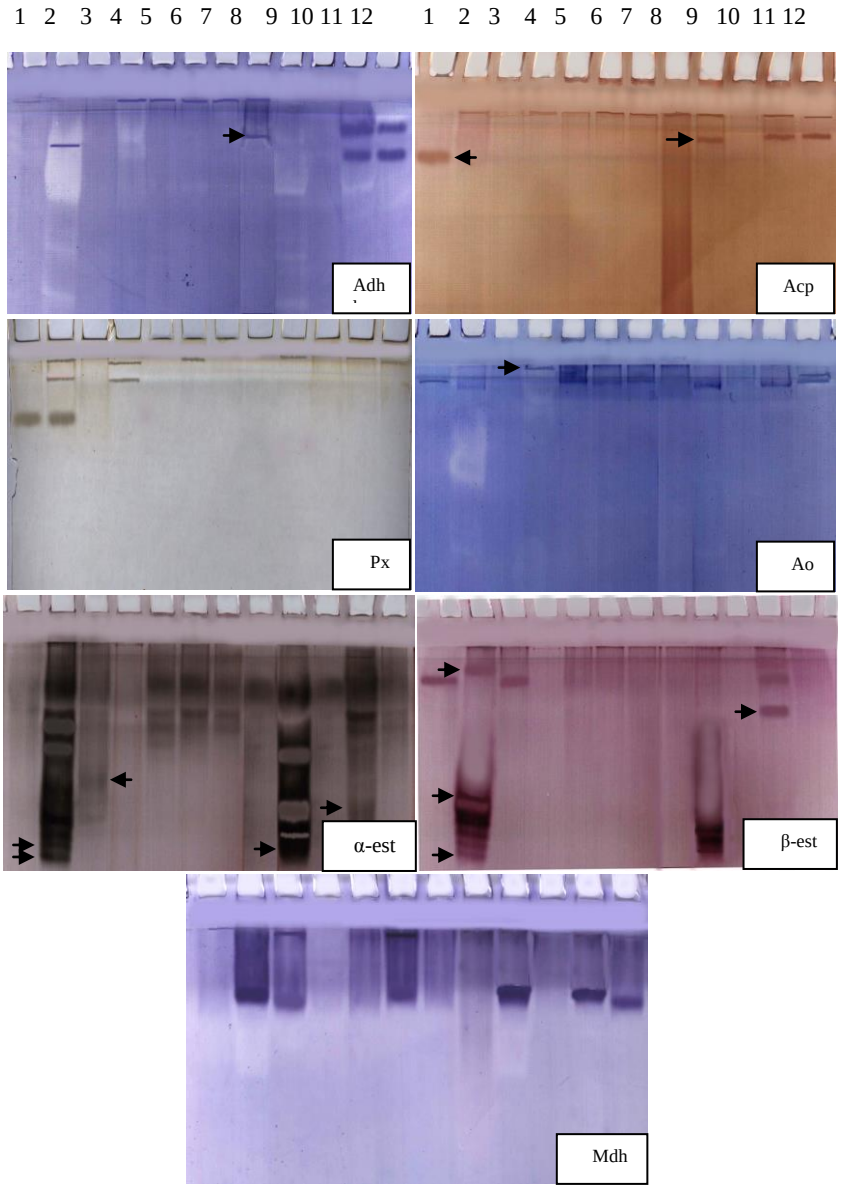


Fig. 3. Zymograms of *Acacia* species using seven isozyme techniques. Arrows indicate unique bands. 1:*A. origena,* 2:*A. asak,* 3:*A. tortilis,* 4:*A johnwoodii,* 5:*A. gerrardii var. gerrardii,* 6:*A. gerrardii var. najdensis,* 7:*A. etbaica,* 8:*A. etbaica ssp. uncinata,* 9:*A. laeta,* 10:*A. seyal,* 11:*A. raddiana* and 12:*A. nubica.*

TABLE 2. Dice similarity coefficient of *Acacia* species based on SEM, SDS-PAGE and isozymes data analysis.

	A. orig	***A. asak***	***A. tort***	***A. john***	***A. g. gerr***	***A .g. najd***	***A. etba***	***A. e. unci***	***A. laeta***	***A. seyal***	***A. radd***	***A. nubica***
A. orig	1.000											
A. asak	.615	1.000										
A. tort	.719	.652	1.000									
A. john	.580	.441	.490	1.000								
A. g. gerr	.817	.632	.752	.563	1.000							
A. g. najd	.807	.682	.707	.510	.838	1.000						
A. etba	.466	.463	.457	.527	.566	.571	1.000					
A. e. unci	.538	.508	.509	.587	.598	.585	.674	1.000				
A. laeta	.452	.692	.479	.505	.492	.530	.547	.598	1.000			
A. seyal	.521	.474	.531	.643	.545	.490	.644	.705	.646	1.000		
A. radd	.436	.484	.518	.490	.531	.500	.515	.549	.496	.553	1.000	
A. nubica	.447	.375	.479	.585	.495	.438	.518	.488	.412	.564	.587	1.000

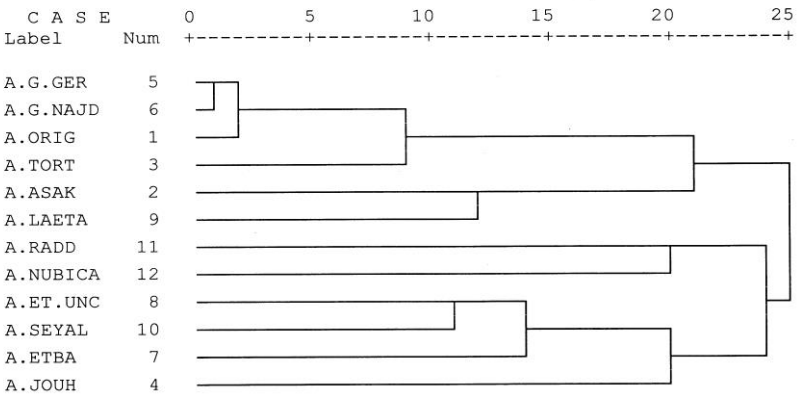


Fig. 4. UPGMA phenogram showing genetic diversity of *Acacia* species based on combination of seed morphology, SDS- PAGE and isozyme characters.

Discussion

The present SEM study displayed diversity in shape, dimensions, color, central aerole features and coat topographic of seeds among different species and characterized each of them. These results were in accordance with that of AL-Gohary and Mohamed (2007), Venier *et al*. (2012) and Karakish *et al*. (2013). The present study thus, supported the importance of seed features for the identification of *Acacia* species. This kind of studies with more species and

populations always help to open a frame work of our knowledge about interspecific and intraspecific relationships in *Acacia*.

SDS-PAGE is a reliable method of genetic characterization because electrophoretic patterns of the protein fractions are directly related to the genetic background of the proteins and can be used to certify the genetic make-up (Rehana *et al*., 2004). In the present study, 62 seed protein bands including 8 unique bands with polymorphism percentage of 80.6%. This revealed a characteristic variability among the *Acacia* taxa with the exception of the two most similar varieties of *A. gerrardii* (*A. gerrardii var. gerrardii* and *A. gerrardii var. najdensis*). Similar findings has been reached by El-akkad (2004) who investigated seed protein patterns in seven *Acacia* species by SDS-PAGE that separated *A. laeta, A. seyal, A. etbaica, A. tortilis spp raddiana* and *A. pachyceras* singly one by one due to their characteristic protein pattern which was unique for each of them. The observed high variability among *Acacia* species may be due to environmental factors which affect the qualitative and quantitative attributes of storage proteins. Thus, it is identified that stable stage and time is required for repeatability of protein profile in crop plants.

Isozyme systems yielded 24 loci ranged between seven in α-esterase pattern and two in both aldehyde oxidase and malate dehydrogenase patterns with distinctive variability among *Acacia* species. These results agreed with those of Casiva *et al.* (2002) who studied the genetic diversity among four Argentinean *Acacia* species with seven isozyme systems and detected 21 loci showing high genetic variability that allowed them to differentiate the species. On the other hand, the seventeen unique bands recorded in the present study were observed in all isozyme patterns that could be considered as biochemical markers for *Acacia* species. This is in accordance with the data of Balasubramanian (2012) who reported *Acacia* specific bands through studying isoenzyme analysis (peroxidase and polyphenol oxidase) for two Indian *Acacia* species. Each studied enzyme gave a different result, and a different level of species separation. Esterases gave the best resolution of *Acacia* species. However, one would hardly expect all species to be separated on the basis of variation in 2 enzyme systems. It is almost certainly that, the more extensive application of the procedure(s) to include wider range of enzyme tests, examination of more populations, examination of plant organs other than seeds, (e.g. leaves and seedlings), would broaden the data base, and therefore would gave better result for taxonomists in providing improved *Acacia* delineation and authentication in Saudi Arabia.

The produced UPGMA phenogram indicated that, the studied *Acacia* taxa could be distinguished by seed morphology characters, SDS-PAGE and isozymes banding patterns. The nine species, one subspecies and two varieties are separated into two main clusters including subclusters based on species specificity. This supported our results obtained from seed morphology and protein components. On the other hand, it confirms the extensive genetic diversity existed in *Acacia* species.

In conclusion, the study provides preliminary database of some *Acacia* species in Saudi Arabia with emphasis on variation patterns which is a major contribution to global biodiversity information system. From the study also, it is evident that seed morphology characters, seed protein and isozyme markers can be used as means of genetic distances to establish *Acacia* taxonomy as well as phylogenetic relationships among taxa. Detection of genetic differences and discrimination of genetic relationship among *Acacia* species are for sustainable utilization and conservation of plant genetic resources.

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توثيق مبدئى لبعض أنواع السنط فى مرتفعات الطائف

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السَنْط أو الطَلْح أو القَرَظ أو الأكاسيا جنس نباتي من الفصيلة البقولية وهو يضم ١٣٠٠ نوع. ويعتبر السنط واحدا من أهم الاشجار والشجيرات في العائلة القرنية فى المملكة العربية السعودية . وتتركز معظم الأنواع في المنطقة الغربية ، ويقل عددها في الأجزاء الشرقية والشمالية من المملكة. معظم أنواع السنط هي مصادر هامة للوقود والأخشاب ، وبعضها مصادر تجارية هامة للصمغ و التانين . ويستخدمه العديد من سكان الريف في الأدوية المحلية والأواني المنزلية والحرف اليدوية . ولكن ثبت مؤخرا أن حوالي ٣٥ ٪ من الأنواع التي تشكل الغطاء النباتي الدائمة الخضرة هي عرضة للإزالة، لذلك كانت هناك حاجة ماسة إلى اتخاذ تدابير مناسبة للحفاظ على تلك الأنواع النباتية في المناطق الصحراوية. لذا تمت دراسة الشكل الظاهرى وأنماط نحت السطح للبذور باستخدام المجهر الإلكتروني الماسح إلى جانب كل من بروتينات البذور و سبع مشابهات انزيمية لتمييز وتوثيق بعض انـــواع الاكاسيا التى تم جمعها من المنطقة الغربية من المملكة العربية السعودية. و قد أظهرت دراسة المجهر الإلكتروني لصفات البذورتنوعا واضحا في الشكل والأبعاد والألوان وملامح المنطقة المركزية وتضاريس السطح للبذور مما ميز كل نوع عن الاخر. وكذلك أظهرت أنماط البروتين والمشابهات الانزيمية للبذور ملامح التباين الشديد بين الأنواع محل الدراسة. ولقد دعمت دراسة علاقات القرابة وتحليل التشابه الجيني التنوع الوراثي واسع النطاق بين الأنواع داخل جنس الأكاسيا.