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Relationships among Phenotypic, Chemical and Genetic Characteristics of Some Selected and Evaluated Carob Strains (*Ceratonia siliqua*)

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Author's contribution

This whole work was carried out by the author AAN.

Original Research Article

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ABSTRACT

This study was conducted to assess the morphological, chemical and genetical diversity among eight carob female strains through two successive seasons (2011 and 2012) in Bourg El-Arab region of Alexandria governorate. The analyzed strains were highly significant for characters of pod weight fruit shape. Strains 2 and 4 always detected the highest approximated yield; this yield ranged from 26 to 28Kg/ tree. The obtained resultsof chemical composition (protein, sugar, tannin, total sugar, flavonoid and total phenol content) exhibit significant differences.

RAPD primers tested with the DNA of the eight carob strain, revealed percentage of polymorphism ranged from 10% (OPZ19) to 84.62% (OPA10). Moreover, eleven unique markers were identified with the tested strains. Dendrograms based upon chemical and genetical data clustered almost the same strains in the same group reflecting a relationship between chemical and genetical characteristics.

Keywords: Carob; strains; evaluation; selection; relationship; RAPD marker.

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1. INTRODUCTION

The carob tree (*Ceratonia siliqua* L), belonging to the family Cesalpiniaceae sub-family of the family Leguminoseae, is widely used in the Mediterranean regions [1], cultivated for ornamental and industrial purposes [2]. World production is estimated at about 315000 ton per year, and the main producers for pulp, seeds, respectively, are Spain (36%, 28%), Morocco (24%, 38%), Italy (10%,8%), Portugal (10%, 8%), Greece (8%, 6%), Turkey (4%,6%) and Cyprus (3%, 2%) of the world production [3].

Applications of carob kibbles are demonstrated din several studies e.g., sugar extraction [4], fermentation for ethanol [5] and citric acid production [6]. Recently, few studies have also referred this by-product of the carob bean gum industry as a good source of polyphenols [7], as well as their antioxidant activity [8]. Attention has been drawn to the health promoting effects of carob pulp mainly due to its poly-phenolic content and dietary fiber [9].The pulp and the seeds are valorized in different applications, the pod fiber content play a role in hypocholesterolemic and hypoglycemic regulation, whereas phenolic compounds can be used as antioxidant additive. Moreover, the locust bean gum (additive E 410) extracted from the endosperm of seeds is used as stabilizer and thickening agents in food industry [10]. Seeds powder can be used in baby foods to prevent vomiting. The locust bean gum is also applied in pharmaceutical industry as drug delivery [11]. It had been observed that this composition is depending not only on technological factors such as the extraction and analytical methodologies, but also on the genotype of the plant, the geographical origin, the climate conditions and the harvesting and storage procedures. However, in spite of the great interest to carob and their use in different applications, few studies are available on Egyptian carob. So to select the best strains, an intensive investigation on the morphological and chemical composition for the different carob strains is needed. These studies will allow understanding correlations between fruit and seed characteristics in order to propose the best characteristics strain that can be helpful for the development of new orchards with the best agro industrial profitability. Based on the above considerations, the aim of the current study was to assess morphological characteristics and chemical composition of different genotypes of carob and to establish correlation between morphological, chemical parameters and genetical characterization.

2. MATERIALS AND METHODS

The present investigation was conducted through two successive seasons (2011 and 2012) in Bourg El-Arab region of Alexandria governorate. Eight female treeswhich are of seed origin (natural population) andsubjected to the same abiotic conditions with no agriculture practiceout of ten were selected and evaluated. Morphological, chemical and genetical characteristics were used to estimate the relationship among these parameters.

2.1 Morphological Parameters

Tree vigor, nature of growth, number of leaves, leaf length,leaflet length and leaflet width. Leaflets measurements were used to generate a morphological based dendrogram.

2.2 Physical Parameters of Fruits

Samples of pods were collected in June from the eight trees; kept in open air and the following parameters have been measured for ten pods of each strain: weight, length, width,

thickness and size index, average number of seeds / pod, average seed weight / pod and seed% / pod. Width and thickness (cm) were assessed by the Vernier caliper (top, middle, and bottom of pod). Size index was determined as the ratio of length over width.

2.3 Chemical Analysis of Fruits

To determine the chemical composition of carob pulp (total polyphenols, total sugars, protein, tannins and flavonoidcontent), samples from seedless pods of morphological measurements were crashed, and then grounded into powder. Extracts were prepared as follows: 1g of carob powder was mixed with 20ml of water and 20ml of acetone to determine the following:

2.3.1 Determination of total phenols

Total phenolic compounds were determined colorimetrically at 660nm and expressed as gallic acid equivalents, according to the method described by [12]. Samples were added to Folin-Cioccalteu reagent and CaCO3 solution and placed in the dark for 15 min before spectrophotometric analysis.

2.3.2 Determination of sugar content

Total sugars were determined colorimetrically at 480nm according to the method described by [13]. Standards were prepared with glucose solutions at different concentrations.

2.3.3 Determination of protein content

Total nitrogen of carob powder was determined according to the AOAC official method 955.04 [14] using a Macro-Kjeldahl digestion and distillation apparatus.

2.3.4 Determination of total flavonoid content

Total flavonoid was determined by using the DMACA methodology, as described previously [15].

2.3.5 Determination of tannins content

The tannins content was determined with the gravimetric method using the cupper acetate such an agent of association with phenol compounds which are extracted from carob pod in three times meaning the boiled distilled water. The determination was carried out according to the method described by [16].

The total phenols, total tannins and total flavonoid were used to generate a dendrogram based on chemical constitutions to assist the relationship among the studied strains.

2.4 DNA Fingerprint

2.4.1 RAPD-PCR reactions

Juvenile leaveswere collected in the spring (March) and kept in ice box until reaching to the laboratory and genomic DNA was extracted according [17]. A set of ten random 10-mer

primers (Table 5) were used in the detection of polymorphism among the evaluated strains of carob. RAPD-PCR was carried out according to the procedure given by [18] with minor modifications. The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5mM $MgCl_2$, 0.2mMdNTPs, 1µM primer, 1 U *Taq* DNA polymerase and 25ng template DNA.

2.4.2 Thermocyling profile and detection of the PCR products

PCR amplification was performed in a Perkin-Elmer/Gene Amp® PCR System 9700 (*PE* Applied Biosystems) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 1 min, an annealing step at 36ºC for 1 min, and an elongation step at 72ºC for 1.5 min. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5ug/ml) in 1X TBE buffer at 95 volts. PCR products were visualized on UV light and photographed using a Polaroid camera. Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0, respectively.

All data obtainedwere tabulated and statistically analyzed according to [19]. Statics program of phonetic, chemical and genetical relationships among populations were evaluated by cluster analysis from the matrix of average values of morphometric, chemical and geneticaltraits. Each accession was considered as an operational taxonomic unit. The relationships among the accessions were estimated by the Pearson coefficient (r). Distance values (D) were defined as follows: $D = 1 - r$, and the distance matrix was represented in a phenogram by the UPGMA clustering method [20].

3. RESULTS AND DISCUSSION

3.1 Morphological Measurements

The studied trees of carob have a straight trunk, however, the canopy growth revealed a spread nature. Number of leaves differ from one strain to another with no significance variance (Table 1), strain4 recorded the highest number of leaves and the lowest number was showed by strain 2. There was no constant trend for leaf length in both of the studied seasons. Strain 4 detected the highest leaflet length in both seasons and leaflet width in the first season and almost in second season, it could be demonstrated that, strain 4 super passed all of the tested strains in vegetative growth.

Regarding date of flowering (beginning of flowering), it was noticed that, three strains flowered in September, e.g., strain 5 and 7 begin in first of September; while, strain 1 flowered in the mid of September. However, strains 3 and 8 flowered at the beginning of October; meanwhile, strains 2 and 4 flowered from 15th to 20th of October. Strain 6 showed delaying in the flowering date; it was started in flowering in the first of November [21]. The means of morphometric characters measured in the studied Moroccan carob accessions showed highly significant differences among the accessions for all the examined characters.

Table 1. Vegetative measurements and flowering date of some carob strains

Strain No.	Canopy nature	Leaf number		Leaf length(cm)		Leaflet length(cm)		Leaflet width(cm)		Date of flowering	
		2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
1	Spread	8.8	7.9	12.5	14.3	6.2	7.3	4.8	3.2	15/9	17/9
2	Spread	6.8	7.0	14.7	15.6	6.8	6.5	3.5	3.9	17/10	15/10
3	Spread	7.3	8.4	17.2	16.9	5.2	5.3	3.1	3.6	1/10	1/10
4	Spread	9.0	8.9	21.2	22.1	8.5	7.5	4.6	4.1	17/10	20/10
5	Spread	8.5	8.2	17.8	16.6	7.5	6.9	2.9	3.6	7/9	10/9
6	Spread	7.8	7.5	14.6	15.9	6.3	7.2	3.9	3.8	1/11	4/11
7	Spread	8.3	8.7	15.7	15.8	5.8	6.2	4.2	4.1	7/9	8/9
8	spread	8.1	8.0	11.9	13.5	5.1	6.3	4.4	3.9	1/10	3/10
LSD 5%	___	2.37	1.90	5.09	8.76	1.05	2.12	1.16	1.04	___	___

3.2 Physical Measurements of Pod Carob Strains

It was observed that pod weight was high in the second season compared with the first one, however, strain 2 showed the highest pod weight in both seasons (Table 2). Regarding pod thickness, strain 5 revealed the highest average in both seasons, meanwhile, strain 2 detected the highest average length of pod in both of the studied seasons. No trend was observed for pod width; however, strain 3 and strain 5 represented the highest average in the first and second seasons, respectively. On the other hand, strains 2 & 8 recorded the highest pulp weight (12.69 and 12.88, respectively) in the first season and (15.87 and 14.30, respectively) in the second season.

Table 2. Physical measurements of pod carob strains

Strain No.	Pod weight (g)		Pod thickness (cm)		Pod length (cm)		Pod width (cm)		Size index		Pulp weight (g)	
	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
1	11.37	12.50	0.67	0.54	15.91	18.11	1.64	1.44	9.70	12.57	9.85	10.17
2	15.21	17.63	0.72	0.71	20.25	22.90	1.37	1.93	14.78	11.87	12.69	15.87
3	6.14	8.96	0.48	0.77	13.19	11.96	2.42	1.59	5.45	7.52	4.71	7.11
4	10.41	10.76	0.81	0.89	17.27	18.56	1.96	1.55	8.81	11.97	8.53	8.95
5	7.25	10.36	0.92	0.96	14.92	14.00	2.00	2.13	7.46	6.57	5.09	9.23
6	13.81	15.27	0.56	0.56	15.34	18.87	1.95	2.10	7.87	8.98	12.05	13.75
7	5.92	9.23	0.49	0.61	12.26	13.97	1.72	1.68	7.13	8.32	4.89	7.97
8	14.21	16.12	0.82	0.85	16.59	19.30	1.35	1.54	8.59	12.53	12.88	14.30
LSD 5%	5.01	7.92	0.09	0.11	9.23	10.75	0.99	0.96	2.45	3.17	1.75	5.38

Strains 2 and 4 always detected the highest approximated yield; this yield ranged from 26 to 28 Kg/ tree in both seasons followed by strain 8 which revealed approximate yield ranged from 25 to 26 kg, Meanwhile, strain 7 yielded approximate weights of pods ranged from 16 to 18 Kg in the first and the second seasons, respectively

The ratio between fruit length (L) and fruit diameter (D) was calculated to determine the differences among the tested strains in shape. This ratio (L/D) was ranged from 5.45 (strain 3) to 14.87 (strain 2) in the first season, and from 6.57 (strain 5) to 12.57 (strain 1) in the second one. It is apparent that fruit shape was influenced by the L/D ratio, e.g. strains 1,6,4 and 5 have almost astraight shape and moreover, strains 3,7 and 8 takes almost a curved shape, while strain 2 showed a twisted shape. Physical measurements of the whole carob

pod indirectly indicate the quality of those pods, furthermore the higher the thickness, the higher the pulp to kernel ratio are the quality indicators of pods [22].

Regarding kernelcharacteristics, it wasnoticed that average number of seeds and average weight of seeds were highest within strain 2in both of the studied seasons; except for, strain 8which revealed the highest average weight of seeds in the second season (Table 3). At the same time, strain 7 showed the lowest average number of seeds in both seasons and lowest average weight of seeds in the first one; however, strain 4 recorded the lowest average weight of seeds in the second one. Meanwhile strains 3 and strain 7 represented the highest percentage of seeds weight per pod in the first and second seasons, respectively. Endosperm production from Iranian locust bean seeds calculated based on seed weight was 66.34% [23].

Table 3. Kernel characteristics and approximate yield

Strain No.	Average number of seeds/ pod		Average weight of seeds/ pod		Seed % / pod		Approximate Yield (Kg)	
	2011	2012	2011	2012	2011	2012	2011	2012
1	8.52	7.95	1.52	1.82	13.37	14.56	24	21
2	12.21	10.73	2.52	1.26	16.57	7.15	28	26
3	6.99	7.11	1.43	1.52	23.29	16.96	20	21
4	8.51	8.87	1.88	1.13	18.06	10.50	26	28
5	7.12	9.02	2.16	1.81	29.75	17.47	18	20
6	8.34	8.56	1.76	1.85	12.74	12.12	24	23
7	6.21	6.31	1.03	1.76	17.39	19.07	16	18
8	8.48	9.28	1.33	2.33	9.37	14.45	25	26
LSD 5%	4.33	2.96	0.65	0.81	3.74	5.83	7.00	10.00

3.3 Chemical Constitutes of Carob Pod Pulp

The proportions of carob pulp and seeds and the contents oftotal sugars, total phenol, total tannins, total flavonoid and total protein in pulp show a great diversity among the testedstrains of carob tree (Table 4). It could be demonstrated that strain 4 super pass the remaining testedones in the contents of total sugar, tannins and total proteins in both seasons. Moreover, strains 2 and 5 also show high content of total sugar and total proteins, respectively. On the other hand, strain 1 revealed the lowest content of total sugar and total phenol.

Table 4. Chemical characteristics of carob pods

Strain No.	Total sugar %		Total phenol %		Tannins%		Total flavonoid g/100g		Total proteins g/100g	
	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
1	45.76	47.89	35.0	44.7	16.0	10.3	4.52	5.53	2.68	2.12
2	54.88	55.65	68.4	65.9	15.7	11.7	1.99	1.87	1.94	2.22
3	51.90	54.90	76.7	69.5	10.7	11.9	1.36	1.65	1.64	1.85
4	55.64	52.63	65.5	66.8	16.5	16.8	3.81	3.99	2.85	2.86
5	49.77	51.03	76.5	74.9	12.4	11.7	2.61	3.13	2.87	2.53
6	50.98	51.10	46.5	50.3	5.3	6.8	2.88	3.32	1.98	1.59
7	53.77	53.67	43.7	44.6	5.7	5.9	2.10	1.98	1.44	2.45
8	47.53	48.53	43.7	45.1	3.5	5.7	3.85	2.64	2.74	2.52
LSD 5%	20.67	27.03	1.09	3.87	1.54	2.34	0.98	0.99	0.32	0.43

meanwhile, lowest content of tannins were detected by strain 8. Furthermore, total phenol and total flavonoid were highest in strain 5 and strain 1, respectively in both seasons.These results showed that carob pod contains appreciable amount of proteins and an important amount of polyphenols that play a significant role in human health as it was reported in literature [22]. Moreover, the composition and quantification of polyphenols in carob fruit has been elucidated [7,24], as well as their antioxidant activity [8,25,26]. Recently, attention has been drawn to the health promoting effects of carob pulp mainly due to its polyphenolic content. A carob pulp preparation rich in polyphenols has shown the potential for a diverse variety of health benefits in humans, namely positive effect on cholesterol metabolism [27], potential regulation effect on blood glucose levels [28]. Carob pods were also used in ancient Egypt, where the pulp of the pods was mixed in porridge, with a little honey, and wax as a treatment for diarrhea and some other diseases [29].

3.4 DNA fingerprint

3.4.1 Polymorphism as detected by RAPD marker

Ten RAPD primers were tested with the DNA of eight carobstrains (Table 5). These primers produced multiple band profile which ranged from 5 to 13amplicons (Fig.1). Total number of amplicons amplified by the ten primers was 85 with an average 8.5amplicon/ primer. The number of polymorphic bands ranged from 1 (OPB02 and OPZ19) to 11 (OPA10), representing percentage of polymorphism ranged from 14.29% and 10% (OPB02 and OPZ19) to 84.62% (OPA10). The size of the amplified bands varied according to the used primers, it was ranged from 200bp to 1500bp. Fifty two RAPD primers tested with carob strains, and these primers yielded a total of 374 bands with an average of 7.2 bands / primer [21]. The number of polymorphic fragments per primer was ranged from one (OPC2) to 18 (OPC1) and fragment size ranged from 40bp (OPD5) to 2000 bp (OPS18).

Table 5. Polymorphism and its percentage as detected by RAPD marker

Primer	Total no. of amplicons	Monomorphic amplicons	Polymorphic amplicons	Percentage of polymorphism
OPA10	13	2	11	84.62
OPA07	8	2	6	75.00
OP A14	5	2	3	60.00
OPA16	11	3	8	72.73
OPB02	7	6	1	14.29
OPB20	11	6	4	36.36
OPC11	11	2	9	81.82
OPZ04	9	7	2	22.22
OPZ19	10	9	1	10.00
Total	85	39	46	
Average	8.5	3.9	4.6	

3.4.2 Genetic similarity among the evaluated strains

Genetic similarity (estimation of genetic distance among accessions within and between species and genera) ranged from 83 (strain 1 and both of strains 7 and 8) to 95 (strain 7 and strain 8). It was clear that strain 1 always detected a low value ofsimilarity with the rest of the tested strains; this could be interpreted by differences of its genetic background from the

other strains. On the other hand, strains 5,6,7 and 8 showed a high genetic similarity (Table 6) it was 93 between strain 6 and all of strains 5,7 and 8; while it was 94 and 92 between strain 5 and strain7 and strain8, respectively reflecting a common genetic background for these strains.

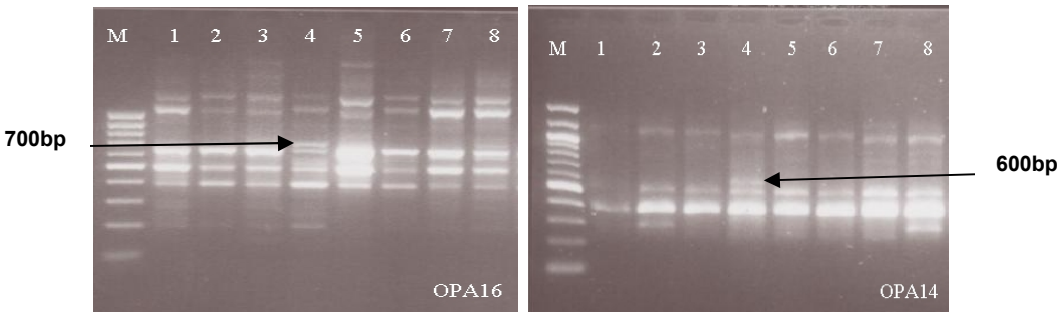

Fig. 1. Polymorphism detected by RAPD marker with eight carob strains
M: Ladder molecular weight marker. Arrows refer to positive unique markers in strain 4

Table 6. Genetic similarity as detected by RAPD marker among some carob strains

1	2	3	4	5	6	7	8
2	85						
3	88	86					
4	88	88	88				
5	85	86	91	87			
6	85	87	91	84	93		
7	83	86	88	86	94	93	
8	83	87	90	86	92	93	95

3.4.3 Unique markers detected in the studied strains

Unique markers are defined as bands that specifically identify an accession from the others by their presence or absence. Eleven unique markers were identified with the tested strains. Strain1 only was characterized by four negative markers with primer OPA07 at (600 bp, 1200 bp, 1300 bp and 1500 bp) and one positive marker with the same primer at 1000 bp. Moreover, strain 2 and strain 4 were identified by two unique markers. Strain 2 identified by OPA10 with two negative markers at 220bp and 380bp. While, strain4 was characterized by two positive unique markers (Fig. 1) with primers OPA14 and OPA16 at 600bp and 700bp, respectively. Meanwhile, both of strain 5 and strain 8 were characterized by negative marker with OPA16 (200bp) and positive marker with OPPo1 (230bp), respectively. A key for 7 blueberry genotypes based on 11 markers amplified by four primers [30].The origin of these unique markers may be attributed to mutation at the priming site of the primers or to insertion/deletion mutation in the distance between the reverse and forward priming site of the primers. The presence of unique RAPD markers among the various *Citrus* genotypes indicates the utility of the approach for fingerprinting purposes. RAPD fingerprinting has a number of potential applications including the determination of cultivar purity, efficient use and management of genetic resources collection, particularly in identification of mislabeled accessions [31].

3.4.4 Relationship and correlation coefficient based on phenotypic, chemical and genetic dendrogram

Regarding phenotypic dendrogram, leaflet length and leaflet width were used to generate phenotypic based dendrogram (Fig. 2). The obtained dendrogram separated strain 4 alone in one cluster; while, the other cluster was divided into two subcluster. The first of these subclusters included two groups; one of them contains strains 2 and 6 however, the other contains strain 3. On the other hand, the second subcluster was similar with first one; it contain two groups, the first group collected trains 7 and 8; meanwhile strain 5 was found in the second group.

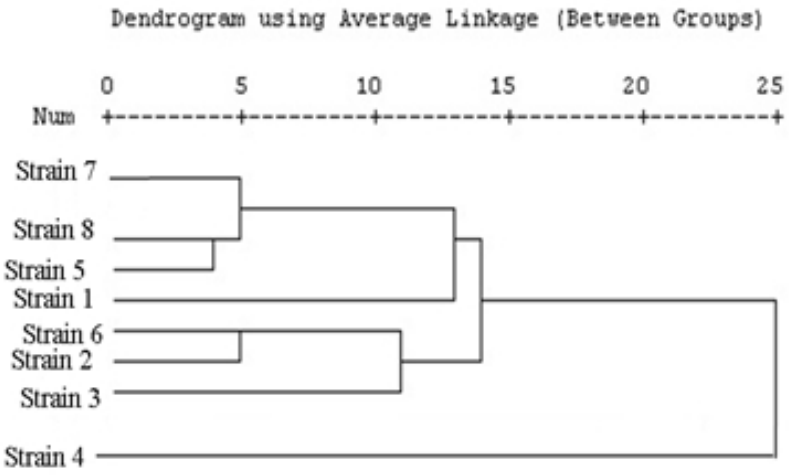

Fig. 2. Phenotypic based dendrogram

The total phenols, total tannins and total flavonoid were used to generate a dendrogram based on chemical (Fig. 3) constitutions to assist the relationship among the studied strains. The resulted dendrogram divided into two clusters, one of them included strains 1, 3 and 4. The second subcluster was divided into two groups; the first one collected strains 5, 6 and 8. While, the second contain strains 2 and 7.

Data of RAPD marker were used to generate genetic based dendrogram (Fig. 4). This dendrogram was separated into two cluster; one of them grouped strains 5,6 and 8. Strains 2 and 7 shared together in one subcluter of the second cluster. Moreover, the second subcluster was divided into two groups; one group included strains 1 and 3 while, the second one contain strain 4.

It is clear that chemical and genetical based dendrogram almost are similar, however, morphological based dendrogram was far from the chemical and genetical based dendrogram.

Morphological plasticity in study is a major weak point in assessment of phenotypic diversity [32]. However, several combined studies in mandarin, both morphological and molecular markers in the past had shown to be independent of genetic diversity [33]. Further, the study on inheritance of agronomic traits of citrus reports them to be controlled by multiple genes which can be assessed only through morphological assessment [34].

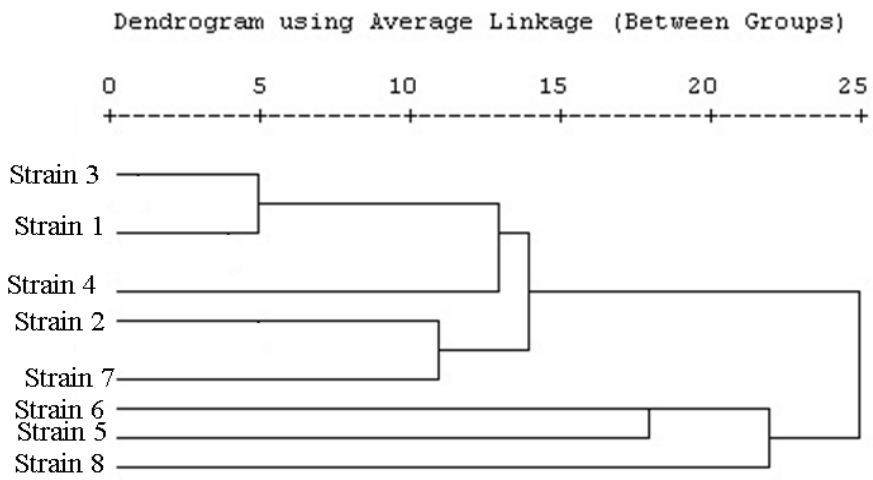

Fig. 3. Chemical based dendrogram

Morphological traits measured to evaluate the polymorphism among carob accession by [21] and stated that morphological traits could be influenced by climatic conditions; by contrast, Clustering based on RAPD data revealed a rough distribution of accessions according to their geographical origin.

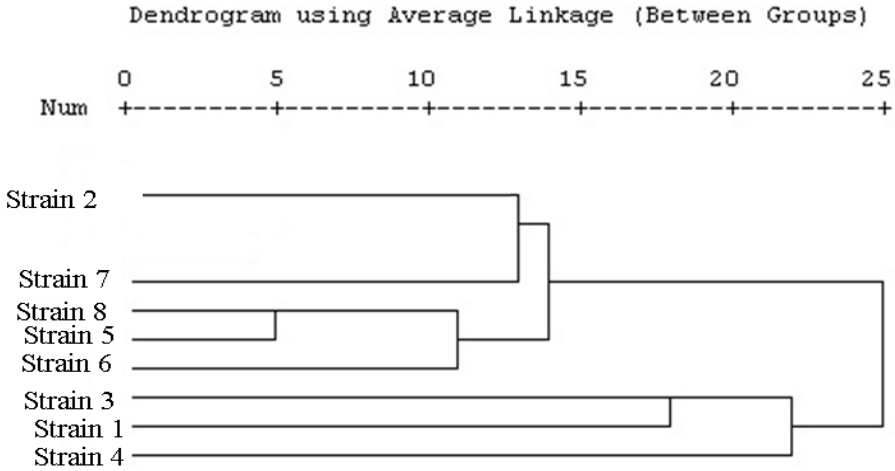

Fig. 4. DNA based dendrogram using RAPD marker

It could be concluded that, strains 2, 4 and 6 are the promising ones regarding yield. Strain 6 flowered lately; this consequently gives an extension for carob harvested season. On the other hand, both genetical and morphological dendrograms areextremely independent and no correlation could be built on them, a vice versa was true for chemical and genetical dendrograms.

4. CONCLUSION

It could be concluded that, strains 2, 4 and 6 are the promising ones regarding yield. Strain 6 flowered lately; this consequently gives an extension for carob harvested season. On the other hand, both genetical and morphological dendrograms are extremely independent and no correlation could be built on them, a vice versa was true for chemical and genetical dendrograms.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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