Molecular genetic polymorphism, Morphological and the effect of peels as natural antioxidants in some squash cultivars

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Abstract: The aim objective of this study was selected the squash cultivars visually according to RAPD Marker to determine the genetic relationships and evaluating similarity among the squash cultivars and also, cultivated and evaluated these genotypes under Egyptian condition in open field experiment. Their good performance levels and quality traits to be continued in the breeding program as parents to produce squash hybrids suitable for open field with high yield. The dried peels of the squash cultivars adding to cake as antioxidants. RAPD markers were utilized to define the genetic connections and estimating likeness between some squash types. Five RAPD primers were utilized to dwell on DNA extracted from the leaves of eight squash types utilizing the CTAB method. A total of 187 bands were developed of which 115 observed that that the polymorphism between the eight species. PCR-RAPD analysis illustrated a number of variables in the volume and number of bands between the species; it means that there are genetic variables between the squash species. Based on these markers, genetic likeness coefficients were calculated and a dendrogram was elevated. The dendrogram analysis described three major clusters. This research was to characterize that the RAPD markers are beneficial in estimating genetic diversity between squash species. The obtained results could be summarized as follow: Results indicated that the genetic differences among genotypes were significant or highly significant for all studied traits, indicating the presence of adequate genetic variability. The genetic differences among the genotypes were highly significant for all studied traits. Generally, the peels of the fourth cultivar were better than the first cultivar as strong antioxidants, while the peels of the first cultivar were the best when compared with the fourth cultivar in raising the cake samples content from sugar.

1. Introduction

Summer squash (*Cucurbita pepo* L.), has comparatively great alimentation and medicinal value than other vegetable crops. Summer squash had contained rich amounts from Vitamin A, C, Niacin, folate and fibers **USDA, (2005)** However; it makes this food of an appropriate dietary source for helping to prevent cancers, heart disease, and inflammation problems. Meanwhile, summer squash is beneficial in weight loss diets may be due to a low-calorie food. Their worldwide value makes this crop great classify between vegetable crops in economic value (**Paris, 1996**). The greatest producers of summer squash involve Turkey, Italy, Egypt, Spain, the U.S.A., and Mexico (**Paris, 2008**).

In Egypt, various not made better squash cultivated, grown and preserved only by local farmers, produce the fruit of low yield, quality, and seed production. Become better squash landraces will assist farmers to secure greater productivity and development quality. The collection, conservation, an estimate of the genetic diversity of Egyptian summer squash cultivated can help establish.

Breeding programs are to become better the studied a local cultivar or to produce new hybrids that meet farm and market requests in periods of the disease impedance and fruit yield and quality. For simplicity, rapidity, and competence, indiscriminate dwell on polymorphic DNA (RAPD) markers have been utilized for the estimate of genetic diversity of more cucurbit plants, like watermelon (**Mujaju et al., 2010**), melon (**Manohar and Murthy 2011**), and cucumber (**Goriunova et al., 2011**) and pumpkin (**Mladenovic et al., 2012**). In the happening research, the genetic difference of squash germplasm in Egypt was determined by RAPD to support in parental chosen for breeding programs.

Random Amplified Polymorphic DNA (RAPD) mechanism based on the Polymerase Chain Reaction (PCR) has been one of the usually generally utilized

molecular mechanisms to improve DNA markers polymorphisms can happen may be caused to base replacement at the primer binding parts or to variations in the areas among the parts. RAPD markers have been used for species identification, analysis of population structure, analysis of genetic impacts of environmental stress and analysis of genetic diversity (**Williams** *et al.,* **1993, Athanasios,** *et al.,* **2009**). RAPD, these short primers were used to reproducibly amplify segments of genomic DNA from a wide variety of species (**Williams** *et al.,* **1990 and Caetano-Anilles** *et al.,* **1991**) Reported that RAPD marker single primer can be used to amplify genomic DNA and that polymorphisms can be revealed among the expansion products of various personages. This type of polymorphism makes well be useful for study genetic diversity, genetic relationships, genetic charting, plant breeding, DNA fingerprinting, and population genetics (**Rebecca and Myers, 2002**) RAPD was used as a molecular tool to assess the diversity and relationship among 20 summer squash *(Curcubita pepo* L.) landraces traditionally used to treat hypertension and prostate hyperplasia (**El-Adl** *et al.,* **2012** and **Mady** *et al.,* **2013**). On squash (*Cucurbita pepo,* L.) **Albrifcany (2015)** used five PCR based RAPD markers to detect genetic variability. under the molecular level among the seven squash genotypes (Eskandarani, Coppi, Saja, Beyaz, Zucchino Ginyoveze, Zucchinotondo di piacenza and Zucchinoromanesco).

Ananthan and Pappiah (1997) found on cucumber that common consolidate efficiency and particular combining ability was considerable for days to first male flower opening. Evidence of dominant gene effects was found. **Ahsan** *et al.,* **(2011)** who studied the analysis of variances which showed significant differences among the parents and hybrids of snake gourd for most of traits, and they found that the lowest hybrid took minimum 81-83 days to produce female flower. **Dogra and Kanwar (2011)** stated in cucumber that the best general combiners for T.S.S were EC173934 and LC-40. **Abdein** *et al.,* **(2017)** studied 4 × 4 complete diallel cross of pumpkin was evaluated with their parents for heterotic manifestation and evaluate the genetic behavior of yield and yield component traits at two locations in Egypt.

Hikal, (2015) studied in eggplant the oxidation of edible oils during processing and storage via autoxidation and photosensitized resulting in a large amount of desirable chemical compounds which have harmful effects on human health. The anthocyanins extracted from eggplant peels by using the acidified aqueous technique to give high yield from anthocyanins. Natural antioxidants extracted are sort after with a view to combat devestating processes may be caused to oxidative stress. The antioxidant of acetone, ethyl acetate and methanol extracts of skin, leaves, and the seed of the variety of Cucurbita maxima are antibacterial activity was studied by **Dissanayake** *et al.,* **(2018)**.

Materials and Methods
Plant materials:
The genetic materials used in the present study included eight cultivars of summer squash (*Cucurbita pepo*, L.) from many countries, as a commonly known variety for comparison. The squash cultivar names are: (SQT1, SQT2, IQ3, IQ5, SR1, SR2, EX1 and SR3). The symbols, names and the origins of squash cultivars showed in **Table 1**.

Table 1: The symbols, names and the origins of the squash cultivars.

No.	Symbol	Name	Origin
1	SQT1	Sakiz Kabgi	**Turkey**
2	SQT2	Siyah Kabuk	**Turkey**
3	IQ3	Erbil Garden	**Iraq**
4	IQ5	Five Star	**Iraq**
5	SR1	Halab	**Syria**
6	SR2	Gabla	**Syria**
7	EX1	Eskandarani	**Egypt**
8	SR3	Hamah	**Syria**

DNA extraction:
Frozen young leaves (500 mg) were ground to powder in a mortar with liquid nitrogen. The powder was poured into tubes containing 9.0 ml of warm (650C) CTAB extraction buffer. The tubes were incubated at 650C° for 60-90 min. 4.5 ml chloroform/octanol (24: 1) was added and tubes were rocked to mix for 10 min., and centrifuged for 10 min. at 3200 rpm. The supernatants were pipetted off into new tubes and 6 ml isopropanol was added. After 60 min., the tubes were centrifuged for 10 min. and the pellets obtained were put in sterile Eppendorf tubes, containing 400 µl of TE buffer of a pH 8.0 (10 mM Tris-HCl, pH 8.0 + 1.0 mM EDTA, pH 8.0). the DNA's from genotypes were, then, extracted and stored at -200C until use **Doyle and Doyle (1990)**.

Determination of the concentration of DNA by UV spectroscopy:

A dilution of DNA by adding 20 µl of DNA solution to 0.98 ml of distilled water in a micro-centrifuge tube, were prepared and mixed well. The UV lamp in the spectrophotometer was warmed up for 20 min. and the wave length of the spectrophotometer was set to 260 nm. Distilled water was added to one cuvette using the distilled water as a blank and set the absorbance to zero. The absorbance of DNA dilution was measured. The concentration of DNA was calculated, according to **Sambrook *et al.,* (1989)**.

PCR amplification:

Five primers (**Table 2**), from Pharmacia Biotech. (Amersham Pharmacia Biotech UK Limited, England HP79 NA), were tested in this Experiment to amplify the templated DNA. Amplification reaction volumes were 25 µl, each containing $1 \times$ PCR buffer with MgCl2 (50 mM KCl, 10 mM Tris-HCl (pH = 9.0), 2 mM MgCl2 and 1% trition x-100), 200 µM each of dATP, dCTP, dGTP and dTTP, 50 PM primer, 50 ng template DNA and 1.5 µ of taq polymerase. Reaction mixtures were overlaid with 15 µl mineral oil and exposed to the following conditions: 94°C for 3 min, followed by 45 cycles of 1 min. at 94°C, 1 min. at 36°C, 2 min. at 72°C, and a final 7 min. extension at 72°C.

Amplification products were visualized with DNA marker on 1.6% agarose gel with $1\times$TBE buffer and detected by staining with an ethidium bromide solution for 30 min. Gels were then de stained in deionized water for 10 min. and photographed on gel documentation device under UV light.

Table (2): The sequence of the RAPD primers used in the study.

No. of Primer	Nucleotide Sequence (5` to 3`)
OPAO-03	AGTCGGCCCA
OPAO-05	TGGAAGCACC
OPAO-07	GATGCGACGG
OPAO-08	ACTGGCTCTC
OPAO-10	GACATCGTCC

Experimental design:

These experiment were conducted during the summer seasons of 2015 and 2016.

In summer season of 2015, The seeds of squash cultivars were grown in pots. All genotypes were selected visually according to RAPD Marker to determine the genetic relationships and evaluating similarity among the squash cultivars.

All these genotypes were cultivated and evaluated under Egyptian cultivation in an experiment in summer season of 2016. Their good performance levels and quality traits to be continued in the breeding program as parents in future studies.

The experimental design used was a randomized complete block design with three replications each replicate consisted of eight plots. Each plot was one ridge of 5 meters in length and 2 meters width so the plot area was 10 m^2, the distance between plants was 0.5 m. apart, each plot contained 20 plants (one plant per hill). The squash cultivar seeds were sown on 1st March 2016 to evaluation trial at El-Nubaria region, Albehira governorate, Egypt.

Data recorded

The following traits were recorded on five plants in each plot:

1- Vein length (cm), Number of branches/plant.

2- Number of fruits per plant, average fruit weight (kg), fruit length (cm.), total fruit yield kg/plant and days to harvest (after 300 day from sowing).

1.Vegetative traits:

For studying the differences between genotypes in the plant performance, five plants were uprooted from each plot after 40 days from sowing and the following data were recorded: Main vein length (cm). and Number of branches /plant.

2. Yield and its components:

This trait was calculated as the average number of fruits counted on the plants. Number of fruits/plant, average fruit weight (kg.), total fruit yield (kg.) /plant and No. of days to harvest.

Statistical analyses:

A regular analysis of variance of a Complete Randomized Block Design was conducted. L.S.D was used for the comparison between all genotypes means. **(Kempthorne, 1957).**

Appropriate L.S.D. values were calculated to test the significance of these genotypes.

Where**t:** The tabulated value at a stated level of probability for the experimental error degrees of freedom.

Data handling and cluster analysis:

Data were scored for computer analysis on the basis of the presence or absence of the amplified products for each primer. If a product was present in a genotype, it was designated "1 ", if absent it was designated "0"after excluding the inreproducible bands. Pair-wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic products, were used to regenerate similarity coefficients, according to **Jaccard (1908)**. The similarity coefficients were then used to construct dendograms, using the unweighted pair group method with Arithmetic Averages (UPGMA)

Employing the SAHN (Sequential, Agglomerative, Hierarchical, and Nested clustering) from the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 1.80 (Applied Biostatistics) program (**Rohlf, 1993**).

Cake Materials:

1-2 cups of raw flour to make cake: 2 cups flour - 1/2 cup butter - 1/4 cup sugar powder - 4 eggs - 3 tablespoons baking powder - vanilla - 1/2: 3/4 cup milk. (**Fennema *et al*., 2004**).

Preparation and drying of peels:

The fruits were peeled after cleaning well and then sliced into thin peels and dried in the oven drying at 60°C for 2-4 hours according to the different varieties of peels. **Krokida *et al.*,2003.**

Cake Making:

1. Sampling of the control cake was prepared (**Hamlyn, 1989**).

2. Cake was made with the addition of peels of four varieties, individually each time by 5g and another frequency of 10g. These quantities are divided by the weight of the flour used in preparation each time.

The percentage of sugars and the strength of the activity:

The percentage of sugars and the strength of the activity of the contents of the peels of vital compounds as antioxidants were estimated by all the samples of the cake produced according to the method (**Ashoush and Gadallah, 2011**) in the next formula:

$$\text{Activity rate as oxidants}\% = \frac{\text{absorption strength of the control sample} - \text{absorption strength of the tested sample}}{\text{absorption strength of the control sample}} \times 100$$

3. Results and Discussion

Table (3): Presence (1) versus absence (0) of PCR –amplified fragments from eight genomic DNA isolated of squash landraces using five random primers.

Name	SQT1	SQT2	IQ3	IQ5	SR1	SR2	EX1	SR3
Band 1	1	1	1	1	1	1	1	1
Band 2	1	1	1	1	1	1	0	0
Band 3	1	1	1	1	1	1	1	1
Band 4	0	0	0	0	0	0	1	1
Band 5	1	1	1	0	0	1	1	0
Band 6	0	0	0	1	0	0	1	0
Band 7	0	0	1	1	0	1	0	0
Band 8	0	0	1	0	0	1	1	1
Band 9	1	1	1	1	1	1	1	1
Band 10	0	0	0	1	0	1	0	0
Band 11	0	0	0	1	0	0	1	0
Band 12	1	1	0	0	0	0	0	0
Band 13	1	1	1	1	1	1	1	1
Band 14	0	0	1	1	1	1	1	1
Band 15	1	1	0	1	1	1	1	1
Band 16	0	0	0	1	0	1	1	0
Band 17	0	0	0	1	0	1	1	1
Band 18	0	0	0	1	1	1	1	1
Band 19	1	1	1	0	1	0	1	1
Band 20	1	0	1	1	1	1	1	1
Band 21	0	1	0	1	1	1	1	1
Band 22	0	0	0	0	0	1	0	0
Band 23	0	0	0	0	0	1	1	1
Band 24	1	0	0	0	0	0	1	0
Band 25	1	1	1	1	1	1	1	1
Band 26	1	1	1	0	0	0	0	0
Band 27	0	0	1	0	1	0	1	0
Band 28	1	0	1	0	1	0	0	0
Band 29	1	0	0	0	1	0	1	0
Band 30	1	0	0	0	0	0	0	0
Band 31	1	1	1	1	1	1	1	1
Band 32	1	0	0	0	1	1	0	0
Band 33	1	1	1	1	1	1	1	1
Band 34	1	0	0	0	0	0	0	1
Band 35	1	0	1	1	0	0	1	0
Band 36	1	1	1	1	0	1	1	1
Band 37	0	0	0	0	1	1	1	0
Band 38	1	1	1	1	1	1	1	1
Band 39	1	1	1	1	1	1	1	1
Band 40	1	1	0	0	0	1	1	1
Total	**25**	**18**	**21**	**23**	**21**	**27**	**30**	**22**

Results in **Table (3)** showed a total of forty PCR-amplified fragments over the eight squash cultivars using five different random primers. EX1 cultivar showed to have the highest number of amplified fragments (30) followed by the cultivar SR2 (27) then the cultivar SQT1 (25). Data showed that only nine out of a total of forty PCR-amplified fragments were monomorphic mean while thirty- one were polymorphic with a ratio of77.5 %.

Data in **Table (4)** showed that the highest genetic similarity (0.80) was observed between the cultivars SR2 (6) and IQ5 (4), that were shared the same cluster (Fig.1). Also the cultivars SQT1 (1) and SQT2 (2) shared a separate cluster where they have a significant genetic similarity of (0.79). Meanwhile, another cluster which involved both cultivars EX1 (7), and SR3 (8) since they had a genetic similarity of 0.78(Table3 and Fig.1).

The genetic similarity values between IQ3 (3) cultivar and each of the cultivars SQT1 (1), SQT2 (2) and SR1 (5) were 0.74, 0.72 and 0.71, respectively, (Table3 and Fig.1). These results suggest that the four cultivars, SQT1 (1), SQT2 (2) IQ3 (3) and SR1 (5) could be considered as one cluster. The dendrogram in (Fig.1) assumed the results that the eight squash cultivars could be separated to three clusters. These clusters were cluster I which involves the two cultivars, SR2 (6) and IQ5 (4), Cluster II involved the cultivars SQT1 (1), SQT2 (2) IQ3 (3) and SR1 (5) and cluster III involved the cultivars EX1 (7) and SR3 (8).

Similarly, the first a group of similar things had contained one group which included SR2 and IQ5 at a level of 80% genetic likeness. The second a group of similar things had contained three groups: the first group involved SQT1 and SQT2 at a level of 79% genetic likeness. The second group included the closely concerning types IQ3 at a level of 73 % genetic likeness to the first group. The third group included SR1with about 71% genetic likenesses to the second group. The third cluster consisted of only one group comprised EX1 and SR3 at a level of 78% genetic likeness. The three groups of similar things were likes to each other at a level of 58% genetic likeness. Genetic likeness ranged among 58% and 80 %.

Table (4): Genetic similarity values among eight squash landraces.

Lane	1	2	3	4	5	6	7	8
1	1	0.79	0.74	0.58	0.7	0.62	0.64	0.63
2	0.79	1	0.72	0.63	0.67	0.67	0.61	0.68
3	0.74	0.72	1	0.68	0.71	0.67	0.65	0.64
4	0.58	0.63	0.68	1	0.68	0.8	0.74	0.7
5	0.7	0.67	0.71	0.68	1	0.71	0.69	0.68
6	0.62	0.67	0.67	0.8	0.71	1	0.76	0.76
7	0.64	0.61	0.65	0.74	0.69	0.76	1	0.78
8	0.63	0.68	0.64	0.7	0.68	0.76	0.78	1

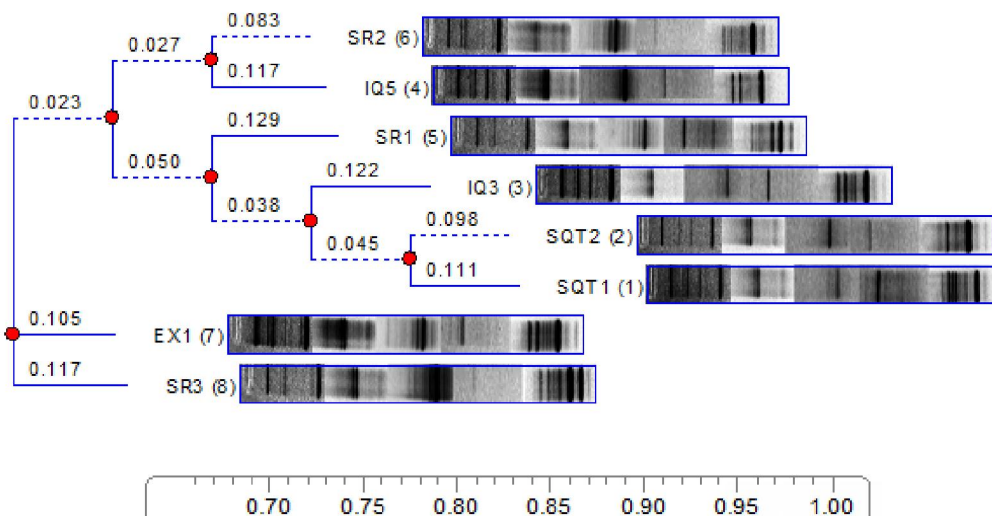Cultivars:-1- SQT1, 2-SQT2, 3-IQ3, 4-IQ5, 5-SR1, 6-SR2, 7-EX1 and 8-SR3.

Fig. (1): Dendrogram of the phylogenetic relationships and the resulted clusters for eight squash cultivars.
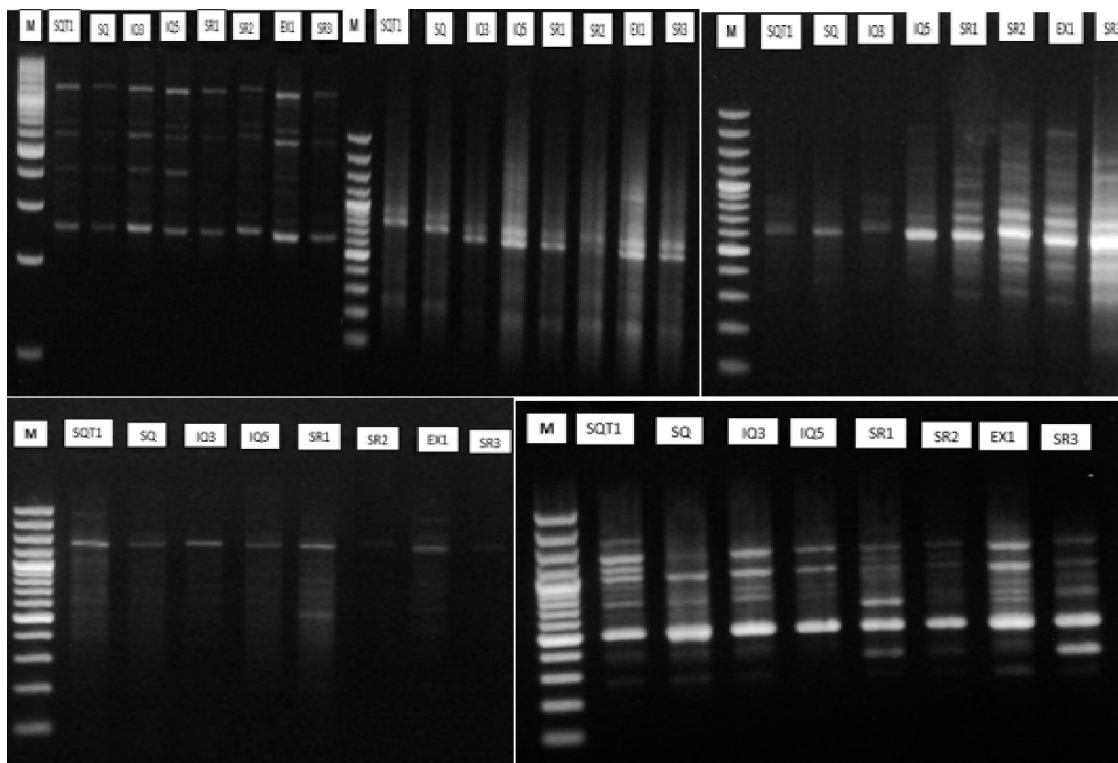Cultivars:-1- SQT1, 2-SQT2, 3-IQ3, 4-IQ5, 5-SR1, 6-SR2, 7-EX1 and 8-SR3.

Fig. (2): RAPD polymorphism in eight squashcultivars.
(1- SQT1, 2-SQT2, 3-IQ3, 4-IQ5, 5-SR1, 6-SR2, 7-EX1 and 8-SR3)
Primer No. 3, Primer No. 5, Primer No. 7, Primer No. 8, Primer No. 10, Respectively.

The performance of cultivars.
1. Vegetative traits:
    The results in **Table (5)** observed that the mean performance of cultivars for various traits in squash.
1.1 Vein length:-
    Data presented in **Table (5)** indicate that vein
    length of the variety eight had the highest number (230 cm).
1.2. Branches number:-
    Data presented in **Table (5)** indicate that number of branches of the variety four had the highest number.

Table (5): Mean performance of cultivars for various traits in squash.

Genotypes	Vein Length (cm.)	No. of branches / plant	No. of fruits / plant	Fruit weight (gm.)	Fruit length (cm.)	Total fruit yield / plant (Kg.)	No. of days to harvest
1	90	4.0	21.7	150.0	15	2.5	40
2	110	4.0	24.0	180.0	20	3.2	38
3	120	1.6	8.0	112.0	13	1.2	50
4	180	4. 6	20.0	146.7	17	2.2	41
5	160	4.3	18.0	140.0	15	3.3	44
6	170	3.6	23.7	126.7	15	2.4	45
7	200	3.6	27.0	156.7	21	2.5	42
8	230	1.6	24.3	146.7	21	2.2	45
L.S.D 0.05	**2.2**	**1.9**	**1.9**	**2.3**	**2.0**	**1.6**	**1.7**
0.01	**2.9**	**2.5**	**2.5**	**3.0**	**2.6**	**2.1**	**2.3**

Cultivars:-1- SQT1, 2-SQT2, 3-IQ3, 4-IQ5, 5-SR1, 6-SR2, 7-EX1 and 8-SR3.

1.3. Yield and its components

-Number of fruits:

Data (**Table 5**) show that cultivar seven (27 fruit/plant) had the highest number of fruits/plant.

-Fruit weight

Data in **Table (5)** show that The cultivar No.7 (156.7gm) and cultivar No.1 (150.0 gm) had the highest number of fruits /plant.

-Fruit length

It is evident from **(Table 5)** that the cultivars seven and eight had the longest fruits (21 cm).

-Total fruit yield / plant:-

Data (**Table 5**) show that the highest yield was produced by the cultivar two and cultivar five was high yielded (3.2and 3.3 kg. /plant).

-No. of days to harvest:

Data (**Table 5**) show that, cultivar No.2 (38 day) had earlier cultivars. The cultivar No.3 (50 days) had later than the other cultivars.

The effect of dried squash peels as antioxidants

Data presented in **Table (6)** showed that all the treatments have a remarkable rate as antioxidant, especially when duplicating their addition. The best treatments were the third cultivars peels followed by the second then the fourth and finally the first cultivar. **Ashoush and Gadallah, 2011.**

Generally, the peels of the fourth cultivar were more better than the first cultivar as strong antioxidants, while the peels of the first cultivar were the best when compared with the fourth cultivar in raising the cake samples content from sugar. **Dorta *et al*., 2011.**

Table (6): The activity rate of the biological compounds in dried squash cultivars peels in the cakes samples as natural antioxidants.

Peels additives	Squash peels as antioxidants							
	SQT1	SQT2	IQ3	IQ5	SR1	SR2	EX1	SR3
5g/250g flour	44.0	80.8	81.5	66.0	61.4	73.6	65.7	58.7
10g/250g flour	56.7	82.6	83.1	72.14	70.2	81.4	74.8	62.4

Conclusion

Considering the genetic diversity observed in the present analysis, it can be concluded that: RAPD-PCR technique showed considerable potential for estimating genetic diversity among our squash cultivars. These molecular markers could be used for characterizing squash genotypes in molecular breeding. This study demonstrates that RAPD markers are useful in assessing genetic diversity among squash species. The use of RAPD technique is shown to be useful in differentiating and characterizing the studied landraces which will be useful in establishing breeding programs for summer squash.

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