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PRODUCTION OF SOME NEW PEACH ROOTSTOCK THROUGH HYBRIDIZATION AND TISSUE CULTURE TECHNIQUE

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ABSTRACT

This study was carried out through three succeeded years (2005-2007) in almond- peach orchard aimed to grow plantlets of new hybrids coming from hybridization between both of Om El-fahm X Hansen by using tissue culture technique and to determine some molecular markers related to root-knot nematode resistance were detected. In field experiment, height of hybrids seedlings ranged from 53.3-70.1 cm, 2.21- 2.65 cm for stem thickness and 41 -63 for leaves number per one. As for tissue culture experiment, stem node was superior than shoot tip on bud development and shoot length in establishment stage. Meanwhile, the third subculture records the greatest significant number of produced shoots and shoot length in multiplication stage. for rooting stage, all genotype hybrids raised mediated ranged from 53.3-73.3 &4.16-5.33 and 3.14-5.11 for rooting %, root number/ shoot and root length, respectively. Hybrid genotype data confirmed that hybrid no.4 was superior than the other hybrids. By using four primers pairs related to root knot nematode resistance by two techniques. Pchgms primer by SSR and OPA11, OP834B and OPAP4 by STS were used. Four molecular markers appeared in hybrid no. 4 only which may be the most hybrid can be resistant to root knot nematode

Keywords: propagation, peach rootstock, hybridization, tissue culture, root-knot nematode resistance.

INTRODUCTION

Prunus belongs to the Prunoideae, subfamily of Rosaceae which includes several species. Producing edible drupes with important economic traits. In 2001, the worldwide annual production of Prunoideae exceeded 28.3 million metric tons, including almost 13.5 million tons of nectarines and peaches (*Prunus persica* [L.] Batsch) **(Martínez-Gómez *et al.*, 2003).** The genus Prunus- often called the stone fruits is one of the most important genera of woody plants.The genus Prunus is comprised of approximately 400 species of trees and shrubs.

Almond (*Prunus dulcis* Miller) is one of the most important nut crops worldwide **(Kester *et al.*, 1991)** and one of the oldest nuts tree. Today it represents the largest production of many nut tree products. Almond grows in regions of the world that are characterized by having a subtropical Mediterranean climate with wet winters and warm dry summers **(Kester 1990; Micke 1994; and Thrope and Roper 1995).** The total world production was 1.679,444 tones according to **(FAO.2004)**

The root-knot nematodes *Meloidogyne spp*., especially *M. incognita* and *M. javanica* cause severe damage to several species of prunus, damage can seriously affect early stages of plant development in the nursery or when rootstocks are transplanted into the field, also the extent of growth reduction caused by these pathogens has been documented for many prunus rootstocks used for peach and nectarine varieties **(Pinochet *et al.*, 1996).**

Rootstocks play a major role in modern orchards. Recently the importance of the recognized rootstock has an essential value for fruit yield. The rootstock, together with the grafted cultivar, influences the vegetative and generative mass and the profitability of fruit production **(Racskó *et al*., 2004).** On the other hand, the most important agricultural traits and the tree as a biotic unit; such as vigor, blossom initiation, fruit set, fruit size and fruit flavor, etc.; may be, substantially, influenced by the rootstock **(Tubbs, 1974; Dozier *et al.*, 1984; Holb *et al*., 2003 and Racskó *et al*., 2004).**

Tree breeding will benefit from the use of DNA molecular markers associated with genes for horticultural traits through marker-assisted selection (MAS). MAS would also allow screening for economically important traits in seedlings, which is especially useful for traits expressed only in fully mature trees. Additionally, MAS could expedite difficult screening procedures such as testing for disease or insect resistance or searching for genes that may express only partial penetrance or expressivity **(Warburton *et al.*, 1996).** The application of MAS can greatly improve the efficiency of peach breeding for resistance to root-knot nematodes **(Lu *et al.*, 1999).**

PCR-based, SSR markers (microsatellites) are becoming the marker of choice for fingerprinting and genetic diversity studies for a wide range of plants. Because of their high polymorphism, abundance, and codominant inheritance, they are well suited for the assessment of genetic variability within crop species, and of the genetic relationships among species. SSR markers proved to be efficient and reliable for the molecular characterization of Prunus spp. rootstocks **(Bianchi *et al.*, 2004).**

To overcome some of the difficulties related to RFLP analysis which has important limitations: it is laborious and time-consuming and it often involves the use of radioisotopes, an alternative called STS has been developed which is PCR-based but not requiring radioactive probing. Different STS markers tightly linked to the resistance genes to root-knot nematodes have been successfully developed and can be utilized for introgression of new root-knot nematode resistance genes into peach rootstocks **(Yamamoto and Hayashi, 2002).**

Tissue culture technique alternative to the traditional methods of propagation since it had made it possible to obtain large quantities of plant material in a short time **(Malavasi and Predieri, 1988).**

Accordingly, this work aimed to evaluate tissue culture techniques for propagation of the new hybrids coming from hybridization of Hansen and Om-Elfahm rootstocks, study different methods of molecular identification to determinate genetic diversity of the Prunus rootstocks under study, and detection of molecular markers related to root-knot nematode resistance.

MATERIALS AND METHODS

This study was carried out through three succeive years (2005-2007) in almond- peach orchard. Two rootstocks namely; Hansen and Om-Elfahm, were used as each of them has a relatively advantage compared to the others. Om-Elfahm (almond) is a common local rootstock, but not resistant to root-knot nematodes; Hansen (peach-almond hybrid) is one of the most suitable rootstocks used in calcareous soils to overcome lime-induced chlorosis, it is resistant to root-knot nematodes (*M. Incognita* and *M. Javanica*) and has relatively low chilling requirements (650 hr at < 7°C) **(Kester and Asay1986)**; The trees were grown in a sandy soil in a private orchard at El-Khatatba region (Menofia governorate), Egypt. Planting distance was about 4× 6m. While the lab. work was carried out in the Tissue Culture &Germplasm Conservation Researches Laboratory and also in Biotechnology Laboratory, Horticulture Research Institute, Agriculture Research Center, Giza, Egypt.

I-Field Experiment:-

Om-Elfahm almond rootstock were selected as mother plants which are vigorous and free from pathogenic symptoms, and subjected to the ordinary horticultural practices.

A cross pollination has been achieved using pollen grains of Hansen peach rootstock. About 60 flowers at balloon stage were selected and bagged with pergamin bags.

Initial fruit set and fruiting percentage were determined as follows:-

Initial fruit set% were recorded that 8.33% after 21 days of pollination.

$$\text{Fruiting percentage} = \frac{\text{Number of final remained fruits (before harvest date)}}{\text{Total number of flowers}} \times 100$$

Seeds after full ripping at the end of July were collected and stratificated by keeping in refrigerator in layers with wet sand until first of December (for almost 4 months) and planted in glass house for 6 months for evaluation. The following data were recorded in the five produced hybrids; seedling length, stem thickness and leaf number.

II-*In Vitro* culture

Shoots had about 5 vegetative buds of the new hybrids were collected and cuted into either shoot tip or stem node explants with

length of about 10 mm for each. The explants washed with running tap water for an hour, surface sterilized by soaking in 10% commercial bleach "Clorox" (5.25% sodium hypochlorite) for 10 min. and rinsed three times in sterilized distilled water ten minutes for each. Explants were cultured on MS establishment medium supplemented by 3% sucrose, 0.7% agar. PH adjusted to 5.6- 5.8 and incubated at 25±2ºC under 16 hrs light and 8 hrs dark.

For multiplication, excised shoot tips (1.5-2 mm length) were cultured on full strength medium supplemented with 0.5mg/L BA, 0.1 mg/L IBA and 100 mg/L myo-insitol.

Half strength MS medium containing 0.4mg/L IBA, 0.8mg/L NAA and 2% sucrose for rooting was used. After four weeks from culturing on rooting media, the following characters were recorded: 1- Rooting percentage. 2- Number of roots / plantlet. 3- Average of root length (cm.).

III-Molecular experiments:-

III-1- DNA extraction

Young fresh leaf samples were collected separately from each genotype (4 week old). Plant tissues were ground under liquid nitrogen to a fine powder, bulked DNA extraction was performed using DNeasy plant Mini Kit (QIAGEN). DNA isolation was achieved according to **Mohamed (2006).**

Simple sequence repeats (SSRs)

PCR reaction was conducted using one primer pair. Its name and sequence is shown as follow

Table (1): list of name and nucleotides sequence of the used SSR primers

Name	Primer sequence
Pchgms1	F -GGGTAAATATGCCCATTGTGCAATC
	R –GGATCATTGAACTACGTCAATCCTC

Amplification was performed according to **Yamamoto and Hayashi (2002)** with 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, for denaturation, annealing, and primer extension, respectively.

Sequence Tagged Site (STS)

Table (2): list of names and nucleotides sequence of the used STS primers

Name	Primer sequence
	5 □——————————————————›3□
OPA11	F= TGCAACGTCACATTTTAACC
	R= GATCCAGCAGAGAAAACGAG
OP834B	F=GCAGTCAAAAATTTCAAACC
	R=TCCGATTCGAGCCCACTACA
OPAP4	F=TTAAGACACCCAAACGATTTCA
	R=TGGGCATTTTGAGGTATCTG

The amplification was carried out according to **Sosinski *et al.* (2000)** as follows: PCR reactions were performed by an initial denaturation for 4 min at 94°C, followed by 32 cycles of: 45 s at 94°C, 30 s at 52-55°C , 30 s at 72°C; and a final extension of 5 min at 72°C.

The bands were visualized by ethidium bromide under UV florescence. Gels were photographed and scanned with Bio-Rad video densitometer Model 620, at a wave length of 577.

III-2- Data analysis

Statistical analysis of variance according to Snedecor and Cochran, 1976. The significant differences among means were determined by Duncun's multiple range test **(Duncan, 1955).**

The similarity matrices were done using Gel works ID advanced software UVP-England Program. The relationships among stone fruit's as revealed by dendrograms were done using SPSS windows (Version 10) program. DICE computer package was used to calculate the pairwise difference matrix and plot the phenogram among rootstocks **(Mohamed, 2006).**

RESULTS AND DISCUSSION

1-Field experiment

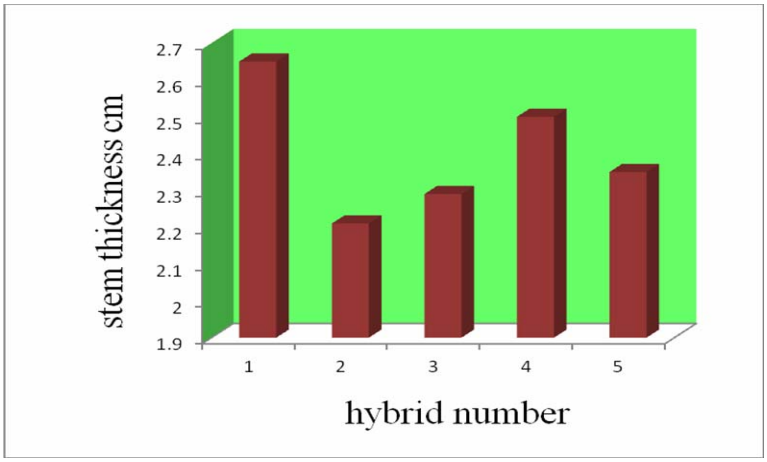
Seedling characters after six months of seed germination in the nursery are shown in table (1) and fig.(1)

Table (3): Seedling characters of Om El-fahm X Hansen hybrid genotypes after six months of seed germination in the nursery.

Om El-fahm X Hansen	Hybrid seedling length cm	Stem thickness cm	Leaves number
1	59.7	2.65	56
2	59.8	2.21	48
3	67.1	2.29	51
4	70.1	2.50	63
5	53.3	2.35	41

Means followed by the same letter(s) are not significantly different from each other at 5% level

As shown in Table (1), vegetative characteristics were affected by hybrid genotype (Om El-fahm X Hansen), e.g, hybrid no.4 proved that it is the most vigour one vegetatively, the contrast was true with hybrid no.5.



Fig(1): Effect of Om El-fahm X Hansen hybrid genotypes on stem thickness after six months of seed germination in the nursery

2-*In vitro* experiment

Effect of different genotypes and explants type during establishment stage

Table (4): Effect of hybrid genotype (Om El-fahm X Hansen), explant type and their interaction on bud development percentage and shoot length (cm.) during invitro establishment stage.

Om El-fahm X Hansen	Bud development%		Mean	Shoot length (cm)		Mean
	Shoot tip	Stem node		Shoot tip	Stem node	
1	66.67cd	88.9b	77.8A	1.53d	1.81bc	1.67C
2	**55.6d**	**66.7cd**	**61.15B**	**1.97ab**	**1.95ab**	**1.96B**
3	55.6d	100.0a	77.8A	1.65cd	2.04ab	1.85BC
4	77.8bc	88.9b	83.4A	2.05a	2.19a	2.13A
Mean	63.92B'	86.13 A'		1.80B'	2.00 A'	

Means followed by the same letter(s) are not significantly different from each other at 5% level

Insignificant differences were present among hybrids no.1, 3&4 which revealed the highest significant percentage of bud development (77.8, 83.4 and 77.8%, respectively). Meanwhile, the lowest significant value of bud development was achieved by hybrid no.2 (61.15%). Stem node was superior than shoot tip with values (86.13 and 63.92%, respectively). Referring to the interaction between the two factors on bud development percentage of stem node hybrid no.3 reached the highest significant percentage (100%), while shoot tip resulted from both hybrids no. 2&3 reached the lowest significant value (55.6%)

Concerning the specific effect of the different hybrids on shoot length (cm) during the establishment stage, data showed that hybrid no.4 was the highest significant value (2.13cm). Meanwhile, the least significant (1.67 cm) was found with hybrid no.1. As for the specific effect of explants type, stem node was superior than shoot tip on shoot length (2.00 and 1.8 cm, respectively).The interaction between the two studied factors showed that hybrid no.4 resulted from both explants types developed the longest significant shoot (2.05 cm for shoot tip and 2.19 cm for stem node). Insignificant difference were found between hybrids no.4 & no.2 (shoot tip 1.97 and stem node 1.95 cm.) and stem node of hybrid no.3 (2.04cm.). On the other hand, hybrid no.1 shoot tip recorded the lowest significant value (1.53 cm). These results go in line with **Fouad *et al.*, (1995)** who cleared that shoot tips of peach rootstocks Nemaguard and Meet- Ghamr sterilized with 0.5%

sodium hypochlorite gave a higher percentage of shoot survival than 10% calcium hypochlorite or 0.2% mercuric chloride.

Effect of different genotypes and number of subculture on number of produced shoot per explant and shoot length during multiplication stage

Table (5): Effect of hybrids (Om El-fahm X Hansen), number of subculture and their interaction on number of shoots/ explants during proliferation stage:

Om El-fahm XHansen	Number of shoots/explants			
	First subculture	Second subculture	Third subculture	Mean
1	2.56 g	4.22 cd	5.73a	4.17A
2	3.11 fg	4.11 de	4.80 bc	4.01A
3	2.89g	3.67 ef	4.93 b	3.84A
4	3.22 fg	4.33cd	5.20 ab	4.25A
Mean	2.95C'	4.08 B□	5.17A'	

Means followed by the same letter(s) are not significantly different from each other at 5% level

It is quite clear, as shown in Table (5), that hybrids derived from crossing between Om El-fahm X Hansen had no significantly differences in number of proliferated shoots. Meanwhile, the highest significant mean number of produced shoots were achieved by the 3rd subculture (5.17) followed by the second one (4.08). While the first subculture showed statistically the lowest significant value of proliferated shoot number (2.95).

The interaction between the different hybrids and the number of subculture showed that, the highest significant number of shoots per explants were achieved with hybrid no.1 at the third subculture (5.73) and hybrid no.4 at the same subculture (5.2) compared with the other treatments. While, hybrids no.1 & no.3 at the first subculture achieved the lowest significant number of shoots per explants (2.56 and 2.89, respectively).

Effect of hybrids (Om El-fahm X Hansen), number of subculture and their interaction on number of shoots/ explants during proliferation stage

Table (6): Effect of hybrids (Om El-fahm X Hansen), number of subculture and their interaction on number of shoots/ explants during proliferation stage

Om El-fahm XHansen	Shoot length (cm.)			Mean
	First Subculture	Second Subculture	Third Subculture	
1	2.10f	3.12 de	3.82 bc	3.01B
2	2.67 ef	3.60 cd	4.14 bc	3.47AB
3	2.64 ef	2.97 de	4.43 ab	3.34AB
4	3.15 de	3.82 bc	4.92 a	3.96A
Mean	2.64C'	3.38B'	4.33A'	

Means followed by the same letter(s) are not significantly different from each other at 5% level

As for the effect of different hybrid on shoot length, it is quite clear as shown in Table (6) that, hybrid no.4 gave the highest significant value of shoot length among the four hybrids. Insignificant differences among the three hybrids (2, 3 and 4) were observed, while the first hybrid recorded the least value (3.01). Tabulated data in Table (6) evaluated the specific effect of the 3 subculture number on shoot length. There were completely coincident in the trend in response to subculture number. The third subculture was the superior in shoot length as the tallest significant shoot (4.33 cm.). The third subculture was in concomitant the aforesaid superior subculture. Meanwhile, the first subculture was the least for shoot length (2.64 cm).

Table (6), displays the interaction effect of the two studied hybrids (Om El-fahm X Hansen) and number of subculture. The third subculture showed significant difference for shoot length with hybrid no.4 (4.92) or hybrid no.3 (4.43) with insignificant differences between them. Moreover, the first subculture achieved the shortest significant shoot length with hybrid no.1 (2.10). Such results are in general agreed with those founded by **Ruzic *et al.*, (1984)** who gave support to the present result pertaining the effect of IBA as added auxin to the MS medium during proliferation stage, and with **Wanas (1999)** who cleared that number of multiplied shoots and shoot length increased with 1.5 fold by the 3rd and 4th subcultures were achieved in MS medium plus 0.4 mgl^{-1} IBA and 1.5 mgl^{-1}BA.

Effect of different genotypes on rooting stage on rooting percentage, root number per shoot and root length (cm)

Table (7): Effect of different hybrid genotypes (Om El-fahm X Hansen) on rooting percentage, root number per shoot and root length (cm) on rooting stage.

Hybrid number	Rooting%	Roots number/shoot	Root length(cm.)
1	73.33 A	5.17 AB	4.39 AB
2	53.33 B	4.16 C	3.14 C
3	53.33 B	4.50 BC	4.16 B
4	66.67 A	5.33 A	5.11 A

Means followed by the same letter(s) are not significantly different from each other at 5% level

It is clear that the highest significant value was achieved with hybrids no.1 (73.33) and no.4 (66.67), while hybrids no.2 &3 got the shortest significant one (53.33) for rooting percentage. As for the effect of different hybrids on roots number, it is clear from the mentioned data that hybrid no.4 recorded the highest significant value (5.33). On the other hand hybrid no.2 showed the lowest significant one (4.16).

Data in the above table cleared also the effect of different genotypes on root length. It is quite clear that the greatest significant value was reached by hybrid no.4 (5.11). Meanwhile, the least value was achieved by hybrid no.2 (3.14). These results are in general agreement with the findings previously by **Wanas (1999)** who rooted almond × peach hybrid rootstock (Hansen 536) on one-half strength MS medium supplemented with 0.5 mg/L PP333, 0.2 mg/L IBA and 0.4 mg/L NAA that resulted in 75% rooting with thick roots and vigorous shoots suitable for transferring to the soil

3-Molecular experiment

Detection of molecular markers related to root-knot nematode

The results generated from STS-PCR and SSR-PCR profiles were used to illustrate the genetic relationships among the studied rootstocks as shown in Table (6) and Figure (2).

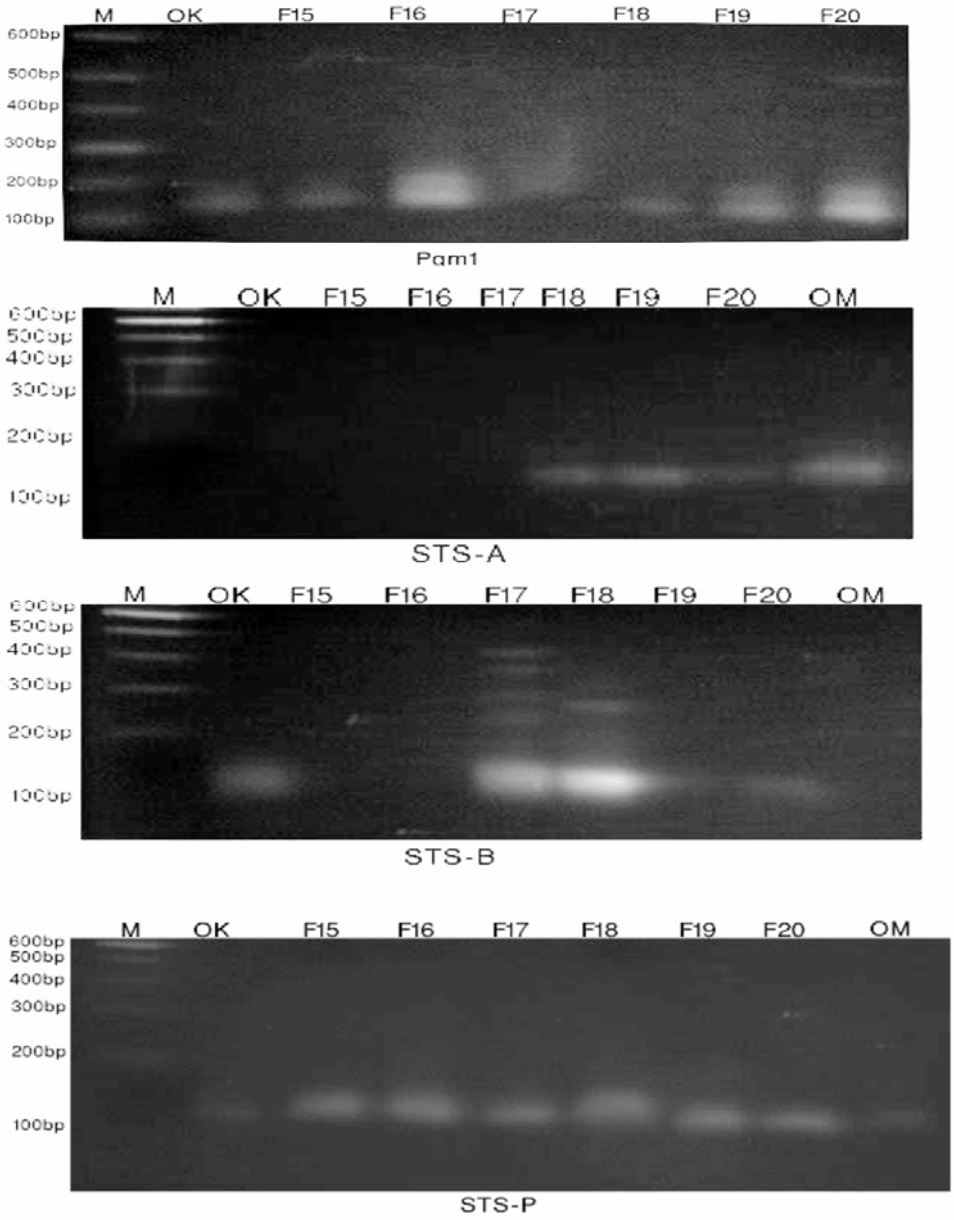


Fig.2. DNA polymorphism using SSR-PCR of the new hybrid rootstocks amplified with primer Pchgms1 and STS-PCR of the new hybrid rootstocks amplified with primer STS-OPA11,OPB and OPP

(M) DNA ladder marker (bp) (OK) Hansen (OM)Om Elfahm
(17) hybrid no.1 (18) hybrid no.2 (19) hybrid no.3 (20) hybrid no.4

The four primer pairs were employed to screen for nematode resistance. As shown in Table (8) and figure (2) SSR with primer pair Pchgms amplified the specific marker with the Hansen stock and Hybrids no.2, no.3 and no.4, this marker revealed the expected size (194bp) as reported by **Yamamoto and Hayashi, 2002.** On the other hand of the three primer pairs belonged to the STS marker only the OP4 has the ability to introduce the marker linked to nematode resistance in all of the tested hybrids at molecular size of 283bp. At the same time, OPA11 revealed the corresponding marker at molecular size of 166bp with hybrids 2, 3, 4 and Hansen. Moreover, OP834B amplified band of 227bp with only the two hybrids (1 and 2) and Hansen rootstock.

Table (8): SSR and STS markers linked to nematode resistance, size of the corresponding bands and the hybrids revealing the markers.

Marker type	Primer	Band size (bp)	Hybrid number and parents
SSR	Pchgms1	194	2, 3, 4, Hansen
STS	OPA11	166	2, 3, 4, Hansen
	OP834B	227	1, 2, Hansen
	OPAP4	283	1, 2, 3, 4, Hansen

The presence of these four bands confirmed that hybrids 1, 2, 3 and 4 can be considered as resistant genotypes for root knot nematode beside the original rootstock Hansen as shown in table (8).

To determine the genetic relationship among the four tested hybrids, Om El-fahm and Hansen stocks, the scoring data (1 for presence and 0 for absence) resulting from tested primers was used to compute the similarity matrices (1) as shown in Table(8).

Table (9):Similarity index (Pair wise comparison) among the new hybrids rootstocks based on STS and SSR-PCR

Rootstocks	Om El-fahm	Hansen	Hybrid no.1	Hybrid no.2	Hybrid no.3
Hansen	000				
Hybrid no.1	000	0.667			
Hybrid no.2	000	1.00	0.667		
Hybrid no.3	000	0.857	0.400	0.857	
Hybrid no.4	000	0.857	0.400	0.857	1.00

The genetic similarity ranged from 20%between hybrid no.1 and both of hybrid no.3 and no.4 to 60% between Hansen and hybrids no.1, no.3 and no.4. The genetic similarity matrices based on the Dice co-efficient were used in the cluster analysis to generate a dendrogram using the UPGMA analysis Fig (3).

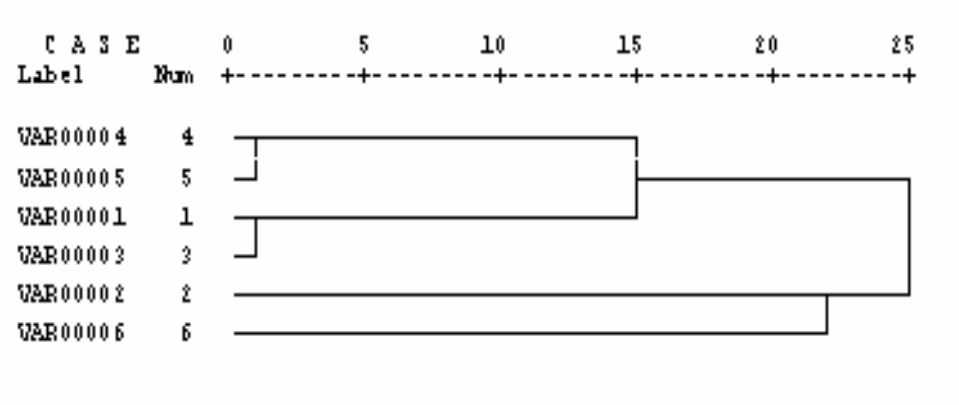


Fig. (3). A dendogram showing the genetic distance among the new genotypes and the parents using SSR and STS-PCR data
1-Hansen 2-hybrid (1) 3-hybrid (2) 4-hybrid (3) 5-hybrid (6) 6- Om El-fahm

The dendrogram in Fig. (3) reveled that Om El-fahm was separated from the remaining six genotype. The cluster containing5 genotypes was divided into two main subcultures, one including hybrid no.2, meanwhile, the other one was divided into two groups. One of them included the Hansen and the hybrid no.3. While, the other group contain hybrids no.4 and no.5. These resulted indicating that, the presence of hybrids no.3, no.4 and no.5in the same subcluster reflecting their parentage relatedness.

Evaluation of the root-knot nematode resistance is a time- and labour-consuming process. Therefore, molecular markers tightly linked to the nematode resistance genes are of special interest for breeding and improving peach rootstocks **(Yamamoto and Hayashi, 2002). Pinochet (1997)** and **Di Vito *et al.* (2002)** indicated that, resistance of prunus to root-knot nematodes is controlled by several different genes.

With Lovell × Nemared family, Pchgms1 marker was appeared to link with linkage group 1 which contains important rootstock

characters for resistance to the root-knot nematode, *Meloidogyne sp.* **(Lu *et al*., 1998).**

In contrast, **Yamamoto and Hayashi (2002)** revealed that, the DNA marker Pchgms1 (SSR) did not show any linkages with the resistance loci to *M. incognita* and *M. javanica* of 'Juseitou' (Japanese peach source).

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انتاج بعض اصول الخوخ الجديدة من خلال التهجين و تقنية زراعة الأنسجة

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تمت هذه الدراسة فى مزرعة لوز و خوخ خلال ثلاث سنوات (٢٠٠٥-٢٠٠٧). كان بهدف الحصول على نبتات من هجن جديدة ناتجة من التهجين بين كل من ام الفحم والهانسن باستخدام تكنيك زراعة الانسجة.وتحديد الاختلافات الوراثية لاصول الخوخ تحت الدراسة وكذلك اكتشاف المعلمات الجزيئية التى يرجع لها مقاومة النيماتودا.

تراوح ارتفاع الشتلات بين ٣, ٥٣–١,٧٠سم و٢١, ٢–٢,٦٥سم لسمك الساق و ٤١-٦٣ لعدد الاوراق. بالنسبة لمرحلة التأسيس سبقت العقلة الساقية القمة النامية فى تطور البرعم وطول الفرع.

بينما سجلت النقلة الثالثة زيادة معنوية فى عدد الافرع المنتجة وطول الفرع فى مرحلة التضاعف. فى مرحلة التجذير ارتفع متوسط كل التراكيب الوراثية للهجن من٣٣, ٥٣– ٧٣,٣٣ % و ٤,١٧-٥,٣٣ سم و ٣,١٤-١١, ٥ سم لنسبة التجذير وعدد الجذور لكل فرع و طول الجذور على التوالى.

نتائج التراكيب الوراثية للهجن اوضحت تفوق الهجين رقم اربع على باقى الهجن . تم استحدام اربع من الكاشفات الجزيئية يرجع لها مفاومة النيماتودا بطريقتين. فقد استخدم الكاشف Pchgms بتكنيك SSRو المعلمات OPA11, OP834B ,OPAP4 بتكنيك STS ظهرت كل الحزم فقط فى الهجين رقم 4ولهذا فهو اقرب الهجن لمقاومة النيماتودا.