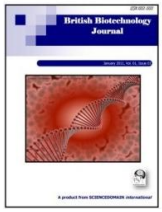

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Production of New Almond-Peach Hybrid Rootstocks Resistance to Root-Knot Nematode

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This research was conducted to hybridize almond (Om El-fahm and M. Dalet) female parent with Okinawa peach rootstock as a male parent to introduce new almond rootstock resistance to root-knot nematode.
Methodology: Okinawa peach rootstock used in crosses with both of Om El-fahm and M. Dalet almond cvs. and the resulting hybrids identified by SSR and STS markers for the presence and/or absence of the expected resistance marker. Inoculation by *M. incognita* with suspension of 2500 eggs per plants through holes in the soil to evaluate hybrids resistance.
Results: Fruit set percentage was higher with Om El-fahm than M. Dalet while, the opposite was true for fruit drop. No significant differences were observed for seed germination with the used females. Hybrid no.3 resulting from M. Dalet x Okinawa crosses showed the highest significant value of vegetative growth parameters. The STS marker; the OPA11 primer pair with Okinawa x Om El-fahm showed the resistant marker at the expected size (166 bp) for all of the tested hybrids except for hybrids no. 7 and 8. Meanwhile, only one hybrid (no. 8) of Okinawa x M. Dalet failed to amplify the resistant marker. The Okinawa parent was clustered with hybrids no. 1, 2, 3, 4 and 14 for Om El-fahm and hybrids no. 1, 2, 3, 4, 5 and 6 for M. Dalet indicating a common genetic resistance for root-knot nematode.

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Conclusion: Hybrid rootstocks slightly varied in their resistance to the root-knot nematode *Meloidogyne incognita*. However, line no. 6 (Okinawa × Om El-fahm) could be considered as highly resistant (HR).

Keywords: Prunus; peach; almond; crosses; nematode; resistance; molecular markers.

1. INTRODUCTION

Prunus belongs to the *Prunoideae* subfamily of the *Roseaceae* family, which includes several species edible drupes with economic importance. The worldwide annual production of *Prunoideae* exceeded 28.3 million tons in 2002 [1].

The invasion by nematodes causes the formation of the syncytia or knots limiting markedly water uptake. Therefore, heavily infested plants suffer from water stress. Once nematodes become established in an orchard, it's difficult to eradicate. Crop losses caused by nematodes are very high, with 20% annual yield losses worldwide. The existing practice of chemical control of nematodes results in residues that contaminate the environment. Hence, there is a need to develop commercially acceptable types of rootstocks with resistance/tolerance to this biotic stress [2].

Breeding of rootstocks plays a major role in modern orchards. The rootstock performance together with the grafted cultivar influences the vegetative and generative mass and the profitability of fruit production. However, the most important agriculture traits and the tree as a biotic unit; such as vigor, blossom initiation, fruit set, fruit size and fruit flavor, may be substantially influenced by the rootstock. Thus, rootstock improvement requires much more time and more resources as scion cultivar breeding [3-5]. Therefore, breeding through conventional methods could not meet the demand of new rootstocks resistant to biotic and abiotic stress. New technologies such as micro-propagation and molecular markers as a marker assisted selection (MAS) will be useful for breeding program. The application of MAS can greatly improve the efficiency of peach breeding for resistance to root-knot nematodes *Meloidogyne* spp. [6]. It is very difficult to observe morphological traits of rootstocks after grafting, so that DNA markers greatly facilitate rootstock identification. Molecular markers are of interest to plant geneticists and breeders as a source of new genetic information on plant genomes and for use in trait selection [7].

The aim of the present investigation is to introduce a new hybrid of almond x peach rootstock and evaluate its resistance to *M. incognita*.

2. MATERIALS AND METHODS

This study was carried out through three successive seasons (2008, 2009 and 2010) in an almond-peach orchard. The trees were grown in sandy soil in a private orchard at El-khatatba region (Menofia governorate), planting distance was 4x6 m. While, the laboratory work was carried out in the Tissue Culture and Germplasm Conservation Research Laboratory, Horticulture Research Institute. Agriculture Research Center, Giza- Egypt.

2.1 Field Experiment

Vigorous mother plants were selected free from pathogenic symptoms and subjected to the ordinary horticultural practices. Pollen used for pollination was prepared by collecting flowers of peach cv. Okinawa at balloon stage just a day before anthesis.

Flowers of almond cvs. Om El-fahm and M. Dalet emasculated by a forceps (60 flowers/branch) were pollinated by peach pollens with a soft hairbrush immersed in pollen vial, after which pollinated branches were enclosed in paper bags labeled with the number of pollinated flowers/bag and hybridization date.

The following crosses were done:

a) Om El-Fahm x Okinawa;
b) M. Dalet x Okinawa and
c) Self-pollination (Om El-Fahm and M. Dalet)

About 60 flowers were used in the cross pollination per combination for each treatment which represented 3 replicates, each replicate contained 20 flowers. Initial fruit set and fruiting percentage were determined as follows:

Initial fruit set percentage=

$$\frac{\text{N. of fruit set (21 days after pollination)}}{\text{Total number of flowers}} \times 100$$

Fruiting percentage=

$$\frac{\text{N. of final reminning fruit (before harvest date)}}{\text{Total number of flowers}} \times 100$$

After full ripping of seeds, theywere collected at the end of July and stratificated by keeping them in a refrigerator in layers with wet sand until the first of December (almost 4 months) after which they were, planted in polyethlene bags filled with a soil mixture of sand: Peatmoss: Vermiculite 1:1:1. The obtained seedlings were then planted and left for six months in glass houses for evaluation.

The following measuerments were taken:

Seedling length (above soil surface to the highest point of seedling), stem thickness (trunk girth above soil surface at 10 cm) and leaf number (total number of leaves for each seedling) was counted.

2.2 Molecular Analysis

Hybrids were tested for its resistance to *M. incognita* at the molecular level using four specific markers [8], one SSR (Pchgms 1) and three STS (OPA11, OP834B and OPAP4) (Table 1). Young fresh leaf samples were collected from each genotype (four weeks old) and DNA was isolated from these leaves according to [9].

PCR reaction for SSR and STS was performed in 25 µl volume containing the following reagent: 2.5 µl DNTPs (2.5 mM), 2.5 µl $MgCl_2$ (25 mM), 2.5 µl Buffer (10 X), 1.0 µl of both forward and reverse primers, 1.0 µl Taq DNA polymerase (1U/1 µl), 2.5 µl Template DNA (25 ng) and 12 µl H_2O (d.w). The amplification was conducted according to [8] for SSR marker and [9] for STS markers.

2.3 Evaluation of *Meloidogyne incognita* Resistance

Infected roots of tomato were cutted into small pieces (2-3 cm) and covered with NaOCl (0.25%). Samples were vigorously shaken for 2-3 minutes. Suspension was poured through three sieves; from top to bottom 80 mesh, 200 mesh and 500 mesh. Eggs on the 500 mesh sieve are gently washed with a slow stream of cold tap water to rinse off residual NaOCl. Eggs from the 500 mesh sieve were collected into a beaker.

Three cuttings were prepared from seven seedling hybrids and inoculated with 2500 *incognita*, which were obtained from a culture of *incognita* (Kofoid & White) maintained in a screenhouse on tomato plants (*Lycopersicone sculentum* L. Var. Castel Rock) the cuttings were grown in plastic pots (15-cm diameter) with sterile sandy loam soil and were inoculated through holes in the soil from the base of the plant. Evaluation of galls was performed 50 days after inoculation at the Laboratory of Nematology and Acarology, Plant Protection Dep. Fac. of Agric. Ain Shams Univ, Egypt.

Gall indices were established using a 1-6 scale of [10], 1=0 galls; 2= 1-10 galls; 3= 11-30 galls; 4= 31-70 galls; 5= 71-90 galls and 6= 91-100 galls/plant. The resistance rate of each hybrid was estimated according to the scale of [11,12].

2.4 Data Analysis

Statistical analysis of variance according to [13] was used for data analysis. The significant differences among means were determined by Duncan's multiple range tests [14].

Similarity matrices for molecular data were estimated using Gel work ID advanced software UVP-England Program. The relationships as revealed by [8], dendrograms were determined using SPSS windows (version 10) program. DICE computer package was used to calculate the pairwise difference matrix and plot the phenogram among rootstocks.

3. RESULTS AND DISCUSSION

3.1 Field Experiment

3.1.1 Initial fruit set, fruit drop percentage and seed germination

Data displayed in Fig. 1 clearly shows the effect of different pollination treatments on the initial fruit set and fruit drop percentage. The highest fruit set was observed by Okinawa as a male parent with Om El-fahm (56.67%) followed by Okinawa x M. Dalet (31.67%); the reverse was true for fruit drop. For self-pollination treatment, Om El-fahm revealed the highest fruit set (53.33%). While, M. Dalet recorded the lowest fruit set percentage (10%). Again, the opposite was true for fruit drop.

No significant differences in germination percentage were observed by Okinawa pollinator with both mother trees (Om E-lfam and M. Dalet) which recorded 74% and 68%, respectively and

self-pollination of each mother tree rootstock 74% for Om- El-fam 66.67% for M. Dalet use same order.

Several authors [15,16] claimed that the percentage of harvested fruit from the 9 crosses between peach and plum cultivars ranged from 5.4% to 25.4%, while the rate of perfect seeds ranged from approximately 70% to 90% as observed in open-pollination plum cultivars [15].

3.1.2 Vegetative parameters of the new hybrid genotypes

Highest seedling length was obtained by hybrid no.11 (64.4 cm), while, hybrid no. 10 showed the lowest length (28 cm). On the other hand, hybrid no.8 and 12 recorded the highest value of stem thickness; while, hybrid no. 10 detected the lowest value. A greatest value of leaf number was observed by hybrid no. 7 (Table 2); meanwhile, the least significant value was obtained by hybrid no. 3.

The same parameters of eight hybrids resulting from M. Dalet x Okinawa were recorded in Table 3. Hybrid no. 3 showed the highest seedling length (61.5 cm), while, the opposite was true for hybrid no. 2 (20.4 cm). For seedling thickness, hybrid no. 3 recorded the highest value (0.43 cm), hybrid no. 2 showed the lowest value (0.21 cm). Hybrid no. 3 showed the highest number of leaves (57) while, hybrid no. 8 had the lowest number of leaves (14).

3.2 Molecular Analysis

3.2.1 Identifying of the hybrids resistant to nematode

This study is based on the analysis of the SSR and STS markers for the parents and their hybrids result from di-allel crosses providing a high and durable resistance to nematodes. One SSR and three SSR primers pairs were mployed to screen for nematode resistance. The sequences of these primers were obtained from published data by [9].

As shown in Fig. 2, hybrids resulting from Om El-fahm x Okinawa amplified the specific SSR marker in the expected size (194 bp) as reported by [9] in all the tested hybrids except for hybrid no. 6. Table 4 summarizes the efficiency of each hybrid to detect the nematode resistant marker in respect to STS marker, OPA11 revealed the resistant marker at the expected size (166 bp) for hybrid no. 1, 2, 3, 4, 5, 6, 9, 10, 11, 12, 13, 14 and 15. However, OP834B amplified the resistant marker (227 bp) for nine hybrids (1, 2, 3, 4, 5, 6, 12, 13, 14 and 15) and failed to identify the marker with the hybrids (7, 8, 9, 10 and 11). OPAP4 amplifies the marker responsible for

Table 1. Names and nucleotide sequences of the used primers

Name	Primer sequence
OPA11	F: TGCAACGTCACATTTTAACC R: GATCCAGCAGAGAAAACGAG
OP834B	F:GCAGTCAAAAATTTCAAACC R:TCCGATTCGAGCCCACTACA
OPAP4	F:TTAAGACACCCAAACGATTTCA R:TGGGCATTTTGAGGTATCTG
Pchgms1	F:GGGTAAATATGCCCATTGTGCAATC R:GGATCATTGAACTACGTCAATCCTC

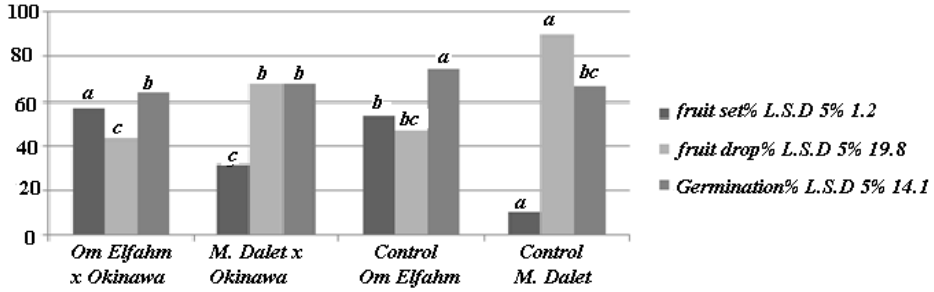

Fig. 1. Effect of Okinawa as a male parent on fruit set%, fruit drop% and germination% of Om El-fahm and M. dalet female parents

nematode resistance for all the investigated hybrids at molecular weight (283 bp) except for the hybrids no. 10, 12 and 13. The failure of the primer pair to amplify the marker of interest in the studied hybrids reflects the fact that these markers were not found in these hybrids. On the other hand, (Fig. 3) the SSR primer pair has the ability to amplify the expected marker (194 bp) for all tested hybrids resulting from crosses between Okinawa x M. Dalet. Meanwhile, the hybrid no.8 did not reveal the interested marker with the OPA11, OP834B and OPAP4 primer pairs. Moreover, the OPAP4 primer pair failed to amplify the specific marker with hybrid no. 7.

Evaluation of the root-knot nematode resistance is a time and labor consuming process. Therefore, molecular markers tightly linked to the nematode resistance genes are of special interest for breeding and improving peach rootstock [9,10]. Moreover, studies indicate that, the resistance of *Prunus* to root-knot nematode is controlled by several different genes. With Lovell x Nemared families, Pchgms1 marker appeared to link with linkage group 1 which contains important rootstock characters for resistance to *Meloidogyne* spp. [15,17]. In contrast, [9] revealed that, the DNA marker Pchgms1 (SSR) did not show any linkage with the resistance loci to *M. incognita* and *M. javanica* of "Juseitou" (Japanese peach resource).

3.2.2 Cluster analysis detected by Okinawa as a male parent and their hybrids

The STS and SSR based dendrogram obtained from UPGMA cluster analysis of genetic distance is presented in Fig. 4, which separated the Om El-fahm from the other genotypes, thus demonstrating the distinctiveness of the genetic background of Om El-fahm from the other genotypes.

The remaining hybrids and the Okinawa parent were separated in another six clusters, the first of them include hybrids no. 7 and 8. The second cluster contain hybrids no. 9, 10 and 11, however, the third cluster include the hybrid no. 6. The fourth cluster comprised hybrid no.12 and 13, the fifth cluster contained only hybrid no. 15. The last cluster was divided into two groups, one group included only hybrid no. 5; while, the second group contains Okinawa and hybrids no. 1, 2, 3, 4 and 14.

Table 2. Vegetative growth parameters of Om El-fahm x Okinawa hybrids genotypes after six months of seed germination in the nursery during season 2009

Hybrid number	Seedling length (cm)	Stem thickness (cm)	Leaf number
1	64.2	0.38	61
2	59.9	0.39	35
3	30.4	0.25	17
4	57.1	0.34	57
5	52.6	0.34	44
6	58.6	0.37	36
7	51.2	0.38	68
8	46.2	0.4	37
9	44.7	0.34	49
10	28.0	0.18	23
11	64.4	0.34	56
12	50.6	0.4	47
13	56.0	0.3	49
14	53.1	0.38	54
15	42.8	0.3	43
L.S.D 5%	9.40	0.07	3.59

Means of significant differences were measured at p≤0.05

Table 3. Vegetative growth parameters of M. Dalet x Okinawa hybrids genotypes after six months of seed germination in the nursery during season 2009

Hybrid number	Seedling length (cm)	Stem thickness (cm)	Leaf number
1	58.8	0.38	46
2	20.4	0.21	17
3	61.5	0.43	57
4	60.2	0.42	47
5	58.2	0.39	51
6	54.4	0.34	38
7	60.0	0.37	52
8	27.1	0.27	14
L.S.D 5%	7.93	0.13	4.23

Means of significant differences were measured at p≤0.05

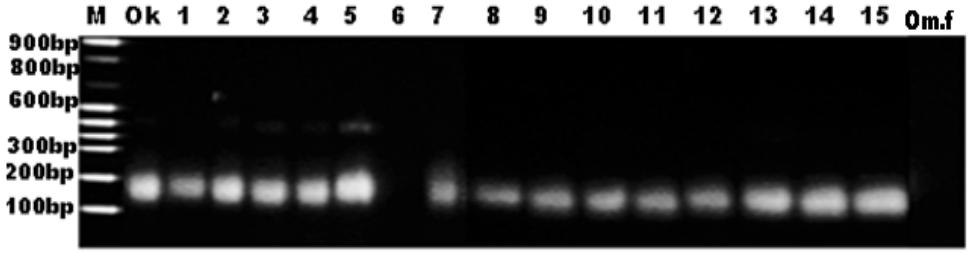

Fig. 2. Nematode resistant markers of the new hybrid rootstocks (Om El-fahm X Okinawa) amplified with primer Pchgms 1. M: ladder molecular weight marker

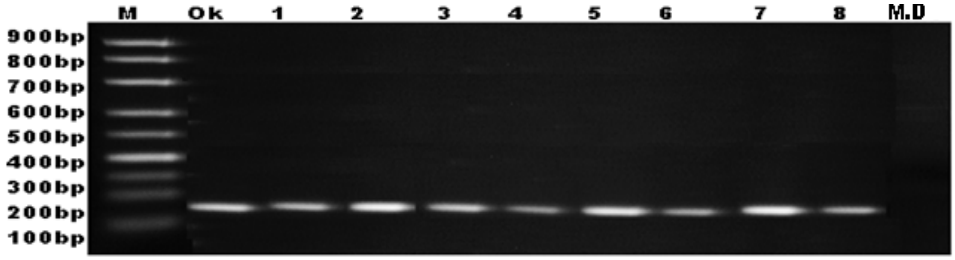

Fig. 3. Nematode resistant markers of the new hybrid rootstocks (M. Dalet x Okinawa) amplified with primer Pchgms 1. M: ladder molecular weight marker

Table 4. SSR and STS markers linked to nematode resistance, size of the corresponding bands (bp) and the hybrid numbers revealing the markers

Marker type	Primer	Band size (bp)	Hybrids of Om El-fahm x Okinawa	Hybrids of M. Dalet x Okinawa
SSR	Pchgms1	194	1,2,3,4,5,7,8,9,10,11,12,13,14&15	1,2,3,4,5,6,7& 8
STS	OPA11	166	1,2,3,4,5,6,9,10,11,12,13&15	1,2,3,4,5,6 & 7
	OP834B	227	1,2,3,4,5,6,12,13,14&15	1,2,3,4,5,6 & 7
	OPAP4	283	1,2,3,4,5,6,7,8,9,11,14&15	1,2,3,4,5,6

The dendrogram resulted from M. Dalet x Okinawa and their hybrids (Fig. 5) revealed that M. Dalet was separated in one cluster from the other hybrids. Two other clusters were obtained, one of them contain the hybrid no. 8; while, the third cluster was divided into two groups. The first group contains the hybrid no. 7, while the second group comprised of Okinawa and hybrids no. 1, 2, 3, 4, 5 and 6.

3.3 Evaluation of Selected Hybrids of Almond-peach Rootstock to *Meloidogyne incognita* Resistance

To ensure the resistance of the resulting hybrids, six randomly chosen hybrids of Okinawa × Om El-fahm and one hybrid of M. Dalet x Okinawa were inoculated by *M. incognita* using suspension contains about 2500 eggs per plant. Data given in Table 5 indicates that the seven hybrid lines of almond-peach hybrid rootstocks were slightly varied in their resistance to *M. incognita*. Line no. 6 (Okinawa × Om El-fahm) can be considered as highly neither resistant (HR), since neither galls nor J_2s were detected in soil or root tissues at the end of evaluation. On the other hand, the rest of tested lines were considered resistant (R) there were low numbers of galls and J_2s. No J_2s were observed in the roots of the hybrids of Om-Elfahm x Okinawa. Only on M. Dalet x Okinawa were J_2s observed on the roots although no galls were observed. Most hybrids were able to sustain J_2s in the soil 50 days after inoculation.

The obtained data is strongly supported by finding of [18]. In this study 'Greenpac' is a new peach hybrid rootstock (*Prunus persica* x *P. davidiana*) x (*P. dulcis* x *P. persica*) developed by Agromillora Iberia, S. L. Barcelona, Spain, for

use mainly as a rootstock for peach and nectarine cultivars but can also be used for almond. 'Greenpac' is root knot nematode-resistant to the main species found in the Mediterranean area such as *M. incognita* and *M. javanica.*

However the low number of galls or the juveniles stages in the root tissues or soils could be due some chemical constituents or histological characters which prevent or delay nematode development [19]. This complete absence of gall symptoms is associated with cell necroses and corresponding hypersensitive-like reaction (HLR) phenotypes occurring either in the stele or in the meristematic apical region or in the cortex [17].

It could be concluded that most of the tested hybrids in this study incorporate new sources of resistance in interspecific peach and almond against *M. incognita*. Molecular investigation detected the responsible marker for nematode resistance at the expected size for most of the tested hybrids. The hybrid rootstocks detected specific differential response to *M. incognita*, hybrid no. 6 reveals the highest degree of resistance. Such sources of nematode resistance will allow a higher diversification in stone fruit production zones.

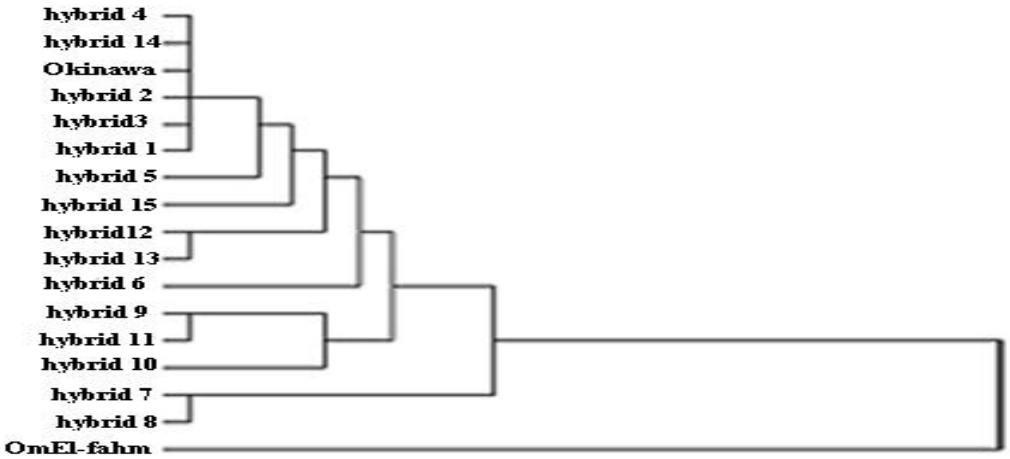

Fig. 4. Dendrogram showing the genetic relationship among the new hybrid rootstocks and their parents Om El-fahm X Okinawa using SSR and STS-PCR data

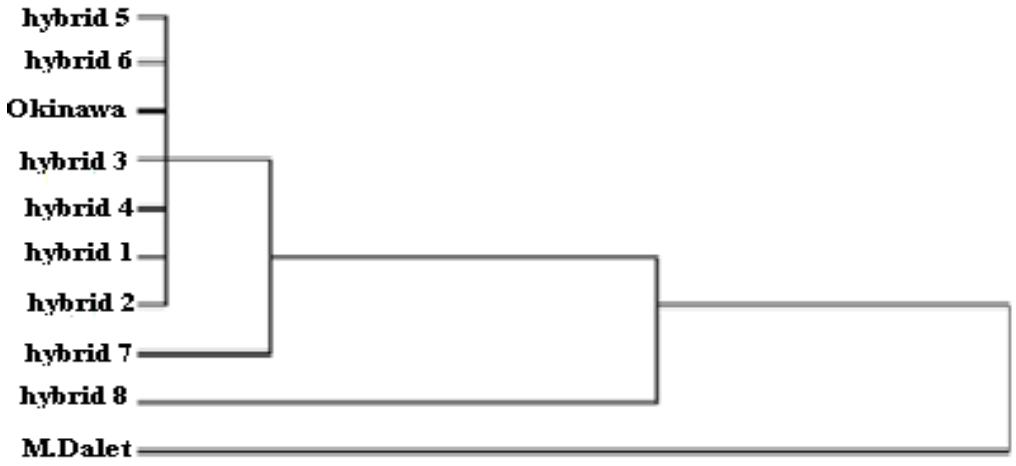

Fig. 5. Dendrogram showing the genetic relationship among the new hybrid rootstocks and their parents M. Dalet X Okinawa using SSR and STS-PCR data

Table 5. Number of galls and population of *Meloidogyne incognita* on some almond- peach hybrid rootstocks 50 days after inoculation

Used parents	Hybrid line	Nematode parameters (number/plant)				Reproduction rate	Resistance rating
		No. of gall	No. of J_2s in soil	No. of J_2s in roots	Final production (pf)		
Om El-fahm x Okinawa	2	0	22	0	22	0.022	R
	3	0	54	0	54	0.054	R
	4	1	25	0	25	0.025	R
	5	0.5	24	0	24	0.024	R
	6	0	0	0	0	0	HR
	12	0	75	0	75	0.075	R
M. Dalet x Okinawa	2	0	46	20	66	0.066	R

HR: highly resistant; R: resistant

4. CONCLUSION

Hybrid rootstocks slightly varied in their resistance to the root-knot nematode *Meloidogyne incognita.* However, line no. 6 (Okinawa × Om El-fahm) could be considered as highly resistant (HR).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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