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מדינת ישראל / משרד החקלאות
מינהל המחקר החקלאי

Budget Department

היחידה לתקציבים

May 22, 2008

USAID Morocco
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Souissi, Rabat
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Dear Mr./Ms.

Re: Project TA-MOU-00-M20-072

Please find enclosed a copy of the Final Report for the above mentioned grant.
Covering period: June 1, 2001 to December 30, 2007

Sincerely,
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FINAL REPORT

Covering Period: June 1, 2001 – December 30, 2007

Submitted to the U.S. Agency for International development; Bureau for Global Programs, Field Support and Research; Center for Economic Growth and Agricultural Development

TITLE OF PROJECT

EVALUATION, MASS-PROPAGATION AND CONSERVATION OF SUPERIOR, VIRUS-FREE PRUNUS LANDRACES

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Grant Number: TA-MOU-00-M20-072

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3. Executive Summary

Almond is rapidly becoming a highly demanded crop of significant commercial value. Improving the knowledge on growing almonds with yield increase, high quality fruit, health beneficial components etc. will contribute significantly to the economy of the Mediterranean region which is highly suitable for its cultivation. In this context, almond growers in the region, including Morocco and Israel, will benefit greatly from the results of this research.

The overall aim of this multidisciplinary project was to advance the stone fruit growing industry in Morocco and Israel by facilitating production of certified propagation material of locally selected rootstocks and cultivars. To this end, the following objectives were proposed: (a) selection of almond genotypes in Morocco and evaluation for drought and salinity tolerance, (b) developing in vitro and /or in vivo rooting and propagation methods, (c) development of in vitro virus elimination methods for stone fruit rootstocks and cultivars, (d) validation of PCR techniques for detection of viral pathogens in post therapy in vitro shoot cultures, (e) optimization of protocols for mass micro-propagation of the selected clones (f) establishment of cryopreserved and in vitro maintained germplasm stone fruit collections of selected Moroccan almond genotypes. Two additions were made to the above listed objectives, after the project had started: DNA finger printing of almond clones and the salt tolerance objective was extended and included limestone.

The majority of the planned research work plan was completed with significant achievements. In brief: A collection of superior almond clones (budwood and seeds) rescued from seedling populations in remote areas of drought and high salinity in Morocco was established. Other collections (stone fruit selected varieties, rootstocks and indicators) were also established. Salt and limestone tolerance of several Moroccan selections has been studied and needs to be continued. Performance of Moroccan rootstocks has been evaluated in comparison to traditional ones. Micropropagation of almond selections and the indicator GF305 was studied with focus on improving rooting. Cold storage and cryopreservation of almond meristems was studied extensively and generated a protocol for cold storage at 4C. Cryopreservation was not successful in our hands. Molecular detection assays (mainly RT-PCR) was adapted for the major viruses in stone fruits. In vitro virus elimination (thermotherapy and shoot tip grafting) and regeneration of virus free plants (overcoming the rooting problem) was studied. Plants are being evaluated for trueness to type. DNA fringerprint analysis using RAPD, SSR and S-allele was started for almond types.

The research teams from Morocco and Israel met at least five times within the lifetime of this project. Due to the political climate, meetings took place in Europe. The annual meetings were dedicated to plan future work, discuss results and problems and broaden the knowledge relevant to this project by visiting labs and meeting scientists in Spain, France, Italy, Portugal and Greece. Protocols and plant material were exchanged between Morocco and Israel and contributed to the interaction between the teams. As a result of the political situation, planned mutual visits of Moroccan and Israeli scientists did not take place. However, the annual meetings and email correspondence kept the bridge of joint work open and active.

By virtue of USAID-MERC funds, both UCP and EB laboratories were equipped and expanded their capacity to accommodate the activities performed in the frame of this project. Domaines Agricoles (Morocco) has gained immense benefits by developing capabilities for setting up a modern nursery for stone fruits in Morocco and on a regional level.

4. Research Objectives

The overall aim of this project was to advance the stone fruit growing industry in Morocco and Israel by facilitating production of certified propagation material of locally selected rootstocks and cultivars. To this end, the following objectives were proposed:

- Development of in vitro and/or in vivo methods for clonal propagation, rooting and hardening of mature almond types.
- Selection of almond genotypes from various regions of Morocco and evaluation for drought and salinity and limestone* tolerance.
- Development and application of effective in vitro virus elimination methods for the targeted viruses.
- Adaptation and validation of sensitive detection methods for the targeted pathogens in post-therapy shoot cultures, aiming to shorten the production of virus-tested plants.
- Adaptation and optimization of tissue culture protocols for mass propagation of the selected local plant material.
- Establish a cryopreserved / in vitro germplasm collection of almond genotypes.
- DNA finger printing of almond clones

The latter objective and the addition of limestone (indicated by * above), were not part of the original proposal and were included at a later stage by the research teams.

The objectives detailed above reflect on the multidisciplinary character of this project. In most cases activities were performed independent of each other. Several activities were studied by research groups in both countries and results were compared and discussed jointly. A flow chart indicating the titles of the main activities branched according to horticulture, tissue culture and virology topics is attached in Appendix 1.

5. Methods and Results

The main goal of this project was achieved by carrying out research activities in various disciplinary areas focusing mainly on almond as the major targeted crop. The activities are listed by topics and the participating countries are indicated by (M) Morocco and (IL) Israel.

A. Plant collections

The aim of this activity was to collect almond landraces and cultivars, other stone fruit rootstocks and cultivars in Morocco and establish them in collections for studies in this project and conserve them for possible future use. In addition, various indicators were collected for virus indexing. Samples collected were established by grafting on rootstocks in UCP. The virological status of plants was determined by ELISA and RT-PCR assays.

i. Collection of local almond selections (M)

A survey has been conducted to cover the major almond seedling populations in various geographic locations of Central and South Morocco. A map of the collection sites is presented in Fig. 1.

During two consecutive years of intensive collection we noticed the extent of the dramatic devastation which occurred in almond seedling populations in these areas due to ongoing drought. A dilemma regarding strict application of criteria set for tree selection and the low number of trees that could be selected in these areas was faced. We agreed to be strict in applying the criteria of selection, namely high vigor and good performance under harsh conditions. Only 47 well performing trees could be selected from these areas (Table 1). All samples collected were established by grafting, or by in vitro culture or by both methods except for three sources (I-6, I-12 and I-13). Fifteen to 20 cultures were established from each source and maintained in vitro for further use. The collection of Moroccan accessions is maintained in UCP. Two selections: I-7 and TOV have been studied extensively in the frame of this project (described below). I-7 was collected from Imintanout and TOV was collected from Toundout valley (indicated in Fig. 1).

Table 1. Selection of local almond seedlings in Morocco

Location	No. of selections
IMINTANOUT	15 (I-7)
TAFRAOUT	3
TOUNDOUT (montagne)	7
TOUNDOUT (vallée)	7 (TOV)
AGLMOUSS	1
NFIFA	1
BIN EL OUIDAN	2
AZZIZAL EST	5
OUZZOUDE	6
Total No. of selections	**47**

Fig. 1. Collection sites of almond landraces and cultivars in Morocco

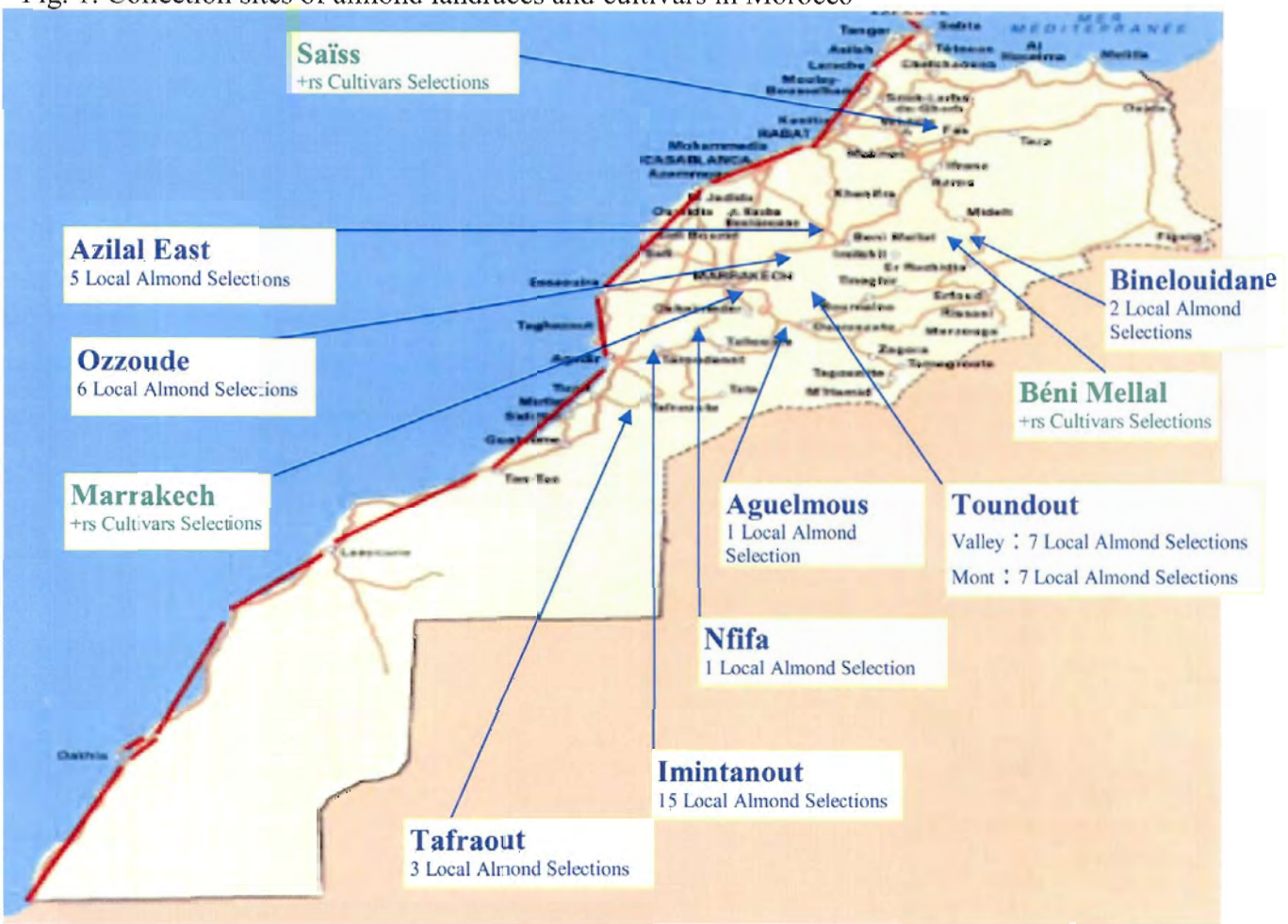

Fig. 2. Varietal collection of Stone Fruit selections grafted on rootstocks in UCP

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ii. Stone fruit rootstocks and varieties collections (M)
Local rootstock selections were collected from various locations. Other selections of rootstocks and varieties were also included (Fig. 2, above).
Among the local roostocks, Pêcher de Missour (PdM) and Missour Apricot (MA) are of great interest. Three selections of MA, designated MA-I, II and III, among a population of MA seedlings were selected for their vigor and apparent resistance to powdery mildew. PdM has been studied extensively in this project (detailed below). Selections of rootstocks and varieties are listed in Tables 2 and 3, respectively. These selections were tested by ELISA for the viruses PNRSV, PDV and ACLSV. To verify the results, virus specific RT-PCR assays were performed for all ELISA negative selections.

Table 2. Collection of various stone fruit rootstocks in UCP

Rootstock	Collection site
GF677	Douiat, EL Ouata, Chlihat (origin Star Fruit)
Pêcher de Missour (PdM)	Missour
Myrobolan	Chlihat (origin Star Fruit)
Myrobolan 1-3	EL Ouata
Kirschensaller	Chlihat (origin Star Fruit)
Abricotier ? (à partir de semis)	Pépinière privée (Kalrat Srarna)
Abricotier de Missour	Missour
Cadaman	Chlihat (origin Star Fruit)
Garnem	Chlihat (origin Star Fruit)
Adesoto	Chlihat (origin Star Fruit)
Montclar	Marrakech (SOPROFEL)
Prunus persica Cv ''Lovell ''	Caroline du sud (Clemson University)- USA
Nemagard almond	Caroline du sud (Clemson University)- USA
Gardian	Caroline du sud (Clemson University)- USA
GF305	INRA- Bordeaux Caroline du sud (Clemson University)- USA
BA-29	El Ouata
Cherry Root-Stock	Italy

Table 3. Collection of cultivars of five stone fruit crops established at UCP

Species	Total no. collected
Almond	77
Apricot	45
Plum	23
Peach	37
Nectarine	25

iii. Addition of Moroccan almond clones to the germplasm field plot in Newe Ya'ar (IL)
Cuttings from 21 different seedlings imported from Morocco to Rahan Meristem (RM) were obtained from RM and recently grafted on GF677 rootstocks in the germplasm field plot in

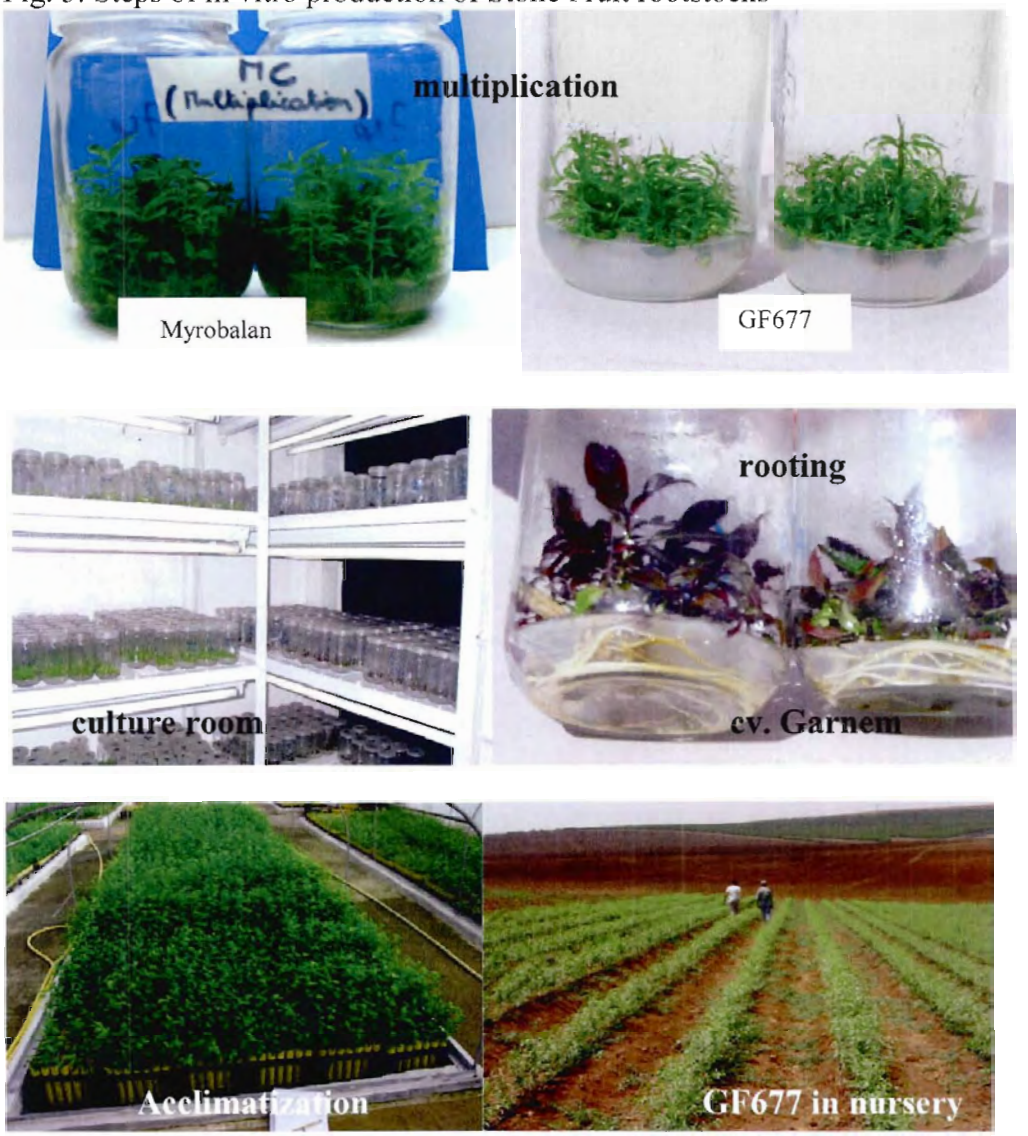
Newe Ya'ar (North Israel). The resulting trees will serve for future breeding, search for new self compatibility alleles, characterization of fruit quality, growth habits, resistance to diseases, salinity tolerance and other desired traits.
In vitro cultures of PdM from UCP were hardened in Israel (VC) and resulting plants were planted in Newe Ya'ar and evaluated as a rootstock under local conditions (see below). This is a significant addition to the existing collection in Newe Ya'ar.

This activity resulted in establishment of new collections in Morocco and an addition to the Israeli collection. This plant material was the main basis for activities described below.

B. Mass-propagation of stone fruit rootstocks and indicators

i. Stone fruit rootstocks (M)
This activity was started at UCP with rootstocks Myrobolan and GF677 needed for establishment and conservation of collected material. Optimization of the protocol for multiplication of collected rootstocks (Table 2) was realized during this project (Fig. 3). The optimized protocol has already been applied for nursery activities in Morocco.

Fig. 3. Steps of in vitro production of Stone Fruit rootstocks



ii. Stone fruit indicators (M)

GF305 is a universal woody indicator plant for stone-fruit virus indexing. It is not readily available in Morocco and is a limitation for the continuity of the certification program. Efforts focused on developing a mass-propagation protocol of this indicator.

Initiation: Certified seeds obtained from Clemson University (USA) were germinated and grown under green-house conditions. These plants served as donors of shoots for initiation of in vitro cultures on a nutrient medium (MS salts + 1/2 strength AP + BAP 2 mg/l + 2iP 0,01 mg/l).

Multiplication: Several media were assayed and the best results were obtained with MS salts + BAP 2 mg/l, IBA 0,05 mg/l) with up to 5X multiplication per transfer.

Rooting: The highest number of rooted shoots was obtained with 1/2MS + IBA 0,1 mg/l + NAA 0.1mg/l.

The development of an efficient protocol for micropropagation of GF305 is of great importance to fruit tree indexing in Morocco and in other countries where it is not readily available. However, its reaction to virus inoculation needs to be verified in comparison to seed germinated plants.

iii. Developing an improved protocol for in vitro propagation and rooting for almond and almond X peach hybrids (IL)

Several protocols were tested for *Prunus* propagation in RM. The rate of multiplication was above 4 for most clones but success in rooting was limited to genotypes with a peach background. In genotypes of pure almond background rooting was low (0-35%).

A new protocol was developed at RM for almond rooting in vitro and applied to in vitro grown shoots of PdM, GF677 and bitter almond. The protocol includes two steps.

Step 1: MS macro + MS micro + MS vitamins + 150% MS FeNaEDTA + 10 ppm NAA + 10 ppm IAA + 10 ppm IBA + 3% sucrose + 0.6% agar. pH 6.00.

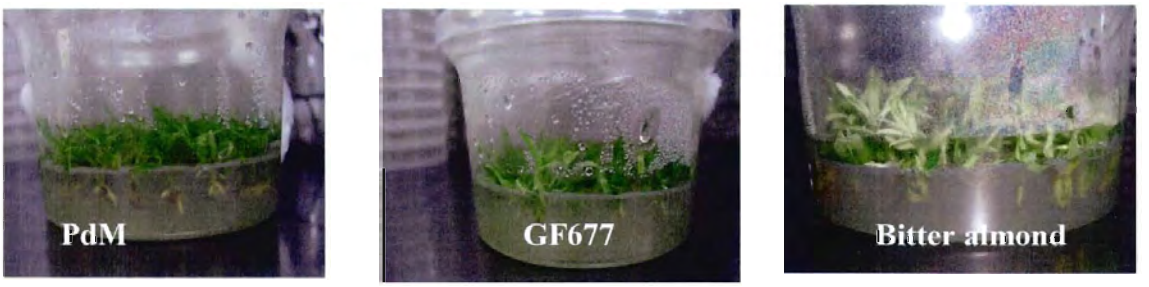
Step 2: MS macro + MS micro + MS vitamins + MS FeNaEDTA + 3% sucrose + 0.6% agar + 100 ppm phluoroglucinol, pH 6.00. Results are summarized in Table 4 and Fig. 4.

Table 4. Percentage rooting of Prunus rootstocks using an improved protocol

Code	Nr. of initial plants	Nr. of rooted plants	Rooting percentage	Results after
PdM	51	38	74.5	10 days in rooting medium
GF677	50	34	68	
Bitter almond	10	10	100	3 weeks in rooting medium

Improving the protocols for propagation and more so for rooting almond shoots grown in vitro allowed to overcome an obstacle in mass micropropagation of almonds.

Fig. 4. In vitro rooting using an improved protocol in RM



C. Cold storage and cryopreservation of in vitro maintained stone fruit cultures

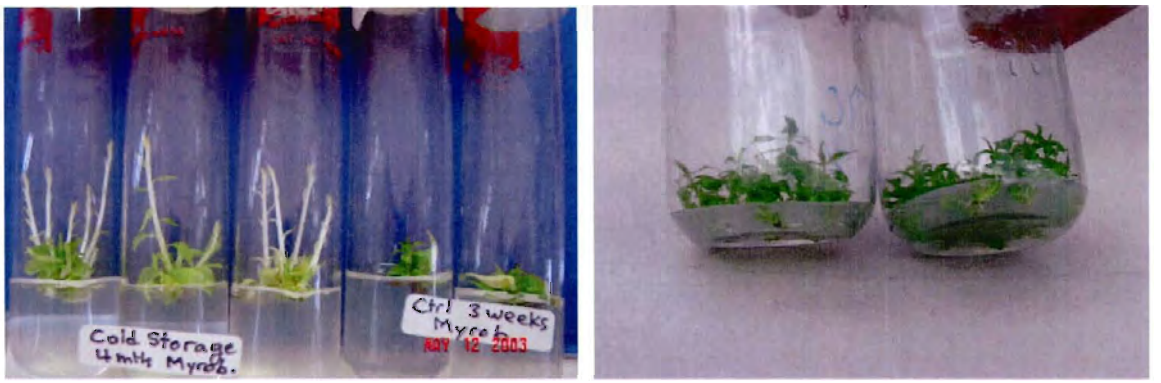
Efficient storage of in vitro cultures of stone fruits including the germplasm and other collections established in the frame of this project was required. This activity was studied in parallel in Morocco (UCP) and Israel (VC and RM). Results were discussed extensively in correspondence and coordination meetings.

i. Cold storage (M, IL)

Storing cultures at ~ 4C reduces the frequency of sub-culturing to once in 5-6 months instead of every 3-4 weeks and decreases labor, glassware use, culture media etc. The following plant materials were selected for cold storage. In UCP - GF 677; Prunus serrulata cv Shirofugen and Myrobolan. Virus infected - apple Malus 70 (ACLSV); and almonds 'Samish' (PNRSV) and 'Hazanov' (PNRSV) and virus free cultures of the above. In RM – Bitter almond and GF677. In VC – almonds Samish, Hazanov and Kochav (54). The adapted cold storage protocol was previously described. In brief, the following medium was selected:
AP + BAP 1mg, IBA 0.01 mg; AP+1/4MS, BAP 0.5 mg). Sucrose (3%) and Sorbitol (4%) were added for evaluation of their effect on cold storage. Duration of storage at 4C was tested for 2, 4 and 6 months.
Based on the results obtained in the three labs over a period of several years, the following was concluded:

- AP-medium supplemented with 3% Sucrose ensures the best cold storage conservation up to 6 months (Fig. 5).
- Virus-infected cultures were stored for 6 months at 4°C without loosing infection.

Fig. 5. Cold storage of Myrobolan for 4 months (left) and at 24C after cold storage (right)



(ii) Cryopreservation (M, IL)
This technique was adapted for long term storage of plant material including shoot tips / meristems. An attempt to apply this technique for stone fruit species was undertaken in this project by UCP (M) and RM (IL).
In UCP, assays were carried out using vitro shoots of GF677, Garnem and Myrobolan, being propagated massively in the laboratory.
The protocol (following Boucaud et. al, 2002) included several steps (1) cold hardening of in vitro plantlets, two weeks after subculture, at 4°C for one week; (2) shoot tips were excised (Size ~ 1 mm) and grown at 4°C for 24h on shoot tip medium supplemented with 5% of DMSO; (3) shoot tips were transferred with 1 ml cryoprotectant (+ 12.5% w/v DMSO + 3% PEG 8000 + 0.4 M sucrose) to cryotubes on ice; (4) cryotubes were placed at room temperature for 1 h and 0.5 ml cryprotectant was removed; (5) samples were cooled at -20°C and then immersed in liquid nitrogen. The total number of cryopreserved shoot tips was 102 for GF677; 75 for Myrobalan and 54 for Garnem.
Meristems could not be regenerated after cryoconservation from any of the rootstocks used. Lack of means to control progressive reduction of temperature is thought to be the major constraint. Another possibility is a vitrification problem which was apparent and could have been the cause for this result.
In RM, a similar experiment was performed with embryos rescued fro I-7 seeds. The success rate of regeneration following the protocol detailed above was very low.

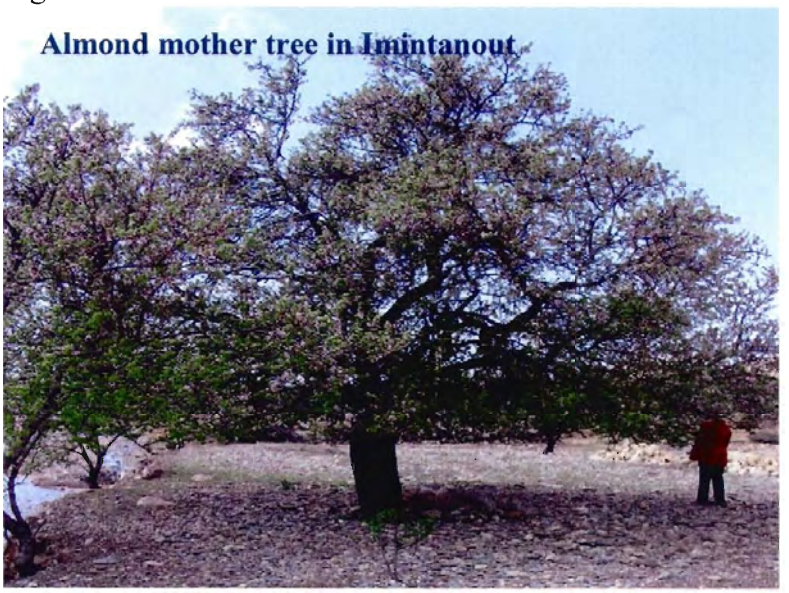
In this project, cold storage (~4C, up to 6 months)) was adapted for in vitro cultures in the three participating labs. In our hands cryopreservation was not a successful procedure.

D. Assessment of almond selections made in Morocco

Selecting the optimal rootstock for almond (and other fruit trees) is important for cultivation of trees. Performance may vary in different areas and therefore an assessment has to be done prior to a decision. Study of almond selections was performed in parallel in Morocco and in Israel.

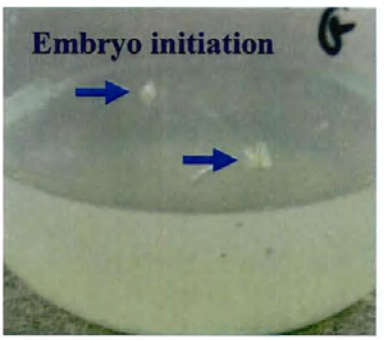
i. Assessment of selection Imintanout 7 (I-7) (M)
The selection identified as I-7, made in the course of this project, is attractive by its mother tree vigor, flowering mode and remarkable production under stressful environment (Fig. 6). Its fruiting mode seems to be similar to that of self compatible varieties. To further characterize I-7, embryo cultures from seeds of I-7 were initiated using the optimized protocol.
Improvement of multiplication and rooting was one of our objectives to be accomplished in the frame of this project. LP medium supplemented with 0.5 mg/l BAP and 0.01 mg/l NAA allowed shoots to have a high vigor with a multiplication rate of 5X.
Rooting was improved using AP medium supplemented with 4 mg/l NAA and reached 70%. The best results were obtained with clone 9. Therefore, plants of I-7, clone 9 were micropropagated, rooted, hardened and used for further studies detailed below. Unless otherwise stated, from this point on, I-7 will refer to clone 9.
The various stages from selection of I-7 to preparation of the clone 9 plants are demonstrated in Fig. 6 below.

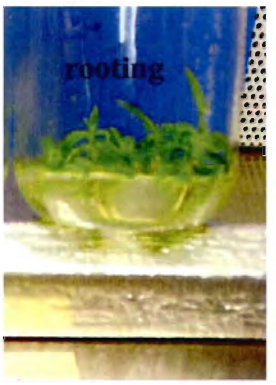
Fig. 6



I-7 selection at flowering

Fruitification of I-7





ii. Graft compatibility of I-7 (M)

Grafting trials were carried out on plants regenerated from I-7 in comparison to GF677, PdM and Garnem. All plants used in this experiment were produced in vitro and grafted 6 months after transplanting. Buds for grafting were taken from cultivars Lauranne (Amond), Sun Snow (Nectarine) and Florida Glo (Peach). Blocks of 30 plants per variety / rootstock with 2 replicates were grafted.

Grafting success rates of I-7 was in general comparable with those of the other rootstocks used in this experiment (Table 5). I-7 showed a good compatibility with almond while Garnem showed good compatibility with nectarine.

Table 5. Success rates of grafting almond, nectarine and peach varieties on I-7 in comparison to other rootstocks.

Variety / Rootstock	Lauranne (Almond)	Sun Snow (Nectarine)	Florida Glo (Peach)
GF677	50%	60%	60%
Garnem	80%	90%	55%
PdM	80%	70%	70%
I-7 (Clone 9)	90%	70%	40%

iii. Salt tolerance of I-7 (M)

Sodium Chloride solutions in a range of concentrations (25; 50; 100; 150; 200;250, 300, 350 mM) were applied to 6 months old plants of I-7, GF677, Aglmous, Garnem, Cadaman and Adesoto. The following protocol was followed:

- For each treatment, 30 plants were irrigated every fourth day with 500 ml Hoagland solution containing increasing concentrations of salt. The treatment with 350 mM was applied twice.
- Controls were watered with Hoagland solution with no salt.

Effect of salt on plants growth was evaluated by main shoot growth and general aspect of the plants.

Plant height was recorded at 3 stages: (M0) before applying 25 mM NaCl; (M1) before applying 150 mM NaCl; (M2) before the first application of 250 mM NaCl. Leaf wilting (LW) was recorded for the various treatments and control plants. General plant reaction was scaled as follows:

1, very tolerant plants: minor leaf burning with no chlorosis;
2, tolerant plants: minor leaf burning with minor chlorosis;
3, sensitive plants: systemic chlorosis and limited leaf wilting;
4, very sensitive plants: complete wilt of leaves.

I-7 selection exhibited a higher tolerance to salinity in comparison to the other tested rootstocks. Results are presented in Table 6.

Table 6. Salt tolerance of the Moroccan selection I-7 in comparison to other rootstocks

Rootstocks	Difference between M0 & M1 (cm)		Difference between M1 & M2 (cm)		Observations*	
	Control	Treated Plants	Control	Treated Plants		
GF 677	5	4.6	5 .3	2.8 (-2.5)	Initial LW at 200 mM Generalized LW at 300 mM	**3**
GARNEM	4.8	3.5	5.6	1.9 (-3.7)	Initial LW at 150 mM Generalized LW at 250 mM	**4**
ADESOTO	3.5	2.7	3.9	1.5 (-2.4)	Initial LW at 200 mM Generalized LW at 300 mM	**3**
CADAMAN	5.8	5.1	5.4	3.9 (-1.5)	Initial LW at 250 mM Generalized LW at 300 mM	**2**
AGLMOUS	3.1	2.8	3.4	1.9 (-1;5)	Initial LW at 250 mM Generalized LW at 300 mM	**2**
I-7 (Clone 9)	3.4	3.1	3.5	2.8 (-0.7)	Initial LW at 250 mM partial LW at 350 mM	**1**

* details on grading plants are detailed in the previous page.

iv. Caracterization of Nfifa and Aglmous selections (M)

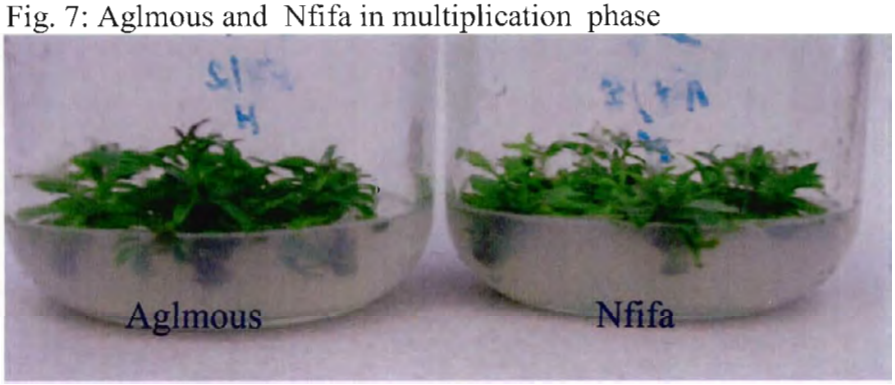
Nfifa and Aglmous selections looked interesting to us, especially with respect to their tolerance to drought (area of selection in Morocco). As done for I-7 selection, Nfifa and Aglmous were introduced in vitro for mass propagation. Multiplication protocols are still being improved to scale up production of uniform plants.

Rejuvenation of Nfifa and Aglmous was done by grafting on young seedlings of PdM rootstock. This is aimed at improving rooting ability of in vitro cultures. We have accomplished the 3rd rejuvenation cycle.

For multiplication in vitro, explants obtained from plants of the 3rd rejuvenation cycle were initiated in vitro. First initiation was done using AP medium containing 1mg/l BAP and 0.01 IBA. Various medium formulations were assayed for multiplication. The best results in terms of shoot vigor and multiplication rate (4X) were obtained with the LP-medium containing 0.5 mg/l BAP and 0.01mg/l NAA (Fig. 7).

For rooting, the best results were obtained with the LP-medium containing 3 mg/l NAA.

Fig. 7: Aglmous and Nfifa in multiplication phase



v. Tolerance of I-7, Aglmous and Nfifa to limestone (M)

Tolerance to high salt is significant for almond production. In areas with calcareous soils, tolerance to limestone is also important and therefore it was added to this study.

Assays were carried out using in vitro propagated plants 6 months after transplanting. The selections I-7, Aglmous, Nfifa compared to GF677, Garnem, Cadaman and Adesoto were used as rootstocks and grafted with nectarine (var. Sun Snow).

Plants (15 per treatment with 3 replicates) were watered every 4th day during 4 months with 500 ml of a ½ Hoagland Solution, CaCO3 1g/l pH 7,8 to 9. Controls were irrigated with ½ Hoagland Solution, pH 6.

As the effect of limestone takes time to show marked symptoms on the fairly tolerant rootstocks used, no clear differences were yet noticeable between the rootstocks assayed, although a yellowish top growth starts to be seen. The experiment is still on going.

vi. Assessment of PdM as an almond Rootstock (IL)

The potential of the Moroccan originated rootstock PdM for commercial use was evaluated in Newe-Ya'ar Research Center. An experimental plot was established in 2004 and PdM was compared with rootstocks available in Israel, such as GF677 and Adafuel. All rootstocks were grafted with the Israeli cultivar 'Um el Fahem', the most important commercial almond cultivar in Israel. The orchard is now in its fourth year. Data on the performance of the rootstocks were taken since 2006. Results obtained for PdM and GF677 are presented in Tables 7 and 8.

All of the trees in the orchard have flowered and set fruits. Data collected during two consecutive years show that fruit yield, weight and size of kernels produced by trees grafted on PdM is significantly smaller than GF677. PdM tree trunk circumference, a measure for the tree size, is significantly smaller than GF677. Although the orchard is only at its fourth year, the conclusion at this time is that the PdM rootstock is inferior to other rootstocks such as GF677. We will continue to monitor the performance of the trees for at least three years before drawing final conclusions.

Table 7. The performance of 'Um el Fahem' trees grafted on GF677 in comparison to the Moroccan originated rootstock PdM (Newe-Ya'ar Research Center, **2007**, planted in 2004)

Root stock	Data	kernel yield kg/h	picking date	% kernel	almond weight gr	kernel weight gr	kernel length mm	kernel width mm	kernel thick. mm	trunk circum. cm	trunk growth cm
GF 677	MEAN	1751	30/7/07	70.5	2.06	1.45	2.66	1.43	0.83	42.6^{A}	27.1
	±S.E.	24		0.2	0.05	0.04	0.05	0.02	0.01	1.9	1.4
PdM	MEAN	601	23/7/07	69.3	1.81	1.25	2.55	1.34	0.82	28.2^{B}	15.5
	±S.E.	8		0.2	0.03	0.02	0.06	0.04	0.00	3.4	3.7

Table 8. The performance of 'Um el Fahem' trees grafted on GF677 in comparison to the Moroccan originated rootstock PdM (Newe-Ya'ar Research Center, **2006**, planted in 2004)

Root stock	Data	kernel yield kg/d	picking date	% kernel	almond weight gr	kernel weight gr	kernel length mm	kernel width mm	kernel thick. mm	trunk circum. cm	trunk growth cm
GF 677	MEAN	76.0	23/7/06	71.6	2.19	1.57	2.87	1.53	0.87	36.3	7.2
	±S.E.	14.0		0.2	0.02	0.02	0.02	0.03	0.01	1.8	0.4
PdM	MEAN	16.5	23/7/06	70.6	1.98	1.40	2.78	1.51	0.81	24.7	4.2
	±S.E.	0.2		0.5	0.06	0.03	0.06	0.01	0.03	2.8	0.6

Fig. 8. Almond trees grafted on different rootstocks in Newe Ya'ar. Trees were planted in 2004, photographed in 2007.

vii. Salt tolerance of Israeli bitter almond seedlings and Moroccan landraces (IL)

Salt tolerance trials were performed in RM with Israeli Bitter almond (BA), six TOV and one I-7 from Morocco, imported to RM in the frame of this project. The studies were performed following the flow chart detailed below (Fig. 9). BA seedlings were traditionally being used in Israel as rootstocks for almonds and therefore were used in a preliminary test for salt tolerance in RM.

BA seedlings were grown in the presence of increasing salt concentrations from 50 to 200 mM NaCl solutions. Six selected clones were tested in a field plot for three consecutive years. These plants were tolerant despite a 5 folds increase in the level of salt accumulation in their leaf tissue (described in a previous report). These clones are currently being used as parental lines for hybrids.

Seven trees, selected in two locations in the Atlas mountains of Morocco for drought and salinity resistance were included in this study - TOV I, II, III, IV, V, VI and I-7.

Seeds (25-65) from each tree were selected for salinity resistance. Twenty six lines were selected for field trials.

Clones of I7 and TOV (as above) were used in comparison with PdM and GF677.

Seeds, except for I-7, were germinated in a greenhouse. Seeds of I-7 did not germinate in standard greenhouse conditions. The seeds from I-7 collected during summer 2003 were germinated in vitro and subsequently grown in the greenhouse. A protocol was developed for embryo excision and in vitro germination. The protocol was used to germinate and store in vitro I-7 seedlings at 4^0C. Plants that have been stored at 4C were transferred to pots.

Plants were irrigated with ¼ Hoagland solution and increasing concentrations of salt (NaCl 50-250 mM). Seven days of drenching with salt solutions were followed by 3 days irrigation without salt. This cycle was repeated while the salt solution was each increased by 50 mM per cycle. Most plants showed severe salt stress at 250 mM NaCl or died. A number of seemingly more tolerant clones (Table 9) were chosen from each group and will be tested in the field. The possibility of using them in a breeding program will be considered as well.

Fig. 9. A flow chart describing the salinity tolerance trials.

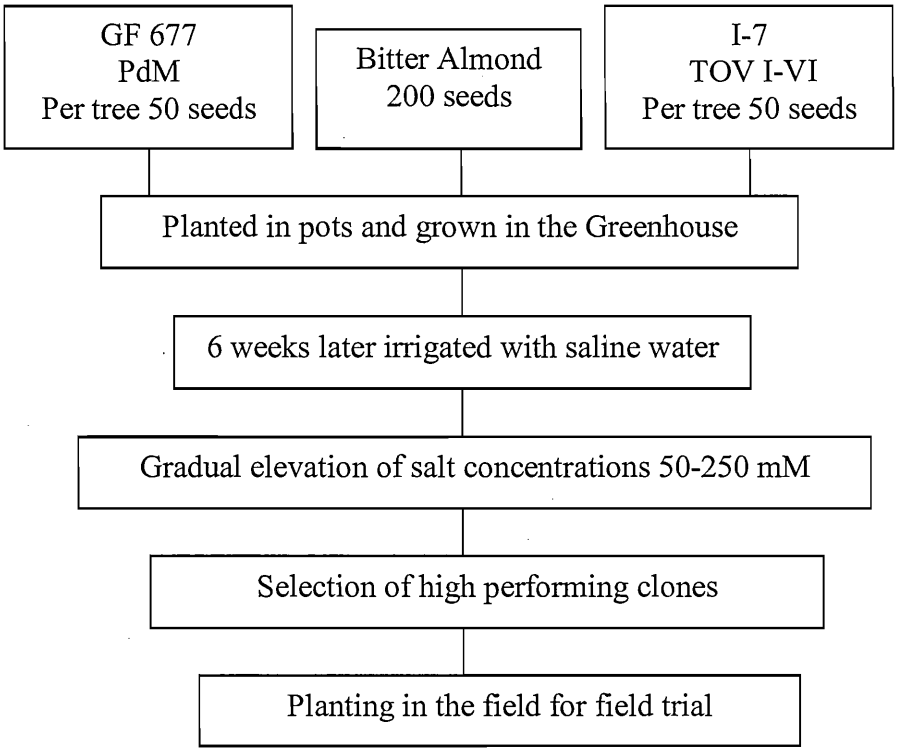


Table 9. Clones of TOV and I-7 selected for salt tolerance in the greenhouse experiment.

Code	*Nr. of initial plants*	*Nr. of tolerant clones*
TOV I	55	4
TOV II	58	4
TOV III	22	2
TOV IV	60	11
TOV V	28	1
TOV VI	25	2
I-7	25	2

Selected lines of PdM were micropropagated in RM laboratory and planted in an experimental field plot in the Negev desert in south Israel for evaluation with the almond variety Um El Fahem. The evaluation is for vigor, salinity tolerance, water deficiency tolerance and eventually for yield under Israeli conditions. This experiment is inspected and monitored by the Ministry of Agriculture (Mr. Reuven Birger –Almond extension specialist). Results are not available yet.

The germination problems encountered for I-7 in Morocco and Israel were very similar. The results of this study in both countries are in agreement. Both groups agree that a follow up study on salt tolerance of almond selections is required and might generate good results.

E. DNA Fingerprint Analysis of almond clones

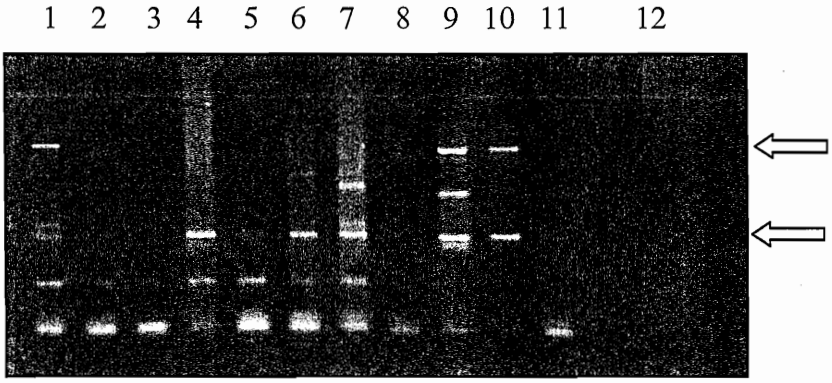
Given that almond plants are self incompatible the polymorphic diversity in the genome is relatively wide. Consequently, genetic markers would be a useful tool for identification of interesting clones.

i. Analysis of *S-allele* composition (IL)
The S-locus controls the specificity of the self-incompatibility interactions. The lab at Newe Ya'ar has analyzed the composition of *S-alleles* for 7 different types of TOV and I seedlings collected in Morocco and provided to us by the Moroccan partners. The above mentioned types and others are grown in an orchard of RM In Rosh Hanikra (Fig. 10). The trees that were analyzed are: I7-2; Tov III-2; Tov 3; Tov I-3; tov IV-6; Tov IV-1; Tov VI-1 and Tov IV-8. None of the types that were analyzed contained the *Sf* allele. The *S-alleles* that were found are *S3*; *Sd*; *Sa*; *Sb* and *S6*. Tov IV-8 contains *S3* and possibly *Sa*. Tov VI-1 contains alleles *Sa* and *S3*. Tov I-3 putatively contains alleles *S3* and *S6*. We are now completing the analysis of the rest of the types.

Fig. 10. Selection I-7 and PdM in the field collection in Rosh Hanikra (Sept 05)

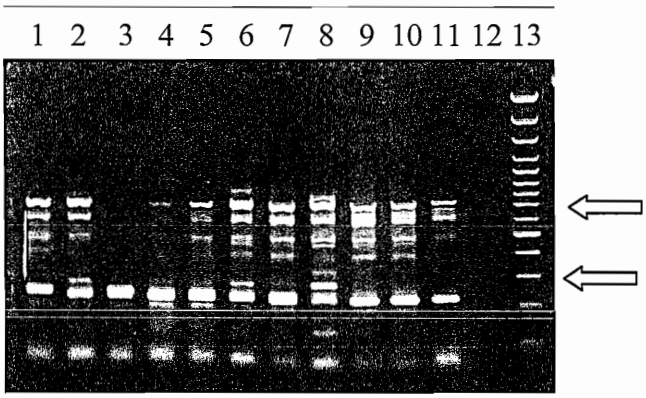
ii. RAPD and SSR analysis of almond clones (IL)
RM has employed two types of markers: RAPD (random amplified polymorphic DNA) and SSR (simple sequence repeat). The RAPD is relatively simple and inexpensive to develop but is low in specificity. SSR is more complex but produces a higher resolution of differential banding pattern. The reproducibility of both marker systems depends on the DNA extraction procedure. We therefore chose a two step simple method that is apparently highly reproducible. In order to identify each parental line we have devised a network of marker sets composed of RAPD and SSR that jointly reproduce specific identification for each clone in our plantation. We have developed DNA fingerprint polymorphic markers to identify the various parental lines and determine polymorphic characteristics of the offspring lines. Examples of results are demonstrated in Fig. 11 and 12, below.
We concluded that RAPD and SSR technologies are effective for polymorphic analysis of almond and peach X almond clones. We have devised a method for identification of parental lines and progenies. The identification of parental lines is based on a matrix of primer sets that differentiate between the various Prunus genotypes in our experimental plantation.

Fig. 11. RAPD analysis with primer OPA 19
RAPD analysis of genomic DNA extracted from TOV, GF677, PdM and bitter almond selections using primer OPA 19 primer.



Lanes- 1: TOV IV-2; 2: TOV III-2; 3: TOV IV-3; 4: 677-13; 5: GF677-12; 6: B.A. 110; 7: B.A. 78; 8: I-7-2; 9: PdM-6; 10: PdM-12; 11: PdM-8; 12: Water.

Fig. 12. SSR analysis with primer pchgms 17 (r+f).
SSR analysis of genomic DNA extracted from TOV, GF677, PdM and bitter almond selections using primer pchgms 17 (r+f). 2.5



Lanes- 1: TOV IV-2; 2: TOV III-2; 3: TOV IV-3; 4: 677-13; 5: 677-12; 6: B.A. 110; 7: B.A. 78; 8: I-7-2; 9: PdeM-6; 10: PdM-12; 11: PdM-8; 12: Water; 13: marker – 100 bp ladder.

F. Validation of rapid virus rapid assays in stone fruit cultivars

Applying sensitive detection assays for the main viruses affecting stone fruit cultivars is a prerequisite for establishing a certification system. It is also significant for post therapy regenerated shoots to allow screening for virus infection at an early stage after heat treatment. An example for monitoring PNRSV in post therapy Um El Fahem shoots is demonstrated in Fig. 14 (below).
In this project ELISA and mainly RT-PCR assays were adapted for PNRSV, PDV and ACLSV. Several pairs of primers were tested and the best results were used and information transferred to UCP as well.

i. Identification of stone fruit cultivars / selections of local or regional significance (IL)
The stone fruit collection consisting of local selections collected in Israel and imported cultivars is maintained in Neve Ya'ar (Dr. D. Holland).

In the frame of this project local almonds, apricot and plums were tested for PNRSV, PDV and CLSV. The first test was done by ELISA. RT-PCR assays were performed for samples with inconclusive ELISA results due to low virus levels (known in the ilarvirus group) and to verify ELISA results. This is the first evaluation of the virus status of this collection. Results are summarized in Table 10.

Table 10. Results of ELISA and RT-PCR tests indicating the presence of PNRSV and PDV in the stone fruit collection in Neve Ya'ar (Israel).

Stone fruit crop	No. of cultivars / selections tested	Origin	No. of plants positive for	
			PNRSV	PDV
almond	14	Israel	4	0
apricot	13	Israel	1	1
plum	6	Israel	2	
Total	**33**		**7 (21%)**	
almond	29	Imported	16	0
apricot	13	Imported	2	0
Total	**42**		**18 (43%)**	

PNRSV was quite common in both local and imported almonds, PDV was rare and CLSV was not detected by the ELISA assay. In the course of the work we discovered that detection of CLSV in almonds is problematic and not reliable both by ELISA and RT-PCR. Results were published (Spiegel et al., 2003, Ann. Appl. Biol. 144:229-236).

The following cultivars and rootstocks were selected and established in vitro for virus elimination.
Almond 'Um El Fahem' (UEF) – a local selection made years ago, considered the most important variety in Israel. The trees in the collection did not exhibit disease symptoms.
Almond 54 (Kochav) – a local variety resulting from an Israeli breeding program in the 1960s (head Prof. P. Spiegel-Roy), a good variety and the major pollinator of UEF. The trees in the collection suffer from bare shoots and condensed growth, similar to 'bud failure' described in the literature.
Rootstock 749 – a peach x almond hybrid. Considered a good rootstock and is of interest in Israel and Morocco. The plants in the collection are relatively young and appear normal.

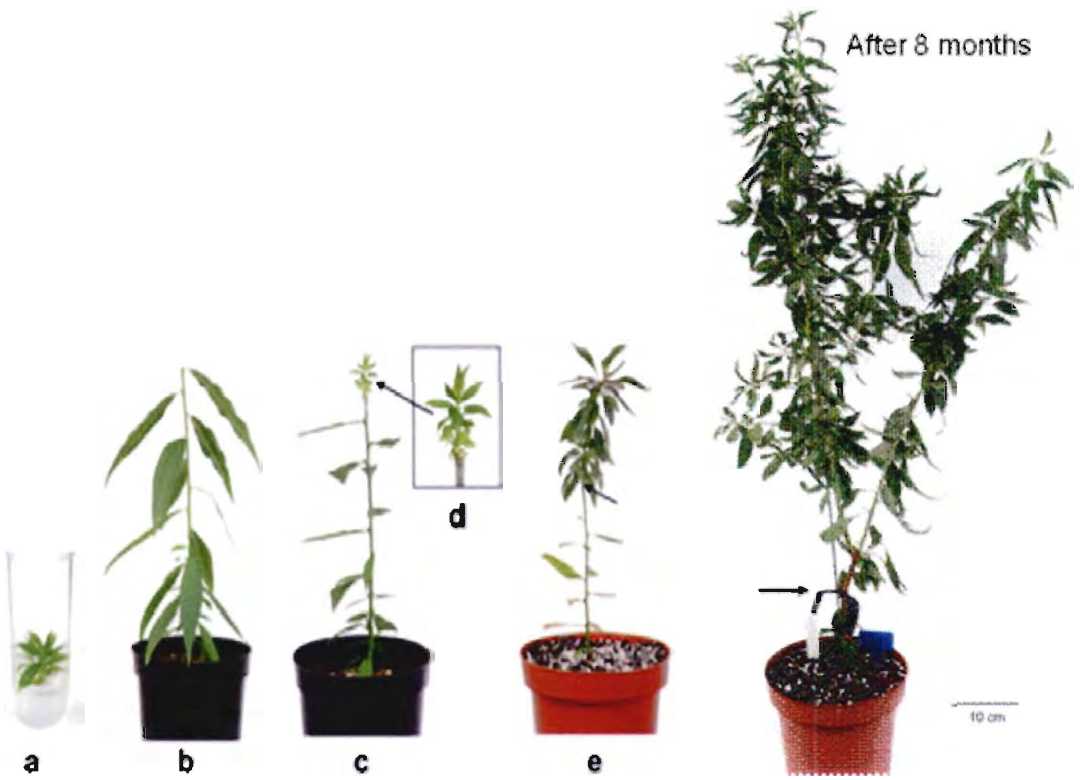
G. Virus elimination and regeneration of virus free plants

Thermotherapy has been the major procedure developed in this project for elimination of viruses from in vitro grown almond shoots and generation of a virus free plant stock for propagation purposes. Shoot- tip grafting (STG) has been also applied in combination with thermotherapy for the same purpose.

i. Thermotherapy (IL, M)
In Israel, the following varieties were heat treated: almond cv. Kochav (54), currently the major almond pollinator; Cv. Um el Fahem, the most important commercial cultivar locally; rootstock 749, a cross between almond x peach. These cultivars were infected with PNRSV. In Morocco, the following varieties were heat treated: almond selcction Nfifa; cv. Ferraduel and cv. Hazanov (PNRSV) and cv. Marcona (PNRSV and PDV).

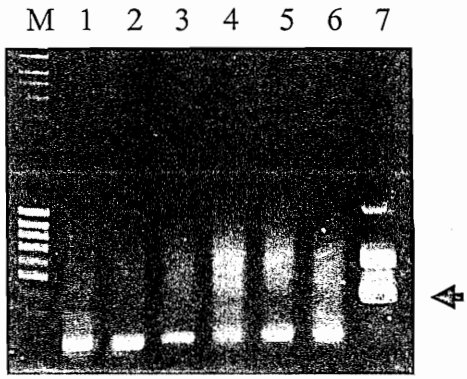
The problems encountered were (i) poor survival following exposure to high temperature (ii) regenerated shoots remained infected with PNRSV (iii) rooting of almond shoots.
The protocol based on alternating high and low temperatures, was developed in Israel and used both in VC and in UCP. Details were given in previous reports. Regenerated shoots were tested for PNRSV and PDV by ELISA (2X) and RT-PCR (2X), at least, repeatedly.
The regeneration of virus free plants was achieved by the following steps shown in Fig. 13. In vitro grown single shoots with a firm stem (a) and decapitated, young, greenhouse grown rootstock plants (b) were prepared for top grafting (c). Grafted plants were immediately transferred to a growth chamber with a fogging system and the grafted shoot was developed (d). After reaching a length of 5-7 cm the grafted plant was gradually acclimatized to greenhouse conditions (e). Mature plants were later grown in a screenhouse and following a dormant period were transferred to a field plot in Newe Ya'ar for horticultural evaluation. The plants developed into trees and are observed for horticultural traits and for freedom from PNRSV. Preliminary observations indicated that the fruit shape and taste is typical of cv. Kochav. Tests for PNRSV were so far negative.

Fig 13. The various steps of regeneration of virus free Kochav almond plants



The grafting point is indicated by an arrow.

Fig. 14. Heat treated, Um el Fahem shoots tested for PNRSV by RT-PCR.



Lanes: M: DNA molecular marker; 1-6: heat-treated, ELISA negative shoots, 7: ELISA positive shoot. The arrow indicated a PNRSV-specific 351 bp amplified product.

ii. Shoot-Tip Grafting (STG) (M)

A two step procedure in which STG is performed on in vitro grown shoot after thermotherapy has been developed in Morocco. This increases the percentage of regenerated virus free shoots.

Thirty shoots of each of the almond varieties - Nfifa, Hazanov, Marcona and Ferraduel were micrografted on in vitro shoots of rootstock Garnem at rooting stage. The success rate of regeneration ranged from 5% (Ferraduel) to 15% (Nfifa).

The virus elimination tools developed and applied in this project in VC and UCP is a pre requisite for production and use of certified plant material.

6. Impact relevance and Technology Transfer

Almond, a traditional component of the Mediterranean cuisine, has become a highly demanded crop with considerable commercial significance. The high nutritional value, health benefits and long shelf life of kernels in addition to its relatively low demand for irrigation make it a very attractive crop. Improving the knowledge on growing almonds with yield increase, high quality fruit and health beneficial components will contribute significantly to the economy of the Mediterranean region which is highly suitable for its cultivation. The use of salt / drought tolerant almond selections will most likely result in an increase in the total area of commercial almond orchards including areas with marginal soil and minimal rainfall.

This multidisciplinary project improved considerably the stone fruit crops growing industry in Morocco. To list a few major points in which advancements were made:
cultivation practices of nurseries and orchards, germplasm collections of almond landraces, micropropagation (in vitro) including rooting and acclimatization of Prunus sp., (including rootstocks, cultivars and woody indicators), virus detection (year round indexing and molecular assays) and elimination. In addition, it laid the basis for setting up a reliable certification program for stone fruit crops.

Both UCP and EB labs have expanded their capacity and were upgraded to accommodate the activities undertaken in the frame of this project. Recruiting scientific and technical personal, provided by means of the project permitted the performance of extensive planned work. Domaines Agricoles (DA) has gained immense benefits by developing capabilities for setting up a modern nursery for stone fruits in Morocco for local farmers and for neighbouring countries. DA made a commitment, in the frame of this project, to distribute almond plants to small farmers in south Morocco at no or minimal cost. This act will have a positive socio-economic effect on rural areas. The information generated from this project has been disseminated by the Moroccan and Israeli scientists to local growers in annual growers meetings and during visits to farms.

The scientists involved in this project from Morocco and Israel worked very closely to establish the most appropriate technologies for each topic of this research. Annual meetings took place in European countries in which results were reported and discussed, problems were raised and future plans made. Mutual visits did not take place due to the political climate. Transfer of plant material from Israel to Morocco and vice versa took place and allowed evaluation trials to be conducted in parallel by two groups under different conditions.

In summary, this USAID funded MERC project yielded considerable gains to the traditional and intensive stone fruit industries in Morocco. The scientists from both countries were deeply involved in this project. Beyond the scientific work which was involved in this project, there has been a feeling of fulfilling a mission of bridging differences, sharing, interacting and working together towards a good cause.

7. Project Activities/Outputs

Coordination meetings within the context of the project

Five annual coordination meetings too place during the life of the project. The meetings were held in European countries. For each meeting the chosen location was in proximity to a facility dealing with subjects relevant to the topic of this project. Each meeting included:

- A Report on activities presented by each participant
- A group discussion on results and problems
- A detailed workplan and specific assignments for each group
- A visit of local facilities and discussions with scientists.

Photos are enclosed in Appendix 2.

1. Meeting in Barcelona on February 13-18, 2002

Participants: M. Aaouine, M. Zemzami, I. Alamy, E. Khayat, S. Spiegel.
The meeting agenda was -
1. Administration and management of the project based on the contract and sub-contracts.
2. Discussions and detailed planning of the technical workplan for year 1.
3. Preparing report procedures and future meetings.
4. A one day visit to IRTA (Cabrils, Barcelona) and discussions with scientists working on almonds and other Prunus sp. Host: Dr. Pere Arus.

2. Meeting in Bordeaux, France on December 9-14.02, 2002

Participents: M. Zemzami, I. Alamy, M. Ait Chitt, S. Spiegel, E. Khayat, D. Holland.
The meeting agenda was -
1. Reports given to the group by each participant; discussions of results and future workplan.
2. Preparations for the Annual Report.
3. Visit of Bordeaux INRA labs - Fruit Tree Virology, Molecular Biology and Genetics and meetings with scientists (host: Dr. Olivier Legall).
4. Visit the CTIFL facility Lanxade, (about 1.5 hrs driving from Bordeaux) - vitropropagation of indicators, GH grafting etc (host: Dr. Pascal Gentit).
5. Lecture on Cryopreservation of embryonic axes of Prunus sp. by Dr. M.T de Boucaud.

3. Meeting in Bari, Italy on December 15-20, 2003

1. Reports given to the group by each participant; discussions of results and future workplan.
2. Preparations for the Annual Report.
3. visit of Istituto Agronomico Mediteraneo, Bari, discussions with Italian scientists involved in Med Network on Virus Disease Assessment and Sanitation of Stone Fruit Trees (MNFT) (Host: Dr. Arben Myrta).
4. Day trip organized by MNFT to local nurseries involved in the certification program for fruit trees.
5. Visit and discussions with scientists in the Univesrity of Bari with focus on in vitro cultures of almond (host Dr. G. Bottalico).

4. Meeting in Faro, Portugal on March 14-18, 2005

1. Reports given to the group by each participant; discussions of results and future workplan.
2. Preparations for the Annual Report.
3. Meetings and discussions with scientists dealing with almond on cultivation, breeding and sanitation, in the University of Algarve (host: Dr. Gustavo Nolasco).
4. Day trip to local nurseries and commercial almond orchards.

5. Meeting in Athens, Greece on December 4-7, 2006
1. Reports given to the group by each participant; discussions of results and future workplan towards the end of the project.
2. Preparations for the Annual Report.
3. Visit of the Benaki Phytopathological Institute.
4. Seminars and discussions on phytopathological problems of stone fruits, Benaki Phytopathological Institute (host: Dr. Christina Varveri).

Training:
Dr. Ibtissam Alami (UCP), spent a 6 weeks training period (summer 04) on virus detection techniques in the Virology Laboratory, Clemson University, South Carolina, USA under the supervision of Dr. S. Scott (head of the lab).
A wide range of techniques were covered during this training including bioassays, dsRNA extraction and analysis, RT-PCR, cloning and sequencing of PCR products, nested PCR, Immunocapture RT-PCR and molecular hybridization . Real Time PCR was also approached.
RT-PCR: The training focused mainly on RT-PCR for detection of PNRSV and PDV in field samples. The various steps of the protocols employed at UCP and in Dr Scott's lab were compared. Optimization of the protocols for reliable detection of these 2 viruses at low titers in field samples were achieved.
Nested PCR: This technique was used for detection of ACLSV which is difficult to detect by conventional RT-PCR. The primers used in Dr. Scott's lab were evaluated by Dr. Alamy.
Immunocapture RT-PCR: This technique was used for the detection of PPV in field samples. Although this virus was not found in Morocco, acquiring the technology being used in Dr. Scotts's lab (as part of the survey in the US) was very important.
Molecular Hybridization: This assay is used for detection of PLMVd in peach and other stone fruit species in the germplasm collection at the Fruit Experimental Farm of Clemson University.
The knowledge and techniques acquired in this training is of immediate advantage to the virology work being done in UCP.

The information generated in this project was not yet published jointly in international journals. The co-workers intend to compile data for publications in the near future.

8. Project productivity

The proposed goals of the project, namely, almond collections from the wild, salt tolerance study, rootstock evaluation, tissue culture practices including cold storage, regeneration of plantlets, rooting and hardening, mass propagation, virus elimination and molecular detection were accomplished. In addition to the above, DNA finger printing techniques, not included in the original proposal, were added to the program. Preliminary results were obtained already and presented in the report. Future work is required to prepare a useful tool for clone differentiation.
It should be mentioned that the total number of almond selections from seedling populations was smaller than expected. This is due to several years of on-going drought in Morocco which resulted in death of trees and a decrease in the seedling population in sensitive areas. However, rescuing plant samples from endangered areas and establishing them in the collection at UCP facilitated saving these clones from possible drought and loss.

It should also be noted that molecular biology supplies (enzymes, primers etc.) are not readily available in Morocco and take a considerable time to be ordered and imported. This has been causing delays in the application of molecular diagnosis of viruses at UCP.

9. Future work

Several activities included in this project require several years to be completed and allow sound decisions and recommendations to be made.

- Evaluation of rootstocks like PdM being evaluated in Israel requires 2-3 more years to be completed.
- Regenerated post therapy plants (cultivars) need several years of horticultural and virological testing to ascertain trueness to type and freedom from virus, respectively.
- Selection from the seedling populations showing a potential of salt tolerance need several
- years to be evaluated and / or to be incorporated into a breeding program.
- The Moroccan apricot rootstock (AM) selection identified in this project has not been studied yet.
- Limestone tolerance needs several years for evaluation.
- Virus indexing using micropropagated GF305 should be compared with seed germinated plants and if comparable will be a breakthrough in greenhouse indexing.

Direct beneficiaries from the project

In Morocco, the personnel of UCP – scientists, technicians and farm workers in the Menara and several other locations in Morocco benefited directly from this project. New techniques were introduced in micropropagation, greenhouse, screenhouse and nursery practices, in vitro propagation and virus elimination etc.

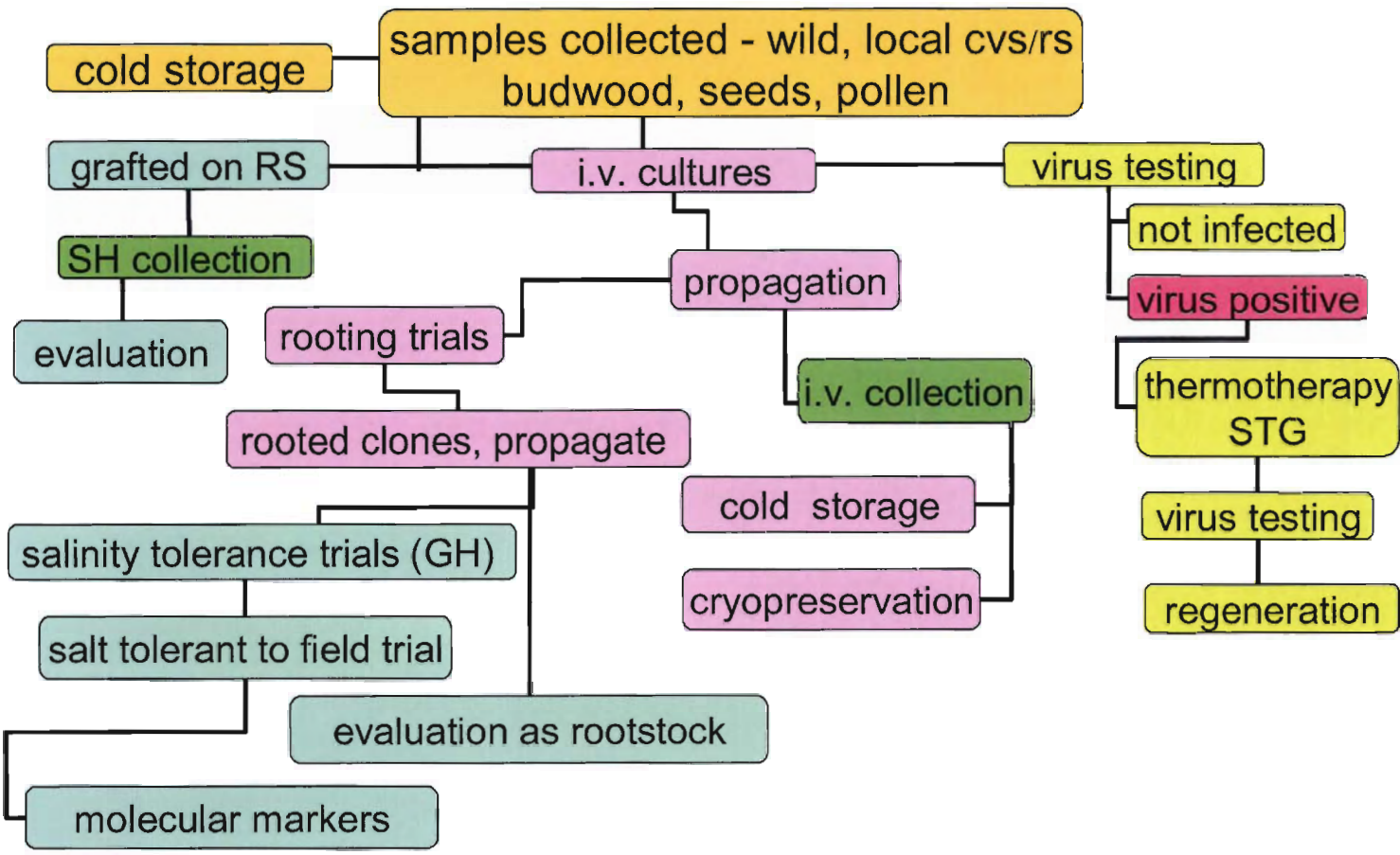
In Israel, the personnel of the Virology lab at VC in Bet Dagan and the fruit tree unit in Newe Ya'ar (scientists, technicians, students, GH and farm workers) benefited from this project through activities described above.

The farmers in south Morocco received almond planting material from DA at minimum cost. Information on improved cultivation techniques has been disseminated through lectures in annual farmers meetings by extension staff.

In Israel, planting material of almond cv. Kochav free of PNRSV will be made available to growers when horticultural evaluation will be completed.

Appendix 1

A flow chart of the main activities performed in the frame of this project. The activities are divided into three branches – horticulture (left), tissue culture (middle) and virology (right)



Appendix 2

Photos from Coordination Meetings

Bordeaux, France (Dec 02)

From left to right: M. Ait Chitt, M. Zemzami, I. Alamy, S. Spiegel, D. Holland, E. Khayat

Bari, Italy (Dec 03)

Athens, Greece (Dec 06)

From left to right: I. Alamy, M. Zemzami, S. Spiegel, E. Khayat