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BARD

FINAL REPORT

PROJECT NO. I-468-82

Somatic Hybridization as a Tool in Citrus Breeding

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Somatic hybridization as a tool in Citrus breeding

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I. ABSTRACT

The overall objective of this project was to establish experimental procedures to utilize protoplast-manipulation methodologies in order to produce cybrid and hybrid *Citrus* trees, as a mean for *Citrus* improvement. Thus, embryogenic nucellar callus lines of *Citrus* cultivars and relatives of *Citrus* were established and it was attempted to produce androgenic (possibly haploid) plants. The latter attempt was discontinued but we succeeded in isolating protoplasts from most types of *Citrus* cultivars as well as from *Microcitrus*. We developed procedures by which these protoplasts could be cultured; the respective calli were established and regenerated into embryos; the latter were further regenerated into fruit-bearing trees. We utilized the protoplast-culture system to ask whether resistance to *Phytophthora* rot could be selected by exposing *Citrus* cultures of several cultivars to fungal filtrates ("toxins"). We found that the cultures had variable sensitivities to the filtrates and that the filtrate had a high auxin content. The answer to this question was thus negative.

We did achieve the main goal of this project. Both cybrid and hybrid trees were established through protoplast-fusion. Most of our efforts were directed towards the establishment of cybrids. Intrageneric as well as intergeneric (e.g. *Microcitrus* as organelle donor, *C. limon* or *C. aurantium* as recipients) cybrid trees were established and their organelle (chloroplasts and mitochondria) constitution was determined by appropriate molecular methods. The respective trees were either grafted on rootstocks or planted in the soil directly. Fruiting is expected in some of the cybrids during the 1989 season.

II. OBJECTIVES

The objectives, as phrased in our original research proposal (June 1982), were as follows:

- a. Production of somatic hybrids
- b. Production of cytoplasmic hybrids (i.e. cybrids)
- c. Provision of additional androgenic plants as source
for haploid protoplasts
- d. Establishment of observation-nurseries from protoplast derived plants
- e. Preliminary evaluation of somatic hybrid plants.

As shall be detailed in the Body of the Report we handled, during our research work, all these five objectives. Moreover the four main objectives were fully achieved. In addition to the original objectives we asked whether protoplast-derived *Citrus* culture can serve to select *Phytophthora* resistant lines through the use of fungal filtrates. This latter research line indicated that the *Phytophthora* filtrates have a very high auxin content and the *Citrus* culture actually reacted to the auxin in the filtrate rather to a specific "toxin". We reached beyond our original objectives: our studies opened the way to others who utilized *Citrus* protoplast-manipulation to produce novel *Citrus* trees. Thus, two research groups, one in Japan (e.g. Kobayashi et al. 1983; Ohgawara et al. 1985) and one in Florida, USA (e.g. Grosser et al. 1988a; 1988b), employed the methodologies published by us and produced protoplast-derived *Citrus* trees and protoplast-fusion derived somatic hybrids between *Citrus sinensis* (orange) and *Poncirus trifoliata*.

In only one research line - production of haploid plants from anther culture - we did not achieve our goal. Although many androgenic plants were produce none were haploid and only very few were apparently derived directly from microspores. This line of research was therefore discontinued.

III. BODY OF THE REPORT

1. Introduction

Citrus cultivars are of major economic importance and rank only second to grapes in world fruit production. *Citrus* fruit productivity has a considerable role in the agricultural economics in the USA and Israel as well as in other countries. The annual value of *Citrus* crops in the USA was recently estimated to be 2 billion US \$. *Citrus* orchards are inflicted with a wide range of diseases such as *Phytophthora* rot, tristeza, several viral diseases and pathogenic nematodes. *Citrus* trees suffer from environmental stresses such as salinity which are major considerations in citriculture in the USA and in Israel (Klotz, 1978; Wallace, 1978; Hutchison, 1977; Hutchison and Cooper, 1973) cultivars of *Citrus* and wild relatives of *Citrus*, having the respective resistances to biotic and abiotic affectors are known. However conventional breeding work in *Citrus* is hampered by problems as heterozygosity, sterility, incompatibility and nucellar embryony (see Iwamasa, 1966, Cameron and Frost, 1968; Frost and Soost 1968; Cameron and Soost, 1979; Vardi and Spiegel-Roy 1978). Also, some of the best known sources for disease - and stress - resistances reside in *Citrus* relatives with which hybrids are difficult to obtain conventionally (Barrett, 1977). Moreover, it is feasible that several *Citrus* characters are affected by cytoplasmic genes located in the mitochondrial and/or chloroplast genomes. Evidence for such cytoplasmic effects is common in other angiosperms (see: Galun and Aviv, 1983; 1986; for review). Transfer of these plasmone-genes only, (i.e. without transfer of nuclear genes) from appropriate "donor" plants to *Citrus* cultivars "recipients" and especially the separation of mitochondria from chloroplasts without impairing the "recipients" nuclear characters are virtually impossible by conventional breeding. Hence, in order to enable the incorporation of new germplasm into *Citrus* cultivars, as well as to manipulate plasmone controlled features, there is an urgent need to supplement the conventional breeding with somatic hybridization.

Cell and tissue culture, and especially protoplast manipulations were suggested as tools for *Citrus* improvement (see reviews: Spiegel-Roy and Vardi, 1984; Vardi and Galun 1988a,b). When such novel plants are produced, of rootstock or scion varieties, they can be propagated by root cuttings or graftings and thus ensure convenient dispersal of elite material.

The present final report deals with the production and the verification of cytoplasmic hybrids (cybrids), production of somatic hybrids, an attempt to obtain haploid androgenic plants as well as with preliminary observations and evaluations of protoplast-derived trees and somatic-hybrid plants.

2. Material and Methods

A. Establishment and maintenance of nucellar calli

Nucellar calli from *Citrus* species and cultivars were obtained and maintained as described by Vardi et al. (1982) and embryogenic callus from *Microcitrus* sp. was obtained as described by Vardi et al. (1986b). Briefly, ovules from the latter species were axenically excised from immature fruits (2-6 weeks after anthesis) of a respective tree grown in the *Citrus* collection at the USDA Horticultural Research Laboratory in Orlando, Florida. About 20 ovules were plated in 5 cm plastic Petri-dishes on solidified Murashige and Tucker (1969) medium (MT) with 500 mg/l malt extract and 4% sucrose without any addition of growth regulators. The dishes were maintained for two weeks in the dark ($25^{\circ}\text{C} \pm 3^{\circ}\text{C}$) and subsequently transferred to dim light.

B. Isolation and plating of protoplasts

Before protoplast isolation, calli were sub-culture at least twice, with 2 to 3 weeks intervals, on nutrient medium (MT) containing 4% sucrose and lacking growth regulators as described by Vardi et al. (1982). For protoplast isolation ca 0.5 g callus was placed in each tissue-culture grade 9 cm Petri dish and mixed with 10 ml maceration medium. The maceration solution consisted (with a few exceptions, see Vardi et al; 1982) of 0.2% Macerozyme, 0.3% Cellulase R-10 and 0.1% Driselase dissolved in a solution composed of half-strength MT macroelements and containing 0.35M sucrose and 0.35M mannitol. The maceration solution was adjusted to pH 5.7 and filter (0.2 μ m) sterilized before use. After overnight incubation ($27^{\circ}\text{C} \pm 1^{\circ}\text{C}$) in the dark, the protoplasts were isolated by sequential filtering through 50 μ m and 30 μ m nylon screens followed by three centrifugations ($\times 100g$, 5 min) in MT containing 0.3M sucrose and 0.3M mannitol (wash-solution). Protoplasts were then plated in MT containing 0.3M sucrose, 0.3M mannitol and 0.6% agar at a final density of $10^5 \cdot \text{ml}^{-1}$, as 4 ml aliquots per 5 cm Petri dish or as 2 ml aliquots at a final density of $10^4 \cdot \text{ml}^{-1}$ above a feeder layer in the same Petri-dishes (see Vardi et al., 1982, 1987).

C. Feeder and donor protoplasts.

A dose of 50 to 60 krad gamma rays (cobalt-60, B.B. 150A, Atomic Energy of Canada) was required for virtually complete division-arrest of *Citrus* protoplasts. Feeder and donor protoplasts were irradiated immediately after isolation, then washed twice by centrifugation in wash-solution. Feeder protoplasts were plated as described by Vardi and Raveh (1976).

D. Recipient protoplasts.

Protoplasts which served as recipients in fusion experiments were washed only once after release from the maceration fluid and then suspended for 20 min in the wash solution containing 0.25mM (recrystallized) iodoacetate. The protoplasts were then washed three times by centrifugation in the wash solution.

E. Rhodamine 6-G treated protoplasts.

Rhodamine 6-G (R6G) was added (15mM) to the maceration fluid. After overnight incubation, the protoplasts were isolated and washed three times by centrifugation in the wash solution (as indicated above).

F. Protoplast fusion

Equal amounts of donor and recipient protoplasts (or iodoacetate treated protoplasts and R6G treated protoplasts - for the production of somatic hybrids) were mixed to obtain a final density of approximately $5.10^5 \cdot \text{ml}^{-1}$. The procedure outlined by Aviv and Galun (1985) was then followed. Briefly, two 0.15 ml drops of a PEG solution (50% polyethyleneglycol "1500" in a solution containing 10mM CaCl_2 , 0.1M glucose) were placed a few mm from each other in the center of a 5 cm plastic Petri dish. Then a 0.3 ml drop containing the mixture of protoplasts was added carefully between the two PEG drops, causing the contents of the drops to mix gradually. After 15 min. 4 ml of $\text{Ca}(\text{NO}_3)_2$ (pH 10.5) were added to the periphery of the PEG-protoplast suspension-drop. Fifteen min later the suspensions from each two Petri-dishes were collected and centrifuged ($\times 100g$, 5 min). The pelleted protoplasts were suspended in 1.5 ml liquid medium (MT with 0.3M sucrose and 0.3M mannitol) and then 1.5 ml of MT medium containing 1.2% agar were added and the mixture was plated over the feeder layer.

G. Isolation of colonies and regeneration of embryos

When colonies reached a diameter of 1-2 mm they were isolated and transferred for further growth on solidified MT medium (containing 4% sucrose). Each colony which reached a diameter of ca 5 mm was transferred to a separate Petri dish and received an identification number. A fragment of each colony was transferred to embryo regenerating medium (containing MT, 1.0% agar and 2% glycerol rather than sucrose; Ben-Hayyim and Newmann, 1983). After about 6 weeks numerous embryos were formed on the glycerol

containing medium. Normally, heart-shaped embryos were individually isolated and transferred to Petri dishes containing solidified MT with 4% sucrose and $1.5 \cdot \text{g} \cdot \text{l}^{-1}$ malt extract.

H. Plant regeneration

Embryos which reached a length of 7-10 mm, and had expanded cotyledonary leaves - were transferred individually to test tubes containing MT with 2% sucrose and 0.05 ppm NAA. This medium promoted axis elongation and root formation. Plantlets which developed in these tubes, having well developed roots, and a few leaves, were planted in autoclaved "Jiffy" turf-pot. The turf-pots were placed in sterile transparent-plastic containers and watered with half-strength macro- and microelements of MT. When roots started to emerge from the turf, the turf-pots with the rooted plants were transplanted into 1 l containers with a soil-perlite mixture. The containers were covered with plastic bags and transferred to the greenhouse. The plants were then acclimatized to lower humidity by gradually opening the bags and maintained in the greenhouse without further protection.

I. Establishment of trees

Protoplast-derived plants and cybrid plants were planted directly in the soil or grafted on either of two root-stocks: Sour orange or Troyer citrange. The plants were then planted in the orchard for further observations.

J. Characterization of the chloroplast genome.

Chloroplast DNA extracts. Chloroplast DNA was isolated by using a modification of the procedure of Frankel et al. (1979). Leaves from 2-4 month old plants or the soft, young leaves from mature trees were used. In both cases the leaves were previously darkened for 2 to 6 days. All procedures until lysis were carried out at 4°C . For each sample 25 g of leaves were washed and finely chopped with 75 ml Buffer A (Sorbitol, 350mM; MES, 50

mM; EDTA 2mM; MgCl_2 , 1mM; K_2HPO_4 , 0.8mM; MnCl_2 , 1mM; NaCl, 50mM; ascorbic acid, 5mM; B-mercaptoethanol, 7mM; spermine 1 mM; spermidine 1mM; PVP 2 mg/ml; pH 6.1) in a Waring Blender, fitted with razor blades, for 5 x 5 s bursts at 4/5 of line voltage. The material was then strained through 4 layers of gauze and 2 layers of "Miracloth" and the filtrate was centrifuged for 2 min at x 250 g (Sorval HG4L). The supernatant was centrifuged for 5 min at x 1.600g (Sorval HG4L). The resultant pellet was resuspended gently with a soft paintbrush in 40 ml Buffer B (Sortibol, 350 mM; hepes, 50mM; EDTA, 2mM; MgCl_2 , 1 mM; MnCl_2 , 1mM; K_2HPO_4 , 0.5mM; NaCl, 50mM; spermine, 1 mM; spermidine, 1mM; PVP, 2mg/ml; BSA 1 mg/ml; pH 7.6) and recentrifuged for 5 min at 1.600 x g (Sorval HG4L). The pellet was then resuspended in 2 ml Buffer B and loaded onto a discontinuous gradient, (25%, 40%, 60% sucrose in Buffer B) in an SW 28 polyallomer tube and centrifuged for 1h x 72,000 g (Beckman).

Two chloroplast bands appeared and were removed separately with wide mouthed Pasteur pipettes into siliconized 30 ml Corex tubes. They were incubated with 50 $\mu\text{g}/\text{ml}$ proteinase K for 4 min at room temperature, then 0.2M NaOH and 2% sarkosyl (final concentration) were added. After 30 min of lysis at 37°C the lysate was centrifuged for 30 min at x 16,300 g (Sorval HB4). The supernatant was extracted twice with freshly distilled phenol, and once with chloroform and isoamyl alcohol (24:1 v/v). The DNA was ethanol precipitated from the aqueous phase. Yields of about 2 μg DNA/g starting tissue were obtained.

Total DNA isolation. Total plant DNA was isolated using the method of Fluhr (1983) with minor modifications. Two hundred mg of young leaf tissue was homogenized on ice, using a mortar and pestle, with 100 mg PVP (P-6755, Sigma) in 5 ml buffer A (as for chloroplast DNA extraction but with 400 $\mu\text{g}/\text{ml}$ ethidium bromide in place of spermine and spermidine).

Sodium dodecylsulphate and proteinase K were added to a final concentration of 0.5% and 50 $\mu\text{g/ml}$, respectively, and lysis was carried out in the dark for 1h at 47°C. The lysate was centrifuged x 12,000 g (Sorval SS34), and the supernatant removed to a Corex tube. It was then extracted once with distilled phenol (freshly neutralised with an equal volume of 0.1M Tris base) twice with chloroform and isoamyl alcohol (24:1, v/v) and once with distilled ether. The ether was totally removed by evaporation with nitrogen and the extract was centrifuged at 4°C for 5h at x 192,000 g in SW 50.1 polycarbonate tubes (Beckman). The pellet was resuspended for 24 h in 100 μl TE buffer. The average yield of total DNA was 1.5 $\mu\text{g/mg}$ starting tissue.

Enzymes and gel electrophoresis. Restriction endonucleases (from Biolabs or Anglian) were used singly or in combination (double digestions), according to the suppliers instructions, to digest 2.0 μg DNA in 60 μl assay volumes. After digestion, a 0.6 volume of liquified sample buffer (0.4% sea plaque agarose, 10% glycerol and 0.02% bromophenol blue) was added to each reaction and the samples were run on agarose gels (0.5 to 1.2% seakem ME type 2) in TAE running buffer (40mM Tris acetate, pH 7.8, 20mM sodium acetate, 2mM EDTA).

DNA transfer and hybridizations. DNA transfer to nitrocellulose was done according to the method of Wahl et al. (1979). Nick translation was carried out with modifications to the procedure of Maniatis et al. (1982). 0.5-1 μg of DNA was digested with 25 μg DNAase for 1 min at 47°C in 20 μl of reaction mixture containing 50mM tris pH7.8, 5mM MgCl_2 10mM B-mercaptoethanol, 3.75mM unlabelled nucleotides and 20 μCi labelled nucleotide (Amersham). Then 5 units of DNA polymerase I (Biolabs) were added and incubated for 6 min at 15°C. This gave an incorporation of 50-90% of the labelled nucleotide into the probe. The reaction was terminated with 80 μl of 0.1% SDS; 40 μl sonicated salmon sperm DNA were then added; the probe was boiled for 10 min and then frozen. Hybridization reactions were in 6 x SSC, 0.08% PVP-360, 0.08% Ficoll 400, 0.08% BSA (Fraction V,

Sigma) 50mM phosphate buffer (pH6.8) and 200 μ g/ml denatured and sonicated salmon sperm DNA. The nitrocellulose blots were soaked in 2 x SSC for 2 min and prehybridized in 5-15 ml of hybridization buffer for at least 2 h at 65°C in heat-sealable bags. The probe was added together with fresh hybridization buffer and hybridization carried out overnight at 65°C. After hybridization the blot was washed, at room temperature, four times for 15 min. in 0.1% % SDS, 2 x SSC then at 50°C twice for 15 min in 0.1% SDS, 0.1 X SSC and finally rinsed in 0.1 xSSC. Several exposures of varying duration were made onto X-ray films.

K. Characterization of the mitochondrial genome

The characterization of the mitochondrial genome (the chondriome) of fusion-partners and cybrids was based on the restriction profiles of their respective mtDNAs. Both fluorography (ethidium bromide staining) and Southern blot hybridization, with heterologous mtDNA, were used to analyse the restriction profiles.

Mitochondrial DNA restriction profiles and Southern blot-hybridization. The procedures for mitochondrial isolation, mtDNA extraction, digestion with endonucleases and Southern blot-hybridization were as described previously (Galun and Aviv 1986). Suspension-cultures in MT with 4% sucrose served as the plant-material source; 10 to 20 g of filtered cells were washed once in water and one volume of cells was suspended in 4 volumes homogenization buffer (0.2M mannitol, 3mM dibasic EDTA, 50mM Tris-HCl, pH8.0; up to 0.1% BSA and 1mM 2-mercaptoethanol were added before use). The suspended cells were passed through a French-press (3,000 lbs/in²) and further extraction and purification were done according to Sparks and Dale (1980) with some modifications as detailed previously (Galun and Aviv 1986). Restriction-endonuclease digestion, gel-electrophoresis and Southern blot-hybridization with mtDNA probes (see below) were performed by standard procedures (see Hanson et al. 1986).

Mitochondrial DNA probes. Two heterologous probes were used in the Southern blot-hybridizations. Plasmid *pmtSylSa-8* is a *SalI* fragment of *Nicotiana sylvestris* mtDNA, cloned in pBR322 (Aviv et al. 1984). Plasmid *bZmE1* is a 2.4kb maize mtDNA fragment, containing the cytochrome oxidase subunit II (COX II) gene (Fox and Leaver 1981) cloned in pBR322. The latter plasmid was kindly provided by Dr. C.J. Leaver (Dept. of Botany, University of Edinburgh, Scotland).

Total DNA extraction. When *pZmE1* served as probe in Southern blot hybridization, endonuclease digested total DNA from leaf tissue rather than mtDNA from suspension cultures was utilized. Total DNA was obtained from 1 to 1.5 g of young-leaves. The leaves were powdered in liquid nitrogen and the DNA extraction of Abbott et al. (1985) was followed with minor modifications as described recently (Breiman et al. 1987).

L. Preparation of *Phytophthora citrophthora* culture filtrate

The culture filtrate (CF) of *P. citrophthora* was prepared as reported by Breiman and Barash (1981). The CF was concentrated by flask evaporation at 40°C to 30% of the original volume and then filter-sterilized. The pH of the concentrated filtrate was 5.9-6.0.

M. Selection procedure with culture filtrate

Nucellar-derived callus lines of four *Citrus* cultivars: Sour orange (*C. aurantium* L), Murcott mandarin (*C. reticulata* Blanco), Shamouti orange (*C. sinensis* (L) Osbeck) and Villafranca lemon (*C. limon* (L.) Burn) were used. About 60 callus pieces (ca 10 mg each) were placed in 9 cm Petri dishes containing either MT or MT supplement with filter-sterilized CF of *Phytophthora citrophthora* at either one of the volume ratios: 1/2 or 1/1 (concentrated CF/MT). The dishes were sealed with "Parafilm" and incubated at 26°±1°C with 16h/day of dim light. The best growing calli were selected for three passages of 4 weeks each, and then propagated for further selection and protoplast isolation.

N. Indole-3-acetic acid (IAA) analysis

Radioactive IAA (50 μ l IAA-2- 14 C, specific activity of 54 mCi/mmol, which contained 0.72 μ g of IAA) was added to the filtrate of the fungus. The filtrate was brought to pH 3.0 with 10N HCl; and extracted three times with ethylacetate; the pooled ethylacetate was extracted with 0.1N sodium bicarbonate which was then acidified with 10N HCl to pH 3.0 and again extracted with ethylacetate as described above. The ethylacetate was dried with sodium sulfate and evaporated to dryness. Further procedures of purification with HPLC, with pentafluorotoluene, TLC and GLC and final calculations were performed as described by Epstein and Cohen (1981).

O. Anther culture

Immature flower buds were collected from plants of *Poncirus trifoliata* (L.) Raf, *P. trifoliata* cv. Flying dragon and 11 accessions of Poorman x *P. trifoliata* (PPT) growing in the orchard in our experimental farm at Bet Dagan. For surface sterilization the floral buds were immersed (10 min) in hypochlorite (1%) and then washed three times in sterilized water. Anthers with microspores at the uninucleate stage were aseptically isolated and cultured in 5 cm Petri dishes on MT medium supplemented with various growth regulators (Hidaka et al. 1979) and sucrose concentration (Hidaka, 1984) as shown in Table 2. About 15 anthers were placed in each Petri dish. The dishes were sealed with "Parafilm" and incubated at $26^{\circ} \pm 1^{\circ}\text{C}$ in the dark until outgrowth from the anthers became visible (usually between 3 to 5 weeks); the dishes were then placed in 16h/day light.

3. Results and Discussion

A. Embryogenic calli

Embryogenic callus, derived from ovular nucelli is the main source of protoplasts which have the capability to divide and to regenerate functional *Citrus* plants (e.g. Vardi et al. 1975, 1982; Kobayashi et al., 1983). To maintain a constant supply of such calli and to broaden our callus-stock collection we renewed existing callus lines (e.g. Murcott mandarin, Sour orange, Shamouti orange) and also established new callus lines (e.g. Rangpur lime, Cleopatra mandarin, *Microcitrus* spp. Poorman x *Poncirus trifoliata* (PPT) by culturing isolated ovules from immature fruits (see Vardi et al, 1982, 1986b). All the established callus lines maintained their embryogenic capacity. However, three of them (Rangpur lime, Cleopatra mandarin and PPT were found to produce abnormal embryos or embryos which lacked the capability to "germinate" and regenerate normal plants.

The collection of nucellar callus lines obtained by us during the two consecutive BARD projects (I-22-79 and I-468-82) are listed in Table 1. It covered different *Citrus* species and cultivars, scion and rootstocks and also the related genus *Microcitrus*.

Our embryogenic calli collection include genotypes known to have pathogen-resistances to tristera virus, *Phytophthora* rot, nematodes and salt tolerance. Although some of the callus lines are not useful for direct protoplast-to-tree regeneration (because abnormal embryos are produced from their respective protoplast-cultures) their protoplasts may serve as fusion partners.

B. Protoplast derived fruiting trees

Trees of several *Citrus* cultivars: e.g. Shamouti orange, Dancy mandarin, Ponkan mandarin, Villafranca lemon as well as of *Microcitrus* which were regenerated from protoplasts and attained the fruiting phase. The fruits and the general characteristics of

Table 1. Established embryogenic callus lines of *Citrus* cultivars (scions and rootstocks), *Citrus* hybrids and *Microcitrus*

Lines	Number of lines	Callus origin		Embryogenic	Plants from protoplasts
		ovules	anthers		

<u>Orange</u> (<i>C. sinensis</i> (L.) Osbeck)					
Nucellar Shamouti	2	+	--	+	+
Shamouti Landau	1	+	--	+	--
<u>Sour orange</u> (<i>C. aurantium</i>) (L.)					
	2	+	--	+	+
<u>Mandarin</u> (<i>C. reticulata</i> Blanco)					
Murcott	2	+	--	+	+
Dancy	2	+	--	+	+
Ponkan	2	+	--	+	+
Cleopatra	1	+	--	+	--
<u>Grapefruit</u> (<i>C. paradisi</i> Macf.)					
Dancan	1	+	--	+	+
<u>Lemon</u>					
Villafranca (<i>C. limon</i> (L.) Burm)	1	+	--	+	+
Rough limon (<i>C. jambhiri</i> Lush)	1	+	--	+	+
<i>C. macrophylla</i> Webster	1	+	--	+	--
<u>Citrus Hybrids</u>					
Poorman x <i>Poncirus trifoliata</i>					
accession No.					
43/8	1	--	+	+	--
44/8	1	--	+	+	--
44/12	1	--	+	+	--
44/30	1	--	+	+	--
45/47	1	+	--	+	--
46/40	1	--	+	+	--
48/20	1	--	+	+	--
<i>Microcitrus</i> spp.	1	--	--	+	+

Microcitrus tree derived from isolated protoplasts do not differ from the trees which were the source of the calli (Figure 1, for further details see Vardi et al. 1986b). Likewise protoplast derived trees from Nucellar Shamouti, Dancy mandarin and Ponkan mandarin were similar to the original protoplast donor trees. The trees yielded similar true-to-type fruits as to shape, color and taste (e.g. Figure 2). We encountered only one case of a Villafranca lemon protoplast-derived tree which bears sweet-lemon type fruits which vastly differ from regular sour Villafranca lemon fruits (Figure 3). This suggests a possibility that the lemon cultivar Villafranca is a chimera composed of a bitter and a sweet lemon combination. The uniformity and true-to-type characters of orange trees, regenerated from cultured protoplasts, were recently reported by Kobayashi (1987). It seems that little, if any somaclonal variation occurs in *Citrus*.

C. Anther culture

Androgenic plants derived from anther culture have a two-fold relevance to *Citrus* breeding: 1. they may furnish hemizygous and subsequently homozygous *Citrus* trees having specific advantageous traits, (e.g. resistance to pathogens); 2. haploid protoplasts derived from such plants may serve in somatic hybridization to establish triploid trees; such trees may have horticultural advantages. Therefore, a great effort, along this research line, was invested by us during the first two years of the present project.

Work in this research line was carried out with anthers of the standard *Poncirus trifoliata*, with anthers of *P. trifoliata* cv. flying dragon, with anthers of Troyer citrange and with anthers of 11 accessions of Poorman x *P. trifoliata* (PPT).

The anther-culture experiments during the 1984 season were performed according to methods reported by Hidaka et al. (1979). Based on the results obtained by us during the 1984 season and on a subsequent publication by Hidaka (1984), in all the anther-culture experiments during the 1985 season, we utilized MT medium in which the growth regulators

Figure 1. A flowering, 3-year-old, *Microcitrus* sp. tree derived from protoplast culture.
Insert: flowers from the same tree (from: Vardi, Hutchison and Galun, 1986). Actual
height is approx. 1.5 m.

Figure 2. Fruits of Dancy mandarin. P - fruits from a protoplast-derived tree; C - fruits from a regular tree.

Figure 3. A branch from a protoplast derived Villafranca lemon tree (x3).

were only kinitin ($2\mu\text{g. ml}^{-1}$) and indoleacetic acid ($0.2\mu\text{.ml}^{-1}$); different levels of sucrose (from 2% to 9%) were added to the culture medium.

The results obtained are summarized in Table 2. A considerable number of anther-culture derived plants were obtained: about 1/5 of the anthers of *P. trifoliata* yielded plants. This rate of success was similar in the two flowering seasons. Furthermore only specific PPT trees yielded anther-derived plants or embryogenic callus, in both seasons.

Isozyme analyses were conducted in collaboration with Dr. Daniel Zamir of the Faculty of Agriculture of the Hebrew University. Two hundred anther-derived plants were analysed by a 4 isozyme system to reveal homozygosis which would indicate that the plants were derived from haploid microspores. Only one plant was found to be homozygous for the 4 isozymes tested. In 80 plants chromosome counts indicated diploidy ($2n=18$). Six anther-derived plants of *P. trifoliata* had the morphology of Flying Dragon, however, no differences in isozymes were found and all of them were diploid. It seems that the anther-derived plants originated from unreduced gametes and are therefore of no breeding advantage. Whether or not our climate is not favorable for the production of haploid-microspore derived androgenic plants is an open question.

D. In vitro selection for resistant to *Phytophthora citrophthora*

Various *Phytophthora* spp. have been shown to secrete phytotoxic metabolites into their culture medium (Behnke 1979, 1980; Plich and Rudnicki 1979; Deaton et al. 1982). *P. citrophthora* is the causal agent of trunk gummosis, collar and root-rot, dumping off and brown rot by *Citrus* spp. A culture filtrate of this fungus has been reported to contain both high and low molecular weight non-specific phytotoxic components (Breiman and Barash 1981; Beiman and Galun 1981). The development of the full sequence from isolated protoplasts to normal plants with various *Citrus* cultivars and species (Vardi et al. 1982) has enabled us to test the reliability of using *P. citrophthora* culture filtrate (CF) as an *in*

Table 2. Anther culture experiments to produce androgenic plants in *Poncirus trifoliata*, Troyer citrange and Poorman x *P. trifoliata* (PPT).

Anther Source	Number of cultured anthers	Number of androgenic plants	Callus with and without embryoids
<u>A. 1984 season</u>			
<i>Poncirus trifoliata</i>	1200	200	+
Troyer citrange	500	none	--
Poorman x <i>P. trifoliata</i>			
43/9	500	none	--
43/18	500	5	+
43/36	500	none	--
44/8	500	2	+
44/12	500	none	+
44/17	500	none	--
44/30	500	none	+
44/51	500	none	--
44/20	500	none	+
<u>B. 1985 season</u>			
<i>Poncirus trifoliata</i>	1600	350	+
<i>Poncirus trifoliata</i> cv Flying Dragon	350	25	+
Poorman x <i>P. trifoliata</i>			
43/18	150	5	+
44/8	500	none	+
44/12	400	none	+
44/30	600	none	--
44/51	600	none	--
44/54	350	none	--
46/40	300	none	+
49/44	300	none	+
48/20	300	none	+
48/21	250	none	--
49/34	20	none	--

vitro selection tool. For this purpose calli from four *Citrus* varieties with known rates of tolerance to the *P. citrophthora* pathogen were chosen for the experiment: Sour orange (resistant), Murcott mandarin (moderately susceptible), Shamouti orange (susceptible) and Villafranca lemon (highly susceptible).

The results obtained were published (see Vardi et al. 1986a) and can be summarized as follows: sensitivity of the four calli to the fungal CF was in reverse order to what is known on the susceptibility of the cultivars *in vivo*. Protoplasts derived from calli selected for tolerance to CF showed a higher plating efficiency with increasing concentration of CF in the medium. Culture filtrate of fungi were reported to be rich in secondary metabolites, growth-inhibiting and stimulating substances (Yoder, 1980), as well as growth regulators. The results obtained with protoplasts-derived selected calli to CF led us to the assumption that an auxin like substance may be present in the CF and that auxin rather than a specific toxin affected the *Citrus* calli. Thin layer chromatography and gas liquid chromatography indeed showed the presence of indole-acetic acid in the CF. The results indicate that CF of *Phytophthora citrophthora* cannot be used as an *in vitro* selection tool.

This part of the project was conducted in collaboration with Dr. Adina Breiman from Tel Aviv University and Dr. E. Epstein from A.R.O. Bet Dagan.

E. The plastome of Citrus

Somatic hybridization and cybridization between different *Citrus* species as well as between *Citrus* species and their relatives are potentially important tools for *Citrus* improvement.

It was reported in several cases that following protoplast-fusion, a selective system, based on selective medium was applicable to prevent division of the non-fused and auto-fused protoplasts. In *Citrus* an efficient selective system is not available. The verification of hybrid nature of calli and plants could be based either on nuclear genes (i.e. by isozyme

analyses, Ben Hayyim et al. 1982) or by analyzing the cytoplasmic composition - the chloroplast DNA (ctDNA) and the mitochondrial DNA (mtDNA).

The first step for using the plastome of *Citrus* for the identification of cybrids and somatic hybrids is to get acquainted with this plastome. For this purpose a physical plastome map was constructed for Sour orange (Figure 4). We then made comparative analyses of several other *Citrus* species and cultivars such as mandarin, pummelo, citron, orange, lemon and grapefruit as well as representative of two other genera - *Poncirus* and *Microcitrus*. The comparative analyses was made by Southern blot-hybridization of labelled total tobacco cpDNA to digests of total *Citrus* DNA. The results obtained (e.g. Figure 5; see Green et al. 1986) were most interesting. The *Citrus* species have only three plastome types: 1. the Sour orange type (*C. aurantium*) which includes also orange, lemon, grapefruit and pummelo (*C. grandis*) ; 2. the mandarin type (*C. reticulata*) ; 3. the citron type (*C. medica*). The plastomes of *Poncirus* and *Microcitrus* are distinct from each other as well as from the three *Citrus* types.

The results obtained, in this part of the project, were of considerable taxonomic interest. They support Barrett and Rhodes (1976) numerical taxonomic study. These authors, using 146 characters, proposed that *C. grandis*, *C. reticulata* and *C. medica* are true "biological" species while *C. aurantium*, *C. sinensis* and *C. paradisi* are basically hybrids between *C. grandis* and *C. reticulata*. They also proposed that *C. limon* is probably a three-way hybrid involving *C. grandis*, *C. medica* and *Microcitrus* sp. All the orange, lemon and grapefruit ctDNA samples analysed by us gave the same plastome restriction patterns as *C. grandis*, indicating that this species was probably the maternal parent or was involved in each of the original crosses, thus providing the "cytoplasm" of each of these *Citrus* types.

CtDNA restriction patterns obtained by using six endonucleases showed no differences between the plastomes of *C. aurantium*, *C. grandis*, *C. paradisi* and *C. limon*. There are,

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Figure 4. The restriction map of chloroplast DNA of Sour orange (*C. aurantium*) based on *Pst*I, *Xho*I and *Pvu*II digestions (From: Green, Vardi and Galun, 1986).

Figure 5. Restriction pattern schemes of chloroplast DNA from *Citrus* and its relatives. Vf - *C. limon*; P - *C. grandis*; So - *C. aurantium*; D - *C. reticulata*; Dn - *C. paradisi*; E - *C. medica*; Mc - *Microcitrus* sp; Pt - *Poncirus trifoliata*.

however, dissimilarities in the restriction patterns of other species examined. Of the three enzymes used for the mapping, PvuII gave the greatest number of differences in restriction patterns. Comparison of these differences with the pattern of fragments in the *C. aurantium* ctDNA restriction map indicated that *N. tabacum* ctDNA probes (Ps2A and Ps1) could be used to distinguish between plastomes of the *C. aurantium* type and each of the other two plastome types. Consequently hybridization of these two probes to restriction endonuclease digests of total DNA provided sufficient differences to allow the identification of the plastomes of *C. medica*, *C. reticulata* and the *C. aurantium*.

This part of the project was performed in collaboration with Ms. Rachel Green, as part of her M.Sc. thesis at the Weizmann Institute of Science.

F. Protoplast-fusion derived plants

General considerations. Our initial aim was to attempt the production of various cybrids and somatic hybrids among *Citrus* cultivars as well as between *Citrus* cultivars and related genera. The rationale was first to assure that such cybridizations and somatic hybridizations can be achieved by protoplast fusion and then to utilize fusion partners which may actually be of breeding value. Success in our endeavor should then become a starting point to utilize protoplast-fusion derived trees for *Citrus* improvement, in both rootstocks and in fruit-bearing cultivars. Emphasis was put, as noted in our proposal, on rootstock improvement. Thus, most of our fusion-combinations had at least one rootstock as fusion partner. We devoted much less efforts in the production of somatic hybrids. There were three main reasons to favor the cybridization. First, part of the planned somatic hybridization should have been between anther-culture derived haploids and (normal) diploid *Citrus* types in order to obtain triploid cultivars. As reported above such haploids were not yet obtained. Second, the lack of a reliable selection medium in our callus-derived protoplast systems did not enable efficient isolation of somatic hybrids. Third,

we were very much interested to obtain information on nuclear cytoplasmic relations on information which was previously unavailable. Then, shortly after the start of our experimental work we were informed that two research teams, one in Japan (at Chiba) and one in the USA (at Lake Alfred, Florida) implemented our protoplast-to-tree system in *Citrus* and were attempting somatic hybridization. These efforts actually yielded somatic hybrids between orange and *P. trifoliata* (e.g. Ohgawara et al. 1985; Grosser et al. 1988a) as well as between orange and *Severinia disticha* (Grosser et al. 1988b).

Production of cybrid trees. Our first fusion combinations were performed before we obtained the information on the characterization of the plastome in *Citrus* cultivars and in related genera.

These fusions involved Sour orange, Villafranca lemon and poorman x *P. trifoliata* (PPT). We did not know, at the time of fusion, that all these cultivars share the same plastome - i.e. their respective ctDNA does not differ by restriction analysis (using many endonucleases to obtain restriction profiles). When the fusion-derived plants were then analyzed we could not use ctDNA analysis to verify the cybrids but had to turn to the more elaborate mtDNA analysis. Nevertheless we found (Table 3A) that indeed our donor-recipient protoplast fusion was effective: γ -irradiation donor protoplasts and iodoacetate treated recipient protoplasts yield the respective cybrids. The detailed information on three such cybrid was recently published (Vardi et al. 1987). Figure 6 shows two of these cybrids when they reached the stage of young plants. Most of the cybrid threes had normal (recipients') morphologies.

There is a known graft-incompatibility between the Troyer stock and lemon cultivars as scions. Therefore we grafted a sample of 40 cybrid plants, regenerated from combinations 2 and 4 in Table 3 (both having Villafranca lemon nuclei) on both sour orange and Troyer stocks. These 80 graftings were performed in the Spring of 1986. The grafted plants were placed in a screenhouse for observations. Evaluation could not yet be performed at the time of writing this Report (July 1988).

Figure 6. Two cybrid plants derived from the fusion of gamma-irradiated protoplasts of PPT (donor) with either Sour orange or Villafranca lemon iodoacetate-treated protoplasts (actual height about 30 cm) (From Vardi, Breiman and Galun, 1987).

The Cleopatra mandarin has certain virtues as rootstock (but also disadvantages). The ctDNA of mandarins differs clearly from that of all the other *Citrus* cultivars. We thus tried to produce cybrids between Cleopatra (as organelle donor) and either Sour orange or Villafranca lemon. In both cases (Table 3) colonies were formed and embryos were induced but no further differentiation, even into leafy structures were obtained. Whether this lack of differentiation stems from the inability of Cleopatra protoplasts to differentiate into plants (Table 1), to incompatibility between Cleopatra organelles and Sour orange (or Rough lemon) nuclei, or for another reason - could not be decided.

One next question was whether it would be possible to transfer *Microcitrus* organelles into either Sour orange or Rough lemon. The respective protoplast-fusions were performed, embryos were induced and plants were produced. CtDNA and mtDNA analyses indicated that the regenerated plants (Figures 7 and 8) were indeed cybrids (Table 3). These results are now prepared for publication. As reported in other cases, most of the cybrids had novel types of mtDNA. We concluded from these results that intergeneric cybridization is possible. None of the cybrids produced by us reached the flowering stage. In some cybrids, flowering is expected in 1989. We shall watch the flowering with great interest: alien mitochondria could cause *male sterility* if this will happen and be combined with parthenocarpy - cybridization could become a method to produce seed-less cultivars.

Somatic hybridization. As noted above, our main effort was devoted to the production of cybrids. Nevertheless we intended to test a method of protoplast-fusion which should lead to somatic hybrids. Namely, the application of pre-fusion treatment to both fusion partners. Duncan grapefruit protoplasts were thus exposed to R6G during maceration and fused with Dancy mandarin protoplasts which were treated (before fusion) with iodoacetate. Treated protoplasts of either Duncan or Dancy do not divide because these inhibitors arrest metabolic activities (Aviv et al. 1985, and see review: Galun and Aviv 1986). But after fusion of these treated protoplasts cell division did occur. Embryos were induced and

Table 3. Derivatives of protoplast-fusion

Fusion partners		Results
A. Production of cybrids		
Donor (-irradiated)	Recipient (JA* treated)	
1. Poorman X P. trifoliata	Sour orange	Cybrid trees were established (not grafted)
2. Poorman X P. trifoliata	Villafraanca lemon	Cybrid trees were established and grafted on Troyer and Sour orange stocks, some trees were not grafted
3. Villafraanca lemon	Sour orange	Cybrid trees were established (not grafted)
4. Sour orange	Villafraanca lemon	Cybrid trees were established and grafted on Troyer and Sour orange stocks, some trees were not grafted
5. Cleopatra mandarin	Villafraanca lemon	Cybrid cultures reached only embryo stage - no further differentiation
6. Cleopatra mandarin	Rough lemon	Cybrid cultures reached only embryo stage-no further differentiation
7. Microcitrus sp.	Sour orange	Young cybrid plants, presently at different stages of development (not yet grafted (Fig. 7)).
8. Sour orange	Microcitrus sp.	Cybrid cultures reached embryo stage- then malformed plantlets appeared with Microcitrus -like leaves.
9. Microcitrus sp.	Rough lemon	Young cybrid plants, presently at different stages of development (not yet grafted (Fig. 8))
B. Somatic hybrids		
R6G treated	JA* treated	
10. Duncan grapefruit	Dancy mandarin	Three young somatic hybrid trees in pots, additional plantlets in vials

*Iodoacetate - to cause transient metabolic arrest and prevent division of unfused and autofused protoplasts

Figure 8. Young cybrid plant which resulted from a fusion between gamma-irradiated *Microcitrus* protoplasts and iodoacetate-treated Rough lemon protoplasts (actual height about 15cm).

Figure 7. Young cybrid plant which resulted from a fusion between gamma-irradiated *Microcitrus* protoplasts and iodoacetate-treated sour orange protoplasts (actual height about 18cm).

finally a few plants were obtained which have the morphology of somatic hybrids and are tetraploids. The method of fusing treated protoplasts can thus be applied for somatic hybridization in *Citrus*.

4. Literature

- Abbott, A.G., O'Dell, M. and Flavell, R.B. 1985. Quantitative variation in components of the maize mitochondrial genome between tissues and between plants with different male-sterile cytoplasms. *Plant Mol. Biol.* 4: 233-240.
- Aviv, D., Arzee-Gonen, P., Bleichman, S. and Galun, E. 1984. Novel alloplasmic *Nicotiana* plants by "donor-recipient" protoplast-fusion: cybrids having *N. tabacum* or *N. sylvestris* nuclear genomes and either we both plastomes and chondriomes from alien species. *Mol. Gen. Genet.* 196: 244-253.
- Aviv, D. and Galun, E. 1985. An *in vitro* procedure to assign pigment mutation in *Nicotiana* to either the chloroplast or the nucleus. *J. Hered.* 76: 135-136.
- Barrett, H.C. 1977. Intergeneric hybridization of *Citrus* and other genera in *Citrus* variety improvement. *Proc. Int. Sci. Citriculture*, Vol. 2: 586-589.
- Barrett, H.C. and Rhodes, A.M. 1976. A numerical taxonomic study of affinity relationships in cultivated *Citrus* and its close relatives. *Syst. Bot.* 1:105-136.
- Behnke, M. 1979. Selection of potato callus for resistance to culture filtrate of *Phytophthora infestans* and regneeration of resistant plants. *Theor. Appl. Genet.* 55: 69-71.
- Behnke, M. 1980. Selection of dihaploid potato callus for resistance to the culture filtrate of *Fusarium oxysporum*. *Z. Pflanzenzucht.* 85: 254-258.
- Ben-Hayyim, G. and Neumann, H. 1983. Stimulatory effect of glycerol on growth and somatic embryogenesis in *Citrus* callus cultures. *Z. Pflanzenphysiol.* 110: 331-337.
- Ben-Hayyim, G., Shani, A. and Vardi, A. 1982. Isozyme systems as a possible tool for identifying fusion products in *Citrus*. *Theor. Appl. Genet.* 64: 1-5.

- Breiman, A. and Barash, I. 1981. Partial characterization of phytotoxin compounds in culture filtrate of *Phytophthora citrophthora*. *Phytopathol. Z.* 102: 1-9.
- Breiman, A., Felsenburg, T. and Galun, E. 1987. *Nor* loci analysis in progenies of plants regenerated from the scutellar callus of bread-wheat. *Theor. Appl. Genet.* 73: 827-831.
- Breiman, A. and Galun, E. 1981. Plant protoplasts as tools in quantitative assays of phytotoxic compounds from culture filtrate of *Phytophthora citrophthora*. *Physiol. Plant Pathol.* 19: 181-191.
- Cameron, J.W. and Frost, H.B. 1968. Genetic, breeding and nucellar embryony. In: Reuter, W., Batchelor, L.D. and Webber, H.J. (eds.). *The Citrus Industry*, Vol. II, University of California Press, Berkeley, Ca, pp. 325-381.
- Cameron, J.W. and Soost, R.K. 1979. Sexual and nucellar embryony in F_1 hybrids and advanced crosses of *Citrus* and *Poncitrus*. *J. Amer. Soc. Hort. Sci.*, 104: 408-410.
- Deaton, W.R., Keyes, G.J. and Collins, G.B. 1982. Expressed resistance to black shank among tobacco callus cultures. *Theor. Appl. Genet.* 63: 65-70.
- Epstein, E. and Cohen, J.D. 1981. Microscale preparation of pentafluorobenzyl esters. Electron capture gas chromatographic detection of indol-3-acetic acid from plants. *J. Chromatogr* 209: 413-320.
- Fluhr, R. 1983. Molecular genetics of the *Nicotiana tabacum* chloroplast. Ph.D. Thesis Weizmann Institute of Science, Rehovot.
- Fox, T.D. and Leaver, C.J. 1981. The *Zea mays* mitochondrial gene coding cytochrome oxidase subunit II has an intervening sequence and does not contain TGA codons. *Cell* 26: 315-323.
- Frankel, R., Scowcroft, W.R. and Whitfield, P.R. 1979. Chloroplast DNA variation in isonuclear male-sterile lines of *Nicotiana*. *Mol. Gen. Genet.* 169: 129-135.
- Frost, H.B. and Soost, R.K. 1968. Seed production: development of gametes and embryos. In: Reuter, W., Batchelor, L.D. and Webber, H.J. (eds.). *The Citrus Industry*, Vol. II, University of California Press, Berkeley, Ca pp. 292-334.

- Galun, E. and Aviv, D. 1983. Cytoplasmic hybridization. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (eds.): Handbook of Plant Cell Culture, Vol. I. MacMillan, New York, pp. 358-392.
- Galun, E. and Aviv, D. 1986. Organelle transfer. In: Weissbach, A. and Weissbach, H. (eds.). Plant Molecular Biology. Methods in Enzymology. Vol. 118. Academic Press, Orlando, pp. 595-611.
- Green, R.M., Vardi, A. and Galun, E. 1986. The plastome of *Citrus*. Physical map, variation among *Citrus* cultivars and species and comparison with related genera. Theor. Appl. Genet. 72: 170-177.
- Grosser, J.W., Gmitter, F.G. Jr and Changler, J.L. 1988a. Intergeneric somatic hybrid plants of *Citrus sinensis* cv. Hamlin and *Poncirus trifoliata* C.V. Flying Dragon. Plant Cell Report 57: 5-8.
- Grosser, J.W., Gmitter, F.G., Jr. and Chandler, J.L. 1988b. Intergeneric somatic hybrid plants from sexually incompatible woody species: *Citrus sinensis* and *Severinia disticha*. Theor. Appl. Genet. 75: 397-401.
- Hanson, M.R., Boeshore, M.L., McClean, P.E., O'Connell, M.A. and Nivison, H.T. 1986. The isolation of mitochondria and mitochondrial DNA. In: Weissbach, A. and Weissbach, H. (eds.) Plant molecular biology, Methods in Enzymology, Vol. 118. Academic Press, Orlando, pp. 437-453.
- Hidaka, T. 1984. Effect of sucrose concentration, pH of media, and culture temperature on anther culture of *Citrus*. Japan. J. Breed. 34: 416-422.
- Hidaka, T., Yamada, Y. and Shichijo, T. 1979. *In vitro* differentiation of haploid plants by anther culture in *Poncirus trifoliata* (L.) Raf. Japan. J. Breed. 29: 248-254.
- Hutchinson, D.J. 1977. Influence of rootstock on the performance of Valencia sweet orange. Proc. Int. Soc. Citriculture, Vol. 2: 523-525.
- Hutchison, D.J. and Cooper, W.C. 1973. Recent advances in the search for improved citrus rootstocks in Florida. Proc. First Int. Citrus Congress, Murcia, Spain. Vol. 2: 181-184.

- Iwamasa, M. 1966. Study on the sterility in the genus *Citrus* with special reference to the seedlessness. Bull. Hort. Res. Station, Japan, Ser. B., 6: 1-77.
- Klotz, L.J. 1978. Fungal, Bacterial and non-parasitic diseases and injuries originating in the seedbed, nursery and orchard. In: Reuther W. Calavan E. and Carman G.E. (eds). The Citrus Industry, Vol. IV. Division of Agricultural Sciences, University of California Press, Berkeley, Ca. pp. 1-66.
- Kobayashi, S., Uchimiya, H. and Ikeda, I. 1983. Plant regeneration from "Trovita" orange protoplasts. Japan J. Breed. 33: 119-122.
- Kobayashi, 1987. Uniformity of plants regenerated from orange (*Citrus sinensis*) protoplasts. Theor. Appl. Genet. 74: 10-14.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982. Molecular cloning. Cold Spring Harbor, N.Y., pp. 100-112.
- Murshige, T. and Tucker, D.P.H. 1969. Growth factor requirement of *Citrus* tissue culture. In: Chapman, H.D. (ed.). Proc. First Int. Citrus Symp., Vol. 3, Riverside Univ. Calif., pp. 1155-1161.
- Ohgawara, T., Kobayashi, S., Ohgawara, E., Uchimiya, H and Ishii, S. 1985. Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata*. Theor. Appl. Genet. 76: 1-4.
- Plich, M. and Rudnicki, R.M. 1979. Studies of the toxins of *Phytophthora cactorum* pathogenic to apple trees. 1. Isolation, some of the properties and activities of a toxin produced by the fungus cultured *in vitro*. Phytopathol. Z. 94: 270-278.
- Sparks, R.B. and Dale, R.M.K. 1980. Characterization on ³H-labelled super-coiled mitochondrial DNA from tobacco suspension culture cells. Mol. Gen. Genet. 180: 351-355.
- Spiegel-Roy, P. and Vardi, A. 1984. Citrus. In: Ammirato, P.V., Evans, D.A., Sharp, W.R. and Yamada, Y. (eds.). Handbook of Plant Cell Culture, Vol. 3, MacMillan, New York, pp. 355-372.

- Vardi, A., Breiman, A. and Galun, E. 1987. Citrus cybrid: production by donor-recipient protoplast-fusion and verification by mitochondrial-DNA restriction profiles. Theor. Appl. Genet. 75: 51-58.
- Vardi, A., Epstein, E. and Breiman, A. 1986a. Is the *Phytophthora citrophthora* culture filtrate a reliable tool for the *in vitro* selection of resistant *Citrus* variants? Theor. Appl. Genet. 72: 569-574.
- Vardi, A. and Galun, E. 1988a. Citrus. In: Sink, K.C. (ed.). Recent Advances in Protoplast Culture of Horticultural Crops (in press).
- Vardi, A. and Galun, E. 1988b. Isolation and culture of Citrus protoplasts. In: Bajaj, Y.P.S. (ed.), Biotechnology in Agriculture and Forestry, Vol. III, Springer-Verlag, Berlin (in press).
- Vardi, A., Hutchison, D.J. and Galun, E. 1986b. A protoplast-to-tree system in *Microcitrus* based on protoplasts derived from a sustained embryogenic callus. Plant Cell Reports 5: 412-414.
- Vardi, A., P. Spiegel-Roy and E. Galun. 1975. Citrus cell culture; isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos. Plant Sci. Lett., 4: 231-236.
- Vardi, A. and Raveh, D. 1976. Cross feeder experiments between tobacco and orange protoplasts. Z. Pflanzenphysiol. 78: 350-359.
- Vardi, A. and Spiegel-Roy, P. 1978. Citrus breeding, taxonomy and the species problem. Proc. Int. Soc. Citriculture, pp. 51-57.
- Vardi, A., Spiegel-Roy, P. and Galun, E. 1982. Plant regeneration from Citrus protoplasts: variability in methodological requirement among cultivars and species. Theor. Appl. Genet. 62: 171-176.
- Wahl, G.M., Stern, M. and Stark, G.R. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyldimethyl-paper and rapid hybridization using dextran sulphate. Proc. Natl. Acad. Sci. USA 79: 2264-2267.

- Wallace, J.M. 1978. Virus and viruslike diseases. In: Reubler, W., Calavan, E. and Carman, G.E. (eds.). The Citrus Industry, Vol. IV, University of California Press, Berkeley, Ca., pp. 67-184.
- Yoder, O.C. 1980. toxins in pathogenesis. Ann. Rev. Phytopathol. 18: 103-129.

IV DESCRIPTION OF COOPERATION

This research project followed a previous BARD project in which the same Israeli and USA investigators cooperated on a program which was planned together. Moreover the Israeli investigators utilized visits at the Horticultural Research Lab (HRL) in Orlando Florida, during the previous project to consult several USDA experts in *Citrus* improvement in order to formulate the details of this project. Throughout the present project there was an exchange of information between the Israeli investigators and the USA collaborator (Dr. D. Hutchison) who is an expert on breeding of *Citrus* rootstocks. Specifically the utilization of *Microcitrus* in this project is a result of collaborative effort: the extraction of ovules from a fruiting *Microcitrus* and the initial culture of of ovules was performed during one of the visits of the Israeli collaborators at HRL.

Both Israeli investigators visited HRL twice during the present project. This was possible because these visits could be combined with attendance in congresses in America. During each of these visits there was a detailed report on the progress of the project as well as a discussion on the future experiments. Moreover, the Israeli Investigators took the opportunity to visit the Lake Alfred Research Center in Florida where a very active *Citrus* research was started. The visits at Lake Alfred were very rewarding because we learned there on the intention to use our *Citrus* protoplast manipulation procedures for the production of somatic hybrids - a subject which was originally included in our proposal. After discussions with Dr. Grosser and others we found that it is advisable for us to focuss on cybrids, since the Lake Alfred team will handle somatic hybrids. This was rather useful for our project because it enabled us to focuss our research.

We hoped that the USA collaborator, Dr. D. Hutchison would come to Israel (during the International Citrus Congress, in Tel Aviv, March 1988) in order to participate in the formulation of the Final Report. Regretfully, due to administrative hurdles, this was not possible.

V. EVALUATION OF THE RESEARCH ACHIEVEMENTS

The main achievement of this project was that we established a novel concept which was only a "dream" when the proposal of the project was submitted. Namely, to augment Citrus breeding by protoplast manipulations. The proposal for this project was based on our previous success to develop protoplast-to-tree systems. Such systems were a novelty for arboraceous plants at the time but since then other investigators followed our lead and came up with similar systems. In the present project we verified the concept that the protoplast system should enable the establishment of functional *Citrus* trees from fused protoplasts. Several cybrid combinations, both within the genus *Citrus* as well as between *Citrus* and another genus, were produced and reached the stage of established trees. Furthermore we produced somatic hybrid plants between two *Citrus* species (*C. reticulata* and *C. paradisi*). Others, using our procedures produced intergeneric somatic hybrids in which *Citrus* was one of the fusion partners.

These studies showed that *Citrus* cybrids and somatic hybrids are realities and can now serve for specific *Citrus* breeding goals. Actually this line of research is already being followed now in the Citrus Research Center at Lake Alfred, Florida, where the Israeli investigators visited and had intensive discussions on future strategies for the utilization of protoplast manipulations for *Citrus* improvement. Finally, if male-sterility will be detected among cybrid trees (e.g. after fusing γ -irradiated *Microcitrus* protoplasts with iodoacetate treated Sour orange protoplasts) a breakthrough could be achieved: the production of seedless *Citrus* cultivars by cybridization.

VI. LIST OF PUBLICATIONS RESULTING FROM THIS PROJECT

- Galun, E. 1988. Application of molecular methods to modern *Citrus* taxonomy. Proc. Inter. Soc. Citriculture (1988) (in press).
- Galun, E., Aviv, D., Breiman, A., Fromm, H., Perl, A. and Vardi, A. 1987. Cybrids in *Nicotiana*, *Solanum* and *Citrus*: isolation and characterization of plastome mutants, perfusion treatments, selection and analysis of cybrids. In: von Wettstein, D. and Chua, n.-H (eds) Plant Molecular Biology (NATO ASI series vol 140), Plenum Press, N.Y. pp. 199-207.
- Green, R.M., Vardi, A. and Galun, E. 1986. The plastome of *Citrus*. Physical map, variation among *Citrus* cultivars and species and comparison with related genera. Theor. Appl. Genet. 72: 170-177.
- Vardi, A. 1988. Cell manipulation in *Citrus*. In: Walker, R.R. (ed) Citrus Breeding Workshop, Merbein, Victoria. CSIRO, Australia, pp. 133-148.
- Vardi, A., Breiman, A. and Galun, E. 1987. Citrus cybrid: production by donor-recipient protoplast-fusion and verification by mitochondrial-DNA restriction profiles. Theor. Appl. Genet. 75: 51-58.
- Vardi, A., Epstein, E. and Breiman, A. 1986. Is the *Phytophthora citrophthora* culture filtrate a reliable tool for the *in vitro* selection of resistant *Citrus* variants? Theor. Appl. Genet. 72: 569-574.
- Vardi, A. and Galun, E. 1988. Citrus. In: Sink, K.C. (ed.). Recent Advances in Protoplast Culture of Horticultural Crops (in press).
- Vardi, A. and Galun, E. 1988. Isolation and culture of Citrus protoplasts. In: Bajaj, Y.P.S. (ed.), Biotechnology in Agriculture and Forestry, Vol. III, Springer-Verlag, Berlin (in press).
- Vardi, A., Hutchison, D.J. and Galun, E. 1986. A protoplast-to-tree system in *Microcitrus* based on protoplasts derived from a sustained embryogenic callus. Plant Cell Reports 5: 412-414.