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Citrus Protoplast Fusion

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E. Galun, D. J. Hutchison

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ABSTRACT

Eight new protoplast systems were established in Citrus from new nucellar callus lines with full plant regeneration. By modification in ovular callus induction the full sequence (embryoids-callus-protoplasts-embryoids-plants) was also achieved in Microcitrus.

Chromosome counts showed that protoplast derived plants were diploid, with the exception of Villafranca, in which a mixture of diploid and tetraploid cells were found in the callus.

Procedures developed for isolation and culture of Shamouti orange protoplast were applicable to other protoplast systems, but the composition of enzymes in the maceration fluid were found to influence results. Increased sensitivity of mandarin protoplasts to sorbitol was shown. Plating efficiency in presence of fructose was severely lowered in Citrus limon cultivars and in C. sinensis.

C. LIMON protoplasts proved much less sensitive to the presence of either 2,4-D or NAA in the culture medium. Calli were found to utilize glycerol. This sugar alcohol also enhanced embryogenesis.

Analysis of ten isozyme system in nine nucellar calli and roots from apomictic seedlings disclosed variation in patterns between prospective fusion partners in many cases.

Metabolic inactivation of protoplasts by iodoacetate and sensitivity to the exposure to gamma ray irradiation were investigated.

Fusion experiments between protoplasts from nucellar callus and non dividing protoplasts from leaf tissue yielded an estimate of 8-9% fusants. Fusion experiments were performed also between different protoplasts, all of nucellar origin. Embryos and plants have been developed and identification of fusants and cybrids has been started.

INTRODUCTION

Citrus breeding work is much hampered by heterozygosity, sterility, nucellar embryony and a long juvenile phase (Cameron and Frost, 1969; Vardi and Spiegel-Roy, 1978). Some types of Citrus hybrids are very difficult to achieve, among these are crosses with wild relatives of Citrus. Furthermore, in some cases, in which such hybrids were obtained, the hybrids tend to degenerate (Barrett 1977).

There is an urgent need to extend the range of resistance of rootstocks and Citrus cultivars against pests, viruses, fungal diseases and environmental hazards. Intergeneric hybridization offers the potential possibility of incorporating new traits into the germplasm of Citrus (Barrett, 1977).

A new approach to plant breeding and improvement can be provided by plant cell genetics. Such an approach requires an efficient protoplast system i.e. reproducible techniques for the regeneration of functional plants from isolated protoplasts. In addition protoplast fusion could also extend the range of variability and contribute to cell genetics studies in this economically important genus.

We have reported previously that embryoids (Vardi et al. 1975) and plants (Galun et al. 1977, Vardi, 1977) can be derived from protoplasts isolated from nucellar callus of orange (C. sinensis (L) Osbeck cv. Shamouti). Subsequently, we extended the number of Citrus species and cultivars in which protoplasts could be readily isolated from respective nucellar calli, cultured in vitro and finally be regenerated into functional plants. Up to now Citrus is the only woody angiosperm among the several genera in which reproducible techniques for regeneration of functional plants from isolated protoplast were reported (Binding et al. 1981). Somatic hybrids and especially cybrids resulting from protoplast fusion are of great interest in Citrus breeding. This interest is mainly based on difficulties encountered in conventional methods due to nucellar embryony and the long juvenile phase. Lack of morphological markers and of selective media complicate the isolation and characterization of fusion products. A clear identification of fusion products at either the callus or the whole plant level is an essential part of somatic hybridization. Therefore an attempt was made to disclose variation among the different Citrus protoplast systems in respect to sensitivity to osmotic stabilizers, mono- and disaccharides and their differential reaction to plant growth regulators. In addition, an analysis of electrophoretic mobilities of ten enzymes in nine different Citrus nucellar calli and their corresponding roots, from apomictic seedlings has been performed. Different isozyme patterns should give us the possibility for selecting the right enzyme(s) for a given pair of parents in order to confirm the identity of the somatic hybrids derived.

The present report deals with the establishment of eleven additional protoplast systems, the development of possible selective media, the analysis of ten isozyme systems and first results obtained from fusion experiments.

MATERIALS AND METHODS

1. Establishment and maintenance of calli

Nucellar calli were obtained as described (Kochba et al. 1972) with few modifications. Ovules of 11 species and cultivars, as detailed in Table 1, were axenically excised from young fruits, two to six weeks after anthesis and placed on solidified medium in 5 cm Petri dishes. The medium (BM-ME) consisted of Murashige and Tucker (1969) nutrient medium (BM) containing 5% sucrose and 500 mg.l^{-1} malt extract (ME). Before autoclaving, the pH was adjusted to 5.7 and supplemented with 10 g.l^{-1} Difco agar. The dishes were maintained at $26 \pm 1^{\circ} \text{C}$ with 16h daily of dim light. The nucellar callus derived from the cultured ovules was subcultured on BM-ME for the first three passages and subsequently on BM only with the same temperature and light regimes.

Calli of lemon cultivars, namely Villafranca (C. limon Burm) and Volckameriana (C. volckameriana) were obtained from Ms. S. Saad and the late Dr. J. Kochba of our laboratory and subcultured on BM medium.

Table 1. List of genera, species and cultivars with cultured ovules yielding callus or embryos

Botanical species	Cultivar	Number of Isolated ovules	Number of Ovules producing callus and/ or embryos
<u>Citrus sinensis</u> (L.)	Clonal Shamouti	240	66
Osbeck	Nucellar Shamouti	200	52
	Shamouti Landau	200	32
<u>Citrus aurantium</u> L.	Sour orange	120	10
<u>Citrus reticulata</u>	Dancy	280	28
Blanco	Ponkan	200	35
	Murcott	200	34
<u>Citrus paradisi</u> Macf.	Duncan	240	50
<u>Citrus reticulata</u> x <u>Citrus sinensis</u>	Niva	280	15*
<u>Citrus macrophylla</u>	Alemow	400	111
<u>Poncirus trifoliata</u>		500	60*
<u>Citrus sinensis</u> x <u>Poncirus trifoliata</u>	Troyer citrange	500	56*
<u>Eremocitrus</u> spp.		160	0
<u>Microcitrus hybrida</u>		250	2*
<u>Microcitrus papuana</u>		400	0

* embryos only.

Ovules of Microcitrus spp. were axenically excised from trees at the USDA Horticultural Research Laboratory, Orlando, Florida and were cultured on BM-ME as described above. While all the Citrus species and cultivars employee are polyembryonic, Microcitrus is a monoembryonic species. One of the cultured ovules formed embryos which proliferated and gave rise to a large number of embryos. The isolated embryos were sub-cultured on BM with the addition of 8.9 μ M benzyl-adenine and 30 μ M 3-indoleacetyl-L-alanine (BM-BA-ILA30 medium).

In addition, green calli were obtained from Poncirus trifoliata, Troyer citrange (C. sinensis x P. trifoliata), Eremocitrus spp. and Calamondin as follows: Seeds were peeled, sterilized in 1% sodium hypochlorite for 10 min and washed three times in sterile water. Embryos were excised under aseptic conditions and placed in Petri dishes containing BM without the addition of plant growth substances. When seedlings have reached 1-2 cm in size, the region between the cotyledons and the first leaves was cut into pieces of 3-4 mm length and some longitudinal cuts were also made in order to stimulate callus formation. Epicotyl segments were then placed on BM with the addition of 8.9 μ M benzyladenine and 60 μ M 3-indoleacetyl-L-alanine (BM-BA-ILA60). The emerging calli were sub-cultured several times on BM-BA-ILA60. The level of 3-indoleacetyl-L-alanine was reduced gradually to 15 μ M (BM-BA-ILA15) and subcultures were made at monthly intervals.

2. Isolation and plating of protoplasts

For protoplast isolation calli were subcultured, on EM, every two to three weeks. About 0.5g callus was placed in a 10 cm Petri dish and 10 ml maceration medium was added. The maceration medium consisted of 0.3% pectinase (fungal, Koch-Light laboratories) or 0.3% Macerozyme, 0.2% cellulose (Onozuka R10, Kinki Yakult Mfg. Co.) and 0.1% Driselase (Kyowa Hakko Kogyo Co.) dissolved in 0.14M sucrose and 0.56M manitol (or 0.35M sucrose and 0.35M mannitol) and supplemented with EM macroelements for more consistent results. The maceration solution was adjusted to pH 5.7 and filter-sterilized (0.2 μ m) before use. After overnight incubation (26 $\pm$ 1⁰ C) the protoplasts were isolated by sequential filtering through nylon screens of 50 and 30 μ m pore openings, followed by three centrifugations (about 100xg, 5 min) in a washing solution (EM containing 0.3M sucrose and 0.3M mannitol). The protoplasts were suspended at a final density of 1.5×10^5 cells per ml of EM containing soft (0.6%) agar and plated as 4ml aliquots in 5cm, Parafilm sealed, Petri dishes. the plates were maintained under continuous dim light at 26 $\pm$ 1⁰ C and colony formation was scored as described (Vardi and Raveh, 1976) four to five weeks after plating.

Leaf protoplasts were isolated as follows: young full sized leaves of Troyer citrange (C. sinensis x P. trifoliata) were surface sterilized (washed in water, dropped in 70% ethanol maintained in 1% sodium hypochlorite for 15 min and washed three times in sterile water) the

leaves were then cut into stripes of 1-2 mm and placed in 10cm Petri dishes containing about 15ml maceration solution). The maceration solution consisted of 0.8% Macerozyme, 0.6% Cellulase and 0.3% Driselase dissolved in 0.7M mannitol supplemented with BM macroelements. The maceration solution was adjusted to pH 5.7 filter sterilized (0.45um) and incubated as above. Protoplasts were isolated by filtration through nylon screen as described above, followed by three centrifugation (about 100 xg 5 min) in a washing solution consisting of 0.7M mannitol plus BM macroelements. After the last centrifugation BM containing 0.3M sucrose and 0.3M mannitol was added to the protoplasts.

Protoplasts for feeder layers and fusion experiments were irradiated with gamma-rays at the dose of 50KR. Fusion was performed according to Aviv and Galun (1980).

3. Chromosome counts

For chromosome counts root tips or pieces of logarithmic phase callus cultures were removed and treated with paradichlorobenzene for 4hrs, fixed in ethanol: chloroform: acetic acid (6:3:1) for 24h and stored in 70% ethanol. The root tips were finally stained with aceto-carmin.

4. Analysis of electrophoretic mobilities of the enzymes.

(a) Extraction procedure: One gram fresh weight of callus cells was ground in a small mortar held at 0° C, with 1.5 ml of 0.8% NaCl, 0.2% NaNO₃ and 20% sucrose. The extract was then centrifuged for 20 min at 1.200xg. Aliquots from the supernatant (15-25µl) were layered on top of the gel after the addition of bromophenol blue dye. Roots were first washed with water and dried on blotting paper. Enzymes were extracted by grinding 0.2g roots in 0.5ml of the solution for callus cells. The extract was then centrifuged for 15 min at about 500xg. Aliquots of the supernatant were treated as above.

(b) Electrophoresis: Polyacrylamide slab gel (anodic) electrophoresis was used. A gel concentration of 7% with various buffer systems was run at a constant voltage of 150-160 volt (about 15-20mA) at 4° C (Maurer 1971). All gel separations were run for 3-5h until the tracking dye-bromophenol blue-migrated to a distance of 50-70 mm from the origin of the separation gel. The buffer systems were adopted from Maurer (1971) and are summarized in Table 2.

(c) Staining solutions: Slab gels were twice washed for 20min at 4° C with the buffer of the staining solution prior to staining. Staining took place at room temperature in the dark until clear-colored bands appeared (1-6h). Staining was arrested by 2% acetic acid. As some of the dyes faded quite fast zymograms were drawn to scale without delay.

The staining solutions for the dehydrogenases mostly adopted from the literature (Brown et al. 1978) were as follows: Tris-HCL 0.1 M pH 8.0 0.32mM NAD (or NADP). 5mM MgCl₂, 1mM NaN₃, 0.17mM phenazine methosulfate

Table 2. Methods employed in gel electrophoresis of Citrus enzymes

Enzyme	Buffer system			Staining solution
	Separation gel	Spacer gel	Electrode	
Phosphoglucisomerase (PGI)	Tris-HCl, pH 7.5	Tris-phosphate pH 6.8	Sodium borate 0.034M pH 8.7	Torres et al 1978b
Lactate dehydrogenase (LDH)	Tris-HCL, pH 7.5	Tris-phosphate, pH 6.8	Sodium borate 0.034M pH 8.7	c
Alcohol dehydrogenase (ADH)	Tris-HCl, pH 7.5	Tris-phosphate, pH 6.8	Sodium borate 0.034M pH 8.7	c
Glutamate dehydrogenase (GDH)	Tris-HCl pH 7.5	Tris-phosphate pH 6.8	Sodium borate 0.034M pH 8.7	c
Glutamate-oxaloacetate transaminase (GOT)	Tris-HCl, pH 7.5	Tris-phosphate pH 6.8	Sodium borate 0.034M pH 8.7	Brown et al 1978
Peroxidase	Tris-HCl, pH 7.5	Tris-phosphate pH 6.8	Sodium borate 0.3M pH 8.3	Siegel and Galson 1967
Leucine aminopeptidase (LAP)	Tris-HCl, pH 8.9		Tris-glycine (5mM Tris and 40mM glycine) pH 8.3	Torres et al. 1978a.
Malate dehydrogenase (MDH, NADP) or (MDH, NAD)	Tris, HCl, pH 7.5	Tris-phosphate pH 5.5	Diethylbarbituric acid Tris(30mM diethyl-8mM Tris), pH 7.0	c c
Esterase	Tris-citrate pH 8.5	Tris-phosphate pH 6.8	Lithium borate, 30mM pH 8.2	Scandalios and Sorenson 1977.

c. See text: (c) staining solution.

(PMS). 0.2 mg ml^{-3} 3[4,5 dimethylthiazole-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) were used in all staining solutions. In addition, MDH, GDH, LDH and ADH contained 0.1 M malate pH 7.0, 0.1 mg glutamate pH 7.0, 0.18 lactate pH 7.0 and 8% ethanol, respectively.

RESULTS

1. Establishment and maintenance of nucellar callus lines

During the first two years of the research project an attempt was made to initiate nucellar callus lines from additional genera, species and cultivars. Using methods similar to those described previously (Kochba et al. 1972) with some modifications. We isolated ovules from the species and cultivars listed in Table 1.

Embryoids followed by whitish callus of nucellar origin started to emerge from cultured ovules one, two or even up to four months after start of culture (Fig. 1a). The number of callus producing ovules was variable and usually rather low (see Table 1). The emerging callus was sub-cultured three times on BM-ME and then maintained on BM.

A series of experiments were conducted in order to determine in what stage of development ovules will give of the best results as to initiation of nucellar callus. For that purpose ovules at different

stages from 0 up to 90 days after anthesis, were cultured on BM supplemented by 250, 500 and 1000 mg/l malt extract. The best results were obtained with ovules 30-60 days after anthesis, cultured on BM supplemented by ME 500 mg/l. To stimulate callus initiation we also tried to follow the procedure described by Mullins and Srinivasan (1976) in Vitis by changing the level of plant hormones in the medium during different stages of ovule culture. In another experiment we tested the effect of the conjugated auxin (3-indoleacetyl-L-alanine) as described by Hangarter et al. (1980).

We found that the addition of plant hormones such as NAA, 24-D or 3-indoleacetyl-L-alanine stimulate callus formation from the integuments, therefore they were omitted from the medium during initiation, isolation and subculture. Ovules of Citrus hybrids such as Niva and Troyer and Citrus relatives such as Poncirus, Eremocitrus and Microcitrus gave rise only to embryoids which developed later into plants. Nucellar callus lines from C. sinensis, C. reticulata, C. paradisi and C. macrophylla showed embryogenic capacity even after repeated sub-cultures, as shown in Fig. 1b and 1c for Dancy and Ponkan mandarins. When torpedo shaped embryos were isolated they regenerated into plants. Whitish embryogenic callus was obtained from Microcitrus hybrida by sub-culturing the embryoids on BM containing 8.9 μ M benzyladenine and 15 μ M 3-indoleacetyl-L-alanine for more than a year.

Chromosome counts of all these callus lines, maintained in culture for more than a year are presented in Table 3. All the new callus lines maintained the diploid chromosome number ($2n=18$), while deviation from

Fig. 1. Embryoidal callus from cultured ovules. a. Callus and embryoids emerging from a cultured Ponkan ovule; the larger torpedo embryoids (TE) were moved to the left to reveal the ovular integuments (OI), with the attached callus and globular embryoids (GE); photographed 6 weeks after begin of culture (x 15). b and c nucellar calli derived from cultured ovaries of Dancy and Ponkan mandarins, respectively (x 6.5)

Table 3. Chromosome counts in established nucellar callus lines of several *Citrus* species and cultivars. Data are expressed as number of cells having a given (2n) chromosome number.

Callus lines	Chromosome number					Total no. of counted cells
	18	19	21	36	39-40	
1. <u>Orange</u>						
Old Shamouti line	11	5	1	0	0	17
Clonal Shamouti	0	0	0	0	0	10
Nucellar Shamouti	10	0	0	0	0	10
Shamouti Landau	10	0	0	0	0	10
2. <u>Sour Orange</u>	17	3	0	0	0	20
3. <u>Mandarin</u>						
Murcott	13	0	0	0	0	13
Dancy	10	0	0	0	0	10
Ponkan	10	0	0	0	0	10
4. <u>Grapefruit</u>						
Dancan	3	0	0	0	0	3
5. <u>Lemon</u>						
Villafranca	3	0	0	5	1	9
6. <u>Microcitrus</u>	5	0	0	0	0	4

this norm was observed only with the Villafranca lemon callus line. It should be noted that during initiation and early maintenance of this line 0.5 ppm 24-D was added to the BM-ME Hence the partial tetraploidy of Villafranca lemon callus could be related to the addition of this auxin to the medium (Singh and Harvey 1975).

2. Isolation and culture of protoplasts

Preliminary tests indicated that procedures previously developed for Shamouti orange protoplasts (Vardi et al. 1975; Vardi and Raveh, 1976; Vardi 1977) gave fair results also with each of the other nucellar callus lines. However, the results were inconsistent, especially when a new stock of Pectinase was employed (fungal Koch-Light laboratories). This prompted us to retest the effect of maceration enzymes on the subsequent division capacities of isolated protoplasts. Since the variability in plating efficiency seemed to be related to the pectinase source, we concentrated on the relative effect of Koch Light Pectinase and Macerozyme on the ability of protoplasts from different callus lines to form colonies (Table 4). We found that in all cases except Villafranca, higher plating efficiencies were obtained when Macerozyme rather than Pectinase was included in the maceration fluid. Moreover, Duncan and Dancy protoplasts were severely harmed by Pectinase as well as by a combination of Driselase and Macerozyme. Protoplasts with a

Table 4. Colony formation by protoplasts derived from orange, mandarin grapefruit and lemon callus lines as affected by two maceration fluids. Both fluids contained 0.2% cellulase (Onozuka R-10) and 0.1% Driselase but differed by including either 0.3% Macerozyme or Koch-Light Pectinase, Data present number of colonies per ml counted 5 weeks after plating; plating efficiencies are given in parenthesis as percent of plated protoplasts producing colonies.

Callus Line	Pectinase in maceration fluid			
	Koch-light Pectinase		Macerozyme	
Nucellar Shamouti	4727	(3.78%)	25818	(20.65%)
Shamouti Landau	7818	(6.25)	17636	(14.10)
Murcott	8454	(6.78)	13545	(10.83)
Duncan *	0	(0)	818	(0.65)
Villafranca	16182	(12.94)	7636	(6.10)

* Colony formation following maceration by 0.2% Cellulase and 0.3% Macerozyme (without Driselase) was: 12954 (10.36%).

high division capacity could be isolated from Duncan callus when Macerozyme was substituted for Pectinase and Driselase was omitted, while for Dancy division was maintained only when Driselase was added to cellulase.

When using osmotic stabilizers previously employed in the culture of Shamouti orange protoplasts i.e. a mixture of sorbitol and mannitol (Vardi et al. 1975, Vardi and Raveh, 1976, Vardi, 1977), very low plating efficiencies were found with most of the new callus lines. Therefore additional combinations of osmotic stabilizers were tested (Table 5). Murcott protoplasts (as well as the two other mandarin protoplasts, Ponkan and Dancy, not shown in Table 5) proved very sensitive to sorbitol; lemon protoplasts showed some sensitivity to sorbitol while no sorbitol sensitivity was indicated by orange and sour orange protoplasts. Protoplasts from all the tested callus lines gave the best plating efficiency when plated on sucrose as a sole osmotic stabilizer. However, plating efficiencies above ca 10% are not practical because of the extreme colony density, which causes growth inhibition before colony isolation has become feasible. We therefore adopted the 0.3M sucrose plus 0.3M mannitol mixture as our standard osmotic stabilizer.

Table 5. The effect of sucrose (SUC), sorbitol (SOR) and mannitol (MANN) on the plating efficiency of protoplasts derived from orange, sour orange, mandarin and lemon callus lines. Data are presented as number of colonies per ml, five weeks after plating; plating efficiencies are given in parentheses as percent of plated protoplasts which produced colonies.

Callus line	Osmotic Stabilizers Added to BM				
	0.14M SUC	0.14M SUC	0.3M SUC	0.3M SUC	0.6M SUC
	0.46M MANN	0.46M SOR	0.3M SOR	0.3M MANN	
Nucellar	5727	4636	5091	4454	6909
Shamouti	(3.80)	(3.08)	(3.38)	(2.98)	(4.60)
Shamouti	3273	5864	17954	9682	25727
Landau	(2.18)	(3.90)	(11.97)	(6.45)	(17.15)
Sour orange	10000	10091	13568	12068	15273
	(10.00)	(10.09)	(13.56)	(12.06)	(15.27)
Murcott	1318	500	2635	16454	23454
	(0.87)	(0.33)	(1.75)	(10.96)	(15.63)
Villafranca	7682	6318	13227	8727	11864
	(5.12)	(4.21)	(8.81)	(5.82)	(7.91)

3. The effect of different sugars on plating efficiencies

The effect of some carbohydrates on growth and embryogenesis of citrus ovular callus was investigated by Button (1978), by Kochba et al.(1978, 1982)and recently by Ben Hayyim and Neumann (1983). It was found that lactose, galactose and glycerol have stimulating effect on embryogenesis, while both glucose and fructose are least effective as to callus growth and embryogenesis. Therefore we decided to test the effect of four sugars on the capacity of protoplasts from different callus lines to divide and form colonies. As seen from Table 6, lactose gave the best plating efficiencies except with Murcott mandarin. Glucose gave variable results, while in the presence of fructose protoplasts from orange and sour orange failed to divide while lemon protoplasts gave some growth. In contrast, Murcott protoplasts gave almost similar results with fructose and lactose.

Table 6: The effect of sucrose (SUC), lactose (LAC), glucose (GLUC) galactose (GAL) and fructose (FRUC) on plating efficiency of protoplasts derived from orange, Sour Orange, lemon and mandarin callus lines. Mannitol (MANN) added up to 0.6M with all sugars. Data are presented as number of colonies per ml, 5 weeks after plating; plating efficiencies (in percent) are given in parentheses

Callus line	Sugars + MANN				
	10% SUC	5% LAC	5% GLUC	5% GAL	5% FRUC
Nucellar	14091	22682	11773	15727	0
Shamouti	(14.09)	(22.68)	(11.77)	(15.72)	
Shamouti	11591	21536	7045	9682	0
Landau	(11.59)	(21.53)	(7.04)	(9.68)	
Sour orange	20727	31318	18727	20545	0
	(20.72)	(31.31)	(18.72)	(20.54)	
Villafranca	6182	13727	4091	7545	1364
	(6.18)	(13.72)	(4.09)	(7.54)	(1.36)
Murcott	25545	6545	8455	1727	5091
	(25.54)	(6.54)	(8.45)	(1.72)	(5.09)

4. Differential auxin sensitivity of protoplasts derived from different callus lines.

We reported previously (Vardi et al., 1975; Vardi and Raveh, 1976) that clonal Shamouti protoplasts are unique among other protoplast systems by their ability to divide and form colonies in media devoid of auxin. Moreover the addition of auxin to the medium had a deleterious effect. With the establishment of additional Citrus protoplast systems we intended to answer whether or not the auxin independence and sensitivity is shared by all Citrus protoplast systems.

Several experiments were therefore performed using protoplasts of the various callus lines, with the two synthetic auxins NAA and 2,4-D applied at various concentration. The results, summarized in tables 7 and 8, show that the callus lines used can be sorted into three groups in respect to the sensitivities of their protoplasts to auxins. Murcott protoplasts (as well as Dancy and Ponkan, not shown all three are mandarins) are most sensitive to both growth substances. Nucellar Shamouti (as well as other Shamouti lines) and Sour orange (i.e. the orange types) showed intermediate sensitivity while Villafranca lemon was least sensitive. Villafranca protoplasts formed colonies even when the culture medium contained 8 ppm of NAA or 2,4-D. In all cases NAA was more harmful than 2,4-D and none of the protoplasts required auxin for cell division.

Table 7. The effect of 2,4-D on plating efficiency of protoplast derived from orange, sour orange, lemon and mandarin callus lines. 2,4-D concentration are given in ppm. Data are presented as number of colonies per ml, five weeks after plating; plating efficiencies are given in parentheses

Callus line	Control	2,4-D(ppm)				
		0.5	1	2	4	8
Nucellar	3639	955	545	409	0	0
Shamouti	(2.9)	(0.76)	(0.43)	(0.32)		
Sour orange	7545	1272	0	0	0	0
	(6.03)	(1.01)				
Villafranca	7318	5045	3727	1182	955	773
	(5.85)	(4.03)	(2.98)	(0.94)	(0.76)	(0.61)
Murcott	5682	0	0	0	0	0
	(4.50)					

Table 8. The effect of NAA on plating efficiency of protoplasts derived from orange, Sour Orange, lemon and mandarin callus lines.

NAA concentrations are given in ppm. Data are presented as number of colonies per ml, 5 weeks after plating; plating efficiencies (in percent) are given in parentheses.

Callus line	Control	NAA (ppm)				
		0.5	1	2	4	8
Nucellar	3639	0	0	0	0	0
Shamouti	(2.9)					
Sour orange	7545	454	0	0	0	0
	(6.03)	(0.36)				
Villafranca	7318	2136	3000	1773	636	364
	(5.85)	(1.70)	(3.40)	(1.41)	(0.50)	(0.29)
Murcott	5682	0	0	0	0	0
	(4.50)					

5. Isolation of protoplast-derived embryoids and plant regeneration

After about 10 weeks of culture variable number of protoplast derived colonies started to form green globular embryoids, in most of the Petri dishes, similar to those previously reported for Shamouti protoplast colonies (e.g. Vardi et al. 1975). These embryoids were isolated and replated on solidified BM without osmotic stabilizers). After additional 4 to 6 weeks, embryoids, mostly at the torpedo stage, were transferred individually into test tubes as described by Kochba and Spiegel-Roy (1976). Plantlets were then transferred to Jiffy pots covered with plastic bags and gradually accustomed to lower humidity. Finally, they were transferred into plastic containers with a soil-perlite mixture and maintained in the greenhouse without further protection. The total period between initiation of protoplast culture and transfer to the greenhouse varied from 5 to 7 months. Plants were thus regenerated from protoplasts from each of the nine callus lines listed in Table 3. Plants of six of these lines are shown in Fig. 2a and b. and for Microcitrus in Fig. 3.

6. Identification of different callus lines and roots from the corresponding apomictic seedlings by isozyme patterns

Fig. 2. a and b. Young Citrus plants derived from cultured protoplasts, photographed 5 months after protoplast plating ($\times \frac{1}{3}$).
a. Orange (nucellar Shamouti), Mandarin (Murcott, Dancy, Ponkan) and Lemon (Villafranca) plants; b. Plants derived from Dancy protoplasts - note uniformity.

Fig. 3. Protoplast-to-plant system in Microcitrus. a. A small colony, 4 weeks after protoplast isolation. b. Embryogenic callus from protoplast culture. c. Young regenerated plants. d. Established plant.

Callus lines of nine Citrus species and cultivars listed in Table 9 and roots of the corresponding apomictic seedlings of each cultivar mentioned, were analysed for ten isozyme patterns. The enzyme list includes several dehydrogenases (malate, glutamate, lactate and alcohol), phosphoglucisomerase, glutamate oxaloacetate transaminase, leucine aminopeptidase, peroxidases and esterase. The data are presented in Figs. 4, 5 and 6. The isozyme patterns identified can be divided into three groups.

a) Isozyme patterns similar in both calli and roots

This group consists of the enzymes LDH, ADH, PGI, LAP and MDH (NAD). The isozyme patterns of LDH were almost identical in all tested calli and absolutely identical in the roots. Both in calli and roots there was only one distinct band migrating close to the tracking dye. The isozyme patterns of ADH consisted of three bands which were absolutely identical in all tested calli but no enzyme activity was present in any of the roots. The isozyme patterns of LAP were very similar within both the calli and the roots and the differences between the patterns of the calli and the roots were minor (the patterns are not shown). The isozyme patterns of PGI were identical for all calli except Troyer, and this was almost so for the roots. The same picture was obtained with isozyme patterns of MDH (NAD), where calli and root isozyme patterns were very similar with the exception of Troyer. This group of enzymes with the exception of Troyer, therefore cannot be used as an identification marker for somatic hybrids with PGI and MDH(NAD).

b) Isoenzyme patterns similar in calli but variable in roots

Table 9. Recognition of fusion products by isozyme patterns: protoplast fusion combinations in which parental types are clearly differentiated by their isozyme patterns.^a

Enzymes	Possible Fusion Partners	
	Callus extract	Root extract
PGI	Troyer with any of the others	Troyer with any of the others
GOT	1. Either Duncan or Troyer with any of the others 2. Either Murcott or Ponkan with any of the others	1. Nucellar Shamouti with any of the others 2. Either Sour orange, Murcott, Dancy or Ponkan with Villafranca, Duncan or Troyer
MDH(NADP)	Murcott with any of the others	1. Villafranca with any of the other. 2. Either Sour orange or Dancy with Nucellar Shamouti, Villafranca, Volckameriana or Troyer
MDH(NAD)	Troyer with any of the others	Volckameriana with any of the others
GDH	1. Troyer with any of the others 2. Either Villafranca or Duncan with any of the others	Either Murcott, Dancy or Ponkan with Nucellar Shamouti, Sour orange, Villafranca, Duncan or Troyer
Peroxidase	(none)	Each one with any of the other
Esterase	Ponkan with any of the others	Sour orange with Nucellar Shamouti, Dancy, Villafranca, Duncan, Murcott or Ponkan

^aNucellar Shamouti orange., Sour orange, Murcott, Dancy and Ponkan mandarin, Villafranca lemon, Duncan grapefruit, Volckameriana and Troyer.

Fig 4. Glutamate dehydrogenase (GDH) and peroxidase isozyme patterns in Citrus callus and roots. The schemes represent the patterns of the following cultivars: 1. GDH: A-E patterns in callus; A - Nucellar Shamouti, sour orange; B - Murcott; C - Dancy, Ponkan; D - Villafranca, Duncan; E - Troyer. F-J patterns in roots; F - Nucellar Shamouti; G - Murcott, Dancy, Ponkan; H - Sour orange; I - Villafranca, Duncan; J - Troyer. 2. Peroxidase: A - Dancy, Duncan, Murcott, Ponkan, Nucellar Shamouti, sour orange, Villafranca; B - Volckamariana. C-I patterns in roots; C - Murcott, Nucellar Shamouti; D - Dancy; E - Ponkan; F - Sour orange; G - Villafranca; H - Volckamariana; I - Troyer.

Fig. 5 NADP and NAD specific malate dehydrogenase (MDH) isozyme patterns in Citrus callus and roots. The schemes represent the patterns of the following cultivars:

1. MDH (NADP): A-D patterns in callus; A- Nucellar Shamouti; B - Sour orange; Troyer; C - Murcott. D - Dancy, Ponkan, Duncan, Villafranca, Volckameriana. E-J patterns in roots; E - Nucellar Shamouti; F - Sour orange, Dancy; G - Murcott, Ponkan, Duncan; H - Villafranca; I - Volckameriana; J-Troyer.
2. MDH (NAD): A-C patterns in callus; A- Nucellar Shamouti, Sour orange, Murcott, Villafranca, Duncan; B - Dancy Ponkan, Volckameriana; C - Troyer. D-G patterns in roots; D - Nucellar Shamouti, Murcott, Dancy, Ponkan, Duncan, Villafranca; E - Sour orange; F - Volckameriana; G - Troyer.

Fig. 6. Phosphoglucosomerase (PGI), glutamate-oxaloacetate - transaminase (GOT) and esterase isozyme patterns in Citrus callus and roots. The schemes represent the patterns of the following cultivars: 1. PGI: A-B patterns in callus; A - Nucellar Shamouti, sour orange, Murcott, Dancy, Ponkan, Volckameriana; B - Troyer. C-E patterns in roots; C - Nucellar Shamouti, sour orange; D - Dancy, Ponkan; E - Troyer. 2. GOT: A-C patterns in callus; A - Nucellar Shamouti, Sour orange, Dancy, Villafranca; B - Murcott, Ponkan; C - Duncan, Troyer. D-G patterns in roots; D - Nucellar Shamouti; E - Villafranca; F - Sour orange, Murcott, Dancy, Ponkan; G - Duncan, Troyer. 3. Esterase: A-F patterns in callus; A - Nucellar Shamouti, Sour orange, Dancy; B - Villafranca; C - Duncan; D - Murcott; E - Ponkan; F - Troyer. G-N patterns in roots; G - Nucellar Shamouti; H - Sour orange; I - Dancy; J - Villafranca; K - Duncan; L - Murcott; M - Ponkan; N - Troyer.

This group consists of peroxidases only. The isozyme patterns in calli were almost unvarying, while isozyme patterns of the root seemed highly specific thus, peroxidases isozyme patterns in root from regenerated plants may serve as markers for the identification of fusion products.

c) Isozyme patterns variable in both calli and roots

This group consists of GOT, MDH (NADP), GDH and esterases. The isozyme patterns of these latter enzymes have been grouped according to calli and root extracts respectively. these enzyme systems can be used as markers for the identification of fusion products between parental protoplast types.

The available enzymes for identification of fusion products resulting from various parental combinations are summarized in Table 9.

7. Protoplast fusion

Regular fusion of intact protoplasts requires efficient selective media which will favour the division of hetero-protoplast fusion products rather than the non-fused or auto-fused parental type protoplasts. The method of selection is based on complementation between protoplasts having different nutritional or hormonal requirements or different sensitivities to inhibitory compounds (e.g. see review of Schieder, 1982)* Complete fusion of diploid components usually results in tetraploid products.

Another method, developed by Zelcer et al (1978) is based on the combination of specific nutritional requirements of one protoplast parent and the effect X-irradiation on the other protoplast parent. Such a method termed "Donor-Recipient" fusion will combine two plasmones and one nuclear genome into fusion products and ultimately lead to functional plants having the recipient nuclear genome and novel plasmone combinations.

Although our results indicated a possible choice of selective media based on differential responses of various protoplasts to sorbitol, fructose, NAA and 2,4-D, preliminary experiments, using these selective media, did not differentiate between parent protoplasts and fusion products to a satisfactory degree. Therefore we employed the following strategies:

a) Iodoacetate treatment

As reported by Nehls(1978) and Medgyesy et al (1980) and based on previous studies with mammalian cells, iodoacetate treated protoplasts are irreversibly damaged unless fused with untreated protoplasts, or X-irradiated protoplasts. We thus treated protoplasts derived from several of our callus lines with various doses of iodoacetate. We found that exposure to ca 0.25 mM iodoacetate for 20 minutes is the lowest concentration which will consistently prevent division of unfused Citrus protoplasts. We also retested the dose effect of gamma rays (Cobalt source) for feeder layer and fusion experiments and found that the dose required for most lines is much higher (i.e. about 50 kR) than previously reported (Vardi and Raveh 1976).

b) Use of non-dividing protoplasts.

It was reported that fusion of protoplasts unable to divide under certain conditions, with protoplasts possessing capacity for division may result in division of fusion products and ultimately in hybrid plants (e.g. Izhar and Power, 1978). Because of close taxonomical relatedness within the Citrus genus (e.g. Vardi and Spiegel-Roy, 1978) no morphological markers are yet available in Citrus species to differentiate zygotic seedlings or somatic hybrids from the maternal type. Only Citrus relatives have distinctive leaf shapes such as the trifoliate leaf of Poncirus trifoliata or that of Citrus x Poncirus hybrids e.g. Troyer citrange (C. sinensis x P. trifoliata) Troyer citrange callus line (derived from epicotyl of young seedlings) as well as epicotyls and young leaves produced a large amount of protoplasts, however when cultured under our standard Citrus protoplast culture conditions they do not divide. These protoplasts could be used in fusion experiments with another protoplast partners derived from nucellar callus lines possessing division capacity.

Combinations in which fusion was attempted, are given in Table 10. The two partners were selected according to specifications noted in Table 9 i.e. the possible recognition of fusion products by isozyme patterns.

Fusion combinations between gamma irradiated protoplasts with iodoacetate treated ones (i.e. combinations 1 to 4 in Table 10) have resulted in colonies in all cases, and also yielded recently embryos which regenerated into plants. Roots from 20 of these plants (6 from

Table 10. List of fusion combinations performed between irradiated protoplasts and iodoacetate treated protoplasts, as well as with non-dividing leaf protoplasts respectively.

Combination number	Iodoacetate treated protoplasts	Irradiated protoplast (50KR)	Non-dividing leaf protoplasts
1	Sour orange	Villafranca	-
2.	Sour orange	Volckameriana	-
3.	Villafranca	Sour orange	-
4.	Volckameriana	Sour orange	-
5.	-	Sour orange	Troyer citrange
6.	-	Villafranca	Troyer citrange
7.	-	Volckameriana	Troyer citrange

combination 2 and 14 from combination 3 listed in Table 10) were analyzed for their peroxidase patterns. The isozyme patterns obtained did not resemble in any case the iodoacetate treated protoplast parent (F and G in Fig. 4). All of them, with two exceptions recalled the peroxidase isozyme pattern of Shamouti orange (C in Fig. 4). Roots from the two exceptional plants had a slightly differing pattern from the other plants. One should however consider the fact that isozyme patterns described in Figs. 4-6 have been obtained from apomictic seedlings grown in vivo while the examination of the root peroxidase isozyme pattern referred to in our fusion work concerns roots from embryos originating from protoplasts in culture. A larger number of roots are now developing on the regenerated plants so far tested. At the same time a larger additional number of embryos and plants are now developing from combination 1 to 4 listed in Table 10. It is our intention, to examine as soon as feasible isozyme patterns of at least three additional systems: MDH, GOT and esterase.

Fusion between irradiated protoplasts originated from nucellar callus and non-dividing leaf protoplasts of Troyer citrange (Table 10) were assessed by counting, after plating, the number of cells indicating fusion. This has been facilitated by the fact that leaf protoplasts contain visible chloroplasts, while callus-derived protoplasts are loaded with starch granules. Thus hybrid cells possess both chloroplasts and starch granules. We obtained 8 to 9% of fusion based on counts of 1000 cells in each of the three combination, 5 to 7, listed in Table 10.

While combinations between iodoacetate and gamma-ray treated protoplasts were plated above a feeder layer composed of the same gamma-rays treated protoplasts, in the fusion combination of non-dividing protoplasts with irradiated ones, we added irradiated protoplasts to the fused-protoplast layer to serve as "internal" feeders. This, of course, was done only in plates that did not serve for the purpose of counting and determining percentage of fusion.

The fusions which contained leaf protoplasts as one of the partners were in any case, not long lasting: only a few divisions were observed resulting occasionally in small colonies of about 16 cells. Even these small colonies subsequently declined. Thus, if leaf-protoplasts should serve in future fusion combinations - a change in procedure is obviously required.

DISCUSSION

Protoplast systems in Citrus and Microcitrus. - The scope and possibilities of protoplast fusion work was considerable widened by our attempt to increase the number and the variety of protoplast systems in Citrus and in related genera. Thus the number of successfully established regenerating protoplast systems in Citrus (species and cultivars) has been brought up by us to 10, and further work is in progress, through establishment of additional nucellar calli from other species, hybrids and varieties - a prerequisite for protoplast isolation and culture. In addition we were able lately to establish a regenerating protoplast system from Microcitrus. This is the first case of establishment of a protoplast system in a wild related genus to Citrus. The latter achievement could open up new possibilities in characterizing fusion products via morphological markers, as Microcitrus leaves are very small and distinctly lanceolate.

Cytological stability and auxin autotrophy. Cytological examinations carried out by us have confirmed, save in one case (Villafranca lemon), the consistent diploid state of calli and protoplast derived plants in our systems.

In existing protoplast systems which lead to plant regeneration, except Citrus, auxin is an obligatory requirement for protoplast

division. The absence of auxin in the culture medium used for callus initiation and protoplast culture in Citrus may be related to the long lasting diploidy in our callus and protoplast systems.

Medium components as tools for hybrid selection.- The auxin autotrophy of Citrus protoplasts led us to investigate the possibility of defining selection media on the basis of different sensitivities to synthetic auxins, such as NAA and 2.4-D. Thus, Villafranca (C. limon) protoplasts grew on higher NAA and also 2.4-D concentrations than protoplasts from other sources. The use of these findings to construct an efficient selective medium was so far not possible, due to the persistent (though low) number of divisions occurring in "sensitive" protoplasts with plating efficiency in the control attaining 7% or higher.

Another approach to construct a medium for hybrid selection, explored by us, stemmed from the testing of osmotic stabilizers in varying proportions, for their ability to enhance protoplast division. We found that when an increasing proportion of either sorbitol or mannitol, in addition to sucrose, was added, sorbitol tends to restrict cell division much more severely in mandarins (Murcott, Ponkan, Dancy) than in lemon or orange protoplasts. Therefore, sorbitol could be considered as a possible selection medium, in fusion combinations involving mandarin protoplast. However, so far mandarin protoplasts have not been used in our fusion experiments; moreover since the use of another prospective marker-like synthetic auxins did not prove efficient enough, no additional attempts were made to pursue this direction.

A different approach evolved from experience gained by us (Button et al. 1976; Spiegel-Roy et al, 1977) and others (Esen and Soost, 1976; Torres et al, 1978) in isozyme determination for the purpose of distinguishing between nucellar and zygotic progenies in Citrus.

An analysis of electrophoretic mobilities of ten enzymes in nine different Citrus nucellar calli and their corresponding roots from apomictic seedlings has been performed in order to find the most suitable system(s) for identifying cell hybrids. The system developed met the following requirements: a) constancy of isozyme pattern and independence of age and stage of development. b) a limited number of bands. c) different isozyme patterns of parental cells.

Somatic hybrids versus cybrids. - We planned to perform our fusion experiments essentially between diploid parents. This would give rise to a certain number of tetraploid plants. It seems, however, that tetraploid Citrus cultivars present only limited practical interest (Cameron and Frost, 1968) except perhaps for further breeding by sexual hybridization. While very little experience has been gained with tetraploid Citrus as a rootstock, their use may be largely questioned on the basis of recent evidence (Barrett and Hutchison 1978) concerning very restricted root systems in tetraploid apomictic seedlings. We therefore, were interested in studying and developing cybrids from protoplast fusion. Because of the earlier referred to obstacles in devising dependable selection media, the "donor -recipient" method has been adopted by us. The recipient of cytoplasmic organelles (contributing the nuclear genome) has been treated with iodoacetate and

the donor of the organelles by irradiation with gamma rays. Higher irradiation doses had to be used (50KR) than those reported in our earlier experiments (Vardi et al, 1975; Vardi and Raveh, 1976).

Some indication on the percentage of successful fusion could be gained from fusion experiments between leaf protoplasts and protoplast from the whitish nucellar callus. Percentage of successful fusions varied in the different combinations, between 8-9%. In addition, several fusion combinations between different protoplasts originating from nucellar callus were performed, resulting in colony and plant formation. In spite of progress made, identification of fusion products is still met with difficulty. Identification of cybrids could be based either on expression of nuclear genes e.g. isozyme patterns or by making use of organelle - DNA restriction patterns (see: Galun 1982), a method not yet developed for Citrus. In our efforts to identify cybrids, so far, only peroxidase isozymes were examined. Results have been unsatisfactory since in all cases a pattern differing from the iodoacetate treated parent has been obtained. This may have been due either to the lesser consistency often shown by peroxidase banding (Siegel and Galston, 1967) or moreover one has to take into consideration the fact that isozyme patterns examined in our work included so far only callus and roots from apomictic seedlings. Up to the compilation of this report no roots from plants derived directly from callus or from protoplasts were examined. This will have to be performed in the future in order to obtain a more reliable identification and evaluation of fusion products. Over one hundred embryos derived from fusion experiments are now in the process of root

and plant development and will be available for examination in the near future.

Prospectives of somatic hybridization and and cybridization in Citrus and related genera. - It has been shown in our work that fusion between different Citrus protoplasts can now be achieved with comparative ease. Methods developed with other systems for cybrids seem also to be applicable to our Citrus protoplast systems. However, in the field of identification of fusion products further effort will be required, namely examination of several enzyme systems in roots in addition to peroxidase. There is also a need to search for methods in inducing division in Citrus protoplast systems other than the nucellar one.

Conclusion - The basis has been laid for fusion in Citrus protoplast systems and for the establishment of somatic cybrids. The scope and the genetic diversity of material available for such work has been considerably broadened. There are still unsolved problems in identification of fusion products and the methods leading to protoplast division from sources other than nucellar callus; these can and should be improved.

EVALUATION OF THE RESEARCH ACHIEVEMENT WITH RESPECT TO THE ORIGINAL
RESEARCH PROPOSAL.

Progress has been made along all the main objectives of our original proposal. Thus, many additional nucellar callus lines in Citrus were established -totalling at present ten such lines (three oranges, three mandarins, one lemon, one grapefruit, one sour orange, and one hybrid) in which the protoplast system was successfully established. The cycle involves explant - callus - protoplast - cell division - colony formation - embryogenesis - plant regeneration. In addition, such a protoplast system was also established in a wild relative of Citrus, from another genus, Microcitrus.

We also developed a method for isolation of protoplasts from other plant organs such as epicotyl and leaf. These protoplasts do not divide under standard protoplast culture conditions but can serve as fusion partners and markers in somatic hybridization studies.

Various approaches have been employed in order to work out methods for selection of somatic hybrids after protoplast fusion. Callus lines were found to differ in auxin sensitivity and reaction to several sugars. Nine enzyme systems have been examined in calli and roots in order to find a suitable system for identifying hybrids. On the basis of this

work, recognition of fusion products should be possible in a number of combinations of fusion partners. This work will have to be complemented in the future by an additional analysis of electrophoretic mobilities in enzyme systems in roots from embryos or plants derived from callus and protoplast.

Metabolic inactivation of protoplasts by iodoacetate and the determination of radiosensitivity of the different protoplasts have also paved the way for fusion experiments with the purpose of creating somatic cybrids. This phase of our work was not envisaged in the original proposal, though it presents a natural extension of the program after a certain stage of development has been reached.

In the protoplast fusion phase of our studies, fusion tests have been performed between protoplasts derived from whitish nucellar callus and green nondividing protoplasts derived from leaves. These have given a fair estimate of fusants. Somatic hybridization between Troyer citrange and Sour orange may ultimately yield combinations for root stock characteristics of exceptional interest.

Further successful fusions were performed between different protoplasts originating from callus. Fusion between different genera (e.g. between Citrus and Microcitrus or between Citrus and Poncirus) will be certainly of great practical interest.

In conclusion, all the main objectives of the original proposal have been substantially fulfilled. The scope of material available for fusion has been broadened considerably; methods have been worked out for

fusion of protoplast and for creation of cybrids. While some procedures have been also worked out for identification of fusants and cybrids, we are also aware of the need for enlarging this aspect of the work in the future in order to pave the way for further progress.

DESCRIPTION AND RESULTS OF COOPERATION

In addition to contact by correspondence between the Principal Investigators and the Cooperating Investigator, Dr. Aliza Vardi stayed during the second half of May, 1981 with Dr. D.J. Hutchison (the Cooperating Investigator) at the Horticultural Research laboratory, Orlando, Florida and thoroughly discussed our research with Dr. Hutchison, Dr. H.C. Barrett and other staff members of this laboratory. A possible disease-resistance (mainly to Nematode and Phytophthora) testing collaboration to screen our future somatic hybrid plants was outlined. During part of Dr. Vardi's stay at Orlando, she was joined by Prof. E. Galun. We isolated several hundred ovules (of Microcitrus hybrida and M. papuana) from young fruits of trees growing at the Orlando collection. These ovules were cultured on BM-ME medium and then brought to Bet Dagan. Callus was developed from which during 1982-3, a protoplast system was successfully established from Microcitrus.

Dr. Hutchison has visited the Fruit Tree Breeding and Genetics Department and the Weizmann Institute during November 1982. During his visit various phases of the project were discussed and conclusions reached focussed on the choice of desirable partners for somatic hybridization, especially breeding material for improved rootstocks.

Prof. P. Spiegel-Roy also held consultations with Dr. Hutchison during a short stay in Orlando in December 1982.

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