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Plant Regeneration from *Citrus* Protoplasts: Variability in Methodological Requirements Among Cultivars and Species *

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Summary. Nucellar callus lines were established from two orange cultivars ('Nucellar Shamouti', 'Shamouti Landau'), three mandarin cultivars ('Murcott', 'Dancy', 'Ponkan') one grapefruit cultivar ('Duncan') and sour orange (*Citrus aurantium*). These callus lines were initiated from in vitro cultured ovules of young fruits and maintained an embryogenic capacity. The plating efficiencies of protoplasts derived from these calli, as well as those of protoplasts from lemon (cv. 'Villafranca') nucellar callus were differentially affected by the maceration enzymes and by the sugars used as osmotic stabilizers. Plants with normal morphological features were regenerated from cultured protoplasts derived from each of the nucellar callus lines. The establishment of eight new protoplast systems in *Citrus* paves the way for cell genetics studies and for novel breeding approaches in these economically important orchard trees.

Key words: Citrus – nucellar callus – Maceration enzymes-Osmotic stabilizers-Plating efficiency – Plant regeneration

Introduction

Implementation of novel approaches to plant cell genetics and crop improvement (Melchers 1980) requires efficient "protoplast systems", i.e. reproducible techniques for the regeneration of functional plants from isolated protoplasts. In spite of considerable efforts in the last decade, "protoplast systems" were reported in only a dozen genera (see Vasil and Vasil 1980). Moreover *Citrus* was the only arboraceous genus among these plants.

We previously reported 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 (*Citrus sinensis* (L.) Osbeck cv. 'Shamouti'). Regeneration of plants from protoplasts and prospects for obtaining somatic hybrids by protoplast fusion are of great interest in *Citrus*, as a) *Citrus* belongs to a group of woody plants having a conspicuous and long juvenile phase differing from other existing "protoplast systems" (all are herbaceous plants), in several physiological aspects; b) while sexual reproduction and hybridization in *Citrus* is hampered by heterozygosity, sterility and nucellar embryony (Cameron and Frost 1968; Vardi and Spiegel-Roy 1978), reproduction by protoplast culture and somatic hybridization by protoplast fusion should be useful procedures in this genus. Therefore we intended to extend the number of *Citrus* species and cultivars in which protoplasts could be readily isolated from respective nucellar calli and subsequently cultured in vitro and regenerated into functional plants. The present report deals with the establishment of eight additional protoplast systems in *Citrus*.

Materials and Methods

Establishment and Maintenance of Nucellar Calli

Nucellar calli were obtained as described (Kochba et al. 1972) with few modifications. Ovules of seven species and cultivars, as detailed in **Results** 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. Before autoclaving, the pH was adjusted to 5.7 and supplemented with 10 g · l^{-1} Difco agar. The dishes were maintained at 26±1 °C with 16 h of dim light daily. The nucellar callus which emerged from the cultured ovules was subcultured on BM-ME for the first three passages and then on BM at the same temperature and light regimes.

An additional nucellar callus line derived from the lemon (*Citrus limon* (L.) Burm) cv 'Villafranca' was obtained from the late Dr. J. Kochba.

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Isolation and Plating of Protoplasts

For protoplast isolation calli were subcultured every two to three weeks on BM. About 0.5 g callus was placed in each 10 cm Petri dish and 10 ml maceration medium was added. The maceration medium consisted of 0,3% Pectinase (fungal, Koch-Light Laboratories), 0.2% Cellulase (Onozuka R10, Kinki Yakult Mfg. Co.) and 0.1% Driselase (Kyowa Hakko Kogyo Co.) dissolved in 0.14 M sucrose and 0.56 M mannitol. Recently this medium was modified by the addition of BM macroelements to achieve more consistent results. The maceration solution was filter-(0.2 μm) sterilized before use. After overnight incubation (26±1 °C) the protoplasts were isolated by sequential filtering through nylon screens of 375, 50 and 30 μm pore openings, followed by three centrifugations (about 100×g, 5 min) in a washing solution (BM containing 0.3 M sucrose and 0.3 M mannitol). The protoplasts were suspended at a final density of $1.5 \cdot 10^5$ or 10^5 cells per ml of BM containing soft (0.6%) agar and plated as 4 ml aliquotes in 5 cm, Parafilm sealed, Petri plates. The plates were maintained under continuous dim light at 26±1 °C and colony formation was scored as described (Vardi and Raveh 1976) four to five weeks after plating.

For chromosome counts, pieces were removed from logaritmic-phase callus cultures and treated with paradichlorobenzene for 4 h, then fixed in ethanol : chloroform : acetic acid (6:3:1) for 24 h, stored in 70% ethanol and stained with aceto-carmine.

Results

Establishment of Nucellar Callus Lines

Using methods which were similar to those described previously (Kochba et al. 1972) we isolated ovules of the following:

- *Citrus sinensis* (L.) Osbeck (orange) cv. 'Nucellar Shamouti', cv. 'Shamouti Landau';
- *Citrus aurantium* L. (sour orange);
- *Citrus reticulata* Blanco (mandarin) cv. 'Murcott', cv. 'Dancy', cv. 'Ponkan';
- *Citrus paradisi* Macf. (grapefruit) cv. 'Duncan'.

Embryoids followed by whitish callus started to emerge from cultured ovules one, two or even up to four months after the start of culture (Fig. 1). Microdissections indicated that the embryoids and calli were of nucellar origin. The frequency of callus producing ovules was variable and usually rather low (about 24 percent in 'Murcott' and 'Ponkan' mandarin and two percent in 'Duncan' grapefruit, representing the most and least "efficient" ovaries, respectively). The emerging callus was sub-cultured three times on BM-ME and then maintained on BM. As preliminary tests indicated that nucellar callus was inhibited or callus formation from the integuments unduly stimulated as a result of the addition of growth regulators, the later were omitted from the medium during both isolation and subculture. All above mentioned nucellar callus lines showed embryogenic capacity as shown in Fig. 2 for 'Dancy' mandarin and 'Ponkan' mandarin; even after repeated subcultures embryoidal centers were initiated on the callus and these developed into globular or torpedo shaped embryos. When the torpedo shaped embryos were isolated they regenerated into plants.

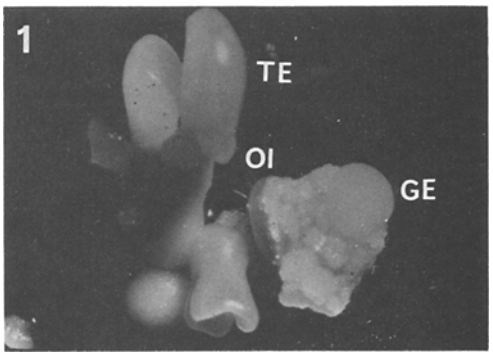

Fig. 1. Callus and embryoids emerging from a cultured 'Ponkan' ovule; the larger torpedo embryoids (TE) were moved to the left to reveal the ovular integument (OI), with the attached callus and globular embryoids (GE). Photographed 6 weeks after beginning of ovule culture (× 12)

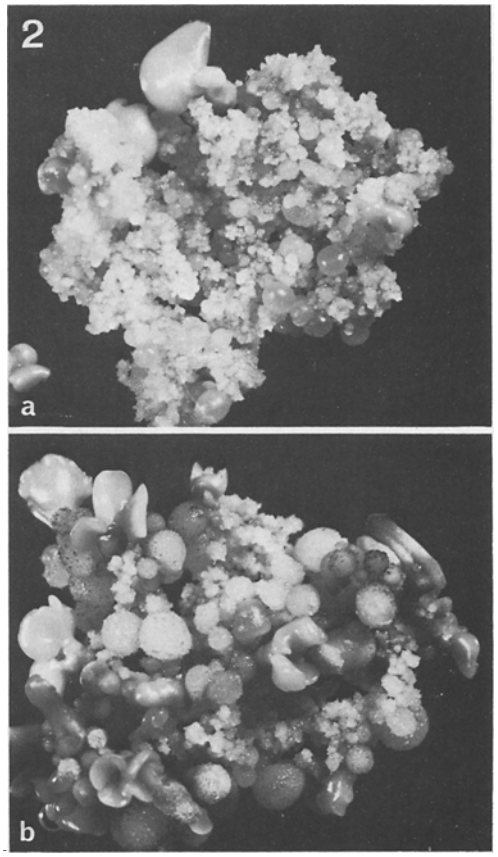

Fig. 2 a and b. Nucellar calli derived from cultured ovaries dissected from young *Citrus* fruits (× 5). **a** 'Dancy' mandarin; **b** 'Ponkan' mandarin

The chromosome numbers of these callus lines, maintained in culture for at least one year, as well as of

a lemon cultivar (cv. 'Villafranca') nucellar callus line are presented in Table 1. All the new callus lines maintained the diploid chromosome number (2n = 18) while a deviation from 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 2,4-D was added to the BM-ME while no auxin was employed during the initiation and culture of the other new seven callus lines. Hence, the partial tetraploidy of 'Villafranca' lemon callus could be related to the addition of 2,4-D to the medium (Singh and Harvey 1975).

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 with each of the eight callus lines listed in Table 1. However, variable results, especially when a new stock of Pectinase was employed (fungal, Koch Light laboratories), prompted us to retest the effect of maceration enzymes on the subsequent division capacities of isolated protoplasts. Since the variability in results seemed to be related to the pectinase source, we concentrated on the relative effects of Koch Light Pectinase and Macerozyme on the ability of protoplasts to form colonies (Table 2). 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 the combination of Driselase and Macerozyme. Protoplasts having division capacity could be isolated from 'Duncan' callus when Macerozyme was substituted for Pectinase and Driselase was omitted, as well as from 'Dancy' callus only following maceration in a macerozyme – Cellulase mixture, in the absence of Driselase.

We tested the capacities of protoplasts from the eight callus lines to divide in BM containing osmotic stabilizers previously used (i.e. a mixture of sorbitol and mannitol) for 'Shamouti' protoplasts. Very low protoplast plating efficiencies were found with some of the new callus lines. Additional combinations of osmotic stabilizers were therefore tested (Table 3). 'Murcott' protoplasts (as well as the two other mandarin protoplasts, 'Ponkan' and 'Dancy', not shown) 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 efficiencies 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 is feasible. We therefore adopted the 0.3 M sucrose plus 0.3 M mannitol mixture as our standard osmotic stabilizer.

Table 1. Chromosome counts in established nucellar callus lines of eight *Citrus* species and cultivars

Lines	Cells with chromosome number				Total number of cells
	18	19	36	39 – 40	
Orange (*C. sinensis* (L.) Osbeck)					
'Nucellar Shamouti'	10				10
'Shamouti Landau'	10				10
Sour orange (*C. aurantium* (L.))	17	3			20
Mandarin (*C. reticulata* Blanco)					
'Murcott'	13				13
'Dancy'	10				10
'Ponkan'	10				10
Grapefruit (*C. paradisi* Macf.)					
'Duncan'	3				3
Lemon (*C. limon* (L.). Burm)					
'Villafranca'	3		5	1	9

Table 2. 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 (± SE) per ml, counted 5 weeks after plating. Plating efficiencies (in percent) are given in parentheses

Callus line	Pectinase in maceration fluid	
	Koch-light pectinase	Macerozyme
'Nucellar Shamouti'	4,727 ± 291 (3.78)	27,818 ± 1,054 (20.65)
'Shamouti Landau'	7,818 ± 654 (6.25)	17,636 ± 645 (14.10)
'Murcott'	8,454 ± 582 (6.78)	13,545 ± 900 (10.83)
'Duncan'[a]	0	818 ± 282 (0.65)
'Villafranca'	16,182 ± 6,091 (12.94)	7,636 ± 545 (6.10)

[a] Colony formation following maceration by 0.2% Cellulase and 0.3% Macerozyme (without Driselase) was: 12,954 ± 1,227 (10.36)

Fig. 3a and b. Young *Citrus* plants derived from cultured protoplasts, photographed 5 months after protoplast plating (×1/3). **a** Orange ('nucellar Shamouti'). Mandarin ('Murcott', 'Dancy', 'Ponkan') and Lemon ('Villafranca') plants; **b** Plants derived from 'Dancy' protoplasts – note uniformity

Table 3. 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 (± SE) five weeks after plating; plating efficiencies (in percent) are given in parentheses.

Callus line	Osmotic stabilizers added to BM				
	0.14 M Suc 0.14 M Mann	0.14 M Suc 0.46 M Sor	0.3 M Suc 0.3 M Mann	0.3 M Suc 0.3 M Sor	0.6 M Suc
'Nucellar Shamouti'	5,727±427 (3.80)	4,636±235 (3.08)	5,091±536 (3.38)	4,454±464 (2.98)	6,909±973 (4.60)
'Shamouti Landau'	3,273±373 (2.18)	5,864±691 (3.90)	17,954±1,036 (11.97)	9,682±554 (6.45)	25,727±918 (17.15)
'Sour orange'	10,000±627 (10.00)	10,091±1,217 (10.09)	13,568±841 (13.56)	12,068±541 (12.06)	15,273±704 (15.27)
'Murcott'	1,318±209 (0.87)	500±145 (0.33)	16,454±609 (10.96)	2,635±318 (1.75)	23,454±700 (15.63)
'Villafranca'	7,682±436 (5.12)	6,318±409 (4.21)	13,227±591 (8.81)	8,727±527 (5.82)	11,864±664 (7.91)

Isolation of Protoplast Derived Embryoids and Plant Regeneration

After 6 to 10 weeks of culture the protoplast derived colonies attained a diameter of 0.3 to 1.0 mm. At least some of the colonies started to form green globular structures 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 an additional 4 to 6 weeks, embryoids, most of which were at the torpedo stage, were transferred individually into test tubes containing 10 ppm GA as described by Kochba and Spiegel-Roy (1976), to promote axis elongation. Plantlets were then transferred to Jiffy pots, covered with plastic bags, gradually acclimatized to lower humidity and higher light intensities in an illuminated culture room and finally transferred to 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 regenerated from protoplasts from each of the eight callus lines listed in Table 1. Plants from five of these lines are shown in Fig. 3.

Discussion

The full sequence from intact ovaries through in vitro cultured callus, isolated protoplasts, protoplast derived embryogenic callus to the establishment of morphologically normal plants was achieved with cultivars of orange, mandarin, grapefruit and lemon as well as with sour orange. In vitro culture did not cause a change from the original diploid state in all but the 'Villafranca' lemon. Maintenance of diploidy in our material is noteworthy since polyploidization was frequently observed in protoplast derived plants (e.g. *Nicotiana sylvestris,* see Zelcer et al. 1978). Some of the 'Shamouti' plants, obtained by us from protoplasts (Vardi et al. 1975; Vardi 1977 and 1978) were grafted on sour orange, reached the fruiting stage and proved to be diploids. Among the existing protoplast systems (Vasil and Vasil 1980) *Citrus* is outstanding in another important aspect: in all the systems but *Citrus,* auxin is an obligatory requirement for protoplast division. The absence of auxin in the culture medium used for nucellar callus initiation and protoplast culture in *Citrus,* may be related to the maintenance of diploidy in the protoplast system of this genus.

Although the protoplasts derived from the eight nucellar callus lines did show variation in sensitivity to certain isolation and culture conditions (i.e. maceration enzymes and sorbitol) they shared the same optimal conditions for the induction of cell division and plant regeneration. On the other hand, *Petunia* species were found to have different medium requirements, for protoplast division and plant regeneration especially with respect to growth regulators (Power et al. 1976). Similarly Bourgin et al. (1979) reported that the conditions for protoplast culture and plant regeneration varied considerably among *Nicotiana* species, and that in some of the *Nicotiana* species neither plant regeneration nor protoplast division could be induced. Uniformity in the culture requirements found in this investigation could perhaps be explained by the taxonomical relatedness within, the *Citrus* genus (Barrett and Rhodes 1976; Vardi and Spiegel-Roy 1978). Further studies by us seem to indicate that uniformity in the standard culture conditions does not preclude intercultivar different isoenzyme patterns as well as differential reactivity to synthetic auxins and to mono- and disaccharides (to be published).

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