PRODUCTION OF SOLID MUTANTS IN CITRUS, UTILIZING NEW APPROACHES AND TECHNIQUES*

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Abstract

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Conditions for embryoid differentiation in Shamouti orange ovular callus were studied. Lines differing in embryogenic capacity were established. Ageing of callus prior to subculture enhanced embryoid development. A pronounced habituation effect has been found in most cultures. Protoplasts have been obtained from callus after treatment with cellolytic enzymes.[1]

Irradiation of any embryogenic line of callus with 12-20 kR seemed to promote embryoid formation, after a time lag in their appearance. Transferrence of embryoids into agar+sucrose and GA_3 and a further transfer into agar+sucrose, GA_3 and adenine sulphate gave best results as to rooting of embryoids and plant survival.

The technique of using decapitated nucellar seedlings for mutagenic treatment was developed further with Shamouti orange and Marsh grapefruit. Irradiation 48-72 hours after decapitation with 2 kR resulted in shoot neoformation and survival not much below that of control, while 6 kR impeded de novo shoot formation in Marsh grapefruit.

A tendency towards mutants with earlier fruit maturity was found in mV_2 plants from material originating from irradiation with the 8-kR dose.

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[1] By Aliza Vardi of our Division and in cooperation with the Senior author and Professor Ezra Galun of the Weizmann Institute of Science, Rehovat.

* Research supported under IAEA Research Contract No. 1273.

Contribution From The Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. 1974 series, No. 219-E.

INTRODUCTION

The use of ionizing radiation for the induction of mutations may be of importance in the improvement of clonally propagated species.

Hybridization efforts are greatly hampered in citrus by polyembryony and sterility (3). Many of the improved citrus varieties originated as natural budsports.

A mutation breeding program, making use of irradiated budwood and the bud isolation technique (production of mV_2 plants), has been initiated by us. At the meeting in Vienna (14) we reported on methods developed by us in citrus, aiming at a reduction in chimera formation and adapted in principle to achieve solid mutants.

This report will deal mainly with two aspects, namely, (a) steps in the production of plants from nucellar callus, and (b) response to decapitated seedling stems through neoformation of shoots from callus.

Sabharwal (13) was the first to show that culture of excised nucelli of polyembryonic species could give rise to adventive embryos, which in rare cases developed into seedlings. Other reports on the establishment of embryoids from citrus nucellar tissue followed (1,6,7,11). The subject has been reviewed recently, along with the related problem of tissue culture and plant propagation (12). In our previous work (6,7) we found that ovules yielded more successful cultures than nucelli with the Shamouti orange. Through culturing ovules on a basal medium containing malt extract and sucrose, a friable embryogenic callus developed (2). This callus, lines of which are being maintained in culture through continuous subculturing, serves as a source for irradiation studies, for production of mutants and for various physiological experiments.

Mendel (10) described regeneration of shoot primordia from decapitated hypocotyls of Annona cherimola. Differentiation of shoot primordia, subsequent to callus formation, takes place readily in decapitated Shamouti epicotyls (14).

We have examined this phenomenon, as well as the response to irradiation of the seedlings after decapitation of stems, in additional citrus varieties. Stems from juvenile citrus seedlings were recently found to yield callus and to regenerate plants in vitro (4). Radiosensitivity of citrus seeds and buds has been reported by Spiegel-Roy and Padova (16).

1. The Callus-Irradiation-Embryoids-Plantlet-Established Plant Sequence.

Based on previous work we have now established in culture numerous lines of callus obtained from culturing Shamoutk (Citrus sinensis) ovules in vitro. Some of these have been grown by consecutivepassages for more than 2-1/2 years. These lines differ in embryogenic capacity, with some of them being devoid of it. Most, but not all lines have become habituated and will grow and differentiate successfully on BM only. Irradiation at the ca-lus stage will give rise, in most cases, to plants of single cell origin (2). Special care must be exercised to insure survival of plants after cessation of culture in vitro and transfer to soil or other media.

The main progress during the last two years has been in the following aspects. (i) A better understanding of the fine structure of the callus: It was found that the callus is made up of proembryoids; embryogenesis occurs in single cells on the periphery and within existing proembryoids. (ii) The establishment of different lines of callus, many of which can be made to differentiate with the aid of a longer period between passages ageing, transfer to BM, and sugar starvation. (iii) Protoplasts have been established with the aid of cellolytic enzymes; these in turn regenerate new callus, with a potential for differentiation. (iv) The effect of irradiation on enhancement of differentiation has been re established with certain dose-rates. (Incidentally, a similar effect was found with callus obtained subsequent to protoplast irradiation.) (v) A notable improvement in rooting of embryoids,and subsequent survival, was found to take place in the presence of GA_3 and adenine (8).

We will now examine, in somewhat more detail,the progress of these investigations, on each point mentioned.

Callus structure. Fig. 1 shows that, unlike most plant cell1 that have a compact or amorphous form, the Shamoutl nucellar callus consists of compact spherical nodules of 0.1-1.0 mm diameter. These give the callus a porous, friable texture. The pro embryoids develop from single cells on the periphery of existing pro embryoids, and sometimes from a single cell or a small group of cells in the interior. This part of the work was performed during Dr. Kochba's stay at Natal University by Button, Kochba and Bornman (2). As plantlets are derived mainly from single cells, this permits development of solid mutants after irradiation directly, or after a further passage in culture.

Factors influencing embryo-genesis. Embryo-genesis has been shown to be influenced by a variety of factors. One of these is the age of callus prior

to subculture (9). The effect is noticeable only upon subculturing and does not depend on the overall age of the callus. Table 1 shows that in this experiment the effect on number of embryoids per culture and percentage of differentiating cultures is largest after ageing the callus for 14 weeks. Callus aged for 20 weeks lost the propensity for embryoid development. Interestingly enough surcrose starvation (no sucrose in the medium) had a similar effect,but the effect was not persistant. Table 2 brings out the overall ageing effect even more clearly.

Subcultures of the embryogenic ovular callus from Shamouti orange have now been maintained for nearly 3 years. During the second year of subculture evidence accumulated concerning a habituation effect in most media. A culture growing on BM+malt extract (500 mg/l) has been established on BM only and has been grown since - for 2 years - on BM alone without any decline in growth potential. Similarly callus from many other media showed much better growth on BM than when grown continuously on the same media. As seen from Table 2, cultures were maintained for eight passages on different media; subsequent growth was determined, for three passages of 6 weeks each, on the same media and compared with the growth made upon transfer to BM only. A considerable increase in growth of tissue was observed on BM alone, with all except three media (ADS 20 mg/l kinetin; 0.1+IAA 1.0 mg/l; and kinetin 1.0+ADS 40 mg/l). The largest difference in favour of BM (which would mean maximization of the habituation effect) was encountered on media containing malt extract 500 mg/l and also for kinetin 1.0 mg/l+ IAA 1.0 mg/l (Table 3). It is remarkable that media with the high kinetin concentration (1 mgl/l showed a very large habituation effect, except when kinetin was combined with adenine. The ovular callus, though autonomous in growth, did not lose the capacity for differentiating embryoids. Cultures of the different buds have now been maintained on BM alone for more than a year without a decline in growth.

Effect of irradiation. The effect of irradiation on embryoid differentiation seems to be more complicated. In our previous report we remarked on the enhancement of embryoid formation after irradiation of the callus with 16 kR (radiation intensity 3.1 kR/hr) (14). The effect of irradiation seemed to be due to a large extent to changes brought about in the growth medium (15). Evidence for this effect is shown in Table 4. The effect of light was studied in another experiment. Callus weight was greater in darkness than in 16-h light. A depressing effect of irradiation on callus growth was found, but callus weight was still satisfactory, with practically no loss in embryoid forming propensity. No appreciable difference in number of irradiated embryoids between callus grown in light or darkness was found (Table 5).

At first it seemed as if normal (auxin- and kinetin-requiring) callus produced more embryoids with certain irradiation doses, while the response of habituated callus did not seem to show such a relationship. Based on these findings a new experiment, in which callus from an embryogenic and a non-embryonic line, at the age of 5, 10 and 15 weeks prior to irradiation and subculture, is being carried out to examine the response to irradiation.

We isolated embryoids appearing at different intervals after irradiation and also subcultured tissue from irradiated cultures in search of new mutant embryoids. Irradiation is performed prior to subculture in a new, unirradiated medium. Table 6 shows the results of irradiation response of the 5- and 10-week-old callus. As a non-embryogenic line did not develop embryoids, results are given for an embryogenic line only. Stimulation of embryoid formation in the same range as observed before, (12-20 kR) is noticeable also with habituated callus tissue. However, irradiation definitely causes a time lag in the appearance of embryoids. This is in line with the known effect of irradiation on temperarily suppressed growth. The higher radiation intensities tested with the 12 kR dose only, seem to stimulate embryoid formation further, with an optimum so far at 85 kR/h. An unexplained decline with low radiation intensities has been found at 4 kR. A somewhat similar phenomenon was found in the experiment reported by us at the IAEA panel in 1972 (14); it concerned callus exposed to 2 kR.

Cytology and protoplast isolation. Our cytogeneticist, Dr. Aliza Vardi, has examined the number of chromosomes in the ovular callus. So far only counts of the diploid number (2n=18) have been noted. This would explain the relative stability of the callus as well as its prolonged capacity for morphogenesis.

By treatment of callus with enzymes causing polysaccharide degradation, protoplasts were established by Aliza Vardi, P.Spiegel-Roy and Ezra Galun.* (Fig.2) The effect of irradiation and mutagens on protoplasts is under study.

Callus from lemon ovules. We have not yet succeeded in culturing lemon ovules and nucelli in vitro. So far, only callus has been obtained from whole and sliced ovules. Addition of 0.5mg/l 2,4-D alone or in combination with 1.0 mg/l kinetin resulted in the best callus development; 10% Valencia orange juice produced less abundant callus, but more than kinetin alone or malt extract.

Rooting of embryoids and survival of plant formed from embryoids. In this part of our work the aims have been to increase the percentage of embryoids forming roots in vitro, and to increase survival of plants derived from

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embryoids of callus origin.

The best method at present seems to be to transfer green embryoids into agar + 5% sucrose + 1 mg/l GA_3 and to effect a further transfer after 10 days into agar + 5% sucrose + 1 mg/l GA_3 + 27 mg/l adenine sulphate. About 60% of the embryoids have rooted in the combined gibberellin + adenine treatment, as compared with only 15% rooting in the control.

In a more detailed study Kochba et al. (8) found that embryoid stages induced by GA_3 and ADS to form roots were those that have no or only a partially developed root zone. Large embryoids have been found to have a fully developed root zone and to form roots to a nearly comparable extent on BM only (Table 7). The need for ensuring better propagation techniques for the survival of tissue-culture-raised plants has been stresaed in a recent review on tissue culture and propagation (12).

2 Adventitious Bud Formation on Cut Stems

We will now turn to another method developed by us, namely, the use of decapitated nucellar seedlings for callus formation and subsequent neoformation of shoots. This technique lends itself well to mutation breeding and is simpler than the recently established method for growing stem segments of juvenile citrus material in vitro (4,5).

We have continued our previous work with Shamouti orange and included Marsh grapefruit in our trials. Based on previous results, doses up to 6 kR were applied and seedlings were decapitated (just below the first two leaves) 72 hours prior to irradiation. Humidity chambers for decapitated seedlings were improved in order to provide optimum and uniform humidity during callus formation and bud neoformation. Better results and survival were usually obtained in experiments carried out during winter and spring, than during summer. Treatments were designed in 3-5 replicated batches of 20 seedlings each. Results are given in Table 8. As mentioned, the response of decapitated seedlings is greatly influenced by temperature conditions in the greenhouse. Radiosensitivity was found to be greater with higher temperatures. Shamouti orange seedlings were more temperature-sensitive than Marsh grapefruit seedlings. This holds also for the unirradiated control. The LD for Shamout orange was in the range of 2-4 kR (closer to 4 kR); the same doses on Marsh grapefruit caused only a time lage im shoot formation, in the earlier experiment. In the later experiment shoot formation was strongly inhibited in Marsh after irradiation at 4 kR and 6 kR; 6 kR was more inhibiting for Marsh than for Shamouti.

Seedlings developing at least two new leaves were transplanted into black polyethylene containers and are growing in a greenhouse with partial shade. At the termination of the growing season, growth, number of leaves and any

aberrant characteristics will be recorded. Evaluation of mutation rate will be made after grafting on rootstocks next spring.

3. Shamouti Orange Plants from Irradiated Budwood

A substantial number of mV_2 Shamouti plants, having their origin in irradiated budwood, started to bear in 1973/74. Fruit from all trees was picked on January 7, 1974, and analyzed. Frist results have to be interpreted with caution, as fruit from Shamouti trees during the first years of fruiting tend to vary to a great extent, especially in shape, size, juice percentage, and peel thickness. A certain tendency to earlier maturity is becoming evident in some mV_2 trees that have been obtained from buds on shoots originating from budwood irradiated with 4-8 kR. This is manifested by a somewhat higher TSS content and a wider TSS/acid ratio. In some cases the skin has a deeper orange colour. Some fruit from these trees also had small protuberances; such protuberances have been found in isolated cases also in the control and other treatments.

Differences in peel smoothness were also found between treatments and between trees of the same treatment. The possible significance of this will be followed up during a detailed analysis of the next crop.

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Table 1. The effect of ageing tissue for various periods prior to subculture, on subsequent embryoid formation in culture (callus cultured on BM)

Age of callus prior to subculture (weeks)	Embryoids per culture (No.)	Differentiating cultures (%)	Avg. fresh weight of cultures (mg)
3	0.8	10	530
6	9.0	30	740
14	108.0	85	520
20	0.0	0	270

Table 2. Effect of callus ageing prior to subculture, on subsequent embryoid formation

Addition to BM	In unirradiated cultures prepared from 6-week-old callus		In unirradiated cultures prepared from 12-week-old callus	
	Embryoids/culture	% differentiating cultures	Embryoids/culture	% differentiating cultures
Kinetin 1mg/l + IAA 1 mg/l	0	0	9.0	40
Kinetin 0.1 mg/l	0	0	4.2	66
Kinetin 1.0 mg/l	1.4	5	38.2	100
IAA 1.0 mg/l	0.8	41	23.4	100
Kinetin 0.1 mg/l + IAA 1.0 mg/l	0	0	11.0	60
BM	0.2	5	25.8	100

Table 3. Growth of Shamouti orange ovular callus culture with and without additions to a basal medium[1]

Additions to BM (mg/l)		Average weight[2] in presence of addition (A)	Average weight[2] in absence of addition (B)	Growth ratio BA
Malt extract	250	250	647	2.58
"	500	120	687	5.72
"	1000	185	297	1.60
"	2000	85	160	2.00
Adenine sulphate	20	360	343	0.94*
"	40	150	380	2.53
"	80	340	480	1.41
"	120	75	180	2.40
Kinetin	0.1	256	434	1.69
"	1.0	278	454	1.63
IAA	0.1	340	487	1.43
"	1.0	200	493	2.46
Kin 0.1 + IAA	0.1	280	460	1.64
"	1.0	404	408	1.00
" 1,0	0.1	160	607	3.79
"	1.0	90	693	7.25
Kin 1.0 + ADS	40	360	387	1.07
IAA 1.0 + ADS	40	200	498	2.49

[1]Cultures were maintained on the same medium for 8 passages of 6 weeks each.

[2]Average weight of culture for three further passages (nos.9-11).

*Underlined values - no habituation.

Table 4. Effect of separate irradiation of growth medium and of callus on the number of embryoids differentiated from Shamouti orange ovular callus.
(Culture medium : basal medium plus malt extract, 500 mg/l; radiation dose, 16 kR; intensity, 3.1 kR/h.)

Treatment	Weight of culture (mg)	Number of embryoids per culture	Percent successful cultures
Irradiation of medium only	580[b]	27.0	87
Irradiation of medium and callus	440[b]	30.0	100
Irradiation of callus only	120[c]	18.0	95
No irradiation	700[a]	18.8	88

Figures followed by different letters differ statistically at P = 0.01.

Table 5. Effect of light and irradiation on callus growth and number of embryoids differentiating from Shamouti orange ovular callus.
(Culture medium: basal medium plus malt extract, 500 mg/l; radiation dose, 16 kR; intensity, 3.1 kR/h; irradiation of both callus and medium.)

Treatment	Weight of culture (mg)	Number of embryoids per culture					Percent successful cultures
		Small globular	Big globular	Small leafy	Big leafy	Total	
16-h light period, unirradiated	1140	13.3	13.0	5.4	7.2	39.4	100
16-h light period irradiated	540	6.0	14.3	6.6	2.4	29.8	100
24-h dark period, unirradiated	1230	13.4	8.6	7.6	4.4	34.0	100
24-h dark period, unirradiated	700	21	5.2	3.2	0.2	29.4	71

Table 6. Performance of irradiated nucellar callus[a]
Embryogenic line (L 1)

Dose[b]	No. of cultures	Differentiation %	Embryoid[e] Index (1)	$\frac{1x\ \%}{100}$[f]	Differentiation %	Embryoid Index (1)	$\frac{1x\ \%}{100}$
		5 weeks after irradiation				10 weeks after irradiation	
0	19	37	1.57	0.58	69	1.45	1.00
2	19	63	1.33	0.84	94	1.73	1.63
4	21	0	-	-	0	0	0
8	19	0	-	-	45	2.50	1.13
12	19	0	-	-	100	2.08	2.08
16	23	0	-	-	100	3.60	3.60
20	20	0	-	-	100	3.22	3.22
12[c]	21	0	-	-	100	4.20	4.20
12[d]	23	0	-	-	100	3.13	3.13

[a]callus was irradiated prior to subculture

[b]Dose rate was 3.1 kR/-h

[c]Dose rate was 85 kR/h

[d]Dose rate was 170 kR/h

[e]Rating of 1-5, from an average of two embryoids to 200 embryoids per culture.

[f]Modified Index taking into consideration the percent of differentiating cultures.

Table 7. Effect of gibberellic acid (GA_3), ADS (adenine sulphate) and development stage on the rooting of Shamouti orange embryoids.

Stage of embryoid development*	BM	Culture medium BM+GA_3** ($1mg\ l^{-1}$)	BM+ADS ($27mg \cdot l^{-1}$)	BM+GA_3 + ADS
1	0	21	10	8
2	33	58	48	50
3	38	67	80	67
4	83	75	94	100

* stage 1: small pseudobulbils, 1 mm or less in diameter;
stage 2: pseudobulbils 2-3 mm in diameter;
stage 3: heartshaped embryoids 3-5 mm long;
stage 4: multi-cotyledonary embryoids larger than 5 mm

** Percentage of rooted plants autoclaved with medium

Table 8. Neoformation of shoots on decapitated seedlings of Marsh grapefruit and Shamouti orange after gamma irradiation. Experiment designed in 3-5 randomized plots of 20 seedlings each.

Dose (kR)	Marsh I[a] No. of seedlings	% shoot-forming seedlings 13 days	20 days	27 days	% Transplanted	Marsh II[a] No. of seedlings	% shoot-forming seedlings 6 days	12 days	27 days	40 days	% Transplanted
0	57	84	89	89	79	100	0	30	90	95	75
2	79	53	85	80	70	100	0	5	60	65	45
4	80	15	68	80	50	100	0	0	10	5	5
6	60	0	5	15	0	100	0	0	5	5	3

Dose (kR)	Shamouti I[a] No. of seedlings	% shoot-forming seedlings 7 days	16 days	22 days	28 days	35 days	% Transplanted	Shamouti II[a] No. of seedlings	% shoot-forming seedlings 7 days	18 days	33 days
0	60	0	80	80	80	80	67	79	0	10	40
2	55	0	11	43	43	50	29	100	0	5	40
4	67	0	18	41	41	53	29	100	0	5	30
6	65	0	3	25	25	31	31	100	0	0	8

[a] Marsh I sown 24.XII, irradiated 7.III; Marsh II sown 25.I, irradiated 18.IV; Shamouti I sown 24.I.74, irradiated 28.III; Shamouti II sown 28.II, irradiated 21.V.