

Induced Mutations in Citrus Shoot Tip Culture In Vitro

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Abstract: This study was undertaken to determine the efficacy of using induced mutation of the callus of citrus clones as a method in citrus breeding. It was observed that the nucellar clones had a higher ratio of callus cell differentiation than the old clones. The callus cells were exposed to gamma irradiation of 7,000 roentgen at 127 R/min.

Callus cells derived from the shoot tip proved to be more appropriate for cytological examination by the smearing technique than the root tip cells. Gamma irradiation of callus cells is quite effective for inducing chromosome aberration and micronucleus inspection.

Introduction

In nature, the frequency of spontaneous mutation is low (approximately 1/40,000) and this limits the potential for selecting spontaneous bud mutations.

Furthermore, citrus breeding is complicated by several factors, such as nucellar embryony, linkage of the gene, and the long cycle of breeding.

Raghuvanshi (3) observed that the wide morphological variation of plants is the consequence of changes in the structure of the chromosomes which results in the evolution of species.

A method of increasing the frequency of mutation by inducing changes in the structure of the chromosome is proposed. However, when working with a multicellular organ as the mutation material, there is interference by diploitic selection or forming a chimera.

Lapins, et al. (4) used repeated bud selection as a method to obtain mutants. This method is time consuming and expensive. More recently researchers have exposed undifferentiated cells to mutagens in order to obtain mutant cells which eventually grow and differentiate into a mutant plant.

Spiegel-Roy et al. (5) used highly differentiated embryogenic callus from 'Shamouti' sweet orange (*Citrus sinensis* (L.) Osbeck) and successfully selected mutants at the cellular level, thus avoiding interference from diploitic selection. The embryogenic materials can easily differentiate, but require years to bear fruit. An alternative to such a long cycle of breeding is to use old clones, but they have low frequency of differentiation (Button and Kochba, 1).

In this study, the vegetative shoot tip of grafted clones of 'Pushicheng,' a chimera cultivar, together with nucellar clones of 'Jin cheng,' 'Tau ye cheng' and 'Gai lian cheng' were screened for superior mutants at the cellular level, and the extent of dedifferentiation was examined.

Materials and Methods

(1) Materials used for tissue culture conditions were: Old clone 'Pushicheng,' (*C. sinensis* (L.) Osbeck), a chimera cultivar which has a long cycle of vegetative reproduction. Buds were selected and grafted onto rootstock and clones were utilized after 4 years of growth. New clones: 'Jin cheng,' 'Tau ye cheng,' and 'Gai lian cheng' (*C. sinensis* (L.) Osbeck) were grown from seeds in test tubes.

The shoots, 3 cm or shorter, were selected and stripped of larger leaves, wrapped in small squares of cheesecloth, and surface sterilized by soaking in 0.25% sodium hypochlorite solution (commercial bleach diluted 20-fold plus 0.1% Tween-20) for 5 min. The disinfected tissue was rinsed 3 times with sterile distilled water. Then the shoot tips were cut into 3 mm segments and used as the explants.

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The base medium was the plant cell culture salt solution of Murashige and Skoog, supplemented with the following organic compounds: 100 mg/l i-inositol, 1 mg/l thiamine hydrochloride, 5 mg/l pyridoxine hydrochloride, 5 mg/l nicotinic acid, 5 mg/l ascorbic acid and 2 mg/l aminoethanoic acid.

The dedifferentiating medium consisted of the supplemented base medium amended with 1 mg/l 2,4-D, 1.5 mg/l NAA, 0.25 mg/ K.T. and sucrose 30 g/l. Inside a test tube (150 mm × 25 mm), the explant was set on a supportive platform made from a folded 9 cm circle of filter paper and placed in the nutrient solution.

The redifferentiating bud medium consisted of the supplemented base medium amended with 0.5 mg/l zeatin, 0.5 mg/l 6-BA, 0.1 mg/l NAA, and sucrose 20 g/l. The test tubes and supportive platform for the buds were the same as above.

Redifferentiating rooting medium consisted of the supplemented base medium amended with 1-2 mg/l NAA, sucrose 50 g/l, and agar 8-10 g/l.

All media were adjusted to pH 5.7 and sterilized by autoclaving at 121°C (15 lbs psi) for 15 min.

The explants were placed into dedifferentiating medium and kept in the dark for 7 days. The explants were then exposed to the following daily light regime: 14 hours, 100 lux overhead illumination supplied by fluorescent tubes. After callus initiation growth of the callus was allowed to proceed for 15-20 days. The callus was then transferred to redifferentiating bud medium and the light intensity was gradually increased from 1000 to 2000 lux. After 6-8 weeks, the callus had redifferentiated, and buds had formed. After buds had grown 1.5-2 cm, they were cut off and placed into rooting medium.

A constant temperature of 27°C ± 1°C was maintained throughout the experiments.

After the shoot had rooted (2-4 weeks), the plantlet was transplanted into pots and placed in the greenhouse.

In some instances, the dedifferentiated buds were difficult to root. In these cases, we took the differentiated bud from the callus and grafted it onto rootstocks. In this way, the quick growth of the scion was obtained.

(2) The effect of exogenous hormones on the shoot tip culture at different stages of development.

Shoot tips from seedlings of new clones grown from seeds in test tubes were selected at different stages of development and placed in dedifferentiating medium. Shoot tips from 'Jin cheng' were selected 7, 13, 23, and 39 days after seed germination. Shoot tips from 'Tau ye cheng' were selected 7 and 13 days after seed germination, and shoot tips from 'Gia lian cheng' were selected 23 and 39 days after germination. All seeds were sown at the same time. Shoot tips were selected from 'Pushicheng,' the old clone, each time the shoot tips were selected from the new clones and placed into dedifferentiating medium.

Thus, at each time of selection, 3 cultivars were selected, 1 old clones and 2 new clones. Each cultivar had 40 tubes with 3 explants in each tube.

(3) Effect of γ -irradiation on chromosome aberration and callus redifferentiation.

The following materials were exposed to γ -irradiation: callus derived from 'Jin cheng' shoot tips selected 13, 23, and 39 days after seed germination, callus derived from 'Tau ye cheng' shoot tips selected 23 and 39 days after seed germination, and callus derived from 'Pushicheng' shoot tips. Thirty test tubes containing callus were exposed to γ -irradiation for each sample. In all cases, the age of

callus was 28 days at the time of exposure.

The source of γ -irradiation was 60 C with a strength of 127 roentgen/min.

Cytological examination was performed using the smearing method, staining with Carbol and Hematorylin. The chromosome zone was made visible by staining with Giemsa.

A Vernier caliper was used to measure callus volume, and analysis of covariance was used to analyze the results.

Results and Discussion

The frequency of callus redifferentiation was higher and the growth of callus was quicker for 3 new clones when compared to the old clone (Table 1).

The callus redifferentiation of old clone 'Pushicheng' was extremely low when compared to the 3 new clones

(Table 2). We tested many old clones of sweet orange representing many cultivars and in all cases, except for 'Pushicheng,' callus redifferentiation was not observed. However, up to now we have not observed root redifferentiation with 'Pushicheng' clones. It is possible that the original source of 'Pushicheng' buds used in this experiment came from a plant which had grown from seed rather than from a grafted plant. We speculate that the stage of development may be an important factor influencing the ability of callus to redifferentiate.

Cheng Zhen Guan (2) recently reported a similar finding working with anther culture of citrus. He reported anthers from new clones had a higher frequency of differentiation than anthers from old clones. Another factor influencing the redifferentiation of callus is the stability of old clones, and whether or not they are chimera.

Table 1. Growth of callus from the shoot tips of 4 cultivars of sweet orange.

Time of shoot tip selection (days after seed germination)	Percentage of shoot tips forming callus			
	Pushicheng*	Jin cheng	Tau ye cheng	Gai lian cheng
7	51.67	58.68	90.48	—
13	55.96	91.80	100.00	—
23	78.30	100.00	—	100.00
39	75.00	100.00	—	100.00
Average	65.00	87.62	95.24	100.00
Diameter of callus (mm ³ /day)	1.30	3.04	3.47	4.84

*Grafted clone plant.

Table 2. Callus redifferentiation of 4 cultivars of citrus.

Source		Cultivar			
		Pushicheng (%)	Jin cheng (%)	Tau ye cheng (%)	Gai lian cheng (%)
First examination	Bud primordium	28.60	77.80	75.00	—
	Redifferentiated	0.0	63.63	86.36	—
Second examination	Bud primordium	33.30	55.60	—	90.00
	Redifferentiated	37.50	100.00	—	79.31
Average	Bud primordium	30.95	66.70	75.00	90.00
	Redifferentiated	18.75	81.82	86.36	79.31

Recently, we have observed callus re-differentiation in old clones grown from seed in other citrus species, such as *C. limon* and navel orange (*C. sinensis* (L.) Osbeck).

The LD-50 for callus was determined by exposing callus from the 'Jin cheng' cultivar to increasing dosages of γ -irradiation (Table 3).

Callus from all 4 cultivars was exposed to 7,000 roentgen and chromosome aberration was observed (Table 4). In all varieties, chromosome aberration was increased as a result of radiation exposure (Table 5). In the case of the old clone, the time of shoot tip selection influenced the frequency of induced mutation.

Callus derived from shoot tip selected in the spring had lower frequency

of chromosome aberration (2.56%) compared to callus derived from shoot tip selected in the summer (3.54-4.7%). With respect to the new clones, the stage of development at the time of shoot tip selection influenced the frequency of induced chromosome aberration. For example, callus derived from shoot tips of 'Jin cheng' cultivar selected 13 days after seed germination had a frequency of induced chromosome aberration of 8.8% compared to 5.91% for callus derived from shoot tips selected 23 days after seed germination. Therefore, to enhance the frequency of induced mutation, callus derived from shoot tip explants should be collected in the spring in the case of the old clone 'Pushicheng,' and at an early stage of development in the case of the new clone 'Jin cheng.'

Table 3. Effect of γ -irradiation dosage on callus from the 'Jin cheng' cultivars.

	Dosage (unit: 1000 roentgen)									
	2	4	6	7	8	10	15	20	25	30
Average survival (%)	93	83	65	25	28	13	0	0	0	0

Table 4. Occurrence of different types of chromosomes and nuclear aberration of citrus callus following γ -irradiation.

	γ -irradiation at 7000 roentgen			Control		
	Total	Number	%	Total	Number	%
Cells examined	10,449			35,435		
Cells with aberration		5445	5.21		350	0.99
Cells with nuclear aberration						
Beady nucleolus		394	7.24		2	0.57
Nucleus with bud		161	2.96		15	4.29
Micronucleus		3141	57.67		125	35.71
Other		461	8.47		36	10.29
Cells with chromosome aberration						
Single bridge		54	0.99		1	0.29
Multiple bridge		31	0.57		0	0.0
Chromosome ring		158	2.90		50	14.29
Backward chromosome		269	4.94		9	2.57
Broken chromosome		50	0.92		8	2.29
Abnormal chromosome		166	3.05		16	4.57
Cells with less chromosomes than the expected norm		559	10.27		88	25.14
Cells with more chromosomes than the expected norm		1	0.018		0	0.0

Table 5. Chromosome aberration frequency of 4 cultivars of citrus.²

	Cultivars											
	Pushicheng				Jin cheng				Tau ye cheng			
	Total number cells	Aberration cells	%	Total number cells	Aberration cells	%	Total number cells	Aberration cells	%	Total number cells	Aberration cells	%
First examination	11479	294	2.56	9015	800	8.87	13663	689	5.04			
May 26	5003	44	0.88	3822	22	0.58	4756	45	0.95			
Second examination	11385	403	3.54	12596	744	5.91				3462	750	5.57
June 10	3713	38	1.02	3724	21	0.56				3386	44	1.30
Third examination	10461	492	4.70	12029	732	6.09				10427	541	5.19
June 18	3329	46	1.38	4217	45	1.07				3485	45	1.29
Average	33289	1189	3.57	33640	2276	6.77	13663	689	5.04	23889	1291	5.40
	12045	128	1.06	11763	88	0.75	4756	45	0.95	6871	89	1.30

²At first, second, and third examination for new clones callus was derived from shoot tips selected 13, 23, 39 days, respectively, after seed germination.

The effect of exposure to γ -irradiation on callus redifferentiation was not the same for the old clone and the new clones (Table 6). In general, the effect of exposure to 7,000 roentgen was to decrease the frequency of callus redifferentiation. Not only was the frequency of bud primordium formation decreased, but the ability of the bud to grow was also impaired. These effects were more apparent with the old clone than with the new clones.

Using the culture conditions described above, we have selected a clone of 'Jin cheng' cultivar with twice the normal number of chromosomes.

After a subculture period of 2-4 months, the percentage of cells with twice the normal number of chromosomes (tetraploids) was 40-70%. In addition, some cells were monoploid, triploid, hexaploid or more than hexaploid. We have also observed polyploid cells in other cultivars. We have verified this tendency for the chromosome number to double, and have obtained a number of redifferentiated buds from such tetraploid cells. The cells of these redifferentiated buds are also tetraploid. This material may have important value in conducting research on inheritance.

Cells were selected in the normal procedure for chromosome examination. We found the cytological examination of callus cells to be more satisfactory than the cytological examination of root tip cells. Not only did the chromosome structure or chromosome aberration appear more clearly, but the chromosome zone did too.

Furthermore, callus can be obtained from any organ of citrus 7 days after incubation, and can be available year around. Cytological examination of callus cells is a practical way to examine the frequency of induced mutation.

There are many problems that remain to be solved. The embryogenesis

Table 6. Effect of γ -irradiation on redifferentiation of citrus callus from 4 cultivars.

Varieties	Cultivars							
	Pushicheng		Jin cheng		Tau ye cheng		Gai lian cheng	
	Treated	Control	Treated	Control	Treated	Control	Treated	Control
Bud primordium (%)	15.05	30.95	45.95	66.70	36.70	75.00	66.70	90.00
Bud growth (%)	8.34	18.75	40.14	81.14	50.00	86.36	53.00	79.31

materials which are used by many researchers instead of vegetative clones are useful in induced mutation breeding.

However, if we can increase the frequency of callus redifferentiation of vegetative clones, the vegetative clones can also be useful due to the shorter cycle of breeding.

Literature Cited

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Cultivar Identification

With the ever increasing number of cultivars and rootstocks being released there is a need for a method to separate and identify them. Researchers at Washington State University and Geneva, New York are using the tech-

nique of isozyme separation to identify and separate by different combinations of isozyme bands, but it is difficult to separate strains of a cultivar with existing techniques.