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EMBRYOGENESIS IN UNDEVELOPED OVULES OF *CITRUS* CULTIVARS IN RESPONSE TO GAMMA RADIATION

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Abstract

Immature fruits of 36 *Citrus* cultivars were exposed to 0 – 12 kr gamma radiation. The maximum average embryo per nucellus (EN) were recorded after 4 months of ovule culture. In control (0 kr), the EN were 6.27, 6.06, 5.62 and 4.86 in Kinnow, Jaffa, Gada dehi and Sweet lime respectively. In 3 kr, EN were 6.85, 6. 23, 5.86, 4.60 in Foster, Kinnow, Valencia and Gada dehi, respectively. In 6 kr, EN were 8.33, 8.12, 5.66 and 3.66 in Foster, Kinnow, Pine apple and Kherna Khatta respectively. In 9 kr, EN were 7.31, 6.76, 5.30 and 3.15 in Kinnow, Foster, Jaffa and Kherna Khatta respectively. In 12 kr, EN were 5.88, 3.92, 3.80 and 2.50 in Kinnow, Foster, Jaffa and Rough lemon respectively. In general, there was suppression of embryogenesis in 9 and 12 kr. The healthy embryos with normal balanced germination were *ex-vitro* grafted on rough lemon seedlings for growth and development.

Introduction

Citrus has first position in the country fruits and is grown in all the four provinces of Pakistan where Punjab produces over 95% crop because of its favourable growing conditions including adequate water. Pakistan has 60% Kinnow and 5% other mandarins, and in the world mandarin production it has 6.50% share. Mosambi is the dominant sweet orange cultivar and is 11% country's *Citrus*. Other sweet oranges contribute to 14%. Sweet lime (4%), acid lime (2%), lemon (2%), sour orange (1%), grape fruit (0.1%) and other *Citrus* types as 2%.

In Pakistan *Citrus* has mostly exotic varieties like Mosambi is from Mosambique, East Africa. Kinnow a leading *Citrus* cultivar of the country is from California. Kinnow is hybrid of King and Willow leaf. King is a seedling origin variety of high heat tolerance and Willow leaf has Mediterranean origin of hot dry climate, so Kinnow is more suited to Punjab climate. Both King and Willow leaf have rich flavour and aroma which is inherited in Kinnow. Sweet lime and grape fruit can have precious traits of parthenocarpy. It is important for *Citrus* industry to have superior varieties adaptable to different agroclimatic conditions, which should increasingly meet the demands of international market.

Since gamma radiation can make changes in DNA, the objective was to study the effects of gamma radiation on immature ovule regeneration of *Citrus* cultivars. Kinnow has good embryogenic response upto 6 kr (Altaf & Ahmed, 1997). Embryos of 33 *Citrus* cultivars studied were responsive to 0 – 12 kr (Altaf *et al*., 2000a). Embryogenesis was not much affected in response to 0 – 6 kr radiation in Mosambi and Valencia (Altaf *et al*., 2000b). All 33 *Citrus* cultivars tested showed suppression of embryogenesis at 9 – 12 kr (Altaf *et al*., 2001). In *Citrus*, somatic embryogenesis has been widely studied as from ovule of cv. Dancy (Coelho *et al*., 1998), for anther culture of cultivars of *C. aurantium*

and *C. reticulata* (Germana, 2003), from undeveloped ovules and stigma/style explants of sweet orange navel group (Carimi *et al*., 1998), from undeveloped ovules of orange cultivar Xinhue (Rong *et al*., 1998). Nucellar embryogenesis in Valencia and rough lemon has been reported (Obukosia & Wailhaika, 2000). It is mostly the nucellus in the ovule which regenerates into embryos because it is least differentiated and has inherited competence for regeneration in culture (Niedz, 2006). Singh *et al*., (2006) raised embryogenic cultures of Kinnow mandarin from unfertilized ovules. Since embryos are from single cells so radiation to pro-embryogenic nucellus can give solid mutants which can provide improvement within the *Citrus* Cultivars.

Materials and Methods

Thirty fruits of each cultivars were collected from orchards in the first week of July. However, 60 fruits each of Chinese lemon, Desi lemon and grape fruit were collected because of low number of available healthy ovules. These cultivars were 10 of mandarins and their hybrids, 12 of sweet oranges and their hybrids, 4 of lemons and 3 rootstocks, 5 cultivars of grape fruit, 1 chakotra and 1 of sweet lime. The fruits were washed thoroughly with tap water and 6 fruits of each cultivar were stored per polythene bag in fridge until used in experiment. Five packages of polythene bags of each cultivar which were gamma radiated as 0 (control), 3, 6, 9 and 12 kr. For Desi lemon, Chinese lemon and Marsh grape fruit 12 in each radiation dose were utilized. The fruits were dipped in ethanol and flamed. The filled and shrunken ovules per fruit were noted. The integuments of ovules were removed and inside masses (nucelli) were cultured in 40 ml sterile MS medium (Murashige & Skoog, 1962) in 100 ml flask which was supplemented with IBA, 2, 4-D and GA (each 1mg/l). The sucrose was 3% and pH adjusted to 5.8. The medium was solidified with 1% Difco Bacto Agar. Four healthy immature ovules were used per flask and 4 flasks of each radiation dose were made. All these operations were carried out in a sterile Laminar air flow cabinet equipped with UV lights. After 2 months of nucelli culture, the whole cultured masses of flask were subcultured for another two months in the same medium as in original culture except 2, 4-D. The cultures were kept at $27 \pm 2^{o}C$ in natural light.

The average number of embryos per nucellus were recorded for 0, 3, 6, 9 and 12 kg after 4 months of culture (Table 1, 2, 3 and 4). The normal healthy embryos were top grafted on soil adapted Rough lemon rootstock seedlings. The humidity in polythene cover was gradually reduced and grafts acclimatized to normal nursery environment in pots.

Results and Discussion

Citrus has variabilities in seed number per fruit within the cultivars in different agroclimatic conditions and in mixed plantings. Fruits from one orchard does not represent the seed number of a cultivar. The average number of ovules per fruit of the cultivar including the shrunken were recorded (Tables 1-4). Ovule origin may be from pollinated or non-pollinated flowers. Also different cultivars ovules were in different stages of development. Complete or high ovule sterility has more economical value in edible *Citrus* fruit, while more seeds with low ovule sterility is of commercial importance for rootstocks.

Table 1. Mandarins, hybrids.

Cultivar	Total ovules	% Shrunken ovules	Control		Average embryos / nucellus in radiated dose (Kr)			
			0 Kr	Embryo weight (g)	3	6	9	12
Kinnow	21	9.52	6.27	0.019	6.23	8.12	7.31	5.88
Feutrell's Early	8	37.5	4.46	0.012	4.39	3.95	3.42	3.82
Ponkan	12	41.66	3.04	0.013	3.55	3.50	2.23	2.34
Honey	18	16.66	3.37	0.016	3.26	3.11	2.67	2.17
Pixie	17	23.52	3.80	0.021	3.07	2.82	2.50	2.30
Tangerine	24	20.83	3.78	0.022	1.98	2.09	2.75	1.83
Orlando	15	13.3	3.92	0.015	4.14	3.72	3.45	2.84
Mediterranean	8	50.0	3.17	0.010	3.05	2.80	2.45	1.58
Seminole	17	17.64	4.37	0.016	4.71	3.88	3.44	2.55
Minneola	26	26.92	4.33	0.013	4.17	3.08	3.33	2.60

Table 2. Sweet oranges, hybrids.

Cultivar	Total ovules	% Shrunken ovules	Control		Average embryos / nucellus in radiated dose (Kr)			
			0 Kr	Embryo weight (g)	3	6	9	12
Mosambi	12	25.0	4.86	0.013	4.95	5.40	4.10	3.45
Blood red	9	22.2	4.83	0.017	4.10	3.80	3.25	2.80
Pineapple	10	40.0	5.16	0.011	4.05	5.66	4.50	2.55
Valencia	14	88.57	5.71	0.013	5.80	5.30	4.30	3.65
Frost navel	13	30.76	4.44	0.014	4.05	3.40	2.80	2.48
Sanguinello	9	33.3	3.51	0.018	3.05	2.73	2.71	2.40
Succari	13	23.07	4.07	0.015	3.85	4.55	4.95	3.10
Washington navel	9	22.22	3.79	0.020	4.20	3.15	2.25	2.20
Moro blood	12	33.33	2.52	0.014	3.01	2.80	2.77	1.80
Jaffa	18	83.33	6.06	0.011	4.50	4.80	5.30	3.80
Hamlin	18	27.77	4.2	0.012	4.25	3.65	3.25	2.70
Tarocco	11	27.27	4.48	0.020	4.10	3.40	3.0	2.33

Table 3. Grape fruits, chakotra, sweet lime.

Cultivar	Total ovules	% Shrunken ovules	Control		Average embryos / nucellus in radiated dose (Kr)			
			0 Kr	Embryo weight (g)	3	6	9	12
Ruby red	14	35.71	4.84	0.019	4.20	3.35	2.9	2.1
Duncan	23	16.0	4.6	0.017	4.10	3.85	2.75	2.22
Marsh	2	100.0	4.06	0.018	4.05	3.39	2.96	2.78
Shamber	22	25.0	4.28	0.018	3.05	2.99	2.87	1.98
Foster	55	12.0	3.93	0.017	6.85	8.33	6.76	3.92
Chakotra	28	17.85	3.02	0.011	2.50	2.83	2.50	1.64
Sweet lime	12	25.0	4.86	0.021	4.50	4.25	3.50	2.75

Table 4. Lemons, rootstocks.

Cultivar	Total ovules	% Shrunken ovules	Control		Average embryos / nucellus in radiated dose (Kr)			
			0 Kr	Embryo weight (g)	3	6	9	12
Desi Lemon	3	33.3	2.61	0.010	3.21	3.05	2.32	1.76
Baramashi	6	33.3	3.08	0.013	3.69	2.94	2.47	1.80
Chinese	4	25.0	3.33	0.017	3.08	2.50	2.83	1.97
Eureka	7	28.57	3.06	0.015	2.66	3.50	2.33	1.76
Jatti Khatti	22	9.0	4.38	0.010	4.06	2.98	2.98	2.50
Kherna Khatta	19	21.05	4.34	0.010	4.15	3.66	3.15	1.83
Gada Dehi	17	11.76	5.62	0.015	4.60	3.07	2.45	2.04

In control (0 kr) nucellus cultures of mandarins, the maximum (6.27) and minimum (3.04) embryos were in Kinnow and Ponkan, respectively. The highest (0.022g) and lowest (0.01 g) embryo weight after 4 months culture was in Tangerine and in Mediterranean. The initial culture was induction medium, some callus growth with embryogenesis occurred after 2 months. The growth initiated from the micropylar end of the nucellus. The subculture was mostly maturation process, growth and development, budding and germination of somatic embryos. Well developed somatic embryos vigorously germinated on solid medium were micrografted on rough lemon seedlings.

In sweet oranges, the maximum (6.06) average embryos per nucellus was in Jaffa and minimum (2.52) in Moroblood. The maximum (0.02g) healthy embryo weight in Washington navel, Tarrocco and minimum (0.011 g) in Pineapple and Jaffa. In sweet lime, chakotra and grape fruit, the maximum (4.86) embryos per nucellus after 4 months were in sweet lime with maximum embryo weight 0.021 g and minimum (3.02) embryos per nucellus were in chakotra with minimum 0.011 g embryo weight after 4 month. The grape fruit cultivars average embryos per nucellus ranged between 3.93 – 4.84 after 4 month and embryo weight ranged between 0.017 – 0.019 g. In control (0 kr), lemons and rootstocks, the maximum (5.62) embryos in Gadadehi and minimum (2.62) in Desi lemon. Chinese lemon embryos had maximum (0.017 g) weight and minimum weight (0.01g) was observed in desi lemon, rough lemon and Kherna Khatta.

In 3 kr, the maximum (6.23) and minimum (1.98) embryos were in Kinnow and Tangerine. Kinnow had maximum 8.12, 7.31 and 5.55 embryos in 6, 9 and 12 kr. The minimum embryos were 3.95, 2.45 and 1.58 in 6, 9 and 12 Kr in Feutrell's early, and Mediterranean both in 9 and 12 kr. Perhaps the culture conditions were more suitable for Kinnow beside polyembryony. The embryos proliferated by budding either directly from nucellus or through a callus intermediary, and several embryos in different stages were usually obtained from the same nucellus. In Avocado cultures irradiations with doses 2.5 Kr stimulated development of heart stage in T362 somatic embryos, however mature somatic embryo development was suppressed at doses of 1 Kr and greater (Sono & Litz, 2004).

In sweet oranges, the maximum embryos per nucellus were 5.80 in Valencia and minimum 3.01 in Moroblood. In 6 kr, the maximum (5.66) embryos in Pineapple and minimum (2.73) in Sanguinello. Jaffa 5.30 and 3.80 embryos in 9 and 12 kr respectively, while Washington navel and Moroblood had minimum 2.25 and 1.80 in 9 and 12 kr respectively. In grape fruits, chakotra and sweet lime, Foster had maximum embryos as 6.85, 8.33, 6.76 and 3.92 and chakotra minimum embryos 2.50, 2.83, 2.50 and 1.64 in 3, 6, 9 and 12 kr respectively. In lemons and rootstocks, the maximum embryos 4.60, 3.66, 3.15 and 2.5 in 3, 6, 9 and 12 kr and minimum embryos in 3 kr (2.66) Eureka, 6 Kr (2.5) Chinese lemon, (2.32) in 9 kr Desi lemon and 12 kr (2.04) in Gada Dehi.

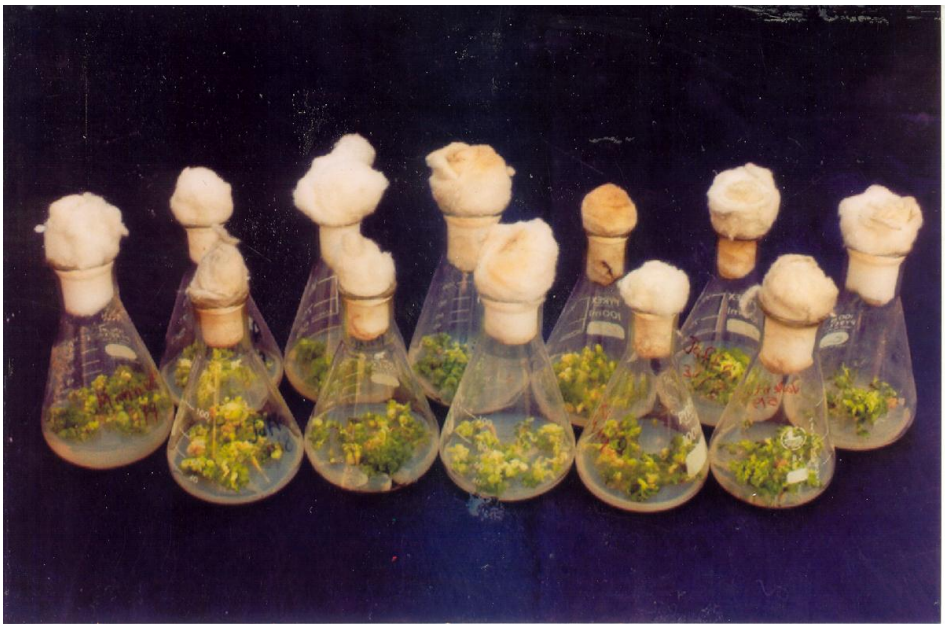

Fig. 1. Embryogenesis of citrus cultivars.

Although embryogenesis obtained in all cultivars (Fig. 1) indicated that all the cultivars were competent to regeneration and the method adopted was successful. The cultivars have different embryonic potential in one medium used which is affected by radiation doses. Ahloowalia & Maluszynski (2001) discussed the use of ionizing radiations and chemical mutagens for inducing variations and tissue culture increases the efficiency of mutagenic treatments (Predieri, 2001) for variation induction, handling of large populations. Gamma rays produce both point mutations (base pair change) and chromosomal alterations (Talon *et al*., 2004).

Embryos that followed normal development were easily converted into plants (Fig. 2). In addition to normal embryo growth, there were embryos in which no further growth was detected or with limited callus formation, cotyledon with shoot primordia without root, abnormal green shoots, embryos with limited growth, shoots meristemless mutants, thin embryonic leaves, thick stem embryos etc. The normal healthy embryos with balanced shoot root system were grafted on rough lemon seedlings. Madhavi (2001) grafted shoot tips of khasi mandarin with 60% success in *In vitro* conditions. Raharjo & Litz (2005) grafted somatic embryos of avacado in ex-vitro conditions. Micro-grafting improves survival of embryos. In our experiment, nearly half of the grafts were successful after acclimatization. The major reason of embryonic plant death were improper connections between the scion and root. Susceptibility of root system was due to damage during high humidity of grafting environment under polyethene cover. There was inability of rough lemon seedlings to produce root hairs with difficulty of small mutant plantlets to cope with harsh summer environment.

Fig. 2. Embryonic plants.

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