# TOWARDS A SEEDLESS CULTIVAR OF KINNOW MANDARIN VIII. *IN VITRO* CULTURE OF UNDEVELOPED OVULES FROM SPONTANIOUS LOW SEEDED MUTANTS AND SEEDED FRUITS

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## Abstract

It is possible to induce embryos from undeveloped ovules of immature fruits of low seeded kinnow natural mutants. Regeneration from undeveloped ovules is from its nucellus tissue which is predetermined as embryogenic cells and BA accelerated somatic embryogenesis. Gibberelic acid stimulated the growth of primary root. Regeneration potential of plant cells can be affected by their epigenetic state and influence of ovular endogenous compounds can cause fluctuation in regeneration ability between repeats in spite of precisely controlled cultural conditions. Regeneration was affected by genotypes. Higher percentages of responsive ovules and a higher mean number of embryos per responsive ovule were present in seedy Kinnow as compared to low seeded mutants. The use of undeveloped ovules with optimum culture conditions might allow large number of plants to be produced throughout the year and can avoid loss of potentially valuable genes because complete degeneration of ovules occur in low seeded kinnow mutants.

**Key Words:** Undeveloped ovules, Low seeded mutants, citrus.

## INTRODUCTION

*Citrus* is the major fruit of Pakistan both in area and production (MINFAL, 2003). Punjab produces 17,51036 tonnes of *Citrus* from 18,3216 hectares which is 95.7% of Pakistan *Citrus* fruit. Higher produce in Punjab is because of its climate, soil and irrigation water is favourable for *Citrus* growth. In Punjab, Kinnow is the leading cultivar which is grown on 105407 hectares of land with production of 1079270 tonnes, contributing 61.7% of *Citrus* fruit.

Kinnow mandarin is chimeric hence a certified, horticultural, ture to type bud wood is not available. The hybrid nature of Kinnow, somatic mutations, large scale propagations without proper bud wood selections and lack of defined strains of rootstock are important reasons of variability. The most important is hybridity of Kinnow (King x Willow leaf) and hybrid nature of its parent King (sweet orange x mandarin).

*Citrus* trees are extremely prone to mutate (Xu-XB *et al*, 2000*)*. which are discovered as limb sports or bud sports (Wu-SL, 1999) and are also detected as nucellar seedlings like early ripening Miho and Okitsu nucellar clones of *Citrus unshiu.* Naval oranges and grape fruit tend to produce more mutation. The famous seedless ‘Washington Naval’ orange in which pollen mother cells degenerate before reduction, is a limb sport of seedy ‘seleta’ sweet orange in Brazil. Numerous mandarin varieties have also arisen from spontaneous mutation. The wide range of clementine varieties in Spain and most Satsuma mandarins in Japan arose from mutations. Majority of seedless *Citrus* varieties have some degree of ovule or pollen sterility. Complete seedlessness under all conditions is rare except Mukaku-Kishiu mandarin which is seedless under all conditions. It is a bud variation strain of seedy Kishiu mandarin (Frost and Soost, 1968). Ovule sterility is a complex phenomenon in which several factors play a role as for example physiological function of three polyamine in shatian pummelo during blossom and fruit setting (Zhong and Zhong, 2000). It is easier to detect a mutation in a clone but difficult to point a mutation in hybrid populations like Kinnow mandarin where all vegetative and fruit characters are variable. It was earlier studied (Altaf and Iqbal, 2002) that ovule number and size were similar in low seeded fruits and in normal Kinnow till 80 DAP (days after pollination). The ovule started shrinking after 90 DAP. At the age of 120 – 150 DAP, there were 0 – 4 developed seeds in spontaneous low seeded mutants and 13 – 34 developed seeds in control and rest were aborted ovules in both kinds of fruits.

The broad objective of overall study was how variation can contribute to seedlessness or very low seed per fruit and the specific objective of the work in this paper is to improve the survival of low viability ovules in spontaneous low seeded Kinnow mutants to avoid the loss of potentially valuable genes, through somatic embryogenesis of nucellus from undeveloped ovules of immature fruits.

## MATERIALS AND METHODS

Natural variability was utilized for seed number per fruit. Immature fruits from normal Kinnow ( C )and low seeded (M) fruit branches were harvested after 60, 70, 80, 90 and 100 DAP (days after pollination). The fruits were cleaned and stored in polythene bags in fridge until used in the experiment. During operation in Laminar Air Flow bench, the fruits were flamed with ethanol and healthy ovules were aseptically taken out in sterile petri plates, because complete degeneration after 12 or more week of pollination occurs in ovules. After removing the integuments, the internal ovular mass was placed on MS medium (Murashige and Skoog, 1962) with additions of BA+GA (each/mg/l) + Glutamine 5 mg/l + 2% sucrose, the medium was solidified with 1% Difco Bacto Agar. The culture medium was sterilized by autoclaving. Hundred ovules per DAP (60, 70, 80, 90, 100) were used. Regeneration responses were recorded (Table 1) after 3 months of ovule culture. The coefficient of regeneration response from undeveloped ovules was estimated as regenerated ovules x average number of embryos per ovule and the product divided by 100. The normal balanced germinated embryos obtained from ovule culture were re-cultured in MS + GA (1 mg/l) + 2% sucrose with pH 5.8. After 2 months in culture, these embryos were grafted onto rough lemon seedlings according to method given in Altaf and Iqbal, (2003).

**Table 1: Normal embryo regeneration from immature ovule culture of control (C) and low seeded fruit (M), recorded after 3 months:**

| Ovlues DAP | % Ovule response | | Average embryos/ responsive ovule | | Coefficient of regeneration | | Normal embryos (%) | |
|---|---|---|---|---|---|---|---|---|
| | M | C | M | C | M | C | M | C |
| 60 | 7 | 15 | 1.3 | 2.5 | 0.09 | 0.38 | 3 (33) | 7 (19) |
| 70 | 9 | 23 | 2.1 | 3.3 | 0.19 | 0.76 | 4 (21) | 11(15) |
| 80 | 11 | 58 | 1.8 | 3.5 | 0.2 | 2.03 | 6 (30) | 31(15) |
| 90 | 13 | 65 | 1.4 | 4.2 | 0.18 | 2.73 | 7 (39) | 43(16) |
| 100 | 12 | 68 | 2.3 | 3.8 | 2.58 | 2.58 | 7 (25) | 44(17) |

## RESULTS

The *in vitro* culture of ovules allows the production of nucellar plantlets from low seeded spontaneous mutants of Kinnow mandarin. Healthy ovules were used in culturing and the age of fruit had definite influence on embryogenesis. It was 7, 9, 11, 13 and 12% ovules responding to regeneration after 60, 70, 80, 90 and 100 DAP respectively, because in 60 – 80 DAP, the ovules are in a process of degeneration, so lower responses were observed despite selection of healthy ovules for culture. Reproduction of seedless orange cultivars from undeveloped ovules has been raised *in vitro* conditions by Starrantino and Russo, 1983.

**Fig. 1: Ovules (100 DAP)**

**Fig. 2: Low seeded mature fruits**

Right plate: Seed shape similarity to sweet orange
Juice with aborted seeds

**Fig. 5: Embryo growth.**

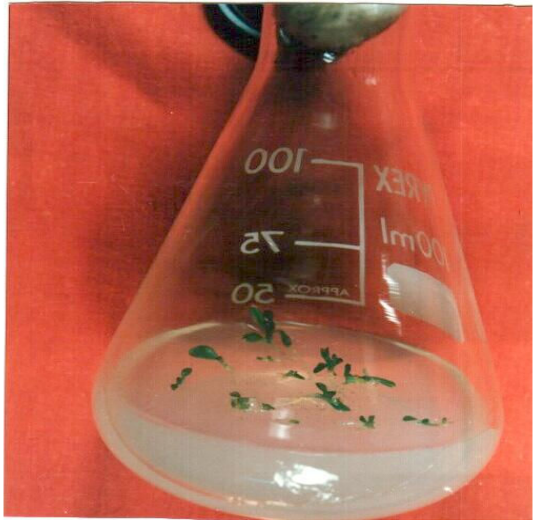

**Fig. 6: Embryos**

**Fig. 7: Grafted plants.**

Glutamine with BA and GA were used in MS medium for efficient utilization of nucellar totipotentiality. The small cotyledonary shaped embryos showed the first primary root. Immediately after that the hypocotyls and two primary leaves grew. The percent of ovules producing one or more embryos was the most important factor in total number of normal embryos produced which was 27 in mutant fruits and 136 in control fruits. Few ovules produced good number of embryos which may be due to the fact that nucellar cells in ovules have meristematic activity in cells called premordium of nucellar embryo. Few ovules produced friable, cream white callus in some nucelli. However, high callus fresh weight accompanied by a greater number of embryos was rare. Majority ovules did not produce any embryos. Regeneration potential of plant cells can be affected by their epigenetic state. Occasionally, one ovule directly originated one normal seedling. Some ovules produced callus, pseudobulbies, embryos and plantlets. In some cultures, embryos either transform into plantlets or into spherical or elongated pseudobulbils, which rarely produce roots and never form shoots. The structures transformed into embryos or neoformation of embryos which then give rise to new pseudobulbils and embryos. The process of embryo proliferation has to be stopped for development of somatic embryos into plants. It is very difficult to produce seedlings from low seeded kinnow mutants using *in vitro* culture of undeveloped ovules, may be genotype of ovules does not allow them to grow in culture or depressive or killing effect of genes results in death of ovules. Somatic embryos developed incompletely so that cotyledons, shoot meristem, and even complete hypocotyls were absent. The requirement of plant regeneration was development of a viable embryo. The second requirement was balanced germination of embryos. Although many embryos germinated, the relative levels of root and shoot development exhibited by individual embryos were not always equal. Plantlet survival was directly related to the root per shoot. The maturation process is not efficient. The genetic control of somatic embryo development is not clear. Somatic embryogenesis and maturation represent a developmental process that pass through recognizable heart, torpedo and mature stages. Apart from diversity in nutritional and hormonal factors, the requirements of different tissues and organs are still largely unknown. Endogenous factors govern the embryoid formation *in vitro* in ovule culture of different origin and endogenous factors controlling embryoid differentiation sometimes come to a better expression in growth media.

The plants from undeveloped ovules had a slow growth both in culture vessels and in initial stages of embryo grafts upto six months and after 8 – 9 months, the grafts developed normally and some vigorously. Eighteen grafts were obtained from low seeded mutants and 106 grafts were obtained from normal kinnow fruits.

## DISCUSSION

Ovule is the undeveloped seed. Ovules arise as an outgrowth of placenta. Outgrowth involves several cell layers. The ovule soon begins to grow upward and finally takes inverted form with micropyle facing axis of ovary. The mature ovule consists of stalk, central nucellus, embryo sac within nucellus and two integuments surrounding nucellus. Micropyle is opening at the apex of nucellus through the integuments (Frost and Soost, 1968).

In *Citrus*, all the ovules cannot develop into seed, rather they abort before maturation process. Complete or high ovule sterility has positive economical impact in edible fruits, while more seeds with low ovule sterility have commercial importance for root stocks, as recently developed high seeded, rootstocks by Forner *et al*., (2003).

Capacity for embryogenesis is widely distributed in *Citrus* and possibly it is a fundamental property of somatic plant cells, especially nucellus in ovule development. The expression of their embryogenic potential *in vitro* is suppressed by the surrounding cells of the tissue. Following culture of nucellus onto medium, the cells divide and organize as somatic proembryos, freed from inhibitory influence that occurs in vivo (Litz and Gray, 1995). Somatic embryos originate from single cells as bipolar structures with well defined shoot and root zones. The genetic factors played an important role in morphogenesis but mutant with altered patterns of embryo development were largely ignored in most of studies. Loss of a single factor through mutation may consequently disturb a wide range of essential functions and result in lethality during embryogenesis (Meinke, 1991). The solution for early degeneration of nucellus in mutants of Kinnow is culture of ovules *in vitro* for establishing plants by grafting embryos on rootstock seedlings. This may give novel variants, not easily available in Kinnow orchards. Plants obtained from somatic embryos can have somaclonal variations. As studied by Navarro *et al*., (1985) that nucellus culture is not admissible for producing virus free, true to type plants of the monoembryonic *Citrus* varieties, because of the high percentage of aberrances. They recommended shoot tip grafting for virus free plants that are true to type without reversion to juvenility. However, nucellus may be used to increase genetic variability in monoembryonic *Citrus* for obtaining new cultivars.

Kinnow embryogenesis and shoot development is best with BA and GA helped in germination of embryos. It is assumed that media is still deficient in some unknown growth factors. Since benefit has been obtained from supplements of cytokinin and $GA_3$, it means that the endogenous levels may be less than adequate. Embryoids formed in the cells and upon subculturing, which developed into normal plants in MS with $GA_3$. This shows that progression from embryo to plant is stimulated by GA3 mainly by encouraging root growth.

Mostly nucellus undergo direct somatic embryogenesis (Obukosia and Waithaka, 2000) which proceeds from already pre-embryogenic determined cells and indirect somatic embryogensis from cells which require redifferentiation before they can express embryogenic competence (Tomaz *et al*., 2001). As a consequence callus formation precedes the formation of embryos. The fluctuation of regeneration ability of nucellus explants between repeats in spite of precisely controlled cultural conditions revealed that there were other factors closely connected with the physiological state of nucelli influencing regeneration (Ikeda *et al*., 2000) with undeveloped ovule age and may be some endogenous compounds could play important role in embryogenesis. This can also be concluded from the fact that nucellar cells are predetermined as embryo genesis. Since embryos germinate and grow into plantlets *in vitro*, (Ribeiro *et al*., 2000) the use of nucellus with optimal concentrations of growth substances might allow an efficient regeneration of plants from undeveloped ovules of mutant fruits of Kinnow mandarin.

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