



## REVIEW PAPER

## OPEN ACCESS

**Innovative breeding methods to develop seedless citrus cultivars****Sajid Ali\*, Ahmad Sattar Khan, Syed Ali Raza, Rana Naveed Ur Rehman***Pomology Laboratory, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan***Key words:** Citrus, flow cytometry, embryo rescue, endosperm culturing, mutagenesis.doi: <http://dx.doi.org/10.12692/ijb/3.8.191-201>

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**Abstract**

Presence of a large number of seed contents in citrus fruits is a big restraint in consumer's acceptability even if the fruit have premium organoleptic characteristics. Consumers are only willing to eat seeded fruits if seedless are not available. Seediness is also a major handicap restricting the use of large number of fruit in processing industries and increases the cost of juice or pulp production. Moreover, seed presence in the fruit may be related with bitterness and certain undesirable aromatic compounds. Various useful breeding approaches have been devised by researchers to reduce total number of seeds or create complete seedlessness in citrus fruits. These consist of both orthodox and modern molecular breeding tools. Conventional techniques used in the past encountered numerous problems due to specific reproductive physiology of citrus and was also time exhaustive. Modern molecular techniques like embryo rescue, protoplast fusion, cybrids, irradiation, inter specific or generic crosses, flow cytometry and citrus transformation are less time consuming and more productive as well as rapid results can be attained in short time period. Molecular techniques have further reduced the difficulties faced in conventional breeding to produce less seeded or completely seedless citrus cultivars.

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## Introduction

Citrus is a collective term used for genus (*Citrus*) belongs to family Rutaceae, esteemed for their glowing colours, fragrances, delightful tastes and premium nutritional values. Seeded citrus, watermelon and grape were accepted over the long years by the consumers. But, during the preceding few decades, interest of consumers in seedless fruits has increased; therefore, seed lessness in seeded fruits has now become an imperative characteristic for several fresh market fruits, including citrus (FAO, 2005; Vardi *et al.*, 2008).

Induced and natural ploidy influences have played a critical role in improvement of specific trait(s). Mutation polyploidy possesses great prospective to bring about sudden changes in genotypes as well as phenotypes. The changes in chromosome number per cell may be produced by application of extremely low or high temperature or by application of auxin as indole-3 acetic acid and endosperm derived calli somatic hybridization, irradiations or interploidal hybridization (Oiyama *et al.*, 1990; Gmitter *et al.*, 1990; Jaskani, 1998). Amongst polyploids, the triploid plants are of supreme importance because these produce seedless fruits (Raza, 2001). Moreover, production of triploid (seedless) citrus fruit can lead to fresh fruit quality improvement seedless varieties have distinctive commercial benefits. High number of seeds is considered as one of the major hurdle in releasing newly developed high quality mandarin cultivars (Vardi, 1996). There are numerous citrus cultivars with desired horticultural traits which have not accomplished commercial significance due to their seediness (Fatta-Del-Bosco *et al.*, 1992).

In the developed countries of the world seeded cultivars are grown when these extend a maturity season gap or for other premium trait(s). Citrus breeders have been exploring various methods which may allow sexual recombination those results in progeny with almost seedless fruits. Several methods have been reported in this regard to develop seedless citrus varieties in the world. Owing to courtesy of molecular breeding tools the development of seedless citrus cultivars will likely to

escalate in near future because early detection of seedless trait(s) is now possible with certain biotechnological markers.

## Factors affecting seed lessness

Parthenocarpic mutant tomato, parthenocarpic pineapples, bananas, some citrus and grape cultivars are major examples of facultative parthenocarpy, as in these crops seedless fruits are produced without fertilization. Consequently, in several of these cases, seed lessness may be attained by combining parthenocarpy with some other factors which inhibit normal fertilization process. Certain environmental factors i.e. low or high temperatures, chromosomal aberrations, chemical treatment, and some genetic factors, like gene(s) controlling meiosis process can also induce seed lessness (Lin *et al.*, 1984; Nuez *et al.*, 1986; Pike and Peterson, 1969; Vardy *et al.*, 1989a, b). On the other hand, self-incompatibility may be combined with trait(s) of parthenocarpy. High endogenous auxins levels and GAs arise in natural parthenocarpic citrus cultivars (George *et al.*, 1984; Talon *et al.*, 1990, 1992). In '*Arabidopsis*', GA3 mainly affects cell division of mesocarp, while, exocarp and mesocarp cell enlargements occur after treatment of auxins. However, in grapes GA3 has been found to prompt cellular expansion in mesocarp tissue (Srinivasan and Morgan, 1996). In tomato, various GA3 treatments have also been reported to induce mesocarp cell enlargement with restricted cell division, where, auxins treatment stimulates cell division process (Bunger-Kibler and Bangerth, 1982). Therefore, the above stated treatments can successfully be used to produce seedless citrus cultivars with premium fruit trait(s).

## Diploid level seed lessness

### *Self-incompatibility*

Phenomenon of self-incompatibility is genetically controlled which prevents seed set in self-pollinated crops and produce functional gametes. Usually in condition of self-pollination, no properly developed pollen tubes were observed in the ovaries of self-incompatible plants (Ton and Krezdorn, 1967). However,

some self-incompatible accession produced seedless fruit when these were self-pollinated (Nagai and Tanikawa, 1928). All pummelos, mandarins and several artificial or natural hybrids are self-incompatible in nature (Hearn, 1969). Some self-incompatible cultivars/varieties are seeded because of their female fertility and absence of parthenocarpy which requires cross-pollination for successful fruit set (Miwa, 1951; Mustard *et al.*, 1956; Krezdorn and Robinson, 1958).

Nevertheless, some self-incompatible accessions can also produce seedless fruit in a solitary planting, and clementine is most famous among these. The fruits are smaller than seeded fruits which tend to have reduced fruit setting percentage. Nevertheless mixed cultivation of these self-incompatible accessions with male-fertile plants frequently yield seeded fruits unless these have female sterility (Soost, 1956; Reece and Register, 1961; Hearn, 1969; Iwamasa and Oba, 1980; Li, 1980). But, seedless fruits may be produced in self-incompatible cultivars without parthenocarpic capability by pollination with tetraploid pollens or by the application of certain growth regulators (Soost, 1961; Yamashita, 1976). The incompatibility system of citrus is of gametophytic in nature and incompatibility 'S' allele(s) are widely distributed; not only in self-incompatible cultivars but also in self-compatibles like satsuma mandarins and 'Dancy' grapefruit (Soost, 1965, 1968; Vardi *et al.*, 2000).

Therefore, self-incompatible individuals may be developed from cross-combinations among self-compatible parents such as Minneola and Orlando arose from the combinations of Duncan and Dancy grapefruit (Swingle *et al.*, 1931). GOT (Glutamate oxalo acetatetrans-minase isozyme) loci and incompatibility locus are interconnected, which may be beneficial for early self-incompatibility screening of cultivars (Wakana *et al.*, 2005).

### Male or female sterility

Various male sterility intensities at diploid level have been found in citrus germplasm. Chromosome aberrations are one of the major phenomena which induce pollen sterility and it is genetically

controlled in Mukaku Yuzu whereas, in Mexican Lime and Eureka Lemon it can be induced by low temperature treatment. Reciprocal translocations are also the major cause of 'Valencia' sweet orange sterility, and this sterility is not usually found in other sweet orange cultivars. Inversions may also be caused by partial pollen sterility in Mexican Lime. Pollen sterility of 'Marsh' grapefruit cultivar is due to the spindle formation failure. Male sterility which may not be caused by chromosome aberrations is also well known. Anther abortion in Satsuma citrus hybrids is may be due to strict male sterility (Iwamasa, 1966).

The sterile stamens appear only as a filament, and due to this sterility no pollen grains are produced. Pollen mother cells degeneration during early stages was found to be present in Tahiti Lime, Washington Navel and few other citrus hybrids. Satsuma mandarin pollen sterility is usually caused by plural sterility i.e. degeneration of pollen grains and abnormal behavior. A close relationship has been found among the free proline contents of citrus anther and pollen fertility (Liu *et al.*, 1995). Generally, cultivars with normal fertility possess high proline contents as compared to partial or complete sterile cultivars. To produce novel seedless cultivars proficiently, genetic exploration of male sterility has been reported to carry out however; genetic analysis of anther abortion has proceeded remarkably. Male sterility is due to gene cytoplasmic interactions which are controlled by one or more than one major gene(s) (Iwamasa, 1966; Yamamoto *et al.*, 1997). Some new seedless citrus cultivars with aborted anther(s) have been released for cultivation in Japan (Nishiura *et al.*, 1983; Matsumoto *et al.*, 1991). On the other hand female sterility is also a very imperative character closely associated with seed lessness in citrus and is a heritable trait (Yamamoto *et al.*, 1995, 2001). Mukaku Kishiu, a bud variation of the seeded Kinokuni Mandarin, citrus cultivar is completely seedless, and is considered to possess strongest female sterility and this female sterility induces zygote abortion, and is controlled by two important genes (Nesumi *et al.*, 2001). Seed contents of both 'Marsh' grapefruit and

'Valencia' orange were less upon hand pollination (Wong, 1939).

Chromosome aberrations, as stated with respect to male sterility, probably occur in embryo sac. On the other hand non-functional lemon pistils are the major reasons for possible blockage of subsequent stigma and style growth is usually related to absence or presence of receptive embryo sacs in the ovules (Wilms *et al.*, 1983).

### Breeding methods for seed lessness

#### *Crossing Diploids (2n) with Tetraploids (4n)*

Crossing diploid (2n) plants as female parents may genetically be able to give a reasonable number of unreduced megaspores is a suitable method to develop triploid plants (Raza *et al.*, 2003). However, these generally possess the limitations of generating a low number of triploid hybrid plants which are more difficult to distinguish and separate from diploids or from the less number of hybrids attained when polyembryonic female parent is used in the crosses (Geraci, 1978). The embryos of triploids from monoembryonic sp. can be identified easily as the seeds carrying embryos are 1/3 to 1/6 times smaller in size as compared to diploid seeds (Esen and Soost, 1973). The major limitation of embryo abortion in the cross of diploid (2n) x tetraploid (4n) has been found a main constraint for developing triploids through this method (Esen *et al.*, 1979).

Several triploids can successfully be developed which may conglomerate the desirable traits of diploid and tetraploid parents provided that if triploid progenies may be rescued by embryo rescue technique (Satarrantino, 1992; Oiyama and Kobayashi, 1990) succeeding in diploid (2n) x tetraploid (4n) hybridization. However, in some crosses, where 'Marsh' seedless grapefruit, 'Avena', 'Sanguigno', 'Sanguinello', 'Tarocco', 'Ovaletto', 'Biondo' sweet oranges, 'Femminello' lemon and 'Hong Kong' Kumquat used as tetraploid polyembryonic male parents had crossed with diploid monoembryonic female parents such as 'Clementine', 'Meyer', 'tangor', lemon and citron the subsequent triploids had not only give seedless fruits but, also produced exceptional organoleptic characteristics and were also

easy to peel (Starrantino, 1992). It has also been perceived that immature embryos culturing of underdeveloped seeds were triploids whereas, embryos of fully developed seed were found to be tetraploids (Oiyama *et al.*, 1991).

#### *Crossing Tetraploids (4n) with Diploids (2n)*

Citrus triploids can also be developed between the cross of tetraploid with diploid parents (Cameron and Burnett, 1978; Esen *et al.*, 1979). Triploids have been developed by excising the seeds from matured fruits of tetraploid (4n) x diploid (2n) crosses. Vigor of the developed triploids was very high but initial survival of germinating seeds was quite low. Moreover, it was also perceived that tetraploid (4n) x diploid (2n) crosses had relatively high seed set as compared to reciprocal crosses (Esen and Soost, 1972; Cameron and Burnett, 1978). This is attributed may be due to unfavorable embryo ratio of 4:4, to endosperm chromosome number in such triploid plants than that of a typical ratio, 4:6, in tetraploids (Esen and Soost, 1973).

### Technique of embryo rescue

Embryo cultures have been used by various plant breeders for more than half a century. In recent past, practical contemplations related to excision of embryo and composition of the medium has enhanced the facility to use culture of zygotic embryo to rescue intervarietal, interspecific or intergeneric crosses in citrus crop improvement (Bhajwani and Razdan, 1983).

Hybrid seeds frequently abort as endosperm flops to develop (Cocking, 1986). Therefore, rescue of hybrid embryos *in vitro* first validated by Laibach and currently is a well-established extensively used technique in plant breeding. The citrus embryo abortion problem in diploids (2n) x tetraploids (4n) is a restrictive element for the recovery of triploids by this technique. This is attributed may be due to different ratios of chromosome number in embryo or endosperm and may also be instigated by unknown reasons in tetraploid pollen parents (Raza, 2003; Oiyama *et al.*, 1991). However, zygotic embryo cultures are regularly employed in the rescue of

immature hybrid embryos which ensued from the interspecific or

other crosses in which pollination and fertilization take place normally. This also includes excision of triploid hybrids from immature seeds usually after 100 to 120 days of pollination which usually results in heart shape zygotic embryos (Oiyama and Kobayashi, 1990; Soares *et al.*, 1992).

#### *Spontaneous Triploids Selection*

Spontaneous triploids and tetraploids exist as sexual zygotic seedlings in citrus. Triploids frequency has been found as high as 5% among seedling of several citrus progenies (Lapin, 1937). In spite of the fact that low seed contents or seed lessness, only 'Tahiti lime' has achieved commercial importance among various natural triploids (Vardi and Spiegel-Roy, 1978; Vardi, 1996).

In open pollinated citrus varieties, 'Eureka Lemon', 'Imperial' grapefruit, 'Lisbon Lemon' and 'Star Ruby' sweet orange triploid progenies were reported suggesting unexpected triploids are prevalent among various citrus progenies (Esen and Soost, 1971). Moreover, spontaneous triploids may be produced by the production of diploid (2x) egg cells by diploid seed parent or (2x) pollen grains upon their union with monoploid (x) gametes. But, perhaps the double chromosome number may normally be provided by egg (Vardi, 1992). These surprising triploid seeds may be differentiated from diploids with significant precision as the triploids were 1/3 to 1/6 in size, as compared diploid ones.

It has also been found that precocity in the production of their respective embryos also take place early than that of diploid seeds of the same age. In addition to the blessing of seed lessness, triploids plants may also have some additional anticipated characteristics, such as 'Winola', is a natural triploid hybrid from the cross of 'Wilking' and 'Minneola', its fruit is of medium sized, round to oblate with reddish skin and of coarsely seedless, sometimes only one seed per fruit. Moreover, trees of spontaneous triploids are usually spreading in growth habit having few drooping branches with thorns only at the non-

fruiting plant parts while, fruiting branches have a tendency to become nearly thorn less which is another desired character as thorns can damage the fruit in windy conditions (Esen and Soost, 1973; Spiegel-Roy and Vardi, 1992).

#### **Use of irradiation**

Irradiation is considered a valuable tool to improve the occurrence of mutation of asexually proliferated plants. Moreover, mutation breeding by irradiation is also a suitable approach to pursue desired triploids, because natural mutations and bud sprouts has often observed in citrus. Gamma irradiation was employed to obtain somatic mutations to study the evolution patterns of several citrus varieties and species. Irradiation with Cobalt 60 at 7 Ki with 127 R/min has been reported to produce 300 times more mutant progenies of 'Jincheng' orange as compared to natural mutations (Zhang *et al.*, 1988).

#### **Mutagenesis and transformation**

Mutagenesis is another efficient way to develop seedless plants from seedy germplasm as sterility is considered one of the most recurrent effects of treatment with any mutagen (Hensz, 1971). Seedless 'Star Ruby' grapefruit was developed through irradiation of seed of the seeded 'Hudson' by using thermal neutrons. Seedless 'Pineapple' strains of orange and 'Duncan' grapefruit by  $\gamma$ -irradiation of selected seeds. Seedless strain of 'Foster' grapefruit was also developed through  $\gamma$ -irradiation of citrus buds. Seed lessness was produced in 'Eureka' lemon as well as in 'Monreal' clementine was by  $\gamma$ -irradiation of their bud sticks (Hearn, 1984; Starrantino *et al.*, 1992).

Biotechnological tools such as genome analysis and transformation have been lead to produce new seedless citrus cultivars. *Arabidopsis* and tobacco transformants have been regenerated encompassing chimeral gene(s) of soybean 'conglycinin' and certain storage protein gene developers linked to bacterial 'RNA segene barnase'. However, Decrease in seed size was only found in *Arabidopsis* seeds, not in tobacco. Certain transformants of both these species were male sterile and associated with anthers gene

expression. The barnase construction can be expedient in eliciting a reduction of seed size. Therefore, West Indian lime plants transgenic in origin containing gene(s) for reduced seed set percentage have already been reported in hypocotyl and epicotyl seedling segments through *Agrobacterium*-mediated gene(s) transfer (Koltunow *et al.*, 1990). Furthermore, the short juvenile period of limes offers the opportunities to test the desired and introduced gene(s) for their ability to prompt reduced seed set percentage. Biotechnological DNA markers are also suitable to obtain few numbers of seeds from DNA and isozyme markers, and these markers are related to female sterility (Garcia *et al.*, 1984).

### Ploidy manipulation tools

#### *Endosperm culturing*

The endosperm is a distinctive tissue especially in woody plants. It is the fusion product of three haploid; two polar and one pollen nuclei and in most of the angiosperms, it is triploid. Advancement of tissue culture technology, it is now easily possible to develop embryogenesis from cultured endosperms. Moreover, this technique also enables the development of triploid citrus plants. Recovery of triploids from endosperm cultures succeeding controlled pollination process can be a valuable breeding scheme for citrus. It could also overwhelm the barriers faced by sexual hybridization following apomixis or embryo abortion. Moreover, embryo culture has become promising by peeling the nucellus off to remove embryoid or embryo tissues. If it would be carried out two months later anthesis of flowers in MT medium + 2 ppm 2,4-D + 5 ppm Benzyl adenine + 1000 ppm casein hydro lysates results in desired production of citrus hybrids (Wang, 1975). Similarly, endosperm embryogenesis of 'sweet tangor' (*C. reticulata* x *C. sinensis*) has also been induced and triploid plants generated (Mooney *et al.*, 2000).

#### Flow cytometry

Flow cytometry was actually developed for the research of medical science, but it is now extensively being used for DNA analysis of plant (Arumuganathan and Earle, 1991a, b). Flow

cytometry usually allows an estimate of the fluorescence and volume of isolated nuclei or cells.

If fluorescent probe is used in DNA specific or nuclear DNA content it can be quantified successfully. Generally 10<sup>4</sup>-10<sup>6</sup>/min of nuclei under study can successfully be analyzed quickly, and their results are presented in the form of histogram of integral fluorescence of DNA. The peak position of the axis is directly proportional to DNA contents of the nuclei under study. Moreover, if young leaves are analysed by flow cytometry, two peaks will be obtained. Role of flow cytometry regarding ploidy analysis and for polyploidy breeding in citrus was first highlighted by Ollitrault and Michaux-Ferriere (1992). Since then, flow cytometry has opened up new innovative opportunities for citrus ploidy manipulation in breeding of valuable cultivars with anticipated traits. Certainly, this technology allows the selection of rare and imperative events which are very difficult to use for breeding through conventional cytological techniques (Ollitrault *et al.*, 1996).

Flow cytometry has been reported to be employed by numerous breeding teams around the globe as a successful tool for citrus breeding (Tusa *et al.*, 1996; Ollitrault *et al.*, 1998a) and production of improved somatic hybrids (Ollitrault *et al.*, 1996c; Grosser *et al.*, 2000). Flow cytometry has further permitted the genome size variation between cultivated diploid citrus species (Ollitrault *et al.*, 1995). Moreover, large genome sizes (0.81pg/2C) are perceived for *C. medica* whereas, *C. reticulata* exhibited smallest genome size (0.74 pg/2C). However, this differentiation not allow estimate of the precise size of polyploid hybrid genome arising through sexual crosses due to chromosomal segregation phenomenon. Nevertheless, one advantage of flow cytometry is that it is quite simple and permits the analysis of 150 - 200 genotypes/day. The methodology is further modified by Arumuganathan and Earle (1991a) which has direct applications for citrus breeding.

### Conclusion

Information regarding genetic and molecular aspects affecting the gamete fertility and seed development is now increasing rapidly and will definitely enhance the

potential of breeding for seedlessness through sexual crosses at diploid level.

Mutagenesis programmes remained interesting way for breeding teams with essential facilities required for field evaluation of seedless citrus cultivars from elite well known seeded accessions but desired results have not yet been attained. On the other hand transgenic plants with gene(s) affecting meiosis and seed development may also be used if consumers accept eating of transgenic seedless citrus. The current ploidy manipulation programmes will result in an extensive range of triploids (seedless) citrus cultivars and will be utilized to combine trait of seedlessness and other characters i.e. resistances against insect pest and diseases, quality traits or extended harvesting period.

The various methods for obtaining seedless hybrids may be applied to different types of cultivars/varieties (sterile, mono or polyembryonic) and lead to very different populations in terms of genetic segregations and recombinations. Gameto somatic hybridization development would significantly increase the efficiency of the latter strategy. Generally, evaluation of newly developed cultivars in field and market validation in cooperation with various stakeholders of citrus industry is a complete necessity of present time to give a chance for new seedless citrus hybrids and mutants to grasp the market as one of the famous and leading cultivars.

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