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TOWARDS A SEEDLESS CULTIVAR OF KINNOW MANDARIN 2. VARIATION IN SEED SHAPE AND SEED SIZE

NAFEES ALTAF, M. MOHSIN IQBAL, ASIA GULNAZ AND EHSAN ULLAH KHAN

Nuclear Institute for Agriculture and Biology (NIAB), P.O. Box 128, Faisalabad, Pakistan.

Abstract

Narrow embryonic leaves are characteristics of seedless cell lines. Narrow embryonic leaves are related to over 20 seed shapes and in small size seeds which have higher frequency of narrow embryonic leaves. The intensity of leaf narrowness varied in different seeds. It seems that more than one factor is involved in seedless trait in cultivars of Kinnow mandarin.

Introduction

In plant development, only one cell, the zygote is able to divide to allow the expression of full genetic potential of the organism, while in all other cells, the expression is restricted. In *Citrus* genome, the zygote is usually weak because of extreme heterozygosity and because of development of poly embryony over the time period as an alternate means of plant survival. Also, zygote has competition with somatic embryos for space and nutrition. Nucellus which is mother tissue is least differentiated and has inherent ability to form embryos is source of obtaining somatic embryos. The nucellar embryos have better compatibility *in vivo* and hence better growth as compared to the zygote which represents different genotype in the existing tissue system. Kinnow has clonal variability and different nucelli result into different somatic cell lines. Kinnow has variable ovule sterility (Altaf & Iqbal, 2002) with different seed number per fruit (Iqbal *et al.*, 2001). Embryonic leaves have variation in length and breadth and low seeded material have usually higher frequency of narrow embryonic leaves (Altaf *et al.*, 2002). Since Kinnow has extensive variability in all morphological traits, the present report gives an account of different seed shapes and sizes for possible presence of seedless trait using embryonic leaf as marker.

Materials and Methods

Kinnow fruits from orchards and markets were collected and thoroughly washed. Different seed shapes were isolated. From marker (Iqbal *et al.*, 2001) and normal fruits (non-marker), the total seeds were divided into the large seed size ranging between 0.275 – 0210 g, medium size ranging between 0.200 – 0.160 g and small size ranging between 0.150 – 0.05g. Seeds were sterilized with 0.01% $HgCl_2$ for 5 minutes with constant shaking and washed with auto claved water to remove the presence of any disinfectant.

Both the seed coat and seed cover were removed and embryos were cultured according to the procedure mentioned in Altaf *et al.*, (2002). Different seed shapes were separated and cultured in MS medium containing GA 1 mg/l (Murashige & Skoog, 1962). After one month, frequency of narrow leaves was recorded in different seed shapes in normal embryos. In different seed sizes, the types of embryos were recorded. Embryos with normal balanced germination were micro grafted on rootstock seedlings.

Results

The seed shapes 1 – 17 given in Fig. A were isolated from market fresh fruits. Seed shape 18 was isolated from small size marker fruits from market. All these have varying embryonic narrow leaf (ENL) frequency (Table 1). Type 19–28 were isolated from low seeded marker fruits. All these seed shapes have above 50% ENL frequency. Seed shape 29 (Fig. B) was isolated from marker fruits of Faisalabad orchards with curled ENL. Figure C gives number of seeds in lines of 2, 3, 4 and 5 seeded fruits with variation in seed shape and size, which were Large (1), medium (2) and small (3) in a specific range of seed weight mentioned in Materials and Methods section irrespective of seed shapes. A 5-seeded fruit was studied by late Dr. Wasim Farooqi of NIAB in 1988 all with different seed shapes (Fig. E). Figure F shows pooled seeds of 10 low seeded fruits from Faisalabad orchards with clear variability of seed shapes and seed sizes. Figure G shows upper line of one seed per fruit, three sets of 2 seeds each of 3 different 2 – seeded fruits and third line of a fruit with variability in seed shape and sizes. The ENL frequency varies among samples of trees and different orchards in the same seed shape, depending on the origin of the bud wood for plant development. Since seedy fruits have different seed shapes with varying frequency of narrow leaves, it seems that seedless trait is spread over various shapes. There is variation of seed shape with respect to seed weight.

Table 1. Effect of kinnow seed shape on embryogenesis.

Seed Shape	Embryos studied	Embryos with Normal growth	Embryos with narrow leaves(ENL)	% ENL in total embryos	% ENL in normal embryos
1	29	23	8	27.6	34.8
2	31	30	20	64.5	66.7
3	10	5	3	30.0	60.0
4	8	7	0	0	0
5	5	4	2	40.0	50.0
6	13	6	2	15.4	33.3
7	9	7	0	0	0
8	15	11	2	13.3	18.2
9	23	16	5	21.7	31.3
10	2	0	0	0	0
11	4	2	2	50	100
12	15	11	3	20.00	27.3
13	18	11	2	11.1	18.2
14	7	4	3	42.9	75.00
15	9	6	1	11.1	16.7
16	10	9	3	30.0	33.3
17	16	15	3	18.8	20.0
18	2	1	0	0	0

There are remote chances of similar seed shape in a 2 – 3 seeded fruit, even though the sizes are different. Seed shapes represent different genotypes or strains. With time the genotypes isolate and variability becomes clear in different branches, twigs and sprouts etc.

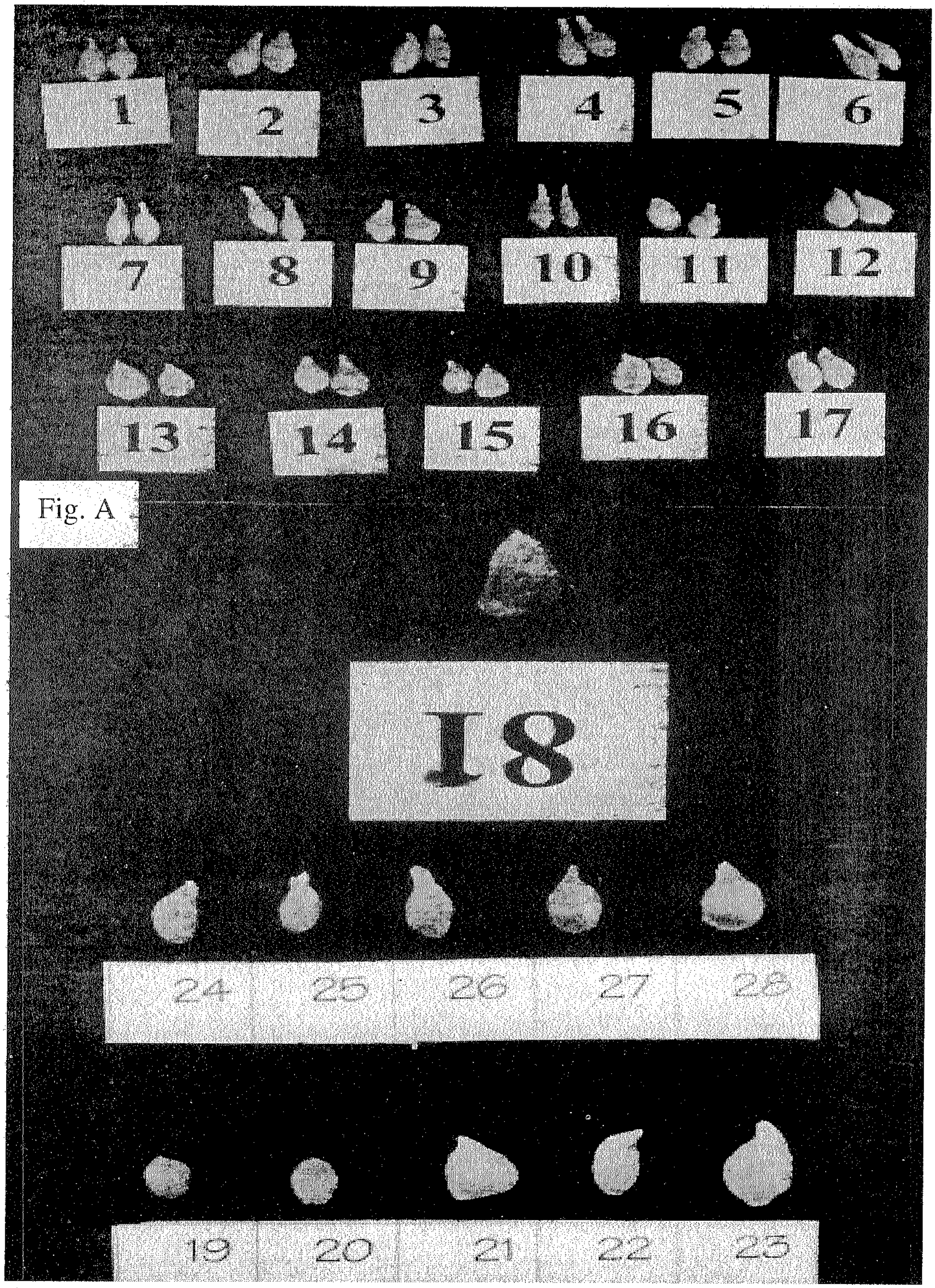

Fig. A. Seed shapes 1-17 from market fruits, 18 from marker market fruit, 19-28 from low seeded marker fruit.

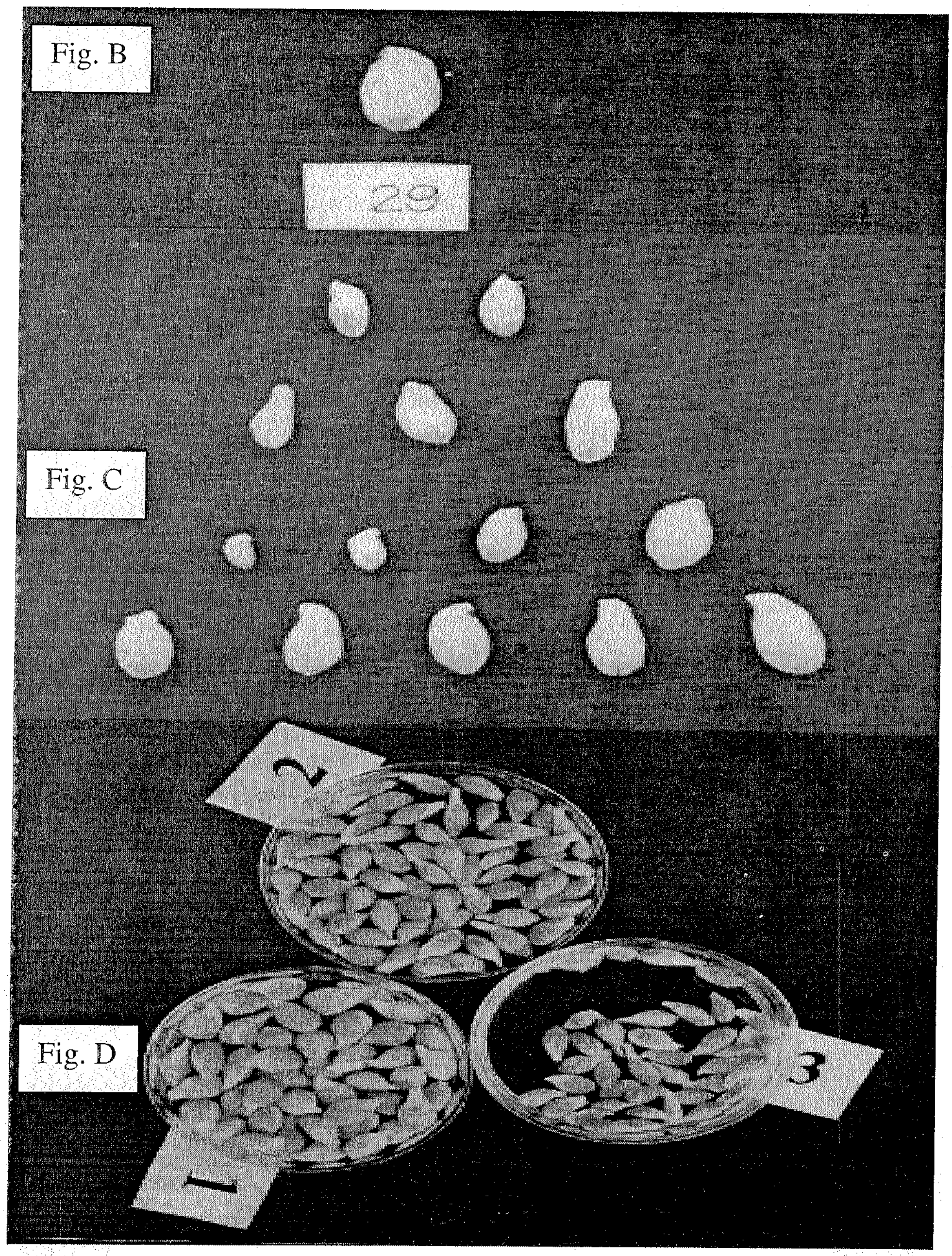

Fig. B. 29 from marker fruit gave curled embryonic leaves.

Fig. C. Number of seeds in lines of 3, 4 and 5 seeded fruits.

Fig. D. Large (1) medium (2) and small (3) size seeds.

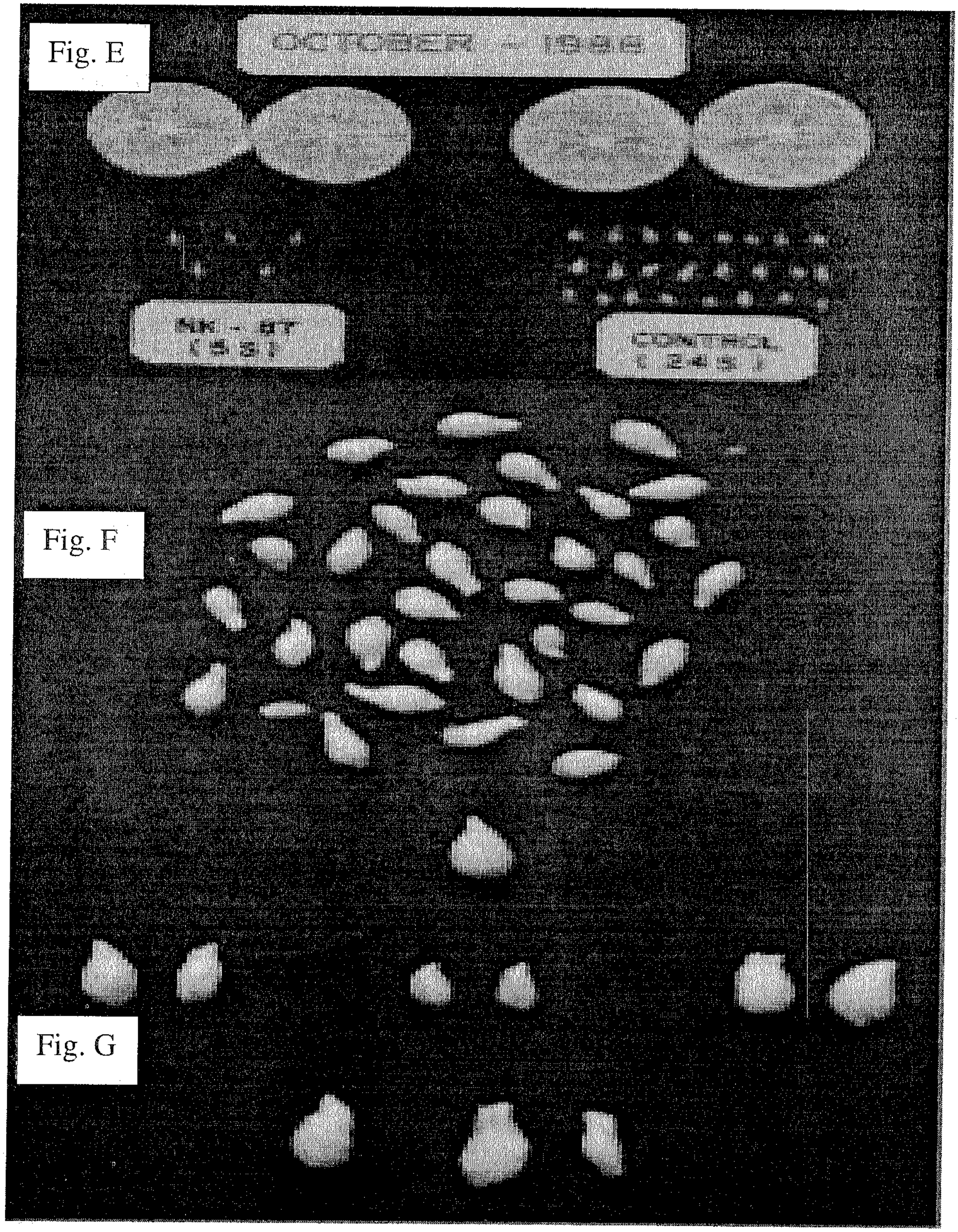

Fig. E. A 5 seeded fruit studied by late Dr. Farooqi in 1988.

Fig. F. Pooled seeds of 10 low seeded fruits.

Fig. G. Upper line of one seed of one seeded fruit middle line of 3 sets of 2 seeds, each of 3' different 2 seeded fruits. lowest line shows 3 seeds of a 3- seeded fruit.

In seedy fruits, usually 3-leaf embryos were in higher frequency as compared to the 2 leaf embryos (Table 2) except the large and small seed size of normal fruits where frequency of 2 and 3 leaves was the same. In meristem less embryos, the stem grew but was unable to grow proper leaves and meristem primordia. Meristem less embryos were in higher frequency in small size seeds irrespective of presence of marker on the fruit. Elongated roots were in higher frequency in marker fruits especially in small size seeds. Also, weak embryos and embryos with distorted shapes were higher in marker fruits as compared to normal fruits. Embryos with very thin leaves or needle like leaves were in small size seeds of all kinds of fruits. One embryo with 2 stems out of 1407 embryos was present in large seed size of normal fruits and was altogether absent in marker fruits.

One-leaf and 4-leaf embryos were consistently present in all seed sizes both in marker and normal fruits. Five-leaf embryos were present in marker fruits, one five-leaf embryo was present in middle size seeds of normal fruits. The large size seeds both in marker and normal fruits had more embryos with cotyledon + root as compared to other sizes. In some cases very strong root development was observed but it was unable to grow proper shoot system with leaf primordia due to unknown reasons. Embryos with one small + one large leaf were consistently present in higher number in middle size seeds from normal fruits. Albino leaves were only observed in embryos from middle size seeds of normal fruits.

Embryos with cotyledon + root (no development of shoot system), one-leaf, meristemless embryos, one small + one large leaves, weak embryos and embryos with various distorted shapes, embryos with very thin or needle like leaves, pale green embryos, albino leaves all are somewhat abnormal with imbalanced germination and with some inherent defects. Even in nutrient agar medium, these embryos with cotyledon + root, meristemless and albino leaves did not survive for longer period while one to five-leaf embryos, one small + one large leaf, elongated roots, two stem embryo were found to survive in culture medium as well as upon grafting on rootstock seedlings. Leaves with 2 mid-ribs also survived in grafts. The best survival was seen with 2 – 3 leaf embryos, elongated roots and 2 stems, although the latter is a less frequent morphogenic structure. One-leaf embryo survive with stagnant growth, rarely second leaf appear. All these embryos have slower growth as compared to control plants especially in the initial six months. The grafted embryos on rootstock seedlings have extensive variability in leaf shapes and plant growth behavior perhaps they are derived from different seed shapes and seed sizes.

Discussion

Seed coat removal improves embryos percentage emergence and emergence time. Kinnow has polyembryony (Altaf *et al.*, 2001). Somatic embryogenesis in *Citrus* with high frequencies of plant regeneration is possible (Hong *et al.*, 1998). Polyembryony causes competition between zygotic and nucellar embryos for space and nutrition during germination, a battle which is usually lost by zygotic genotype (Oliveira *et al.*, 2000). In polyembryonic *Citrus* cultivars, zygote is usually weak. There was a negative association between polyembryony degree and hybrid frequency in controlled crosses of Clementine and Sunki (Soares-Filho *et al.*, 2000). However, fewer number of nucellar embryos also depend on degree of ovule degeneration.

Table 2. Effect of Seed size on embryogenesis of marker and normal fruits.

Embryo types	**Seed Sizes**					
	M1	**M2**	**M3**	**S1**	**S2**	**S3**
Cotyledons + Roots	*32(10.8)	36(9.4)	21(6.4)	26(18.1)	5(3.0)	14(15.9)
Embryos with 3 leaf	59(20.0)	60(15.6)	44(13.4)	34(23.6)	55(32.9)	15(17.0)
4 leaf	26(8.8)	32 (8.3)	21(6.4)	1(0.7)	4(2.4)	4(4.5)
2 leaf	23(7.8)	45(11.7)	28(8.5)	41(28.5)	34(20.4)	15(17.0)
1 leaf	14(4.7)	29(7.5)	27(8.2)	7(4.9)	15(9.0)	6(6.8)
5 leaf	12(4.1)	15(3.9)	11(3.3)	---	1(0.6)	---
Meristem less	25(8.5)	34(8.8)	44(13.8)	11(7.6)	4(2.4)	11(12.5)
Elongated Roots	33(11.2)	45(11.7)	44(13.8)	9(6.3)	9(5.4)	6(6.8)
Weak Embryos	17(5.8)	26(6.8)	29(8.8)	4(2.8)	2(1.2)	5(5.7)
No Germination	29 (9.8)	21(5.5)	19(5.8)	6(4.2)	3(1.8)	3(3.4)
1 small + 1 large leaf	14(4.7)	22(5.7)	16(4.9)	2(1.4)	16(9.6)	4(4.5)
Embryos with distorted shapes	11(3.7)	17(4.4)	19(53.8)	2(1.4)	4(2.4)	2(2.3)
2 Stem embryos				1(0.7)		
Embryos with very thin leaves		2(0.5)	6(1.9)			3(3.4)
Albino leaves					15(9.0)	
Total embryos	295	384	329	144	167	88
(%)	(21.0)	(27.3)	(23.4)	(10.2)	(11.9)	(6.3)
G. Total=	**1407 embryos**					

M1, M2, M3 Large, medium and small sized seeds in Marker fruits
S1, S2, S3 Large, medium and small sized seeds from normal fruits.
* embryos (%)

Various mutant types were observed in Kinnow mandarin. Some mutations were lethal like meristemless embryos, embryos with needle like leaves and albino embryos. Normal but different from control like root elongation more than double the size of shoot, leaves with 2 mid-ribs, various degrees of narrowness of leaves with more length as compared to control, 2 stem embryo all these survived on grafting on rootstock seedlings.

In Punjab, rough lemon is the dominant cultivar. The excessive vigor imparted to the scion by rough lemon generally produces poor quality fruit and mandarin fruit in particular for trees grown on rough lemon rootstock tends to be puffy and holds poorly on tree. However, there are exceptions. There are Kinnow fruits with strong holding potential to tree with excellent quality on rough lemon only with seedy draw back. We used grafting for (i) propagation of plants from embryos which otherwise have no growth power in soil (ii) to decrease the time required for flowering and fruiting and (iii) to adjust plants against stresses which rough lemon is capable of.

Embryonic narrow leaves are characteristics of low seeded fruits and embryonic leaves have resemblance to orchard plant leaves (Altaf *et al.*, 2002). This work clearly indicated that there is probability of narrow embryonic leaves in most of the seed shapes. Since embryos have single cell origin the low seeded/seedless trait can be cloned by growing these embryos into plants. The genetics of seedlessness is not clear in *Citrus*. The genetic control of fruit number, fruit size and seed number in progeny of cross *Citrus volkameriana x Poncirus trifoliata* are controlled by QTLs some of which are located in the same genomic regions suggesting that undesired associations could be broken to some degree by recombination (Garcia *et al.*, 2000). Cit. Dad-1-1 gene which is involved in programmed cell death was isolated from Satsuma mandarin fruit. The studies suggest that Dad-1-related sequences are present as a small gene family in the *Citrus* genome. Expression of cit dad-1-1 was progressively down regulated in leaves as they matured but not in juice sac/segment epidermis (edible part) towards fruit ripening. (Moriguchi *et al.*, 2000). For seedlessness, genes for parthenocarpic ability, male/female sterility, pollen incompatibility and genes that directly target seed destruction after fertilization to leave an imperceptible residue. Koltunow *et al.*, 2000 observed that in different seed types specific tissues need to be targeted in order to reduce seed size effectively. They are trying these genes in West Indian lime. Vardi *et al.*, 2000 suggest that S (self incompatibility) allele of Satsuma differs from two S alleles of Ellendale and one of the S allele of Clementine. In addition, they suggested that obligatory parthenocarpy in *Citrus* depends on 3 dominant complementary genes. The various degree of narrowness in embryonic leaves from low seeded fruit samples of Punjab orchards indicated that it is not one factor involved in low seeded/seedless trait but a complex of unknown factors. Clones from somatic cell lines of various seed shapes and seed sizes can help in understanding the complexity of seedless trait in Kinnow mandarin.

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