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TOWARDS A SEEDLESS CULTIVAR OF KINNOW MANDARIN IV. EMBRYOGENESIS OF 1–12 SEEDED FRUITS

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Abstract

Kinnow mandarin has nucellar polyembryony. Embryogenesis of fruits having 1–12 seeds was studied. There were 24 types of embryonic structures with most normal as 2–3 leaf with balanced germination. The normal looking embryos have best plant growth while meristemless embryos have no survival upon top – grafting on rootstock seedlings.

Introduction

Clonal variation in Kinnow mandarin was utilized to enhance and release somatic cell lines carrying seedless trait. Seed number variability is not whole sole environmental as the narrow new emerging leaves of branches/sprouts carrying low seeded/ seedless trait also appear in embryos (Altaf *et al.*, 2002), indicating the genetic stability of the newly formed leaves character. Exact reasons of seed number variability are not known but hybrid nature of Kinnow, large scale propagation as dominant cultivar and no control on quality selection of scion budwood with undefined rootstock seedlings are definitely responsible factors. Similarly, there are examples of clonal variation as seedless form of Santra (Mud Khed seedless) was selected from the local cultivar in India (Chakrawar & Rane, 1977). At Nagpur, India, seedless Santra has been selected which has commercial potentiality (Chadha & Singh, 1990). Selection of improved cultivar from existing well spread cultivars has been the practice of most of the *Citrus* breeders.

The present paper describes the embryo morphogenic structures derived from the low seeded fruits. The normal embryos from different somatic cell lines are utilized for future studies of low seeded trait by making plants on top-grafting embryos on rootstock seedlings.

Materials and Methods

Fruits were collected from Punjab orchards as follows:

1. Fruits (Fig. 1A) carrying marker (Iqbal *et al.*, 2001) have 5 – 20% probability of low seeded/seedless trait. There is variation in structure of marker and in size, shape of marker fruits.
2. Fruits were collected from branches having narrow new emerging leaves of sprouts (Fig. 1B), stem or terminal portion of branches. However, there is variation in intensity of narrowness of newly formed leaves. All fruits were screened for developed seed number and embryos of individual fruits having 1 – 12 seeds cultured according to the method described by Altaf *et al.*, (2002). Morphogenic structures of all embryos of individual fruits were recorded after one month in culture medium. The data was arranged under total fruits with a specific developed seed number from marker and normal fruits (Table 1). The normal embryos were top-grafted on rootstock seedlings of 4 – 6 months age for future studies.

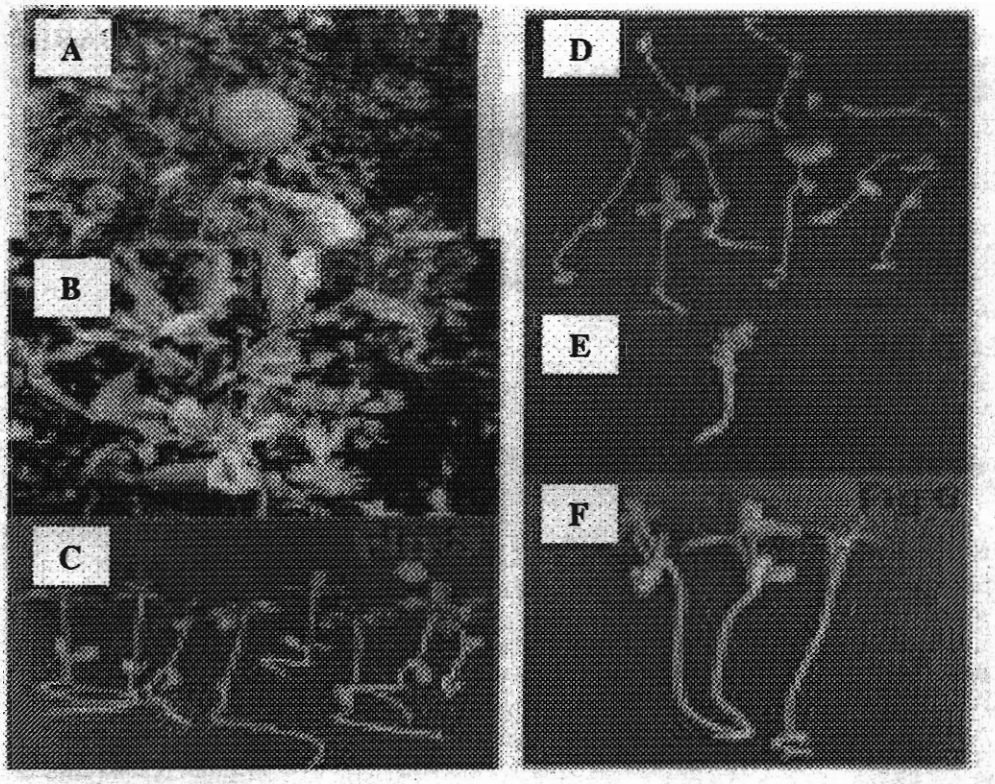

Fig. 1. Kinnow mandarin fruits collected from Punjab orchards.

A. Marker fruit.
B. Narrow new emerging leaves of branch carrying seedless trait.
C. Embryos from low seeded fruits, also have narrow new emerging leaves like parent.
D. Different embryonic structures from low seeded fruits.
E. Multiple embryos.
F. 2 Stem embryos.

Results

Kinnow mandarin nucellar polyembryony developed embryos of various growth stages. The following morphogenic structures were found in 1 – 12 seeded fruits (Table 1, Figs. 1C–F).

1) Meristemless, 2) No germination, 3) Meristemless + large root, 4) 2 leaf embryos, 5) Embryos with very thin leaves, 6) 2 leaf elongated roots, 7) 3 leaf elongated roots, 8) Elongated 2 leaves, 9) One leaf embryo, 10) One elongated leaf, 11) 1 small + 1 large leaf (with 2 mid-rib), 12) 1 small + 1 large leaf without root, 13) 1 small + 1 large leaf, 14) Weak embryos, 15) Distorted embryos, 16) 4 leaf embryo, 17) 5 leaf embryo, 18) Cotyledon + root, 19) 2 small leaves + leaf with 2 mid-rib, 20) Without root embryos, 21) Elongated roots, 22) Thin wrinkled leaves, 23) One leaf with 2 mid-rib, 24) Embryo with 2 stem.

Table 1. (Cont'd.). Effect of seed number on embryogenesis.

	Fruit (n)		Fruit (m)		Fruit (n)		Fruit (m)		Fruit (n)		Fruit (m)		Fruit (n)		Fruit (m)	
Seed No./fruit	7		7		8		8		9		9		10		10	
Fruits observed	35		9		24		3		12		11		12		5	
Embryo types:	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
1. 3 Leaf embryos	128	(19.7)	27	(20.1)	157	(24.2)	14	(16.3)	76	(16.6)	51	(17.3)	69	(17.6)	26	(21.7)
2. 2 Leaf embryos	211	(32.4)	47	(35.1)	197	(29.6)	37	(43.0)	150	(32.9)	91	(30.8)	12	(32.3)	27	(22.5)
3. One leaf	35	(5.4)	5	(3.7)	48	(7.4)	2	(203)	20	(4.4)	11	(3.7)	25	(6.4)	7	(5.8)
4. Elongated roots	77	(11.8)	9	(6.7)	60	(9.3)	10	(11.6)	35	(7.7)	49	(16.6)	39	(9,9)	14	(11.7)
5. Weak embryos	99	(15.2)	31	(23.1)	40	(6.2)	22	(25.6)	108	(23.7)	14	(4.7)	54	(13.7)	32	(26.7)
6. Meristemless	15	(2.3)	1	(0.7)	24	(3.7)			29	(6.4)	9	(3.1)	15	(3.8)		
7. Cot-+root	26	(4.0)	6	(4.5)	43	(6.6)			24	(5.3)	11	(3.7)	17	(4.3)		
8. Embryos with V thin leaves	8	(1.2)			9	(1.4)					7	(2.4)			1	(0.8)
9. Distorted Shapes	17	(2.6)			92	(9.6)					47	(15.9)	40	(10.2)	3	(2.5)
10. 1 leaf with 2 mid-rib	7	(1.1)									2	(0.7)			2	(1.7)
11. No germination	10	(1.5)							3	(0.7)			1	(0.3)	4	(3.3)
12. 1 small + 1large leaf	13	(2.0)	2	(1.5)	6	(0.9)	1	(1.2)	5	(1.1)	3	(1.0)	5	(1.3)	4	(3.3)
13. 4 leaf	3	(0.5)			5	(0.8)			3	(0.7)						
14. Without root embryos	2	(0.3)	4	(3.0)												
15. 3 leaf, 1 leaf with 2 mid-rib			2	(1.5)												
16. 5 leaf embryo					1	(0.2)										
17. Embryos with 2 stem					1	(0.2)			3	(0.7)			1	(0.3)		
18. Embryo types	14		10		13		6		11		11		11		10	
Total embryos	**651**		**134**		**648**		**86**		**456**		**295**		**393**		**120**	

n: normal Kinnow fruit.
m: ring on proximal end of fruit.

Table 1. (Cont'd.). Effect of seed number on embryogenesis.

	Fruit (n)		Fruit (m)		Fruit (n)		Fruit (m)		Fruit (n)		Fruit (m)	
Seed No./ fruit	4		4		5		5		6		6	
Fruits observed	54		10		45		10		38		12	
Embryos types:	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
1. Embryos with 2 leaf	161	(29.3)	38	(36.5)	211	(30.3)	47	(42.3)	205	(36.9)	34	(23.9)
2. 1 small leaf + large leaf	5	(0.9)			4	(0.6)	5	(4.5)	14	(2.5)	1	(0.7)
3. Embryos with 3 leaf	116	(21.1)	11	(10.6)	104	(14.9)	23	(20.7)	90	(16.2)	32	(22.5)
4. Embryos with elongated roots	56	(10.2)	18	(17.3)	82	(11.8)	7	(6.3)	63	(11.3)	7	(4.9)
5. Multiple embryos	1	(0.2)										
6. No Germination	9	(1.6)	4	(3.8)	16	(2.3)			14	(2.5)		
7. Distorted embryos	22	(4.0)					3	(2.7)	28	(5.0)		
8. Cot. + root	25	(4.6)	2	(1.9)	47	(6.7)	6	(5.4)	26	(4.7)	5	(3.5)
9. Weak embryos	62	(11.3)	28	(26.9)	127	(18.2)			62	(11.2)	41	(28.9)
10. 1 leaf with 2 mid-rib	2	(0.4)									2	(1.4)
11. 1 small leaf + 1 leaf with 2 mid-rib	5	(0.9)			6	(0.9)	1	(0.9)				
12. Meristemless	32	(5.8)	2	(1.9)	28	(4.0)	8	(7.2)	21	(3.8)	13	(9.6)
13. Embryos with V. thin leaves	11	(2.0)			10	(1.4)	1	(0.9)	2	(0.4)	1	(0.7)
14. 1 leaf	37	(6.7)	1	(1.0)	48	(6.9)	8	(7.7)	25	(4.5)	6	(4.2)
15. 4 leaf	4	(0.7)			5	(0.7)	2	(1.8)	5	(0.9)		
16. 5 leaf	1	(0.2)										
17. Embryo with 2 stem					1	(0.1)			1	(0.3)		
18. Thin wrinkled leaves					8	(1.1)						
Total embryos	**549**		**104**		**697**		**111**		**556**		**142**	

Table 1. (Cont'd.). Effect of seed number on embryogenesis.

	Fruit (n)		Fruit (m)		Fruit (n)		Fruit (m)		Fruit (n)		Fruit (m)	
Seed No./ fruit	4		4		5		5		6		6	
Fruits observed	54		10		45		10		38		12	
Embryos types:	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
1. Embryos with 2 leaf	161	(29.3)	38	(36.5)	211	(30.3)	47	(42.3)	205	(36.9)	34	(23.9)
2. 1 small leaf + large leaf	5	(0.9)			4	(0.6)	5	(4.5)	14	(2.5)	1	(0.7)
3. Embryos with 3 leaf	116	(21.1)	11	(10.6)	104	(14.9)	23	(20.7)	90	(16.2)	32	(22.5)
4. Embryos with elongated roots	56	(10.2)	18	(17.3)	82	(11.8)	7	(6.3)	63	(11.3)	7	(4.9)
5. Multiple embryos	1	(0.2)										
6. No Germination	9	(1.6)	4	(3.8)	16	(2.3)			14	(2.5)		
7. Distorted embryos	22	(4.0)					3	(2.7)	28	(5.0)		
8. Cot. + root	25	(4.6)	2	(1.9)	47	(6.7)	6	(5.4)	26	(4.7)	5	(3.5)
9. Weak embryos	62	(11.3)	28	(26.9)	127	(18.2)			62	(11.2)	41	(28.9)
10. 1 leaf with 2 mid-rib	2	(0.4)									2	(1.4)
11. 1 small leaf + 1 leaf with 2 mid-rib	5	(0.9)			6	(0.9)	1	(0.9)				
12. Meristemless	32	(5.8)	2	(1.9)	28	(4.0)	8	(7.2)	21	(3.8)	13	(9.6)
13. Embryos with V. thin leaves	11	(2.0)			10	(1.4)	1	(0.9)	2	(0.4)	1	(0.7)
14. 1 leaf	37	(6.7)	1	(1.0)	48	(6.9)	8	(7.7)	25	(4.5)	6	(4.2)
15. 4 leaf	4	(0.7)			5	(0.7)	2	(1.8)	5	(0.9)		
16. 5 leaf	1	(0.2)										
17. Embryo with 2 stem					1	(0.1)			1	(0.3)		
18. Thin wrinkled leaves					8	(1.1)						
Total embryos	**549**		**104**		**697**		**111**		**556**		**142**	

Table 1. Effect of low seeded fruit on embryogenesis.

	Fruit (n)		Fruit (m)		Fruit (n)		Fruit (m)		Fruit (n)		Fruit (m)	
Seed No./ fruit	1		1		2		2		3		3	
Fruits observed	6		6		27		6		51		13	
Embryo types:	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**	**No**	**%**
1. Meristemless	3	(25.0)	6	(75.0)	7	(5.9)			6	(1.7)	10	(15.2)
2. No germination	2	(16.7)	1	(12.5)	6	(5.1)	2	(15.4)	27	(7.7)		
3. Meristemless + large root			1	(12.5)								
4. 2 leaf embryos	5	(41.7)					3	(23.1)	122	(34.5)	18	(27.3)
5. Embryos with V. thin leaves	2	(16.7)			9	(7.6)	1	(7.7)				
6. 2 leaf elongated roots					32	(27.1)			5	(1.4)		
7. 3 leaf elongated roots					14	(11.9)	3	(23.1)	74	(20.9)	13	(19.7)
8. Elongated 2 leaves					6	(5.1)						
9. One leaf embryo					10	(8.5)			16	(4.5)	2	(3.0)
10. One elongated leaf					1	(0.8)						
11. 1 small + 1 large leaf (with 2 mid-rib)					4	(3.4)					1	(1.5)
12. 1 small + 1 large leaf without root					1	(0.8)						
13. 1small + 1 large leaf					5	(4.2)			2	(0.6)		
14. Weak embryos					6	(5.1)	1	(7.7)	58	(16.4)	10	(15.2)
15. Distorted embryos					4	(3.4)	1	(7.7)	10	(2.8)	4	(6.1)
16. 4 leaf embryo					3	(2.5)			4	(1.1)		
17. 5 leaf embryo					1	(0.8)						
18. Cot. + root					9	(7.6)	2	(15.4)	30	(8.5)	7	(10.6)
19. 2 small leaves + 1 leaf with 2 mid-rib											1	(1.5)
Total embryos	**12**		**8**		**118**		**13**		**354**		**66**	

n = normal , m = marker

Table 1. (Cont'd.). Effect of seed number on embryogenesis.

	Fruit (n)		Fruit (n)	
Normal fruits				
Seed number	11		12	
No. of fruits observed	18		11	
Embryo types:	**No.**	**%**	**No.**	**%**
1. Weak + distorted embryos	85	(17.8)	51	(16.8)
2. 1 small leaf + 1 leaf with 2 mid-rib	2	(0.4)		
3. Elongated roots	53	(11.1)	19	(6.3)
4. Embryos with V. thin leaves			12	(4.0)
5. 4 leaf embryos	3	(0.6)		
6. No germination	14	(2.9)		
7. 5 – leaf embryo			1	(0.3)
8. 3 – leaf embryo	84	(17.6)	89	(29.4)
9. 2 – leaf embryo	174	(36.5)	106	(35.0)
10. 1 leaf embryo	21	(4.4)	11	(3.6)
11. 1 small + 1 large leaf	5	(1.1)	3	(1.0)
12. Meristemless	19	(4.0)	11	(3.6)
13. Cot. + root	17	(3.6)		
Types of embryos	11		9	
Total embryos	**477**		**303**	

Embryos having cotyledons, normal 2–3 leaves and with balanced shoot – root development ranged between 42.3–59.5% with minimum in 1–2 seeded and maximum in fruits having 11–12 seeds. Perhaps the mature embryos have quick growth of 3 leaves, while others have 2 leaves. These were the most normal embryos and upon grafting, they had maximum survival with comparatively faster growth. The meristemless embryos that cannot develop leaves and proper shoot system ranged between 35.3–4% with maximum in fruits with 1–2 seeds and minimum in 11–12 seeded fruits. Only one meristemless embryo with elongated root was found in one seeded marker fruit. Meristemless embryos developed small needle like leaves after 2–3 months growth in the same culture medium where they never gained proper growth even after grafting on rootstock seedlings.

Embryos with narrower leaves were 11% in 1–2 seeded, 1.3% in 3–5 seeded, 0.92% in 6–8 seeded, 1.6% in 9–10 seeded and 4% in 11–12 seeded fruits. Only one embryo with thin wrinkled leaves was found in 5 seeded normal fruits. One embryo with elongated leaf was found in 2,4, 8 and 12 seeded fruits. Two small leaves and one large leaf with 2 mid-rib was found in 3, 7 seeds marker fruits and 8 seeded normal fruit. Two stem embryos were found in 5, 6, 8, 9 and 10 seeded fruit. The maximum seeds (16.7%) could not germinate in one seeded normal fruits followed by 2 seeded (15.4%) and one seeded marker fruit (12.5%). In 4 seeded and in higher number upto 12 seeds, the seed germination was high in some cases 100% as in 3, 5, 6, 7, 8 and 9 seeded marker fruits and also 100% seed germination in 8 and 12 seeded normal fruits.

Embryos with very thin leaves in which usually the new emerging leaves are narrower, 2 stem embryos and embryos with 2, 3, 4 and 5 leaf with balanced germination, embryos with elongated roots and leaves also grew and survived. Difficult to grow were shoot meristemless, one leaf embryos, embryos without root development, leaves with mid-rib, weak and embryos with distorted shapes. Since embryos have single cell origin,

the clones will be studied for new emerging leaves and other plant characteristics derived from low seeded fruit trait.

Discussion

The ideal consumer's demand in Kinnow is attractive appearance with pleasant smell, easy to peel, high sugar content with no seed and most importantly fruit with uniform quality. Commercially unacceptable number of seeds and variability in fruit characteristics including seed number per fruit is an obstruction to good export earnings with the facts that Kinnow is dominant cultivar. *Citrus* is highly heterozygous and Kinnow itself has chromosomes of three cultivars, so somatic variability released through nucellar polyembryony is important to improve Kinnow clone especially for seedlessness. It was observed that narrow new emerging leaves of embryos have similarity to new emerging leaves of the branch from where the low seeded fruits were harvested (Altaf *et al.*, 2002) indicating the genetic origin of seedless trait rather than environmental. So the low seeded/seedless fruit character can be selected to some extent on the basis of new emerging leaves of embryos. In large embryo population from somatic cell lines, selection of mutants is feasible alternative where otherwise improvement is difficult, time consuming with conventional means.

Comparatively fast growing plants developed with 2, 3, 4 and 5 leaves and with 2 stem embryos with normal or elongated roots. Embryos with thin or wrinkled leaves, one leaf or leaves with 2 mid-rib are comparatively slow growing. Weak, distorted shapes, multiple embryonic structure are difficult to grow. Meristemless mutation is lethal, like some can only develop root system without stem and leaves. Embryos with poor root system are not normal as they have tendency of leaf fall after grafting

It is usual that embryos with normal sequence of development were easily converted into plants in sweet oranges, lime and mandarins (Tomaz *et al.*, 2001), though plant regeneration can be obtained by deformed embryoids by culturing them on proper media (Hong-Yong *et al.*, 2000). Nucellar selections have been used for production of seedless fruit (Ruberto *et al.*, 1999).

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