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TOWARDS A SEEDLESS CULTIVAR OF KINNOW MANDARIN VII. NATURAL AND INDUCED VARIABILITY.

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Abstract

Plants were made from natural and induced variability to improve the Kinnow plant and especially the fruit quality. There are differences among fruits in developed and undeveloped seed number and so are differences in various tissue grafting responses. Nine % selected material did not sprout even after several months of grafts and ultimately died. One third of the grafts did not work may be because of nature of selected mutated tissue, incompatibility of tissue to rough lemon etc. Around half of the grafts showed leaf abscission under stress. The successful growing clones in the field will be tried for their fruit characteristics and selected clones will be multiplied and released as cultivars. Five clones proved to be low seeded with 0 – 6 seeds per fruit.

About 80% of Kinnow nucellus has plant regeneration responses. The plants developed from nucellar calli, radiation and 8-hydroxy quinoline were developed. The sprouts selected from different vegetative characteristics branches were grafted on rough lemon to study induced mutations and for selection of quality fruit.

Introduction

International market demand for fruit with increasing quality are difficult to produce because of lack of adequate varieties or impossibility of growing some of the existing higher quality varieties in many areas as a consequence the number of scions varieties and rootstocks that can be used in different *Citrus* growing areas is very limited. Not a single variety originated by conventional breeding has reached wide international use. The varieties presently grown have been mainly originated by bud sport mutations and chance seedlings (Pena & Navarro, 1999). In this situation genetic improvement of existing cultivars has a high priority.

The province of Punjab produces 95–96% *Citrus* and Kinnow enjoys 61–62% share in production. Kinnow fruit has pleasant flavour and loose skin that can be easily peeled, therefore, consumer show an interest in mandarin. Any genetic change through spontaneous mutations cannot be easily detected unlike new cultivars originated by spontaneous mutations in Satsuma and Clementine mandarin as Kinnow show extreme variabilities in all characteristics because of its hybrid nature; also its parent king is a tangor. Therefore, we tried to utilize extreme heterozygosity chimerism of cultivar Kinnow by making natural selections, as it is the dominant cultivar with large-scale production. Induced variability by callusing, gamma radiation and chemicals for creating genetic changes is also tried. Spontaneous and induced mutations, Nucellar selection and soma clonal variation is important in *Citrus* breeding. Three spontaneous mutant clones A, B, C, of Valencia orange were compared by grafting selections and the source cultivar onto *C. aurantium* rootstock. The improved fruit characteristics and altered ripening time of clone A and B warrant propagation of this plant material and broader testing on a commercial scale (Kotsias, 2000).

Irradiation definitely enhances mutation frequency of vegetatively propagated plants. Spiegel-Roy (1988) reported that mutation breeding through irradiation is one of the pathways to seek triploid varieties. Zhang *et al*., (1988) obtained chromosome variation 1X, 3X, 4X and upto 16X mutated cells. Colchicine treatment of axillary buds of Ellendale and Clementine upon grafting on rootstock produced tetraploid plants and plants established in field by double grafting were having larger and stouter thorns on twigs, thicker and broader petals and larger pollen grains than original diploids (Oiyama, 1992). Embryogenesis of nucellar callusing has been tried on sweet oranges, Rangpur lime and mandarins (Tomaz *et al*., 2001). Ikeda *et al*., 2000 used ovules of Trovita orange and Oomishima navel for callus lines developing. Nucellar embryogenesis can provide somatic clones and callusing definitely is a mutagenic process.

The broad objective of this work was to utilize natural and induced variability for improving characteristics of Kinnow mandarin cultivar and the specific objective of the study is to develop Kinnow clones of seedless quality fruit from natural and induced variability.

Materials and Methods

1. Natural mutations: The dominant variety Kinnow mandarin is chimeric and has heritable bud variations like marker fruits, which have 5–20% probability of carrying seedless trait. Normal fruits also have probability of carrying seedless trait (Iqbal *et al*., 2001). The low seeded/seedless fruit bearing branches were selected from Punjab orchards. Healthy apices (Fig. 1) and sprouts were grafted onto one-year rough lemon seedlings. After 6–8 weeks of graft survival, the plants were transplanted into field. At the graft age of 3 months, all the sprouts of root stock seedlings were removed, to facilitate sprouting in scion.

2. Induced mutations: Healthy growing points from vigorous, mutant vegetative sprouts/shoots were selected and grafted on rough lemon seedlings in 2003. The plants used were developed by the methods given below:

a. Callus regenerated plants: Nucelli from fruits 60–90 DAP were cultured on MS + 2, 4 – D (2 mg s/l) for two months. The embryogenic calli were subcultured on MS + 2, 4-D+BA (each 1 mg/l). After 6 weeks, healthy embryos were developed on MS + GA 1 mg/l for one month. The plants were transferred to pot soil by hardening procedure of gradual reduction in humidity and finally one-year plants were transferred to field in 1997.

b. Use of $AdSO_4$ (Adenine Hemisulfate) $C_5H_5N_5$ .1/2 H_2SO_4 for development of embryogenic callus: Nucellus embryogenic callus cultures were made in $AdSO_4$ (25 – 40 mg/l) in MS medium. After 2–3 months, the cultures were transferred to MS + $AdSO_4$ 2 mg/l for 6 weeks. Healthy normal embryos were transferred to MS + GA 1 mg/l medium for 4 to 6 weeks. After passing a hardening phase, they were transferred to nursery and finally at the age of 2 years to field in 1997.

c. Use of Gamma radiation (0, 30, 60, 90 and 120 Gray) to pro-embryogenic nucellus: Embryogenic cultures were developed using nucellus after radiation as explant in MS + BA (1 mg/l) medium. After 3 months, the healthy, normal, balanced germinated embryos were grown in MS + GA (1 mg/l) medium for one month and then were

transferred to pots with gradual reduction in humidity. After passing a hardening phase of 2 months, the plants were transferred to nursery. The plants were transferred to field at the age of 2 years in 1998.

d. Use of 8-Hydroxychinolin (C_9H_7NO): Healthy uniform medium sized sterilized seeds were soaked in different doses of 8 – Hydroxychinolin (10–50 mg/l) for various time period (2–48 hours). After removing both testa, the seeds were germinated in MS salt medium. The developed plants were transferred to field in the year 2000.

Results

The low seeded/seedless tissue is mutant, which is lethal to ovule development and can be different from normal wild type vegetative tissue especially in grafting responses. Around one third of total grafts were not successful. Graft failure means no cellular union between scion and rootstock. This may be because of manual error. The small size of grafted tissue has low survival as compared to larger size. Sometimes the mutated tissue is very slow in growth. There is variability in mutated tissues. The physiological state of grafted tissue, the season of grafting and the compatibility between scion and rootstock are important factors. The change of apical vegetative meristem into floral meristem, which never reverts back into vegetative state and mostly, dies after grafting. There is union of grafts (Fig. 2), which not proliferated by sprouting and died at different stages. Since low seeded/seedless fruit bearing branches were selected, we experienced half of the vegetative tissue (Table 1) with leaf abscission under stressed condition of grafting (Fig. 3).

With a cycle of new emergence of leaves and drop, this high association of abscission trait with seedless fruits is important to understand as it eliminates large valuable germplasm because of linkage of negative character of fruit drop. Seedless clones also suffer harsh temperature both hot (above 45°C) and cold (below 2°C) seasons in NIAB field. Successful grafts in field are those, which have no leaf abscission that helps the plants to maintain growth.

There are always damages because of mishandling by labour including breakages in field and nursery etc., because the seedless clones are delicate and mutant tissue need extra care for at least one year. The nursery of mutant's grafts (Fig. 4) is essential to study the mode of sprouting, vegetative growth characteristics of plant etc.

Kinnow nucellus 60 – 90 DAP is responsive (70.9%) to callusing (Table 2). The embryogenic callus lines (Figs. 5-7) were utilized for obtaining plants with variable vigorous morphological characteristics by grafting mutant branch on rough lemon seedling. Nucellar embryos (Figs. 8-11) can also be obtained (Table 3) in adenine hemisulfate containing medium. The variable characteristics healthy branches were selected and grafted on rough lemon seedling.

Nucellus before embryogenesis was radiated. There was increase in regeneration in radiated nucelli as compared to control. The regeneration increased upto 60 gray which dropped in increasing dose of radiation. The embryos were grafted on rough lemon (Figs. 12-13). The coefficient of regeneration lowered as compared to control in response to 90 gray (Table 4) and further reduced in response to 120 gray, the coefficient of Nucellar embryogenesis was calculated by multiplying % nucelli responded to embryogenesis with average number of embryoids formed per nucellus and dividing the product by 100.

Fig. 1. Shoot apical meristem graft.

Fig. 2 Seedless plant

Fig.3 Plant showing abscission in one branch.

Fig. 4. Seedless fruit.

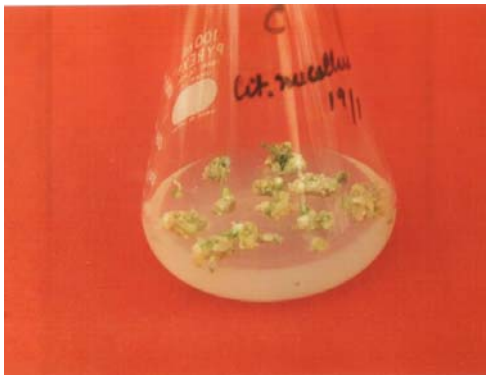

Fig. 5. Nucellar embryogenesis.

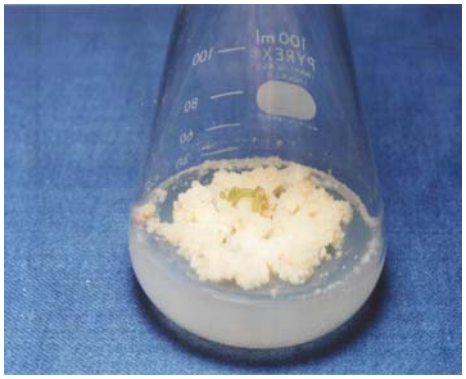

Fig. 6. Nucellar callus.

Fig. 7. Nucellar embryogenesis.

Fig. 8. Nucellar callus.

Fig. 9. Regenerations.

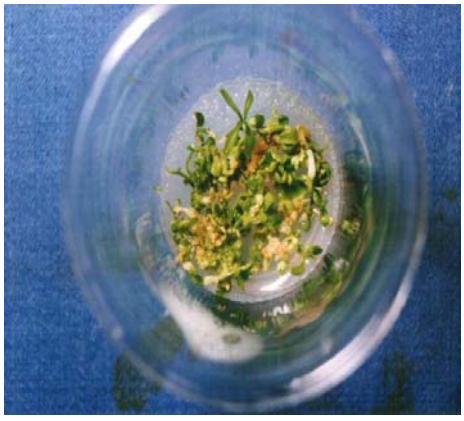

Fig. 10. Embryo development.

Fig. 11. Embryo development.

Fig. 12. Embryo micrograft.

Fig. 13. Embryo from radiated nucellus.

Fig. 14. Plant from 8-hydroxy quinoline in pots.

Figs. 15-16. Plants selected from variable branches.

Discussion

The genetic improvement of quality of Kinnow fruit in particular and Kinnow plant in general is on high priority for development of better economy by export, industry and fresh fruit market as citrus is major fruit of Pakistan with Kinnow mandarin as the leading cultivar. Kinnow has low seeded/seedless fruits (Altaf & Iqbal, 2003) with strong holding potential to tree available in cultivar that were utilized for developing new strains.

The chemical 8-hydroxy chinolin showed overall 33% seed germination (Table 5). It is 27, 24, 19, 16 and 14% for 10, 20, 30, 40 and 50 mg/l. Increasing the quantity of chemical and time of soaking, seeds stored reduced germination (Fig. 14).

Table 1. Shoot apical meristem/sprout grafting from low seeded/seedless fruit branches (3 years data).

Sr. No.	Grafting response	Counts (%)
1.	Graft Failure	23519 (33.3)
2.	Not responded to sprouting , Ultimately died	6132 (8.7)
3.	Graft with leaf abscission in stress	35380 (50.1)
4.	Grafts died in hot season	1382 (2.0)
5.	Grafts died in cold season	738 (1.0)
6.	Successful grafts in field	1550 (2.2)
7.	Grafts damaged	1293 (1.8)
8.	Grafts in pots	600 (0.8)
	Total	**70,594**

Table 2. Plants obtained from nucellar embryogenic callus.

Sr. No.	Treatments	Counts
1.	Nucelli cultured in MS + 2, 4-D (each 2 mg/l (for 8 weeks)	1239
2.	Embryogenic nucelli recovered	879
3.	Embryos in MS + 2,4-D + BA (each 1 mg/l) (after 6 weeks)	3627
4.	Healthy embryos transferred to MS + GA (1 mg/l) (for 4 weeks)	1457
5.	Embryos transferred to pots	600
6.	One year plants transferred to field	250

Table 3. Use of $AdSO_4$ for development of nucellar plants.

Sr. No.	Treatments	Counts
1.	Nucelli cultured in MS + AdSO4 (25–40 mg/l) (for 2–3 months)	3000
2.	Embryos developed in MS + AdSO4 (2 mg/l) (after 6 weeks)	6785
3.	Healthy embryos transferred to MS + GA (1 mg/l)	1000
4.	Embryos transferred to pots	89
5.	Plants transferred to field	22

Table 4. Embryogenic response of nucellus (90 DAP) in gamma radiation. Data after 3 months of culture.

Gamma radiation Gray	Nucellus response	% Nucellus response	Embryos produced	Av. Embryos per nucellus	Coefficient of regeneration
Control	341/649	52.54	1296	3.8	2.00
30	352/570	61.75	1479	4.2	2.59
60	381/633	60.11	1944	5.1	3.07
90	291/627	46.41	1077	3.7	1.72
120	143/544	26.29	329	2.3	0.60

Table 5. Germination of seeds after soaking in 8 – OH chinolin (10 – 50 mg/l) for 2 – 48 hours.

	Time (hours)					
C9H7NO (mg/l)	2	8	16	24	48	Total
10	63	49	51	36	21	220
20	67	54	43	19	17	200
30	53	47	35	11	9	155
40	42	38	33	14	6	133
50	39	27	28	13	7	114
			Total			**822**

It is a common practice that seedless fruit cultivars has been obtained by chance seedlings and bud sprout mutation. Mandarin cv. Ganpeng 1 was derived from chance seedling of CV. Penggan discovered in 1991 with quality fruits of 0.1 seeds per fruit (VS. 15–20 seeds per fruit in Penggan). The plants are adaptable to infertile red soils and are productive (Xu *et al*., 2000). Some 119 seedless cultivars of the genus *Citrus*, Fortunella and Clausena were obtained using bud sport selection, chance seedling selection and induced mutation. Reasons of seedlessness are reduced pollen fertility embryo sac abortion, chromosome ploidy variation and self incompatibility (Deng *et al*., 1996). Qianyang Wuhe Ponkan is a new Ponkan (*C. ponkan*) variety derived from a bud mutation. It has seedless fruits, high yields, early maturity and vigorous growth (Wu, 1999).

Our work of large scale screening of kinnow seedless trait and grafting of shoot apical meristem and sprouts from low seeded/seedless fruit branches showed maximum association of leaf abscission under stress. The low percentage of clones was heat or cold sensitive. It is one third losses due to graft failure and around 2% of successful grafts in NIAB field to be tested in coming seasons for clone characteristics. Up till now 5 clones have been tested for 0–6 seeds per fruit. After testing every clone, the best clones will be multiplied and tried for large scale propagation. These selected clones will definitely revolutionize *Citrus* in Pakistan as Kinnow is most soil and climate adapted cultivar. The bud take in mutant tissues needs further investigations. Vander *et al*., 1993 reported that bud take was highest and scion growth began earliest and was greatest from buds grown under hot conditions, bud starch, free abscisic acid, isopentenyl adenosine and zeatin riboside concentrations were generally highest in bud wood from cool conditions and IAA and gibberellic acid concentration were highest in bud wood from warm and moist conditions. Buds from new wood took better than buds from old wood which may be associated to higher IAA levels in new woods. The greater number of dormant buds from new wood may be correlated with higher free ABA. The higher IAA, isopentenyl adenosine and zeatin riboside concentrtation of apical buds most probably resulted in their greater percentage bud burst and faster growth (Vander *et al*., 1992).

Abscission is undesirable as it leads to fruit damages and losses. Abscission is associated with seedless *Citrus* cultivars under stress conditions. Less fruitlet abscission occurs on leafy than on leafless inflorescences in *Citrus* cultivars. The role of leaves in *Citrus* fruitlet abscission is well documented (Mehouachi *et al*., 2000). Since leaf and fruit abscission both have same mechanisms, we eliminated all Kinnow grafts which had leaf abscission in grafting. Grafting is a stress condition and sprouts having fruit drop trait abscise leaves within 3–4 days in low seeded/seedless Kinnow. Some branches have partial leaf abscission. They are probably chimeric for abscission trait. The branches which have strong fruit holding potential to tree characteristics never abscise leaves during grafting in seedless trait carrying Kinnow mandarin. Nucellar embryogenesis (Chaturvedi *et al*., 2001) and Nucellar embryo culture from polyembryonic *Citrus* cultivars (Obukosia & Waithaka, 2000) have been used by several workers. Plants from Nucellar embryogenic calli can provide novel variants (Figs. 15-16). Also that most *Citrus* viruses are not transmitted through the process of embryogenesis and that nucellar plant which are produced by somatic embryogenesis has the same characteristics as the mother plants that produced from seeds. The limitation is that nucellar plants are juvenile and require many years to lose their adverse juvenile characters to become commercially acceptable.

Large number of embryos in callus embryogenesis are abnormal and difficult to establish as plants. Nucellar callus embryogenesis by Nagasawa *et al*., (1999) revealed that water content of somatic embryos is an important index for normal germination and high germination rates were obtained in somatic embryos with 80 to 85% of water content. Tomaz *et al.,* (2001) reported that embryos that followed the normal sequence of development were easily converted into plants.

In our experiments of embryogenic nucellar callus of Kinnow mandarin, we used normal balanced germinated embryos for plant development and not tried abnormal embryogenesis for further studies except one leaf embryos, leaf with double mid-rib heart shaped leaves, rootless mutants which never survived upon grafting. Healthy but different characteristics vegetative leaves and stem were used for further plant development from nucellar callus, callus in the presence of $AdSO_4$, embryogenesis in response to gamma radiation and from plants derived by seed soaking in 8-OH chinolin. For this purpose medium sized seeds were used as tetraploids originate from large and triploids originate from small size seeds mostly. Esen & Soost (1971) reported that all 4X plants arose from large seeds. Tetraploids with an embryo: Endosperm radio of 4: 6 develop normally, as do diploids with a 2:3 ratio while triploids with a 3:5 ratio produced smaller seeds. Colchicine and 8 – OH chinolin have same mechanism of disturbing spindle formation resulting in polyploidy as existence of branches with larger size organs in plants derived from Kinnow seeds treated with 8 – OH chinolin.

All Kinnow plants were developed by top grafting selected material onto rough lemon seedlings. Top grafting is best procedure not only to establish plants in soil as Darjeeling orange shoot tips on root stock seedlings by Mukhopadhyay *et al*., (1997) which maintained tree growth, high yield and high fruit quality in spring twigs top grafted orchard of *Citrus* Changshan – huyou (Du, 2001).

Finally, plants developed through natural selections for seedless trait with elimination of abscission character and plants developed through nucellar callusing and nucellus in $AdSO_4$, plants developed from gamma radiated nucellus and from 8 – OH chinolin treated seeds can be a valuable germplasm for future cultivars originated from Kinnow mandarin.

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