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VARIATION WITHIN KINNOW (*CITRUS RETICULATA*) AND ROUGH LEMON (*CITRUS JAMBHERI*)

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Abstract

Citrus is number one in area and production of fruits in Pakistan. Punjab is the major *Citrus* fruit producing province. Kinnow mandarin (*Citrus reticulata*) is the dominant scion variety grafted on locally well adapted rootstock rough lemon (*C. jambheri*). The seed embryos from randomly collected fruits from all over Punjab province were studied in *invitro* culture and 24 different embryo seedlings shapes were found. In another experiment 10534 seeds from randomly collected fruits were cultured, among which 65% were found to be mono embryonic, 22% were bi-embryonic and 13% were poly embryonic. The explants from poly embryonic seedlings were responsive in culture while the explants from mono embryonic seedlings had weak culture response. The variation in rough lemon rootstock was also studied. It was observed that in one-year-old population of 1512 rough lemon plants, 59% were just normal plants, 18% had large spines, 6% had straight stem, 5% were broad leaved and 12% had dense foliage. It was further observed that among the 3 year old population of 318 plants, 19% individuals had broad leaves, 48 % were with dense foliage, 6% had large spines, 24% possessed straight stems and only 3% plants had leaf abscission character. Both the cultivars have intra varietal variation.

Introduction

The cultivation of *Citrus* has globally flourished due to its fruit and ornamental value. *Citrus* trees are ever green, full of fragrance, flavor, juice and visual beauty. The fruit possesses nutritive, refreshing and medicinal value. There are two markets in *Citrus* sector: fresh fruit *Citrus* market and processed *Citrus* products market like *Citrus* juice. The share of Pakistan in world fresh *Citrus* production is 2% (Anon., 2000-2004). Among the major fruits of Pakistan, *Citrus* holds the leading position both in terms of area and production. In Pakistan during 2005-2006, *Citrus* was cultivated over an area of 192.3 thousand hectares and the production remained 2458.4 thousand tonnes (Anon., 2006). During the year 2006 Pakistan made the highest ever export of around 200,000 tonnes of Kinnow to a number of countries including Middle East and the South East Asia (Syed, 2007). The prevailing climatic conditions in Pakistan are suitable for the successful cultivation of *Citrus*. Kinnow and feutrell's early are popular mandarin varieties, extensively cultivated in the major fruit producing and the largest canal system holder province, Punjab. Among different *Citrus* varieties grown in Pakistan, Kinnow mandarin (*Citrus reticulata*) is the major cultivar that captures more than 70% of the total area under *Citrus* cultivation. Kinnow is King and Willow-leaf mandarin hybrid developed by H.B. Frost in 1915. This miraculous cultivar was introduced in subcontinent in early 1940s and it was recommended for general cultivation in 1960s. Now, average Kinnow production stands over 2 million tonnes per annum (Syed, 2007). Due to its adaptation to the local climates it has become the most widely planted *Citrus* cultivar in Pakistan. It is a heavy yielder than any of other *Citrus* cultivars while they are grown under the same agro-climatic conditions because Kinnow has heat tolerance inherited from cultivar King. Kinnow has pleasant flavor and taste when fully ripe.

Kinnow is the dominant scion variety budded on the rough lemon (*Citrus jambheri*), which is well adapted root stock in the local climatic conditions of Punjab province. Kinnow mandarin (*Citrus reticulata*) possesses variability in vegetative and fruit characteristics like fruit sizes and shapes, peel thickness, fruit color, peelability, time of maturity, number of segments, central axis solid to hollow, sweetness, acidity, flavor, juice contents, number of embryos per seed, seed shapes, seed sizes and fruit abscission and fruit retention on the mother plant. A detailed study of the Kinnow seed embryos was made to study the variation within the embryo population as embryos are derived from cell lines present in fruit bearing branches. Rough lemon is local, well adapted and dominant rootstock. It has extreme variability and different strains. A proper rootstock seedling selection is major consideration because it is the root system of budded tree, supporting the tree, responsible for absorption of water and nutrients, providing storage of carbohydrates in leaves and synthesis of certain growth regulator, adapting the scion to particular soil and potentially providing tolerance to some diseases. The population of the rough lemon was also studied to asses the variability with in the rootstock seedlings. Selection of proper rough lemon seedlings is essential not only to produce healthy Kinnow plant, enhanced yields but also gives strength against stresses. Poly embryony is found in *Citrus*. This results in multiple seedlings most of which are exact clones of the parent tree. Thus many cultivars of *Citrus* are chance seedlings or mutations and not the result of breeding efforts. Apomictic processes can be initiated at several points during gametophyte development. Adventitious embryogenesis initiates from the somatic tissues of the ovule, nucellar and integument cells. These cells develop directly into embryos and compete with the zygote. The apomictic embryos are compatible to mother tissue and growth can be vigorous as compared to zygote. Both poly and mono embryonic plants are found in orchards of Kinnow mandarin.

Materials and Methods

Fruit collection: Kinnow fruit have 0-54 seeds per fruit. Low seeded/ seedless fruits were screened out on the basis of stylar ring and narrow new emerging leaves of the sprouts and the shoots of the orchard plants. The plants having fruits with stylar ring (marker) also have branches with fruits which have no stylar ring at all. The chances of seedless trait in such plants were 5-20%. The fruits were recorded according to the developed seed number 1,2,3,4 and 5. The rest was considered to be seedy. Highly seeded fruits were recorded just to have an idea of extent of the seedy nature. Seedless trait was found in both kinds of fruits with marker and normal (non marker fruits). Fruits were washed thoroughly, dried and stored in fridge and polyethylene bags until used in the experiment.

Seed culture: Seeds were sterilized with 0.01% $HgCl_2$ for five minutes with constant shaking and washed 4-5 times with sterile distilled water to remove any disinfectant. Both the seed testa were removed and embryos were placed on sterilized MS medium (Murashige & Skoog, 1962) containing GA@1mg/l. The pH was adjusted to 5.8. Cultures were kept in normal day light/night cycle at 25°C. The embryo shapes were studied after one month of culture and the data was recorded.

Study of embryony: The Kinnow fruit samples were randomly collected from various locations in Punjab province, both from the orchards and from the market. Fruits were washed thoroughly, dried and stored in fridge in polyethylene bags until their use in the experiment. The seeds were cultured as mentioned above. After 35 days, the embryos per seed were counted and recorded.

Explant culture: The explants from apex, stem, cotyledonary node (CN), and leaf from the developed mono and poly embryonic seedlings were cultured on MS-medium containing BA and 2-4 D (each 1mg/l) and other conditions were similar as mentioned above. The initial weights of the explants were noted at the time of the culturing and the weights of the explants were recorded after three weeks of culture. The increase in weight in *in vitro* growth of the mono and poly embryonic explants was noted.

Establishment of rough lemon nursery: Rough lemon fruits were collected from different orchards of Punjab Province. The seeds were extracted, soaked in water over night and then were sown in pots. The grown seedlings were then shifted in the field. Nursery seedlings were purchased from various locations of Punjab and were also planted in the field. In total there were 10,000 plants in the rough lemon nursery. One year old rough lemon population was divided in many blocks out of which nine blocks were randomly selected, each comprising of 168 plants. Another selected, 3 year old, rough lemon population containing 318 plants was also included in study. The selected blocks were thoroughly studied for morphological characters like straight stem, broad leaves, large spines and leaf abscission. The data recorded regarding the one year plants is given in pie graph-1 while data about the 3 year old plants is shown in pie graph-2.

Rooting in rough lemon: The rough lemon shoot cuttings were tried with rooting powder containing 4% IBA in simple talc at the base of the cuttings which were inserted in holes in soil of the pots. The pots were covered with transparent polyethylene bags to maintain internal humidity. After 6 weeks, humidity was gradually released and the cuttings were left to grow like normal plants.

Results and Discussions

A good Kinnow tree is vigorous, top erect, dense leaves, symmetrical with few thorns, leaves medium size, petioles short to medium. Fruit medium size, 6.5x7.3cm, weight is 200 grams, peel smooth and glossy, color yellow orange, juice 50%, seeds 24 per fruit, TSS 12-13, acidity 0.8, ripening time is February. Average yield per Kinnow tree is 1000 fruits in Sargodha (Ishfaq *et al.*, 2004). Kinnow mandarin has extreme variability. Variability in fruit characteristics itself has a negative economic impact. The merits of Kinnow tree are its vigorous growth, high yield potential, large and attractive fruit size, good blend of sugars and acid, deep orange color, adaptation of Kinnow with soil and climatic conditions of Punjab. Demerits are alternate bearing, seediness, limonin contents (causing bitterness) in juice, acidity, fruit and leaf abscission and low shelf life. Marker fruits have scar at the place of the stylar end of the fruit because of style abscission and are characteristics of all *Citrus* especially the hybrid cultivars (Garcia *et al*., 2001). The tree bearing marker low seeded fruit branches also have low seeded non-marker fruit branches which are on our priority of selection. Such trees are usually 1-4 in orchards of 25 acres of land. The marker is formed by delayed style abscission and this characteristic is inherited in vegetative growth and seedling plants. There is variability in fruit size, shape, structure of marker itself, number of developed and undeveloped ovules etc. The marker fruit trees also have normal fruit branches with seedless trait. The embryogenesis in *Citrus* species has been studied by various researchers: Khan *et al*., (2005) and in Kinnow mandarin (Parveen *et al*., 2003; Altaf, 2006, Singh *et al*., 2006) but no one has compared explant cultures of mono and poly embryonic Kinnow mandarin. Embryos provide single cell clones of mutant branches. Twenty four morphogenic structures were found in low seeded fruits as mentioned in Table 1. 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. So the seedless trait (Fig. 1) was selected to some extent on the basis of new emerging leaves of embryos.

Comparatively fast growing plants were developed with 2, 3, 4 and 5 leaves, 2 stem embryos with normal or elongated roots. Embryos with thin or wrinkled leaves, one leaf or leaves with two midribs are comparatively slow growing. Weak distorted shapes, multiple embryonic structures were difficult to grow. Meristemless mutation is lethal for embryo growth. Embryos with needle like leaves and albino embryo types were also lethal. Two stem embryos were rare. Embryos with poor root system are not normal as they have tendency of leaf fall after grafting. It was usual that normal sequence of development were easily converted into plants. Narrow embryonic leaves are related to over 20 seed shapes and in small seed size, which have higher frequency of narrow embryonic leaves. The intensity of leaf narrowness varied in different seeds. Abnormalities are found both *in vitro* and *in vivo* conditions, some times because of imbalanced endogenous growth regulator concentrations. Concentrations of Abscisic acid lower than 100ng/ ml in *Daucus carota*, a level comparable to the endogenous levels of ABA produced abnormal embryos especially those with extremely elongated roots (Kamada & Harada, 1981). The ABA contents in the flavedo of a novel mutant of Navelate named Pinlate mature fruit was 3-6 times lower than in the parent tissue (Rodrigo *et al.*, 2003). Secondary and abnormal embryos were observed in Valencia *in-vitro* embryogenesis (Lang Tao *et al.*, 2004). Some of the abnormalities observed were lack or dedifferentiation of protoderm and absence of apical meristems and procambial strands. Embryos that follow the normal sequence of development were easily converted into plants in *Citrus* species (Tomaz *et al*., 2001). Meristemless embryos were also found in *Citrus deliciosa* suspension cultures (Cabasson *et al*., 1997). Steinitz *et al*., (2003) deduced that the absence of a shoot in germinating embryos could originate from deviant differentiation at these early stages of embryony. Critical factors influencing plant production and survival were the production of normal viable embryos, balanced germination, and successful acclimatization to the external environment (Gmitter & Moore, 1986). In *Citrus*, zygote is usually weak because of extreme heterozygosity and because of development of polyembryony over the time period as an alternate means of plant survival. The zygote is weak because of competition with somatic embryos for space and nutrition. Nucellus is least differentiated and is source of obtaining somatic embryos. Nucellar embryos have better compatibility *in vivo* and hence better growth as compared to the zygote. Kinnow has clonal variability and different nucellar embryos result in different somatic cell lines. In embryo population from somatic cell lines, selection of mutants is feasible alternative where otherwise improvement is difficult, time consuming with conventional means. Kinnow mandarin has inter and intra orchard variability in all fruit characteristics. Mono embryonic strains are important with pollen self incompatibility for seedless trait. The seed formation may be due to foreign compatible pollen. In polyembryonic strains, even if zygote is a failure, the pollination triggers nucellar embryogenesis resulting in apomictic seed formation which is undesirable if the objective is seedless fruit. Mono and poly embryony segregate in Kinnow populations in varying frequencies. Poly and mono embryony (Fig. 2) must be coming from parental genomes. Mono embryony was 65%, the two embryos per seed were 22% and polyembryony was 13% in total 10534 seeds studied. These percentages depended upon the samples collected. Mono embryony provides new hybrids and nucellar polyembryony gives embryos of the somatic cell lines present in branches.

Mono and poly embryony seems to be under genetic control. Kapiro (2004) found that one gene is essential for nucellar embryony to occur and a second unlinked gene

strongly influences the number of embryos per seed in *Poncirus trifoliata*. Analysis of quantitative trait loci revealed the presence of six genomic positions, two in Poncirus trifoliata and four in *Citrus volkameriana* contributing individually up to 24% of the total variation for apomixis. One of the markers associated to apomixis (Apo-2) is also associated to embryony type. Therefore the genetic control of apomictic reproduction found in *Citrus* nuceller embryony is quite complex. The explant culture of mono and poly embryonic Kinnow seedlings have differences in growth rate (Table 2). The initial growth of explants is always slow which increases with passage of time and growth is always faster in subcultures. In this experiment, the growth of explants was compared after three weeks. Although polyembryonic seedlings are weaker than the mono embryonic ones yet the explant gained relatively more weight in the former in given period. All the four types of the explants gained weight in the culture media and showed the growth indices. Explants from poly embryonic seedlings were responsive to culture conditions studied while explants from mono embryonic seedlings had weak culture response. Genetic improvement can be achieved within Kinnow cultivar by selection and vegetative propagation of natural mutant branches for various degree of embryony. Importance of nucellar embryony for isolation of somatic cell lines having desirable fruit characteristics cannot be ignored.

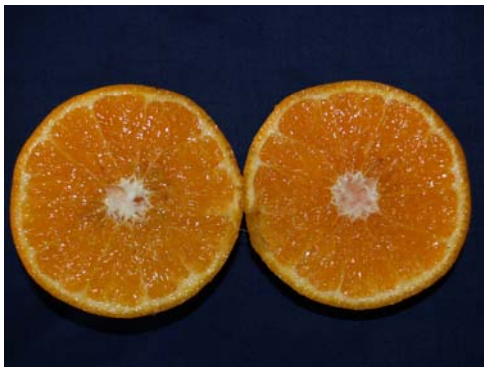


Fig. 1. Seedless trait in Kinnow mandarin.

Fig. 2. Poly (left) and Mono embryonic (right) seedlings.

Table 1. Embryo shapes in Kinnow mandarin.

Sr#	Type of embryos	Counts	%	Sr#	Type of embryos	Counts	%
1.	Meristemless	595	5.82	13.	1 small + 1 large leaf	204	1.99
2.	No germination	663	6.48	14.	Weak embryos	1025	10.02
3.	Meristemless + Large root	02	0.02	15.	Distorted embryos	617	6.03
4.	2 leaf embryos,	3279	32.07	16.	4 leaf embryo	213	2.08
5.	Embryos with very thin leaves	17	2.55	17.	5 leaf embryo	55	0.54
6.	2 leaf elongated roots	18	5.44	18.	Cotyledon + root	906	8.86
7.	3 leaf elongated roots	175	1.71	19.	2 small leaves + a leaf with 2 midribs	02	0.02
8.	Elongated 2 leaves	07	0.07	20.	Without root embryos	161	1.57
9.	One leaf embryos	576	5.63	21.	Elongated roots	636	6.22
10.	One elongated leaf	02	0.02	22.	Thin wrinkled leaves	55	0.54
11.	I small + 1 large leaf (with 2 mid-ribs)	65	0.64	23.	One leaf with two midribs	120	1.17
12.	1 small+1 large leaf without root	44	0.43	24.	Embryo with 2 stems	09	0.09

*Total Embryos studied: 10226

Table 2. Mono and Polyembryony in Kinnow Mandarin.

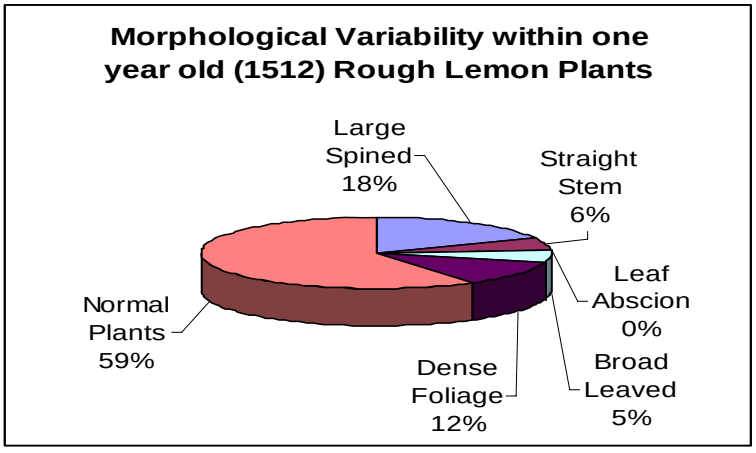
Embryos per seed	No. of seeds	% of seeds
Mono embryonic	6847	65
Bi-embryonic	2318	22
Poly-embryonic	1369	13
Total seeds studied	**10534**	

Table 3. Increase in explant weight after *in vitro* culture.

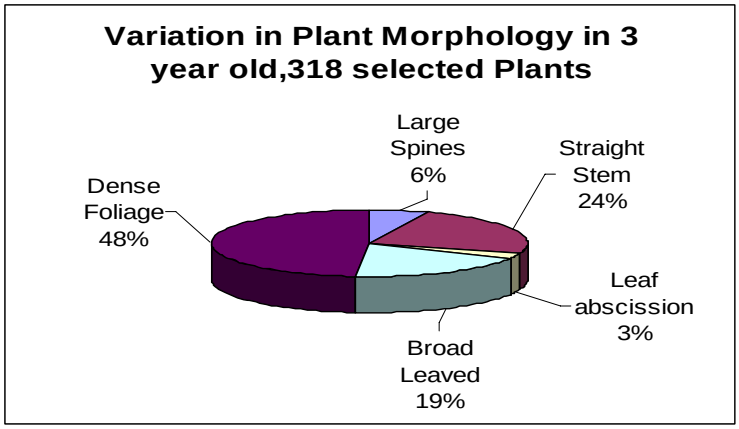
Explant source	Initial weight (mg)	Weight (mg) after 3 wks culture	Increase in weight	% Increase
	Explant culture of mono embryonic seedlings			
Stem	4.66	5.55	0.89	19.10
Apex	5.66	7.19	1.53	27.03
CN	11	14	3.00	27.27
Leaf	17	20	3.00	17.65
	Explant culture of polyembryonic seedlings			
Stem	10	40.83	30.83	308.30
Apex	7	25.5	18.50	264.28
CN	12	17.61	5.61	46.75
Leaf	23.33	111.11	87.78	376.25

Rough lemon as rootstock is good for warm humid areas and also for deep well-drained sandy soils which are subject to marked fluctuations in soil moisture. It is susceptible to gummosis, lemon decline, quick decline and root rot. It develops a deep root system, produces a heavy yield and gives long life to the tree if planted on suitable soil. The root system of the rough lemon is abundant in the upper parts of the soil and widely spread. Under suitable conditions and good culture, rough lemon stock is capable of inducing more rapid growth in *Citrus* varieties budded on it than any other stock commonly used. However Rough Lemon under drastically altered soil and climatic conditions do not seem to be suitable rootstock for *Citrus* because of tree health and quality of fruit (Khan *et al*., 2004). Bhusal *et al*., (2002) claimed rough lemon as drought intolerant and most flooding tolerant. The tree is usually vigorous and large, upright spreading with numerous thorns, leaves medium small, blunt pointed and light green. There are many selections of Rough lemon with morphologically distinct fruit that differ in fruit color from red to yellow. One of the red Rough Lemon selections (57-228-502) produced a high yielding and more manageable compact lemon tree with a large proportion of summer crop fruit (Freeman *et al*., 1986). The variability of rough lemon is documented by Singh (1997) as Mithi, mitha-tulia, Jambheri, Kotagiri, Renuka lemon, Florida Rough, Jatti Khatta, Jullandhuri, Jatti Khatti, Pathankot, Jambhiri Peda gaon, Jambhiri Kodur, Italian-76, Soh-myndong, Khatta Patiala, Jambhiri brown, Rough lemon Wynad,Rough lemon Chethalli, Jambhiri Poona, Jambhiri LocalAngul, Jambhiri Nagpur, Khatta, Jallandar Khatti, Khatta Jamir, Moogunimbe, Brazilian Rough Lemon, Chase Rough, Eastes Rough, Florida Rough-8748, Limoneria, Rough lemon 58-III-4, Rough Lemon Thrassa, Rough Lemon South Africa and Tulia Renga. Variation within rootstock populations is documented with scientist and seems to be a natural phenomenon. In C-32 Citrange marker studies indicate that variation is caused by both genetic and environment factors, with most populations having about 10% zygotic seedlings. Many nucellar seedlings have ‘off type’ leaf morphology presumably due to environmental factors (Roose *et al*., 1992). *Citrus limonia* is used as rootstock in 80-90% of Brazillian orange tree fields and a high polymorphism was detected within this germplasm using both

molecular and morphological markers (Marino *et al*., 1997). Herrero *et al*., (1996) documented two main factors that limit genetic intraspecific variability: apomictic reproduction, where nucellar embryos are much more vigorous than the zygotic ones, and nursery men selecting against variability in the seedling stage of the rootstocks or in propagating the scion cultivars vegetatively. Carlos *et al*., (2002) indicated that the combination of visual selection of leaf apex morphology and SSR analysis for the identification of hybrids derived from the cross of poly embryonic *Citrus* cultivars will improve the accuracy of the selection, save time and reduce the costs involved in the use of the molecular markers alone in *Citrus* breeding programs. On the basis of the results of morphological and phenotypic evaluations and some more specific studies, 29 clones from sour orange having various genetic structures were selected (Tuzcu& Yildirim, 2000). Moore & Castle (1988) did morphological and isozymic analysis of open pollinated 15 *Citrus* rootstock cultivars. They concluded that populations varied in degree of polyembryony and in percentages of the plants that morphologically and isozymically variable. In our rough lemon nursery more than half of the plants (59%) had normal morphology (Graph-1). Since spine is characteristic of juvenility, all the plants have spines. The differences are in the intensity, structure and size of the spines in plants. In this study18% plants were with large spines. Dense foliage in plants was 12% and has positive growth effects on root system as the root sprouting is always in response to leaf sprouting and well developed roots are prerequisite for scion bud growth and development. The plants with broad leaves were 5% and with straight stem were 6%. A root stock plant with straight stem is preferred as compared to branched stem for budding/grafting. Graph-2 shows the selected plants of Rough lemon for grafting the desirable fruit quality plants of Kinnow mandarin. The age of the rough lemon plants at the time of data recording was 3 years. In selected plants, the major share (48%) is of dense foliage plants followed by straight stem (24%). The broad leaf character was in 19% plants. Six percent of these plants have large spines where as the leaf abscission was observed in 3% plants which recovered after the plants got themselves established in the new field. The abscission character may be due to transplantation stress because the selected plants were transplanted from the nursery to a separate block.



Graph-1



Graph-2

In root induction of rough lemon (*Citrus jambheri*) success was recorded with 4% Indole Butyric Acid in simple talc for adventitious rooting in rough lemon stem cuttings. The rooting was good in cuttings with foliage. The rooting in rough lemon cutting can be used for vegetative multiplication of desirable plants. Machado *et al*., (2005) observed an increase in root number per cutting with the increase in IBA concentration in rooting experiments of semi hardwood cuttings of grape wine rootstock VR-043-43.

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