Occurrence of spontaneous polyploids in *Citrus*

M. Usman, T. Saeed, M. M. Khan, B. Fatima

Plant Tissue Culture Cell, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan

ABSTRACT: Seedlings recovered from underdeveloped seeds of different commercial cultivars of *Citrus* were studied for their ploidy level. Cytological examination revealed that maximum triploid plants were found in lime Kaghzi (15.5%) followed by grapefruit Foster, mandarin Kinnow, sweet orange Musambi and mandarin Feutrell's Early (7.3%). Lime also produced the highest percentage of tetraploids (9.1%) followed by mandarin Kinnow and grapefruit Foster, while the minimum was found in mandarin Feutrell's Early. The polyploids were transplanted in pots and after hardening they were grown in the greenhouse for further morphological and genetic assays.

Keywords: polyploids; *Citrus aurantifolia*; *C. paradisi*; *C. reticulate*; *C. sinensis*

The potential of polyploidy as a means of achieving improved *Citrus* cultivars has long been recognized; various origins of *Citrus* polyploidy and their uses in crop improvement and genetic manipulations have been discussed (Lee 1989). Polyploids such as triploids, tetraploids, pentaploids, hexaploids and octaploids are found spontaneously in *Citrus*. Well known are for instance tetraploid Hongkong wild kumquat (Longley 1925), triploid Tahiti lime (Bachi 1940) and tetraploid Triphasia desert lime (Esen, Soost 1972). Triploids show more vigor than tetraploids and have thick round leaves. Only a low proportion of triploids have produced satisfactory yield so far (Soost, Cameron 1975). However, triploids obtained from *Citrus* breeding were found the most resistant to low temperature. Tetraploids grow more slowly, are compact in habit and yield less than diploids of the same cultivar. Leaves of tetraploids are broader, thicker and darker in color than leaves of diploids. Yields vary with selections that might have less economic value but are important as breeding material (Khan, Rehman 1994).

Underdeveloped seeds are found with considerably high frequencies of polyploids in several polyembryonic cultivars of sweet orange and tangor (Wakana et al. 1981). Many other polyploids were produced experimentally and some valuable cultivars were evolved by ploidy manipulation (Spiegel-Roy 1988). The aim of this investigation was to develop a well-defined methodology for a rapid polyploidization and to enhance the existing status of *Citrus* germplasm.

MATERIALS AND METHODS

Fruits of mandarin (*Citrus reticulata* Blanco) cultivars Kinnow and Feutrell's Early, sweet orange (*Citrus sinensis* L. Osbeck) cv. Musambi, grapefruit (*Citrus paradisi* Macf.) cv. Foster and lime (*Citrus aurantifolia* Swing.) cv. Kaghzi were collected. They were cut in halves to extract seeds. Pulp of fruits was washed with tap water and obtained seeds were categorized as developed and underdeveloped. The developed seeds were discarded while the underdeveloped seeds (Fig. 1) were surface sterilized with 75% ethyl alcohol plus 1–2 drops of Tween 20 as surfactant for 3–5 minutes. Then the seeds were dipped in 5% sodium hypochlorite solution for 2 to 3 min and washed in deionized autoclaved distilled water. Plastic pots (7.5 × 8.5 cm) were filled with sterilized sand. Five seeds of each cultivar were sown per pot comprising 20 pots per cultivar that were kept in a growth room in 16h photoperiod, 22 μmol m^2/s and 25 ± 2°C temperature. Pots were watered every 2 to 3 days and the germination percentage was recorded.

At transplanting, after 6–10 weeks of sowing, root tip (greenish part) were taken for cytological studies and root and shoot length (cm) was measured. Root samples were pretreated with 1.4-di-

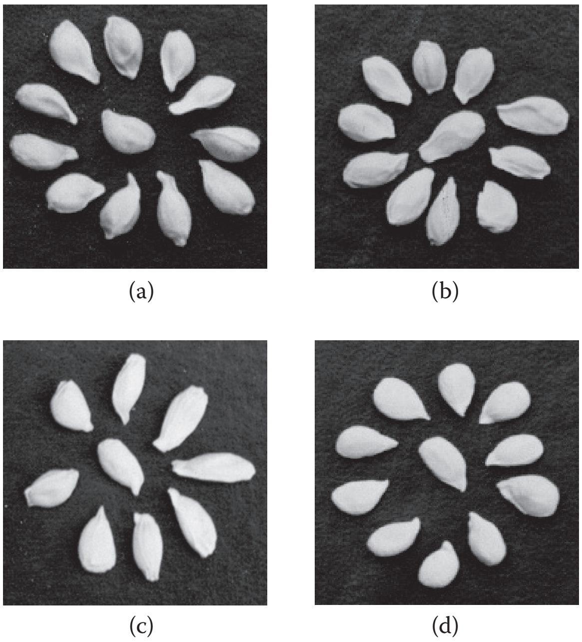


Fig. 1. Underdeveloped seeds of (a) Kinnow, (b) Feutrell's Early, (c) Musambi, (d) Kaghzi

chlorobenzene for 2–3 h at 20–26°C. Then they were dipped in fixative (100% ethyl alcohol, glacial acetic acid and chloroform in 6:1:3) for 10–12 h at 4°C. After fixation samples were washed with sterilized water. For microscopic observation the material was hydrolyzed with 5M HCl at 25°C for 20 min. Samples were dipped in Mordant solution containing 4% $FeNH_4(SO_4)_2$ 12 H_2O for 1 h and washed 4–5 times with sterilized water. Samples were stained with 0.5% hematoxylin dye for 2–4 h (Gmitter et al. 1990). Prepared squashes were observed under Nikon inverted microscope at 10× × 100× magnification. After cytological confirmation diploids were discarded. The polyploids were transplanted in pots filled with sterilized substrate containing sand, silt and farmyard manure (2:1:1). When seedlings obtained 10–15 cm height, they were planted in the greenhouse. The experiment was laid out in Completely Randomized Design (CRD). Every cultivar included 3 randomized plots. Data were analyzed statistically with Duncan's Multiple Range Test (Steel, Torrie 1980).

RESULTS AND DISCUSSION

Seed size and germination percentage

Citrus cultivars were examined for seed size viz. developed and underdeveloped. Significantly higher numbers were found in grapefruit cv. Foster (10 seeds) for developed seeds and in mandarin cv. Kinnow (17 seeds) for underdeveloped seeds (Table 1). The results are strengthened because triploids were obtained from underdeveloped seeds ranging 1/3 to 1/6 of size of developed seeds yielding mostly diploids (Esen, Soost 1973a,b). 1 haploid, 18 diploids and 51 triploids were found in plants originating from 82 underdeveloped seeds out of 2,049 seeds from pummelo cultivar Banpeiyu crossed with grapefruit (Toolapong et al. 1996). Maximum germination percentage was found in Musambi (42.5%) followed by Kaghzi (35.5%), while minimum germination was observed in Feutrell's Early (25.0%), Foster (29.5%) and Kinnow (35.3%).

Plant growth in *Citrus* cultivars

Diploid seedlings

Regarding total plant growth, results were found highly significant for diploid seedlings. Maximum plant growth was observed in Foster grapefruit plants (12.38 cm), followed by Musambi sweet orange, Feutrell's Early mandarin and Kaghzi lime (10.02 cm). Seedlings of mandarin cv. Kinnow depicted the least growth (9.44 cm) of all investigated cultivars (Table 2). Diploids are reported to grow more rapidly (Khan, Rehman 1994). Their leaves have lower dry matter and higher water content opposite to lemon leaves with high dry matter and low water content (Jaskani et al. 2002).

Triploid seedlings

Results of total plant growth of triploid seedlings of *Citrus* were nonsignificant for all investigated cultivars. However, the maximum growth was observed in grapefruit cv. Foster (11.16 cm), followed by Musambi, Feutrell's Early and Kinnow.

Table 1. Seed size of various *Citrus* cultivars

Seed size	Kinnow	Feutrell's Early	Musambi	Foster	Kaghzi	Mean
Developed	8 d	3 g	4 efg	10 c	4 fg	5.6 b
Underdeveloped	17 a	5 e	8 d	15 b	5 ef	9.8 a
Mean	12.5 a	4 c	6 b	12.5 a	4.5 c	

Means sharing the same letters are statistically non-significant

Table 2. Diploid seedling growth (cm) of *Citrus* cultivars after 10 weeks of sowing

Cultivars	Root length	Shoot length	Plant growth
Kinnow	4.49 ± 1.24*	4.97 ± 1.57	9.44 ± 1.43 b
Feutrell's Early	4.72 ± 0.98	5.71 ± 1.24	10.42 ± 1.21 b
Musambi	5.51 ± 1.64	5.05 ± 1.58	10.56 ± 1.62 b
Foster	8.03 ± 7.98	4.37 ± 1.09	12.38 ± 5.97 a
Kaghzi	5.29 ± 1.78	4.74 ± 1.10	10.02 ± 1.50 b

Means sharing the same letters are statistically non-significant;* ± standard deviation

Table 3. Triploid seedling growth (cm) of *Citrus* cultivars after 10 weeks of sowing

Cultivars	Root length	Shoot length	Plant growth
Kinnow	4.26 ± 1.33*	5.43 ± 1.35	9.70 ± 1.45
Feutrell's Early	4.45 ± 1.45	5.54 ± 0.91	10.00 ± 1.31
Musambi	5.10 ± 1.93	5.29 ± 0.91	10.40 ± 1.49
Foster	6.74 ± 2.59	4.43 ± 0.89	11.16 ± 2.24
Kaghzi	4.85 ± 1.65	4.79 ± 0.95	9.64 ± 1.32

Means sharing the same letters are statistically non-significant; * ± standard deviation

The least plant growth (9.64 cm) was found in lime cv. Kaghzi (Table 3). The results are supported as increasing ploidy results in larger cells, thicker and wider leaves which are less pointed from apex, large flowers and fruits, thick branches and stems with shortened internodes and crotch angles (Sanford 1983).

Tetraploid seedlings

Data regarding total growth of tetraploid seedlings showed significant differences for all studied cultivars. The maximum plant growth was found in mandarin cv. Feutrell's Early (12.34 cm), followed by Musambi (12.12 cm), Kaghzi (10.34 cm) and Kinnow (9.00 cm) while Foster showed the last plant growth (8.58 cm) (Table 4). The results are well-founded since tetraploids are reported to grow more rapidly than diploids (Khan, Rehman 1994) and leaves of tetraploids are broader and thicker with dark green colour (Barrett, Hutchinson 1978, 1981).

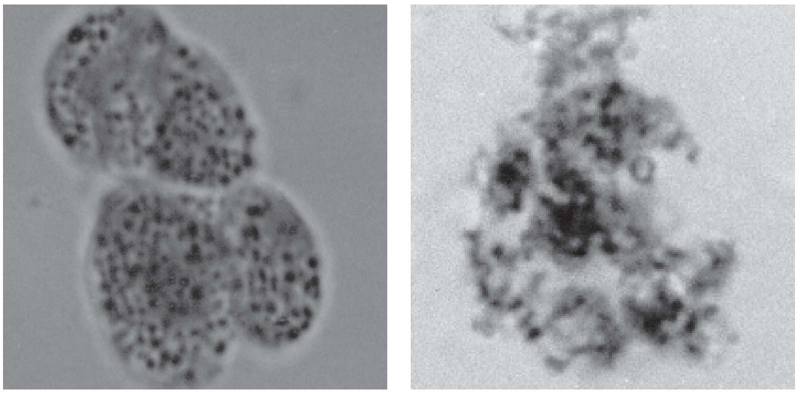
Frequency of polyploids

Root tips of *Citrus* cultivars were carefully excised, treated and prepared. These samples were then cytologically examined to confirm their ploidy level. Maximum percentage of diploid plants was found in cv. Feutrell's Early (90%), followed by Musambi (88%), Kinnow (84%) and Foster (82%) whereas minimum of diploid seedlings (75%) were observed in lime cv. Kaghzi. Lapin (1937) reported that majority of species of *Citrus* are diploid with somatic chromosome number 18. Maximum percentage of naturally occurring triploids was found in Kaghzi (15.45%), followed by Foster (14%), Kinnow (11.33%) and Musambi (9.33%); the lowest percentage (7.33%) of triploid plants was observed in cv. Feutrell's Early (Fig. 2a,b). The results obtained are in agreement with previous work since the frequency of spontaneous triploids was found as high as 5% among the seedling progeny (Frost 1925). Naturally occurring triploid Tahiti lime was reported by Bachi (1940).

Table 4. Tetraploid seedling growth (cm) of *Citrus* cultivars after 10 weeks of sowing

Cultivars	Root length	Shoot length	Plant growth
Kinnow	5.00 ± 0.96*	4.00 ± 2.02	9.00 ± 1.60 c
Feutrell's Early	5.00 ± 0.50	7.33 ± 0.29	12.34 ± 1.33 a
Musambi	5.50 ± 1.73	6.62 ± 0.48	12.12 ± 1.32 a
Foster	4.84 ± 1.47	3.75 ± 0.93	8.58 ± 1.30 c
Kaghzi	5.35 ± 1.20	5.00 ± 1.61	10.34 ± 1.39 b

Means sharing the same letters are statistically non-significant; * ± standard deviation



(a) (b)

Fig. 2. Chromosomal analysis (×1,250) of recovered polyploids (a) tetraploid Kinnow, (b) triploid Kinnow

Similarly spontaneous triploids were obtained among progenies of Lisbon and Eureka lemon, Ruby sweet orange and Imperial grapefruit (Esen, Soost 1971). As for naturally occurring tetraploid seedlings, the highest percentage was depicted in cv. Kaghzi (9.09%), followed by Kinnow (4.6%), Foster (4%) and Musambi (2.66%); while the lowest percentage of tetraploids (2%) was observed in cv. Feutrell's Early (Table 5). Several spontaneous tetraploid cultivars were reported previously such as naturally occurring tetraploid Hongkong of wild kumquat (Longley 1925), tetraploid Triphasia of dessert lime (Esen, Soost 1972), *Fortunella hindsii* and *Clausena excavata* (Froelicher et al. 2000). Spontaneous polyploids were also observed in apomictic seedlings of *Citrus* (Barrett, Hutchinson 1978).

The number of developed and underdeveloped seeds per fruit per cultivar was found to vary. Seeds from open pollinated fruits were polyembryonic (aposporous) with varying number of embryos per seed. Esen and Soost (1973a,b) and Soost and Cameron (1975) noted that small seeds that were thought to be polyploids were 1/3 to 1/6 of diploids seeds size.

Considerable number of shrivelled seeds occurred in *Citrus* fruits (Cameron, Burnett 1978). Empty seeds were observed less frequently in spontaneous polyploids (Oiyama et al. 1980, 1981; Oiyama, Kobayashi 1990), while a lower set of fully developed seeds is a common feature in *Citrus* (Cameron, Soost 1969; Esen, Soost 1972; Oiyama et al. 1981). Many empty and few fully developed seeds were obtained among spontaneous polyploids (Cameron, Soost 1969; Esen, Soost 1972; Cameron, Burnett 1978; Esen et al. 1979; Oiyama et al. 1981; Oiyama, Kobayashi 1990).

A reduction in the development of seed size of underdeveloped seeds might be due to the intrinsic capability of the ovule due to polyploidy, differences in number of pollinations and due to the delayed pollination leading to the shortage or absence of endosperm required for the growth and development of cotyledons. Differences in the frequencies might also be ascribed to certain external factors like environment including temperature, light intensity, etc. (Barret, Hutchinson 1978). Our results are consistent with the previous findings of Toolapong et al. (1996) who selected 82 seeds for their small size and reported that the number of seeds, in general, and in different sizes in particular, is a cultivar dependent characteristic, whereas the plant sector or location of the fruit on the plant did not show any significant impact on seed number and its development also recently supported by the findings of Fatima et al. (2006) in certain mandarin and sweet orange cultivars of *Citrus*. Akragas, Kamarina, Segesta and Selinunte are seedless lemons found as individual plants in commercial orchards, while Erice has 0–4 seeds per fruit (Calabrese et al. 2000).

Table 5. Frequency of polyploids in open pollinated seeds of *Citrus* cultivars

Cultivar	Percentage of polyploids		
	Diploids (2*n*)	Triploids (3*n*)	Tetraploids (4*n*)
Kinnow	84.0	11.3	4.6
Feutrell's Early	90.0	7.3	2.0
Musambi	88.0	9.3	2.7
Grape fruit	82.0	14.0	4.0
Lime	75.0	15.5	9.1

Fig. 3. Recovered spontaneous triploid Kinnow seedling transplanted in a pot

CONCLUSIONS

A significant number of polyploids recovered from underdeveloped seeds of *Citrus* cultivars. Lime Kaghzi yielded maximum triploid plants (15.5%) followed by grapefruit Foster, mandarin Kinnow (9.1%), sweet orange Musambi and Feutrell's Early (7.4%) mandarin. Kaghzi also produced the highest tetraploid percentage (9.1%) followed by Kinnow and Foster while the minimum value was found in Feutrell's Early (2.0%).

Recovered polyploids were transplanted into plastic pots (Fig. 3) and shifted to the greenhouse for hardening. Morphological and genetic characterization of recovered polyploids is planned for future breeding and biotechnology programmes.

References

BACHI O., 1940. Observaceous Citologicalem *Citrus*. I. Numero de cromosomas de algunas especies y variedades. Journal of Agronomy (Piracicaba), *3*: 249–258.

BARRETT H.C., HUTCHINSON D.J., 1978. Spontaneous tetraploidy in apomictic seedlings of *Citrus*. Economical Botany, *32*: 27–45.

BARRETT H.C., HUTCHINSON D.J., 1981. Occurrence of a spontaneous octaploid in apomictic seedlings of a tetraploid *Citrus* hybrid. In: Proceedings of the International Society of Citriculture, *1*: 29–30.

CALABRESE F., DE MICHELE A., BARONE F., 2000. New seedless lemon varieties for Sicily. In: Programs & Abstracts. (P129), IXth Congress of the International Society of Citriculture: 114.

CAMERON J.W., SOOST R.K., 1969. *Citrus*. In: FEWERDA F.D., DE WIT F. (eds.), Outlines of Perennial Crop Breeding in the Tropics: 129–162.

CAMERON J.W., BURNETT R.H., 1978. Use of sexual tetraploid seed parents for production of triploid *Citrus* hybrids. HortScience, *13*: 167–169.

ESEN A., SOOST R.K., GERACI G., 1979. Genetic evidence for the origin of diploid mega- gametophytes in *Citrus*. Journal of Heredity: *70*: 5–8.

ESEN A., SOOST R.K., 1971. Unexpected triploids in *Citrus*: their origin, identification and possible use. Journal of Heredity, *62*: 329–333.

ESEN A., SOOST R.K., 1972. Tetraploid progenies from 2× × 4× crosses of *Citrus* and their origin. Journal of American Society of Horticultural Sciences, *97*: 410–414.

ESEN A., SOOST R.K., 1973a. Seed development in *Citrus* with special reference to 2× × 4× crosses. American Journal of Botany, *60*: 448–452.

ESEN A., SOOST R.K., 1973b. Precocious development and germination of spontaneous triploid seed in *Citrus*. Journal of Heredity, *64*: 147–154.

FATIMA B., USMAN M., KHAN M.M., SADAQAT H.A., 2006. Impact of light on *Citrus* seed development. Turkish Journal of Botany (in press).

FROELICHER Y., LURO F., OLLITRAULT P., 2000. Analysis of meiotic behaviour of tetraploid *Clausena excavata* species by molecular marker segregation studies. In: 9th International Society of Citriculture Congress, South Africa: 116.

FROST H.B., 1925. The chromosomes of *Citrus*. Journal of Washington Academy of Sciences, *15*: 1–3.

GMITTER F.J.J., LING X.B., DENG X.X., 1990. Induction of triploid *Citrus* plants from endosperm calli *in vitro*. Theoretical and Applied Genetics, *80*: 785–790.

JASKANI M.J., KHAN M.M., KHAN I.A., 2002. Growth, morphology and fruit comparison of diploid and tetraploid Kinnow mandarin. Pakistan Journal of Agricultural Sciences, *39*: 126–128.

KHAN M.D., HABIB-UL-REHMAN, 1994. Crop improvement in horticulture. In: BASHIR E., BANTEL R. (eds.), Horticulture. Islamabad, National Book Foundation: 150–152.

LAPIN W.K., 1937. Investigations on polyploidy in *Citrus* work. All-Union Scientific Research Institute Humid Subtropics, *1*: 1–68.

LEE L.S., 1989. *Citrus* polyploidy origins and potential for cultivar improvement. Australian Journal of Agricultural Research, *4*: 735–747.

LONGLEY A.E., 1925. Polycarpy, polyspory and polyploidy in *Citrus* and *Citrus* relatives. Journal of the Washington Academy of Sciences, *15*: 347–357.

OIYAMA I., KOBAYASHI S., 1990. Polyembryony in undeveloped mono-embryonic diploid seeds crossed with a *Citrus* tetraploid. Horticultural Science, *25*: 1276–1277.

OIYAMA I., OKUDAI N., TAKAHARA T., 1980. Studies on polyploid breeding in *Citrus* : Triploid progenies from small seeds of diploid sweet orange cultivars. Japanese Society of Horticultural Science, *3*: 52–53.

OIYAMA I., OKUDAI N., TAKAHARA T., 1981. Ploidy levels of seedlings obtained from 2× × 4× crosses in *Citrus*. In: Proceedings of the International Society of Citriculture, *1*: 32–34.

SANFORD J.C., 1983. Ploidy manipulations. In: MOORE J.N., JANICK J. (eds.), Advances in Fruit Breeding. West Lafayette, Indiana, Purdue University Press: 100–123.

SOOST R.K., CAMERON J.W., 1975. *Citrus*. In: MOORE J.N., JANICK J. (eds.), Advances in Fruit Breeding. West Lafayette, Indiana, Purdue University Press: 507–540.

SPIEGEL-ROY P., 1988. *Citrus* breeding past, present and future. In: 6th International *Citrus* Congress, Middle East, *1*: 9–17.

STEEL R.G.D., TORRIE J.H., 1980. Principles and Procedures of Statistics. New York, McGraw Hill Book Co. Inc.: 232–251.

TOOLAPONG P., KOMATSU H. et al., 1996. Triploids and haploid progenies derived from small seeds of Banpeiyu, a pummelo, crossed with Ruby Red grapefruit. Journal of Japanese Society of Horticultural Science, *65*: 255–260.

WAKANA A., IWAMASA M., UEMOTO S., 1981. Seed development in relation to ploidy of zygotic embryo and endosperm in polyembryonic *Citrus*. In: Proceedings of the International Society of Citriculture, *1*: 135–139.

Received for publication May 30, 2005
Accepted after corrections November 25, 2005

Výskyt spontánních polyploidů u citrusů

ABSTRAKT: Předmětem studia byla úroveň ploidie u špatně vyvinutých semen různých pěstovaných odrůd z rodu *Citrus*. Na základě cytologických zkoušek bylo zjištěno, že nejvíce triploidních rostlin má citroník Kaghzi (15,5 %), následovaný grapefruitem Foster, mandarinkou Kinnow, sladkoplodým pomerančovníkem Musambi a mandarinkou Feutrell's Early (7,3 %). Citroník také vytvářel nejvyšší procento tetraploidů (9,1 %), následovaný mandarinkou Kinnow a grapefruitem Foster, zatímco u mandarinky Feutrell's Early byl nalezen pouze minimální počet těchto rostlin. Získané triploidní rostliny byly přesazeny do květináčů a po otužení budou pěstovány ve skleníku za účelem dalšího morfologického a genetického hodnocení.

Klíčová slova: polyploidy; *Citrus aurantifolia*; *C. paradisi*; *C. reticulate*; *C. sinensis*

Corresponding author:

Dr. Muhammad Usman, Plant Tissue Culture Cell, Institute of Horticultural Sciences, University of Agriculture, Jail Road, Faisalabad, Pakistan
tel.: + 92 030 072 578 26, e-mail: muhammadusman30@yahoo.com