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Citrus Germplasm Enhancement by Interploid Hybridization 1. Reciprocal Crosses of Kinnow and Succari

MUHAMMAD USMAN†, M. RAMZAN, B. FATIMA, MUHAMMAD J. JASKANI AND MUHAMMAD MUMTAZ KHAN
Department of Horticulture, University of Agriculture, Faisalabad-38040, Pakistan
†Corresponding author E-mail: muhammadusm@hotmail.com; Phone: 92-41-9200161-70 Ext. 388

ABSTRACT

Triploid Kinnow was developed through interploid hybridization followed by embryo rescue. Embryos from developed and underdeveloped seeds of reciprocal crosses of 4X x 2X of kinnow mandarin (*Citrus reticulata* Blanco) and succari orange (*Citrus sinensis* L.) were cultured on modified Murashige and Skoog (MS) medium with different levels of GA_3. Significant number of underdeveloped (10.35) and developed seeds (8.5) was found in 2x X 4x and 4x X 2x crosses, respectively. Higher levels of GA_3 (8 mgL^{-1}) gave more germination of embryos from developed (133) and underdeveloped (113) seeds from 2x X 4x crosses. More triploids (40.5) were observed from embryos of underdeveloped seeds in 2x X 4x crosses than developed seeds. In reciprocal crosses, developed seeds gave more number of tetraploids (56.58) than underdeveloped seeds. Seedlings from embryos of developed seeds had a highly significant survival rate (189.4 & 66.6) than underdeveloped seeds in 2x X 4x and 4x X 2x crosses, respectively. Embryo germination behaviour and survival rate was much better when 2x succari was used as seed parent. Such studies might be helpful in enrichment of citrus germplasm sources of the country.

Key Words: Citrus; Kinnow Mandarin; Succari Sweet Orange; Triploid; Interploid Hybridisation

INTRODUCTION

Pakistan is producing about 30 different fruits, out of which the Citrus holds the major position. Kinnow mandarin (*Citrus reticulata* Blanco) provides major stay to Pakistan's citrus industry. The climate of Pakistan is the best for excellent production of Kinnow (Saunt, 1990). It covers 50% of the total Citrus area and 64% of the total Citrus production in the Pakistan (Anonymous, 2000). Besides local consumption, it has high export value due to unique taste, excellent flavour and high nutritional value. Pakistan is earning a handsome amount of foreign exchange by exporting the fresh as well as processed kinnow to the Gulf, Europe and other countries. Kinnow has few drawbacks especially alternate bearing, loose skin and late maturity. More number of seeds per fruit is another problem and is disliked by the consumers as seeds cause bitterness in the juice during processing. Seedlessness in Kinnow is desireable from both industrial as well as consumer's point of view. The triploid cultivars of Citrus were found to be seedless (Webber & Batchelor, 1948) while spontaneous polyploids are also found in nature (Krug & Bacchi, 1993). The triploid cultivars can be produced by crossing tetraploid and diploid cultivars.

Breeding of citrus and many other fruit trees is a long, tedious process complicated by long periods of juvenility, insufficient space, embryo abortion and, in citrus, a high degree of apomixis. Plant tissue culture offers a promising method to overcome these problems (Litz *et al.*, 1985). Embryo abortion can be overcome by embryo rescue technique (Starrantino & Recupero, 1981). The problem of polyembryony can be solved through proper selection of parents and the cytological studies. It is observed that 2X x 4X crosses produce more underdeveloped seeds than the developed seeds as compared with the reciprocal crosses (Khan & Jaskani, 1996). The underdeveloped seeds produce a high number of zygotic (triploid) embryos more than 90% of which are highly abortive (Oiyama & Kobayashi, 1990). These embryos from underdeveloped seeds can be cultured *in vitro* to get the triploid seedlings. Cytological studies of these may help to identify the ploidy level of the seedlings. The objective of initiation of this research venture was to induce triploidy or seedlessness in citrus through interploid hybridization and its confirmation by karyotype studies. The out come of this exploration will be contributive to the genetic resources of Citrus in the country.

MATERIALS AND METHODS

Reciprocal crosses between the tetraploid kinnow (mandarin) and diploid succari (sweet orange) were made as: Succari (diploid) x Kinnow (tetraploid), and Kinnow (tetraploid) x Succari (dipliod).

Controlled hand pollinations were made according to commonly employed techniques for controlled pollination in citrus (Soost & Cameron, 1975). Unopened or partially opened flower buds were collected from pollen parent trees one day before anthesis. In the laboratory, petals and styles were removed with forceps and the flowers were placed under a lamp to promote dehiscence for crosses to be made next morning. Before pollination, the flowers of desired stage i.e., those that were expected to open within the next 24 h, were emasculated on seed parent plants. Emasculated flowers were pollinated by touching the dehisced anthers of the pollen parent gently to the stigma and covered with paper bags to avoid foreign pollen contamination.

Fruits derived from these crosses were harvested after 12-15 weeks of pollination to avoid embryo abortion, washed with detergent and rinsed with the tap water for 10 min. Fruits were then treated with 10% sodium hypochlorite for 10 min followed by a quick dip in 75% ethyl alcohol (C_2H_5OH) (Khan *et al.*, 1992). They were given a round cut with the help of a sterile knife and twisted gently to separate into two halves. The seeds were obtained after washing out the pulp and were categorized as developed and underdeveloped seeds visually. The embryos excised from both types of seeds were placed in culture media using embryo rescue technique (Starrantino & Recupero, 1981). MS medium (Murashige & Skoog, 1962) was used as the basal medium supplemented with thiamine and pyridoxine (10 mgL^{-1} each), nicotinic acid (5 mgL^{-1}), glycine (2 mgL^{-1}), myoinositol (100 mgL^{-1}), casein hydrolysate (500 mgL^{-1}), sucrose (30 gL^{-1}) and GA_3 (0, 4, 8, 10 mgL^{-1}). The cultures were placed in the growth room at 25 ± 2°C and 16 h of photoperiod.

Transplanting and cytological studies. Germinated seedlings having 3-4 leaves were transplanted in plastic pots, 3 x 3.25 inches in size, having sterilized media comprising of sand, silt and leaf manure (1:1:1). During acclimatization, seedlings were sprayed with fungicide Topsin (1 ppm) to protect from the fungus. At transplanting only the greenish part of the root tip (1cm in length) was taken for the cytological studies (Gmitter *et al.,* 1990). Samples were squashed thoroughly in a drop of 95% acetic acid on the glass slide and studied under microscope at 10x X 100x magnifying power. The data was collected for the parameters like: seeds per fruit (%), germination (%), polyploid (%) and survival rate (%) of the seedlings of developed and underdeveloped seeds obtained from the reciprocal crosses. The experiment was laid out in a Completely Randomized Design (CRD) and was analysed by Duncan's Multiple Range test (DMR) at probability > 5% (Steel & Torrie, 1980). Three replications were laid out in each treatment.

RESULTS AND DISCUSSION

Statistical analysis revealed significantly (P>0.05) more underdeveloped seeds (10.35) from the Diploid (2x) X tetraploid (4x) crosses while reciprocal crosses yielded significantly (P>0.05) more number (8.5) of developed seeds (Table I). Similar results were found by Khan *et al.* (1992). The fruit of 2x X 4x cross lead towards sterility or seedlessness. Significantly (P>0.05) higher number of embryos (113.0) germinated from underdeveloped seeds of 2x X 4x crosses on GA_3 8 mgL^{-1} followed by 10 mgL^{-1} (94.43) (Table II). Similar pattern was observed in case of developed seeds. Highest number of embryos germinated from developed seeds of the same cross on 8 and 10 mgL^{-1} (133 & 99.75, respectively) of GA_3. In both developed and underdeveloped seeds control gave minimum embryo germination. Overall embryos from developed seeds showed a better germination compared with the underdeveloped embryos in both type of crosses. These results are in accordance with the findings of Khan *et al.* (1992) and Yaqub (1996), who cultured embryos from both the developed as well as underdeveloped seeds from different crosses of citrus cultivars and found that the developed seeds had better germination rate than the underdeveloped seeds.

Table I. Number of developed and underdeveloped seeds per fruit

Crosses	Developed seeds		Underdeveloped seeds	
	Means	Percentage	Means	Percentage
Kinnow (4x) X Succari (2x)	8.5 b	67.2	4.15 a	32.8
Succari (2x) X Kinnow (4x)	4.0 a	27.87	10.35 b	72.13

Root samples were taken at the time of seedling transplanting which were stained and studied under the microscope according to squash technique (Geraci *et al*., 1975). Significantly (P>0.05) higher number of triploids was found from both the underdeveloped (40.5) and developed (33.07) embryos of 2x X 4x crosses than the reciprocal crosses (Table III).

Table III. Ploidy level of seedlings survived from embryo culture of developed and underdeveloped seeds

Crosses	Developed			Underdeveloped		
	2N	3N	4N	2N	3N	4N
Kinnow (4x) X Succari (2x)	-	10.02a	56.58c	-	20.16b	5.04a
Succari (2x) X Kinnow (4x)	155.92 d	33.47b	-	4.5 a	40.5 c	-

Cytological study of the seedlings from the developed embryos revealed highest number (155.92) of the diploid seedlings. Tetraploids were not found in both the developed and underdeveloped embryos. While reciprocal crosses showed significantly higher number of tetraploids from

Table II. Effect of GA_3 on germination of the cultured embryos from developed and underdeveloped seeds

Crosses	Developed				Underdeveloped			
♀ parent ♂ parent	GA_3 (mgL^{-1})							
	0	4	8	10	0	4	8	10
Kinnow (4x) X Succari (2x)	22.5 a	59.8 b	87 c	76.16 bc	16.32a	32.64a	38.03 a	38.08 a
Succari (2x) X Kinnow (4x)	43.89b	58.52b	133 d	99.75 c	26.6a	66.5 b	113.0 d	94.43 c

developed (56.58) and the undeveloped (5.04) embryos with no diploid plant in both the cases. These results are in line with of Esen and Soost (1971) who cultured the underdeveloped embryos of citrus and got more than 90% triploid seedlings. Geraci *et al*. (1975) also found that the underdeveloped seeds of citrus have more triploid embryos as compared to the developed seeds.

Total number of seedlings survived was significantly (P>0.05) higher from the embryos of developed seeds than the undeveloped seeds in both types of the crosses. Highly significant (P>0.05) number of seedlings (189.4) survived from the 2x X 4x crosses compared to than the reciprocal crosses (66.6) in case of developed embryos and these seedlings were mostly diploids and tetraploids (Table IV). In case of the underdeveloped embryos, 2x X 4x crosses revealed significantly better survival (45) than the reciprocal crosses. These findings are in accordance with Oiyama *et al*. (1991) who reported that the triploid and pentaploid seedlings had less survival rate as compared to the diploid and tetraploid seedlings. Soares *et al*. (1992) also found low survival percentage of the pentaploid seedlings. The character seems to be genetically controlled because all the seedlings from developed and underdeveloped seeds were provided with almost the same environmental conditions.

Table IV. Survival of the seedlings from embryo culture of developed and underdeveloped seeds

Crosses	Developed	Underdeveloped
♀ parent ♂ parent	**Mean no. of plants survived**	**Mean no. of plants survived**
Kinnow (4x) X Succari (2x)	66.6 c	25.2 a
Succari (2x) X Kinnow (4x)	189.4 d	45 b

Use of diploid succari sweet orange (*Citrus sinenesis* L.) as a female parent yielded significantly higher number of underdeveloped seeds per fruit, better germination behaviour and much higher number of triploids in embryos from both the developed and underdeveloped seeds with highly significant number of seedlings survived. The reciprocal crosses (4x X 2x), only, yielded significantly high number of tetraploids from developed embryos while considerable amount of triploids was also observed. It is quite evident from above results that in citrus breeding programs, use of diploid succari as a female parent might be much more beneficial to create triploidy than the tetraploid kinnow. The importance of plant genetic resources in the field of crop breeding and conservation is well understood. Plant breeders have always been appreciating enhancement of the existing gene pool. Therefore, such studies would be helpful in the enrichment of the existing citrus germplasm resources of the country.

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