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IN VITRO GERMINATION OF HYBRID EMBRYOS FROM INTERPLOIDAL CROSSES OF CITRUS

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Abstract

Interploid crosses between Feutrell's early (2x), Kinnow mandarin (2x), Succari sweet orange (2x) and Kinnow (4x) were made for the production of triploid hybrids. Maximum fruit set (100%) was observed between crosses of Kinnow (2x) X Kinnow (4x) and maximum June drop (95.5%) was noted when diploid Succari as female parent was crossed with tetraploid Kinnow. The hybrid fruits harvested after 12–14 weeks or 7 months after pollination produced many underdeveloped and few developed seeds; however, tetraploid Kinnow as seed parent yielded more developed seeds per fruit. Immature embryos of seeds harvested 12–14 weeks after pollination were cultured *In vitro* on MS medium supplemented with adenine sulphate (0.25 g l^{-1}), malt extract (0.5 g l^{-1}) and sucrose (50 g l^{-1}). Maximum germination (58.6%) of hybrid embryos from developed seeds was obtained in 4x X 2x cross of Kinnow strains.

Introduction

Among polyploids, triploids are of utmost importance as they produce seedless fruits and vegetables. Production of seedless (triploid) citrus fruits could lead to the improvement of quality of fresh fruits (Jaskani *et al.,* 2006). Seedless citrus varieties have a distinct commercial advantage. Seediness is considered as an obstacle in releasing newly selected high quality mandarins (Vardi, 1996). There are a number of cultivars with desirable horticultural characteristics which have not attained commercial importance because of their seediness (Fatta-Del-Bosco *et al*., 1992). Presence of a large number of seeds in citrus fruits is a big hindrance in consumer acceptability even if fruit posses high organolaptic properties (Raza *et al.,* 2003).

In citrus spontaneous triploids and tetraploids also exist as sexual zygotic seedling in diploids. The frequency of triploids is as high as 5% among seedling progeny (Jaskani *et al.,* 2007). In spite of low seed content and seedlessness, only the 'Tahiti lime' attained commercial significance among naturally occurring triploids (Vardi & Spiegel, 1978). Mutation breeding through irradiation is also a way to seek triploids, since natural mutations and sports are often found in citrus. Gamma irradiation and chemical mutagens are used to observe somatic mutation as a means to study the evolution of various citrus species and varieties. Irradiation with Co^{60} at 7 Ki with 127 R/min produced about 300 times more mutants of Jincheng orange than the natural mutation (Zhang *et al*., 1988). The endosperm is a unique triploid tissue. Recovery of triploid plants from *in vitro* endosperm culture following controlled pollination could be a useful breeding strategy for citrus, a vegetatively propagated perennial crop, for which seediness is undesirable and unnecessary (Wang, 1975).

Triploids of citrus can also be produced by crossing tetraploid seed parent with diploid pollen parent (Cameron & Burnett, 1978; Esen *et al*., 1979). 4n x 2n crosses had higher seed set than that of reciprocal crosses (Cameron & Burnett, 1978). Hybrid seed often abort because the endosperm fails to develop normally (Cocking, 1986). Embryo culture has been practiced by plant breeders for over half a century. However technical considerations relating to the composition of the medium and excision of the embryo have improved the ability to utilize zygotic embryo culture to rescue intervarietal, interspecific and intergeneric crosses (Jaskani *et al.,* 2005). Hence breeding program was initiated to create diversity and resolve associated problems like embryo abortion.

Materials and Methods

The current study was carried out in Experimental Fruit Orchard square # 9 and Plant Tissue Culture Cell, Institute of Horticultural Sciences, University of Agriculture Faisalabad. Four citrus cultivars i.e., Feutrell's early (2x), Kinnow mandarin (2x) and Succari sweet orange (2x) were crossed reciprocally with Kinnow mandarin (4x). Unopened mature flower buds from the pollen parents were collected and after the removal of petals and stigma, flowers were kept under light for 12-16 hours for pollens dehiscence one day before pollination. Flowers on seed parents were emasculated on the day of pollination. Pollination was made by applying pollens with camel hair brush or simply by touching the anthers on the sticky surface of stigma of the mother parent flower. After satisfactory pollination the pollinated flowers were covered with craft paper bags to avoid from stray pollens. Pollinated flowers were tagged written with 'date' and 'name' of parents.

Embryo rescue: For embryo culture, hybrid fruits were harvested after 3-4 months of cross-pollination and were sterilized by flaming with 95% ethyl alcohol under laminar air flow. The fruit rind was cut with a sterilized scalpel, and was twisted to separate it into two halves. Seeds were removed from fruits with a needle and the embryos were excised from the micropylar end of the seed after removing the seed coat and were cultured *In vitro* on Murashige & Skoog (1962) medium fortified with 0.5 g l^{-1} malt extract, 0.25 g l^{-1} adenine sulphate and 50g l^{-1} sucrose. The pH of the media was adjusted to 5.7 before autoclaving at 121°C for 20 min. All cultures were incubated at 25 ± 2°C with a 16-h photoperiod. Embryo germination and plantlet development and survival rate were recorded. Data was recorded on fruit set (%), fruit drop (%), seed development (developed seeds, underdeveloped seeds and aborted seeds), embryo per seed, embryo germination.

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Statistical analysis: Completely Randomized Design (CRD) was applied with three replication. The data recorded was analyzed statistically using LSD test at 5 % level of significance (Steel *et al.,* 1997) and all the results were significant.

Results and Discussion

Fruit set and drop in reciprocal crosses of citrus: The pollination was made in reciprocal crosses during March. Fruit set data was collected after one month of pollination. When tetraploid Kinnow used as mother parent was crossed with diploid Kinnow, Feutrell's early and Succari, the fruit set was 80%, 70.4% and 64.8%, respectively. The diploid Kinnow as female parent crossed with tetraploid Kinnow yielded 100% fruit set. In crosses between Succari (2x) X Kinnow (4x) fruit set was 73.5% (Table 1).

There were differences for fruit set among the parents (Table 1). It was also noted that low fruit set occurred in crosses where tetraploid was used as seed parents. This agrees with the data of Cameron & Burnett (1978) who reported that use of sexual tetraploid as seed parents is subjected to the usual problems of low fruit set from hand cross-pollination of tetraploid, and a low incidence of viable seeds per fruit. However, high proportion of triploids was obtained. Soost & Cameron (1975) reported that a large percentage of perfect flowers also may fail to set fruit. However, only a small number of flowers on citrus trees will set fruit which develops a maturity. Low fruit set occurred due to fluctuation in temperature, relative humidity and rainfall during the month of March, April and June (Figs. 1-3). Temperature between 15-20^{o}C enhance pollen production in the anthers of Satsuma mandarin flowers while cool temperature result in many non-viable gametes (Yelenosky, 1985). During the crosses the average temperature was 25.3^{o}C (Fig. 1) which might be a reason of low fruit and seed set.

The different pollen parents also showed low fruit set (Table 1). Effect of pollen of different species on fruit set has been reported in the literature. Soost (1975) observed that fruit set in Clementine mandarin with Ruffer pollen was significantly lower than either Pearl or Kinnow pollen and seed numbers were also lower with Ruffer pollen. Similar observation was reported by Kedar & Gopal (1977) that self pollinated Nagpur mandarin showed 60-62% fruit set when cross pollinated with grapefruit pollen.

Factors within the tree (food reserves, mineral nutrition, hormonal balance etc.) as well as environmental factors such as temperature, wind, humidity, soil moisture and, of course, diseases and pests all play their respective role in determining the fate of flowers and developing fruits (Reuther, 1973). For the success of hybridization program involving trees grown in the field, one has to take a direct look on environmental factors and data on fruit set and fruit drop in Kinnow (Jaskani & Khan, 1992).

Table 1. Fruit set and drop in different interploidal crosses of citrus.

♀ Parents	♂ Parents	Fruit set (%)	June drop (%)
Kinnow (2x)	Kinnow (4×)	100	93.6
Feutrell's early (2x)	Kinnow (4x)	100	90.14
Succari (2x)	Kinnow (4x)	73.5	95.5
Kinnow (4x)	Kinnow (2x)	80	94
Kinnow (4x)	Feutrell's early (2x)	70.4	69.3
Kinnow (4x)	Succari (2x)	64.8	82.4

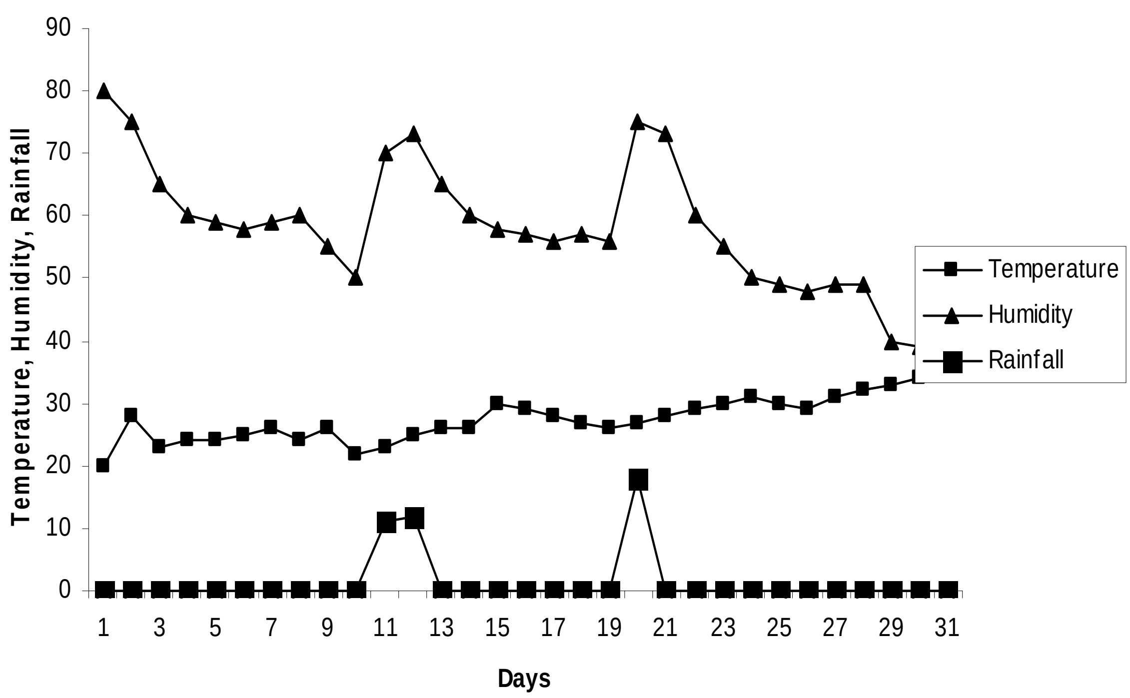

Fig. 1. Mean daily temperature (^{0}C), humidity (%) and rainfall (mm) during March 2007.

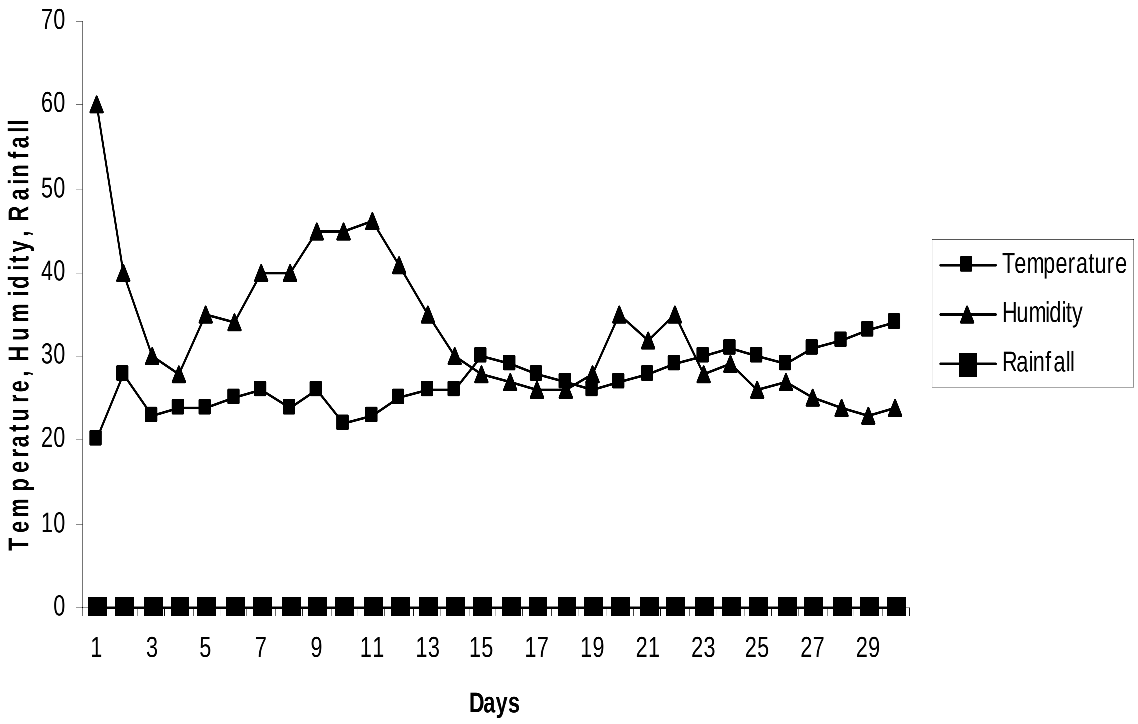

Fig. 2. Mean daily temperature (^{0}C), humidity (%) and rainfall (mm) during April 2007.

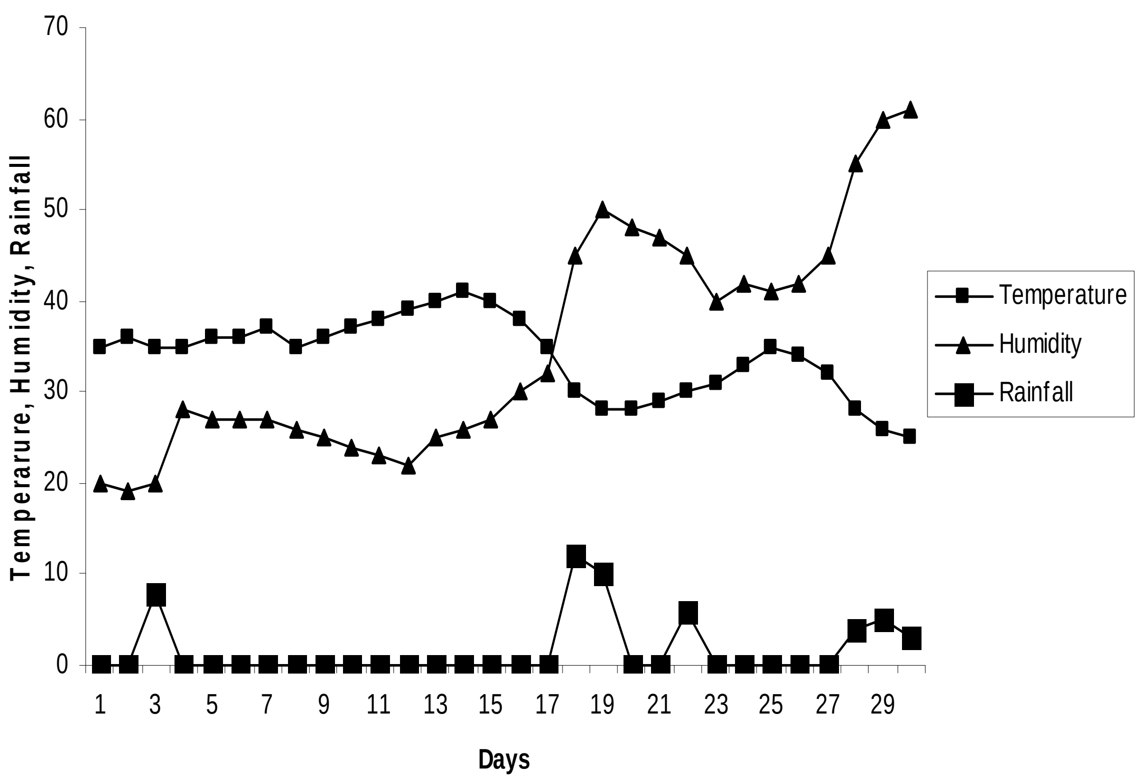

Fig. 3. Mean daily temperature (^{0}C), humidity (%) and rainfall (mm) during June 2007.

Fruit drop was recorded during the month of June which varied in different reciprocal crosses. When tetraploid Kinnow as female parent was crossed with diploid Kinnow, Succari and Feutrell's early the fruit drop was 94%, 69.53% and 82.4%, respectively. Diploid Kinnow, Feutrell's early and Succari as female parents were crossed with tetraploid Kinnow, the fruit drop recorded was 93.3%, 90.14% and 95.5%, respectively (Table 1).

As shown in Table 1, maximum fruit drop occurred when diploid Succari as seed parent was crossed with tetraploid Kinnow and minimum fruit drop was noted when tetraploid Kinnow as female parent was crossed with diploid Feutrell's early. When there are differences in direct and reciprocal crosses it means cytoplasmic factor might involve in shaping the parameter under consideration. So author are suggested that high light this factor beside other a biotic factor. Fruit drop relates to many factors such that pollination and fertilization but climatic conditions are important. Data regarding temperature, rainfall and relative humidity of March, April and June 2007 is presented in Fig. 1, 2 and 3. Data shows that high temperature and low relative humidity prevailed during June which might cause high fruit drop. The existence of several waves of drop particularly at the fruitlet stage has been related to environmental conditions (Zucconi *et al*., 1978).

Several researchers have shown that some climatic factors affect the citrus orchard yield. Jones & Cree (1964) reported that high air temperature during the "June drop" period can be very detrimental to yield. Moss & Muirhead (1971) indicated a negative effect of September high air temperature on Naval yield in Australia. Reuther (1973) stated that a single day with an air temperature above 40^{o}C during May-June cause reduction in yield of Navels. Lack of moisture or high temperature will significantly increase the dropping of fruitlet during early growth stages (Yelenosky, 1985).

Frequency of developed, underdeveloped and aborted seeds: Maximum developed seeds were observed in crosses of Kinnow (4x) X Kinnow (2x) and Kinnow (4x) X Succari (2x) which was 12 and 11, respectively. Diploid Kinnow as female parent was crossed with tetraploid Kinnow, yielded minimum developed seeds (2) which was statistically at par with crosses of Feutrell's early (2x) X Kinnow (4x) and Succari (2x) X Kinnow (4x) in which 4 and 3 developed seeds were observed, respectively (Table 2). Maximum underdeveloped seeds (16) were found when diploid Kinnow as female parent crosses with tetraploid Kinnow which was statistically similar with the crosses of Succari (2x) X Kinnow (4x) (11). Minimum underdeveloped seeds were produced when tetraploid Kinnow as female parent was crossed with diploid Kinnow, Feutrell's early and Succari and yielded 3, 2 and 2, respectively (Table 2). Crosses of Kinnow (2x) X Kinnow (4x) yielded maximum aborted seeds (17). Minimum aborted seeds (2) were noted when diploid Succari as female parent was crossed with tetraploid Kinnow which statistically relates with Feutrell's early (2x) X Kinnow (4x) and Kinnow (4x) X Succari (2x) produced 5 aborted seeds each (Table 2).

Table 2. Number of developed, underdeveloped and aborted seeds per fruit after 6 month of pollination.

♂ Parents	♀ Parents	Developed seeds	Underdeveloped seeds	Aborted seeds
Kinnow (2X)	Kinnow (4X)	2b	16 a	17 a
Feutrell's early (2X)	Kinnow (4X)	4b	6 bc	5 bc
Succari (2X)	Kinnow (4X)	3b	11 ab	2 c
Kinnow (4X)	Kinnow (2X)	12a	3c	6 b
Kinnow (4X)	Feutrell's early (2X)	9a	2c	7 b
Kinnow (4X)	Succari (2X)	11a	2c	5 bc

Low number of developed but high underdeveloped and aborted seeds occurred in different crosses. Similar observations were reported by Soost & Camermon (1975) indicating low incidence of viable seed. The restriction in seed development is due to early termination of endosperm development that appears to be related to deviation in ploidy level from normal embryo to endosperm ratio 2:3 as pointed out by Esen & Soost (1973). It was suggested that the unfavorable ratio changes the amount and timing of protein (enzyme) synthesis and then changes in growth hormone in the endosperm occur, leading to abnormalities in the development of endosperm, embryo and seed.

All the crosses except where Kinnow (4x) was used as seed parent, produced more underdeveloped than developed seeds. Esen & Soost (1973) reported such seeds as triploid. They also reported that seed size of 3x seeds was reduced 3 to 6 times as that of diploid following 2x X 2x crosses. 4x X 2x, 4x selfed and 2x X 4x crosses showed the same magnitude of size differences between 3x and 4x as was observed between 2x and 3x seeds from 2x X 2x crosses. These seeds were devoid of cotyledons and needed to be cultured *in vitro* to recover the possible triploid plants.

Embryo culture: Reciprocal crosses of Kinnow (4x) X Feutrell's early (2x) (58.6%) and Kinnow (4x) X Succari (2x), (45.4%) gave high embryo germination (Table 3). Tetraploid Kinnow as female parent crossed with diploid Kinnow showed low embryo germination (16.6 %) which is statistically at par with Succari (2x) X Kinnow (4x) and Feutrell's early (2x) X Kinnow (4x) which showed 28.5% and 28% embryo germination, respectively (Table 3).

Table 3. Embryo germination (%) in different reciprocal crosses of citrus.

♀ Parents	♂ Parents	Embryo germination
Feutrell's early (2x)	Kinnow (4x)	28 b
Succari (2x)	Kinnow (4x)	28.5 b
Kinnow (4x)	Kinnow (2x)	16.6 b
Kinnow (4x)	Feutrell's early (2x)	58.6 a
Kinnow (4x)	Succari (2x)	45.4 a

The problem of citrus embryo abortion in 2x X 4x crosses has been an obvious limitation for recovering triploids (Esen *et al.,* 1979; Oiyama *et al*., 1981).Many triploid plants can be produced which may combine desirable characteristics of diploid and tetraploid parents if triploid embryos could be rescued by tissue culture.

Rescuing triploid embryos from immature fruits (Starrantino & Recupero, 1981) and mature fruits (Oiyama & Kobayashi, 1990) following 2x X 4x hybridization have been reported.

Guo *et al.*, (1988) stated that embryos from small-sized seeds of *C. reticulata* cultured on MT medium (a modified MS medium) had respective germination and survival rates of 92.3% and 84.6% when the medium was supplemented with 500 mgl^{-1} casein hydrolysate; 92.3% and 69.2% with 2 mgl^{-1} gibberellic acid; 76.9% and 61.5% with 3 mgl^{-1} NAA; and 84.6% and 76.9%, with 1 mgl^{-1} IBA. Root tip cells of the 20 plantlets regenerated had a chromosome number of 2n = 3x = 27.

Conclusion

The problem of low fruit set and fruit drop presented difficulties in obtaining fruit to work further. Response of fruit set for different crosses were dissimilar and in various parental combinations. Diploid Kinnow crossed with tetraploid Kinnow always yielded maximum fruit set and the lowest was observed in a cross of tetraploid Kinnow with diploid Succari. Fruit drop in different crosses was also recorded. June drop range was 82.4% to 95.5% during the study.

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