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EXPLORING CITRUS CULTIVARS FOR UNDERDEVELOPED AND SHRIVELLED SEEDS: A VALUABLE RESOURCE FOR SPONTANEOUS POLYPLOIDY

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Abstract

Citrus cultivars were explored for seed development in response to genotype and plant sides. Number of developed, under-developed and shrivelled seeds per berry per cultivar was found different. Kinnow showed the highest number of shrivelled seeds 3.7 per berry compared with Feutrell's early, Musambi and Succari cultivars that ranged 2.1-2.4 seeds per berry. The interaction of different plant sides i.e., east, west, north and south with seed development and number has shown a significant impact. Sweet orange cultivars showed better root and shoot growth *In vitro* compared to mandarins.

Introduction

Citrus, a member of the family Rutaceae, is a rich bounty of edible fruits of different species. *Citrus* enjoys the first position among 30 other fruit crops with respect to its cultivation in Pakistan and we stand amongst one of the major *Citrus* producing countries of the world having an area of 200 thousand hectares with yearly production of 14.72 million tonnes. About 95% of total *Citrus* production is concentrated in the Punjab province. Increase in area has boosted citrus production but stagnation in yield per hectare (~10 tons/hec) in the last 50 years needs to be pondered. Recent decline of ~5 tons/hec in citrus production compared during 2006 and 2007 is also alarming, suggesting dire need for crop improvement (Anon., 2008).

Diversified germplasm will provide sound base for *Citrus* crop improvement programs. Conventional breeding has had difficulties in *Citrus* varietal improvement, due to the complex reproductive features (Swingle & Reece, 1967; Soost & Cameron, 1975; Grosser & Gmitter, 1990a) including polyembryonic nature, long reproduction cycle, sterility, incompatibility and endogamy depression (Grosser & Gmitter, 1990b, c). Ploidy manipulation is one of the important tools being widely used for germplasm development and crop improvement. One of the methods is to explore hidden potential for hyperploidy leading to germplasm development in citrus. Generally, seedless or nearly seedless *Citrus* cultivars are highly desired by the consumers and have been produced using modern techniques like interploid hybridization followed by embryo rescue and endosperm culture (Varoquaux *et al*., 2000; Fatima *et al*., 2002, Jaskani *et al*., 2007, Usman *et al*., 2002, 2008). Many cultivars have achieved commercial success because of seedlessness in the world and ploidy manipulation plays a key role in evolving and exploring this character. There are a few polyploids available to *Citrus* breeders in the country suggesting need to develop polyploids for *Citrus* breeding (Rao *et al*., 1992) and germplasm development. Spontaneous polyploidy is one of the natural sources of generating polyploids and is directly related to the seed size in citrus. Arrested seed development is characteristic feature of the seedless cultivars as in Mukaku Kishu type (*Citrus kinokuni* hort. Ex. Tanaka) seedless citrus attributed to abnormal embryo sacs and unfertilized ovules. The arrested embryo development is not due to endosperm abortion in this cultivar (Yamasaki *et al.*, 2009). Moreover underdeveloped and shrivelled seeds are postulated to have hyperploid embryos. Keeping in view these facts this pilot study was initiated to develop database of mandarin and sweet orange cultivars for number and type of seeds per berry and effect of plant sides on these characters. This study will help developing heterozygous hyperploids for ploidy manipulation and genetic improvement of citrus cultivars.

Materials and Methods

Site description: Mature *Citrus* fruit was taken from Post-graduate Agriculture Research Station (PARS), Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan. The experimental station is situated 73.06° N of equator at 31.26° longitude and 184 m above the sea level. Ten year old highly vigorous and healthy plants were selected for each of the following commercial cultivars of *Citrus* grown at PARS (10 kilometres south of Faisalabad) under monocrop cultivation system on a well drained moderately fertile sandy loam soil with pH range 7.8-8.2.

1. Mandarin (*Citrus reticulata* Blanco) cultivars Kinnow and Feutrell's early
2. Sweet Orange (*Citrus sinsensis* (L.) Osbeck) cultivars Musambi and Succari

Fruit collection: Randomly ten plants were marked from each cultivar. Ten full ripe fruits were harvested from four different sides of each plant in each cultivar i.e., East, West, North and South from November to January during 1999-2001. Fruits were horizontally cut into two halves and all seeds were extracted and washed in tap water to remove pulp and other ricines material. Seeds were arbitrary categorized as developed, underdeveloped and shrivelled and developed seeds were discarded. Rest of the seeds were considered aborted and were not used for further investigations. Data were taken for total number of seeds per berry per tree side in each cultivar. Number of seeds on each side of plant in each cultivar was further categorized as developed, under-developed and shrivelled seeds per berry.

Media preparation and sterilization procedures: MS (Murashige & Skoog 1962) and MT (Murashige & Tucker 1969) media were used for seed germination *In vitro* as described by Starrantino & Recupero (1981). Both the media were added sucrose at 3% and 8% agar as solidifying agent. Medium pH was adjusted to 5.6 ± 1. Ten ml of the medium was dispensed into each test tube with a dispenser and capped before it was autoclaved at 121°C temperature and 15 psi pressure for 20 minutes. The test tubes containing autoclaved media were kept for 24 hours in a growth room to observe any contamination before explant inoculation. Inoculation room and the Air Laminar Flow Cabinet were sterilized by exposing to ultra violet radiations for at least 4-5 hours. The laminar flow hood and the autoclaved utensils were further sterilized with 95% ethyl alcohol before use to avoid any contaminants (Alves *et al.*, 2000).

Explant sterilization and culture conditions: Fully developed seeds were discarded while underdeveloped and shrivelled seeds were selected, surface sterilized (Saeed *et al.*, 2004), decoated and cultured on both MS and MT medium for germination. The cultures were incubated at 27±1°C with a 16 h photoperiod in 35 microMm^{-2}.s^{-1} light intensity. Data were collected for germination percentage.

Transplanting: All 4-6 months old seedlings were transplanted into plastic pots (3" x 3.25"), containing sterilized media comprised of sand, silt and leaf manure (1:1:1) for further seedling growth and development. At the time of transplanting root tips were excised and processed for cytological studies (data not shown) and data were recorded for root and shoot length (cm) of seedlings grown from underdeveloped and shrivelled seeds in MS and MT media. Data were analysed and means were compared by New Duncan's Multiple Range (DMR) Test (Damon & Harvey, 1987).

Results

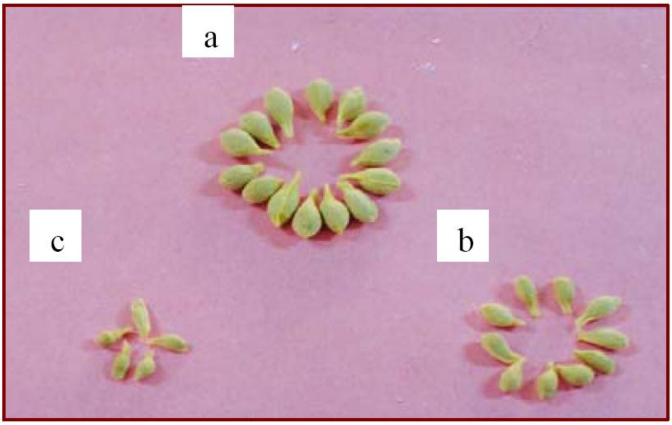
1. Number of seeds per berry per *Citrus* cultivars: Number of seeds and seed size was highly genotype dependent and a significant interaction was observed among these parameters. Trend for developed seeds per berry was variable among cultivars and was found in descending order as Kinnow (12.23) > Musambi (11.20) > Feutrell's early (9.50) > Succari (8.82) at $p \geq 0.005$ (Table 1). Number of developed and under-developed seeds of Succari and under-developed seeds of Kinnow statistically appeared at par. Among under-developed seeds, Kinnow and Succari depicted the highest number (8.7 and 9, respectively) followed by Musambi and Feutrell's early (7.7 and 6.1, respectively). Number of shriveled seeds were also the highest in Kinnow and rest of the cultivars were non-significantly different from each other. Overall among four cultivars explored, Kinnow showed the highest number of seeds per berry followed by Musambi, Succari and Feutrell's early as shown in Fig. 1. Data regarding percentage of under-developed and shrivelled seeds revealed that among mandarins, the percentage was significantly higher in Feutrell's early than Kinnow whereas among sweet oranges, the percentage for both the seed categories was higher in Succari than Musambi (Table 1).

2. Effect of plant sides on number of seeds per berry: Data revealed that plant sides had no significant effect on number of seeds per berry among different seed sizes. However, their interaction was highly significant ($p \geq 0.001$). The number of shriveled and developed seeds from all the four plant sides ranged 2.6-10.6, respectively and were statistically at par to each other. However, the highest number of under-developed seeds was recovered on northern side (8.45) of the plant followed by southern, eastern and western sides (8.0) which were at par to each other. Data analysis regarding interaction of plant sides with seed size for number of seeds among different *Citrus* cultivars was found highly significant ($p \geq 0.001$) as presented in Table 1.

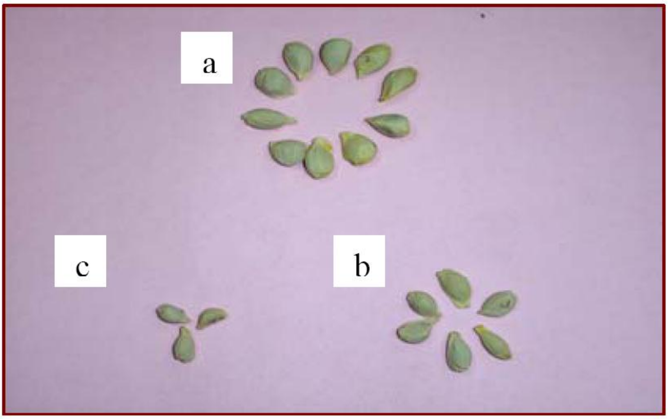
Table 1. Number of seeds per berry in *Citrus* cultivars.

Cultivar	Seed types						Mean number of seeds per berry
	Developed		Underdeveloped		Shrivelled		
	Number	%	Number	%	Number	%	
Kinnow	12.23 a	49	9.00 d	36	3.72 g	15	24.99
Feutrell's early	9.50 c	53	6.14 f	34	2.40 h	13	18.0
Musambi	11.20 b	53	7.70 e	37	2.10 h	10	21.0
Succari	8.82 d	44	8.74 d	44	2.30 h	12	19.83

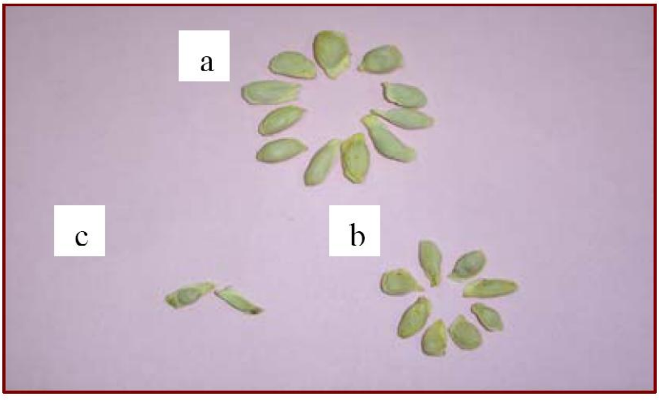
*Means sharing the same letters are statistically non-significant



1. Kinnow



2. Feutrell's early



3. Musambi

Fig. 1. Number of a) developed b) under-developed and c) shrivelled seeds in *Citrus* Cultivars.

3. Effect of plant sides on number of seeds per berry in *Citrus* cultivars

a. Developed seeds: Cultivar response to different plant sides for number of developed seeds per berry was variable. Kinnow gave the highest number of developed seeds on all the four sides of the plant followed by Musambi while Feutrell's early and Succari were likely to be at par to each other (Fig. 1). The highest number of developed seeds was observed on the north (13.10), west and south side of the Kinnow plant and were statistically at par to each other whereas significantly less number of seeds were observed on the east side of the plant. The highest number of seeds was observed on east (10.05), north and south sides of the plant while west side showed the lowest number of seeds (8.85) in Feutrell's early. No significant differences were found (11.70 to 10.55) among all the four plant sides studied in Musambi plants. The highest number of developed seeds were found on east (9.71), west and south sides of the Succari plant while the lowest number (7.84) was observed on the north side (Fig. 2a)

b. Under-developed seeds: Similar trend was observed for the number of under-developed seeds of Kinnow as south and north sides of the plant exhibited more number of seeds (9.50 and 9.40, respectively) followed by east and west sides. East, north and west sides of the Feutrell's early plants showed significantly higher number of seeds (6.94) whereas the lowest number of seeds was recorded on south side. The highest number of under-developed seeds were observed on the north side of the plant (8.10) followed by south side while the lowest number of seeds were on west and east sides (7.66) of the Musambi plant. The under-developed seeds were highest on the north side of the Succari plant (10.15 and 9.00, respectively) and were found statistically at par to each other (Fig. 2b).

c. Shrivelled seeds: Among shriveled seeds, plant sides have not shown any significant difference for all the four *Citrus* cultivars. However, the number of shriveled seeds was the highest for Kinnow while rest of the three cultivars explored showed no significant differences (Fig. 2c).

4. Effect of media on plant growth raised from under-developed and shriveled seeds of *Citrus* cultivars *in vitro*

4.1 Root and shoot length (cm) in Citrus cultivars: Longer root development (5.62 cm) was observed from under-developed seeds compared to shrivelled seeds (4.97 cm). Sweet orange cultivars depicted highest root length compared to mandarins (Fig. 4a). Seed size had a direct impact on shoot length as under-developed seed gave significantly longer shoots than shrivelled seeds (Fig. 4b). More shoot length was observed in Kinnow (6.02 cm), Feutrell's early and Musmabi (5.25 cm each) from under-developed seeds followed by Succari (5.25 cm). *Citrus* cultivar Musambi showed the highest shoot length (4.70 cm) followed by Succari and Feutrell's early (4.40 and 4.32 cm, respectively) while the lowest shoot length was depicted by Kinnow (1.65 cm) from shrivelled seeds *In vitro*.

4.2. Root and shoot length (cm) on different media: Use of MS and MT media for seed germination showed no significant differences for root and shoot length *in vitro* on MS and MT media. However, cvs. Musambi and Succari roots and shoots grew longer than cvs. Kinnow and Feutrell's early (Tables 2-3).

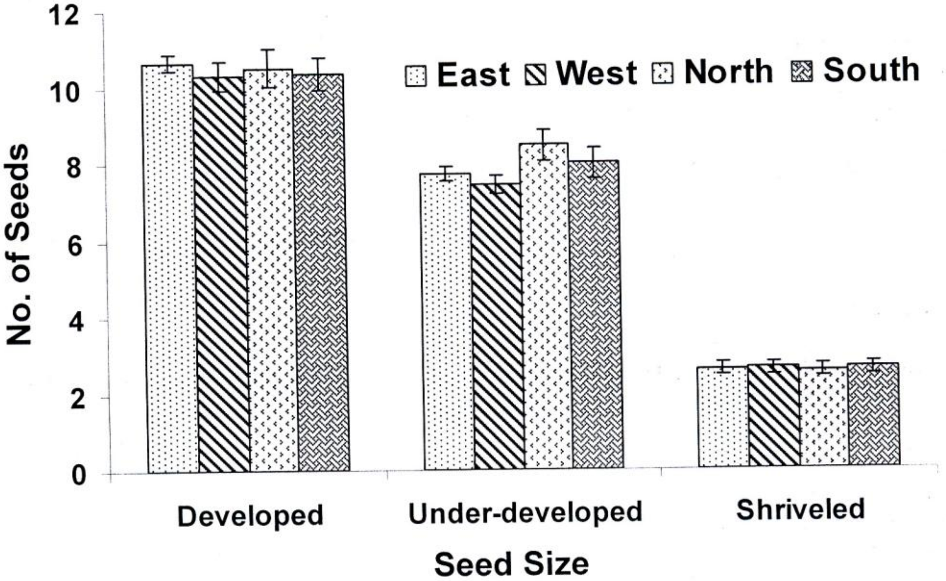


Fig. 2. Effect of plant sides to number of seeds per berry. Data are means of three Reps. and vertical bars (I) show Standard Error.

Table 2. Root length (cm) on different media in *Citrus* cultivars *in vitro*.

Cultivars	Media		Means
	MS	MT	
Kinnow	4.60 c	4.54 c	4.57 d
Feutrell's early	5.31 b	4.64 c	4.98 c
Musambi	5.91 a	5.74 ab	5.82 a
Succari	5.38 b	5.70 ab	5.52 b

*Means sharing the same letters are statistically non-significant

Table 3. Shoot length (cm) on different media in *Citrus* cultivars *In vitro*.

Cultivars	Media		Means
	MS	MT	
Kinnow	3.90 ± 0.39	3.77 ± 0.73	3.84 c
Feutrell's early	4.37 ± 0.77	4.27 ± 0.70	4.32 b
Musambi	4.61± 0.94	4.80 ± 0.66	4.70 a
Succari	4.23 ± 1.01	4.54 ± 1.08	4.40 b

*Means sharing the same letters are statistically non-significant. Data are means ± SE from three reps.

4.3. Plant growth in response to seed size *In vitro*: Citrus seedling growth was found significantly better from under-developed seeds compared to shrivelled seeds. Shoot growth was much less (2.9cm) in shriveled seeds compared to (5.7cm) shoots developed from underdeveloped seeds (Fig. 5).

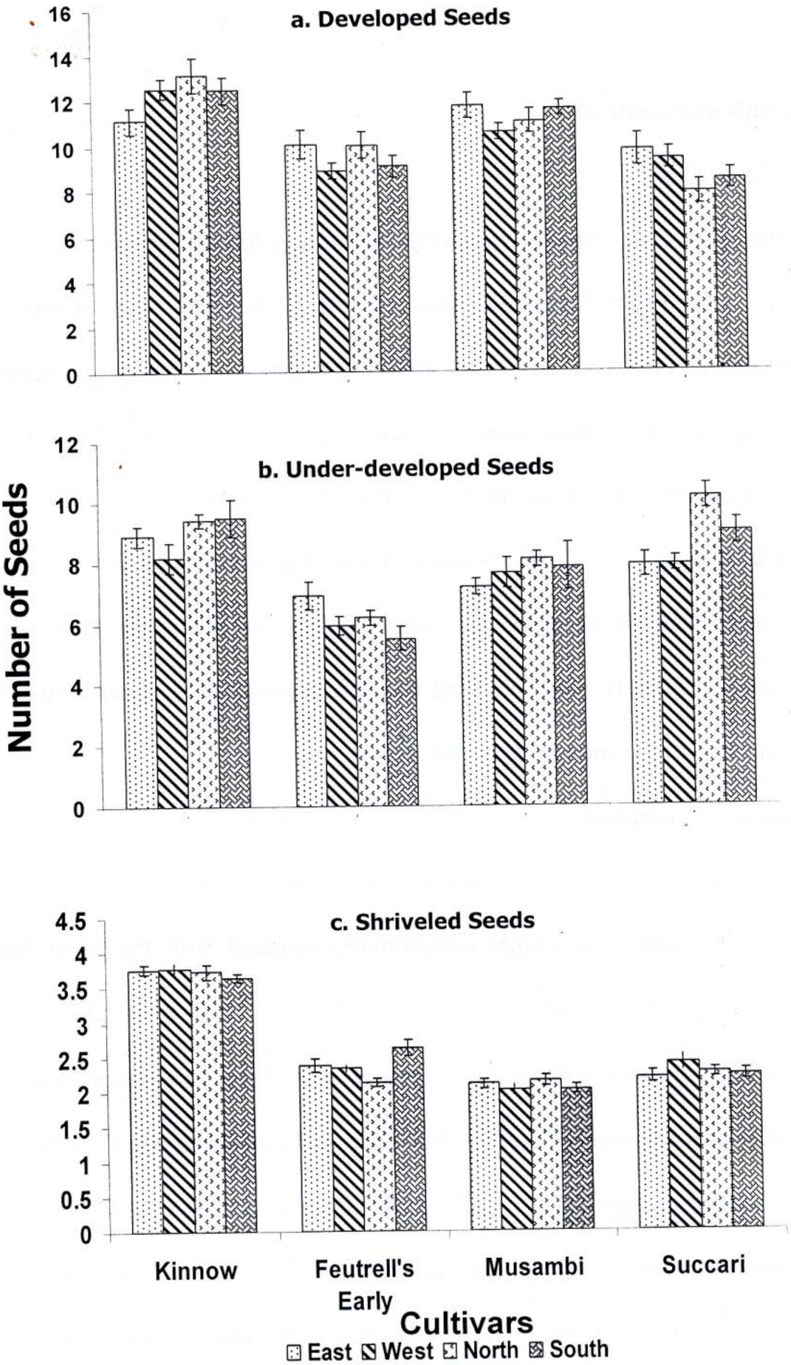


Fig. 3. Effect of plant sides on number of seeds a) developed b) under-developed c) shriveled per berry in Citrus cultivars. Data are means of three Reps. and vertical bars (I) show Standard Error.

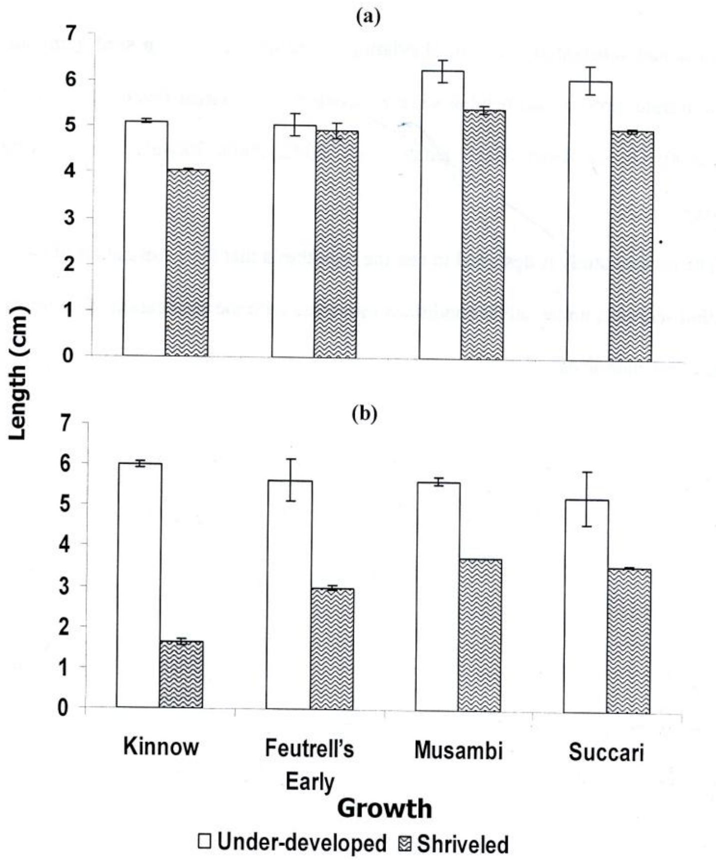


Fig. 4. Seedling length in citrus cultivars in response to seed development viz. under-developed and shriveled seeds where data in fig. a correspond to root length and in fig. b to shoot length). Data are means of three Reps. and vertical bars (I) show Standard Error.

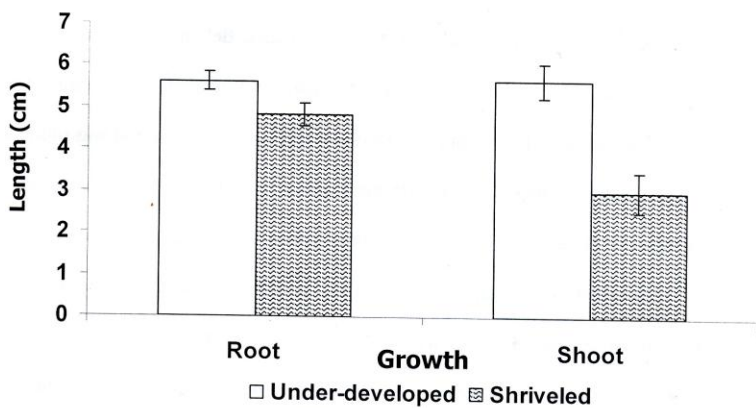


Fig. 5. Seedling growth in response to under-developed and shriveled seeds. Data are means of three Reps. and vertical bars (I) show Standard Error.

Discussion

Number of developed and under-developed seeds per berry per cultivar was variable while cultivar Kinnow depicted significantly higher number of shrivelled seeds per berry compared with rest of the three citrus cultivars. Seeds from open pollinated fruits were polyembryonic (aposporous) with varying number of embryos per seed. Esen & Soost (1973a, b) and Soost & Cameron (1975) found that small seeds that were thought to be polyploids were 1/3rd to 1/6th the size of diploid seeds. Apparently different sides of the plant i.e., E, W, N and S has shown no significant impact on the development of different types of seeds, however, the interaction of the plant sides with the seed size was significantly different from each other. Large differences have been observed in the same cultivar between localities and years (Khan & Roose, 1988a, b). Though considerable number of shrivelled seeds occurred in *Citrus* fruits (Cameron & Burnett, 1978), however, empty seeds were less frequently observed in spontaneous polyploids (Oiyama *et al*., 1981; Oiyama & Kobayashi, 1990), while lower set of fully developed seeds in *Citrus* is common feature (Cameron & Soost, 1969; Esen & Soost, 1972; Oiyama *et al*., 1981).

Many empty and few fully developed seeds were obtained among spontaneous polyploids (Tachikawa *et al.*, 1961; Cameron & Soost, 1969; Esen & Soost, 1972; Cameron & Burnett, 1978; Esen *et al.*, 1979; Oiyama *et al.*, 1981; Oiyama & Kobayashi, 1990). The reduction in the development of seed size of under-developed and shrivelled seeds might be due to the intrinsic capability of the ovule due to polyploidy and due to the delayed pollination leading to the shortage/absence of endosperm required for the growth and development of cotyledons. Our results are consistent with the previous findings of Toolapong (1996) who selected 82 seeds for their small size and reported that number of seeds, in general and in different sizes in particular, has been cultivar dependent whereas the plant side or locality of the fruit on the plant has not shown any significant impact on seed number.

It is concluded that number of developed, under-developed and shrivelled seeds per berry per cultivar was found different. The different plant sides i.e., east, west, north and south has not shown any significant impact on the development of different types of seeds and seed number and is supported by the previous reports of Toolapong (1996) and Saeed *et al*., (2004) in different citrus cultivars. The seeds might have appeared as under-developed and shriveled because of the i) intrinsic capability of seeds that might be due to polyploidy and ii) due to delayed pollination and fertilization by the male gametes (Cameron & Soost, 1969) which lead to the reduction of seed size. The minor differences observed in number of developed, under-developed and shriveled seeds doest not seem to be due to the direction of the plant because within the same cultivar the number differs in different categories. It could more be attributed to the differences in development of seeds in fruit, number of pollinations and to certain external factors as well (Barret & Hutchinson, 1978).

The problem of *Citrus* embryo abortion in shrivelled seeds of spontaneous origin has been an obvious limitation for recovering the probable polyploids and other odd ploidy plants (Esen *et al*., 1979; Oiyama *et al*., 1981). Many polyploid plants might be produced rescuing embryos from immature fruits (Starrantino & Recupero 1981) and mature fruits (Oiyama & Kobayashi 1990; Oiyama *et al*., 1991). Germination response studies of underdeveloped seeds of *Citrus* cultivars revealed mandarins better for root and shoot induction percentage than sweet oranges on MS medium as compared to MT medium.

Among shrivelled seeds, Feutrell's early and orange cultivars Musambi and Succari showed better response for root and shoot induction percentage than Kinnow on MT medium as compared to MS medium.

Our results showed higher germination percentage of embryos from under-developed than shrivelled seeds among all the *Citrus* cultivars. For root and shoot induction on different media explored our results are strengthened by the findings of Chen (1985) and Jaskani *et al*., (2007) who reported better plant regeneration on MT medium than MS medium in *Citrus.* Diploids are usually reported to grow more rapidly than any other ploidy (Khan & Habib-ul-Rehman, 1994) and diploid leaves have lower dry matter and higher water content opposite to lemon leaves with high dry matter and low water contents (Adeishvilli & Bakanidze, 1982). Similar results were observed by Jaskani *et al*., (2002) who reported that diploids were larger than polyploids in height.

The embryos recovered from shriveled seeds were found less developed as compared with the under-developed seeds obtained from the same fruit and this factor might be responsible for the delayed germination and reduced seedling growth from shrivelled seeds. The under-developed and shrivelled seeds are often malnutrited due to the degeneration or abnormal development of the endosperm which acts as the nutritive tissue for the development of zygotic embryos (Coking, 1986). Though this failure in endosperm availability or premature termination of the endosperm development is not yet well understood but it might be caused by the polyploidy which is interlinked with the development of the seed size (Stephens, 1942; Sarker & Coe, 1971; Esen & Soost, 1973a, b; Wakana *et al*., 1981).

It is concluded from the above findings that Sweet orange cultivars Musambi and Succari showed better root and shoot length *In vitro* than the mandarins for both underdeveloped and shrivelled seeds. These inconsistencies in the germination behavior of the under-developed and shriveled seeds of *Citrus* cultivars might also be accredited to their inherited capability that could be due to the hyperploidy. These conclusions are in accordance with the previous studies as the high ploidy levels are often accompanied by the slow and weak growth (Tusa *et al*., 1990), however, we differ in use of cultivars of mandarins and sweet oranges.

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