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EXPLOITATION OF POTENTIAL TARGET TISSUES TO DEVELOP POLYPLOIDS IN CITRUS

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Abstract

One of the rich sources of germplasm development to improve citrus crop is the ploidy management through *in vivo* and *in vitro* techniques. *In vitro* and *in vivo* applications of colchicine change the chromosome number by interfering with chromosomal segregation at anaphase, restricting cell wall formation and yielding polyploid cells. Further, endosperm culture under aseptic conditions also allows regeneration of plants with variable genetic make up. We report here the development of polyploids through *in vitro* culturing of endosperm and application of colchicine. Somatic embryos were developed from 'Kinnow' mandarin (*Citrus reticulata* L.) and 'Succari' sweet orange (*Citrus sinensis* L. Osbeck) on basal medium modified with higher concentrations of BA and NAA ($10mgL^{-1}$ and $2mgL^{-1}$, respectively) from calli induced on Murashige and Tucker (MT) medium supplemented with BA, 2,4-D, KIN, ME and CH. Recovery of polyploids from endosperm culture was significantly low (32%) compared to colchicine induced ploids (53.67%). Bud sprouting percentage and shoot length was found inversely proportional while leaf size was directly proportional to colchicine application. The stomatal studies of the regenerated plants revealed them as polyploids. These strategies are promising techniques for production of citrus polyploids, however; further genetic characterization of the ploids is suggested for future breeding and biotechnology applications.

Abbreviations: BA 6-benzaylaminopurine, CH Casein hydrolysate, KIN Kinetin, ME Malt Extract; NAA 1-naphthaleneacetic acid, 2,4-D 2,4-Dichlorophenoxyacetic acid

Introduction

Ploidy manipulation is a renowned source of germplasm development for citrus crop improvement. *In vitro* culture offers the novel methods of creating polyploid plants (Hannweg, 1999; Starranntino, 1999; Ollitrault *et al.,* 2000; Zhang *et al.,* 2007). Polyploids could be developed using strategies like *in vivo* and *in vitro* application of colchicine for doubling the chromosome number (Hannweg, 1999), interploid crossing followed by embryo rescue (Khan, 1996; Williams & Roose, 2000; Usman et al., 2002; Jaskani et al., 2007), endosperm culture *in vitro* (Mooney *et al.,* 1996), anther and or pollen culture to develop haploids and double haploids and somatic hybridization (Cabasson *et al.,* 2001). Spontaneous polyploids are also found due to development of unreduced gametes (Froelicher *et al.,* 2000).

Endosperm of most diploid angiosperms including *Citrus* is triploid hybrid tissue owing to double fertilization (Frost & Soost, 1968). *In vitro* plant regeneration from endosperm offers a single step approach for triploid production. This strategy also helps to overcome embryo abortion frequent in the progeny of 4x X 2x crosses by somatic

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embryogenesis from endosperm derived calli (Gmitter *et al.,* 1990; 1992). Triploid plantlets have been successfully recovered in several tree genera using endosperm culture *in vitro* including *Citrus* (Mooney *et al.,* 1996; Ollitrault *et al.,* 1996) Malus (Mu & Liu, 1979), Prunus (Liu & Liu, 1980), Actinidia (Gui *et al.,* 1982), Pyrus (Zhao, 1983) and Morus (Thomas *et al.,* 2000). The availability of limited gene pool and polyploid germplasm for breeding and biotechnology programs suggests intensive research work in this important area. Therefore, both strategies were explored for polyploid development in elite citrus cultivars using colchicine application *in vivo* and endosperm culture *in vitro*. The generated polyploids will contribute towards enrichment of Citrus germplasm for future breeding and biotechnology applications.

Materials and Methods

Endosperm culture *in vitro*: Fruit from tagged flowers of two citrus cultivars Kinnow mandarin (*Citrus reticulata* Blanco) and Succari sweet orange (*Citrus sinensis* L. Osbeck) was harvested after 12 weeks of anthesis, washed with tap water, sprayed with ethyl alcohol and flamed for surface sterilization. Fruits were cut into two halves and twisted to avoid any injury to seeds. Immature seeds were excised from segments with sterilized needle and washed to remove adherent pulp with sterilized distilled water. Outer and inner integuments of the seeds were dissected and peeled carefully. The nucellus and visible embryos from micropyler end were discarded and endosperm was excised from seeds and cultured *in vitro* on given media formulations (Table 1).

Sucrose was added in the solution as carbon source @ $30gL^{-1}$. Medium pH was adjusted to 5.7 and 8 gL^{-1} agar was employed for media solidification. Ten ml of the medium was poured in each test tube, caped with plastic sheet and placed in autoclave for 20 minutes at 121ºC under 15psi pressure. Endosperm was excised in the laminar flow hood and cultured on callus induction medium. The cultures were placed under dark condition to induce callus in the growth room with temperature adjusted at 25 ± 2 °C. The induced callus was allowed to develop and the calli were sub-cultured on callus proliferation medium. Proliferating calli were further sub-cultured on embryogenesis medium for embryoid development. These regenerating embryoids were placed on shoot initiation media. The developed shoots were excised and subjected to rooting media. The factorial experiment was laid out in completely randomized design (CRD) with ten replicates. Means significance was defined by least significant difference (LSD) test (Steel & Torrie, 1980).

Colchicine application *in vivo*: Three year old diploid plants uniform in size and vigor, of four *Citrus* cultivars viz. Kinnow mandarin, Feutrell's Early, Musambi and Succari sweet orange grafted on rough lemon (*Citrus jambhiri* L.) and grown in the green house were selected. Plant shoots were bent approximately 12-14 inches above ground into a loop and fastened to the stem. All buds, except three on loop, were cut off. Four plants of each cultivar were served as controls. A small wisp of rolled cotton wool was fastened on each bud. Colchicine (1%) was applied twice daily to each bud with a dropper (Jaskani *et al.,* 1996). This practice was repeated for five consecutive days. The cotton wool was removed and plants were kept for uninterrupted growth under green house conditions. The treated buds were allowed to grow and give new leaves. All bud/branch growth (except from treated buds) was removed. Shoot characteristics and leaf area of untreated

and treated shoots were measured. Five leaves from each sprouted shoot were randomly collected and the mean leaf area (length and width) of each cultivar was recorded. Leaf peels were taken from plants giving at least seven true leaves for chloroplast counting under microscope (Jaskani *et al.,* 1996).

Stomatal studies: The plants obtained from endosperm and colchicine application were subjected to stomatal studies.

Number and size of stomata: A thin epidermal layer of leaf was removed from lower surface and mounted on a clean slide having a drop of distilled water. A drop of safranine solution was added to stain the stomata and covered with cover slip. Gentle pressing helped the dye to penetrate the epidermal tissues. Then a drop of immersion oil (xylene) was placed on cover slip to clear vision and excessive dye.

Table 1: Media formulations used for *in vitro* culture of endosperm tissue

Media Formulations		PGRs in mgL^{-1}									
	Basal media	BA	2,4-D	Kin	ME	CH	IBA	Lactose	NAA	GA_3	IAA
Callus induction (CM)											
CM_1	MT	5	2	5	500	1000	-	-	-	-	-
CM_2		5	2	5	500	-	-	-	-	-	
CM_3		5	2	5	-	-	-	-	-	-	-
CM_4		5	2	-	-	-	-	-	-	-	-
Callus proliferation (PM)											
PM_1	MT	-	-	-	500	-	-	-	-	-	-
PM_2	MT	-	-	-	-	-	-	500	-	-	-
PM_3	MS	0.5	-	-	-	-	0.5	-	-	-	-
Embryogenesis (EM)											
EM_1	MT	5	-	-	-	-	-	-	1	-	-
EM_2		5	-	-	-	-	-	-	2	-	-
EM_3		10	-	-	-	-	-	-	1	-	-
EM_4		10	-	-	-	-	-	-	2	-	-
Shoot development (SM)											
SM_1	MS	-	-	0.5	-	-	-	-	-	-	-
SM_2	MT	10	-	-	-	-	-	-	-	0.5	-
SM_3	MS	-	-	1	-	-	1	-	-	-	-
Root induction (RM)											
RM_1	MS	-	-	-	-	-	1	-	-	-	1
RM_2		-	-	-	-	-	1	-	-	-	2
RM_3		-	-	-	-	-	2	-	-	-	1
RM_4		-	-	-	-	-	2	-	-	-	2

MS stands for Murashige and Skoog (1962) **MT** stands for Murashige and Tucker (1969)

The number of stomata was counted under magnifying power of x1250. Length of the stomata was measured by the ocular micrometer adjusted lengthwise while width was measured placing it in cross sectional position. The divisions were counted and multiplied with the factor `1' micron (Khan *et al.,* 1992a) for length and width of the stomata.

Chloroplast density of colchiploids: Lower epidermis was removed piercing leaf by hand and placing it on the glass slide after addition of one drop of distilled water. The chloroplast density was scored under the microscope at x1250 magnification. Ten

stomata were observed per leaf (Weyes & Lawson, 1997). The experiments were statistically designed using a Completely Randomized Design (CRD) with three replications in factorial arrangement. Data were analysed and means were compared by New Duncan's Multiple Range (DMR) Test (Damon & Harvey, 1987).

Results and Discussion

Endosperm culture *in vitro*:

Callus induction: No genotypic differences were observed for number of days to callus induction (19-20 days) however, the effect of medium was found significant for callus iduction. Earlier callus induction was observed in CM_1 medium (22.38 days) followed by CM_2 (27 days) and CM_3 (29.63 days) medium. The addition of BA and 2,4-D, KIN, ME and CH in the basal medium (MT) induced callus significantly earlier as compared to MT medium supplemented with cytokinin (KIN), ME and CH (Table 2). Free nuclear endosperm excised from *Citrus s*pecies did not develop any callus. Endosperms became cellular in 11-14 weeks post-anthesis and were distinguished by their elastic texture, absence of central vacuoles and size (<20% of available seed volume). Cellular endosperm excised at 12-14 weeks post-anthesis were the most responsive for callus induction. Our deductions are in line with Wang & Chang (1978) and Gmitter *et al.,* (1990, 1992) who also induced calli from cellular endosperm excised 12-14 weeks post anthesis most frequently from chalazal end of endosperm *in vitro.* Response of Succari for callus induction (%) was significantly (P> 0.05) higher (14.16%) on all media formulations than Kinnow which induced very little callus from chalazal end of endosperm in *Citrus* (Fig. A). In *Morus alba* it may take up to 17-20 days after pollination (Thomas *et al.,* 2000) Similar trend was depicted for callus induction percentage that was significantly higher (25%) on MT medium supplemented with BA, 2,4-D, KIN, ME and CH in succari sweet orange followed by CM_2 (17.91%) while no callus induction was observed in medium supplemented with BA and 2,4-D (CM_4). Mostly auxins particularly 2,4-D is necessary for callus induction (Khan et al., 1991; Thomas & Chutervedi, 2008), however, it seems as if addition of BA with 2,4-D inhibited the callus induction (Avenido *et al.,* 1991). Among cultivars Succari proved better for callus induction (14.16%) while among media CM_1 induced significantly more callus (20.14%) while CM_4 depicted no callus induction at all (Table 1). These findings are supported by the results of Wang (1975), Wang & Chang (1978) and Chen *et al.* (1991).

Callus proliferation: Significant (P>0.05) callus proliferation (53.3%) was observed in Kinnow mandarin compared to Succari (21.25%). Media used did not show any significant differences in callus growth percentage except MS medium supplemented with auxins IBA and BA @ 0.5 mgL^{-1} each (PM_3) which did not show any growth in callus of both the citrus cultivars (Table 3). Kinnow endosperm derived calli growth was much better (+++) on MT medium supplemented with ME (PM_1) and was creamy white in color, rough and friable in texture on MT medium supplemented with both ME and Lactose (PM_1 and PM_2, respectively) while calli derived from Succari endosperm was white, smooth and watery on MT containing ME (PM_1) (Table 4). Similar findings were obtained by Wang & Chang (1978) and Chen *et al*. (1991) who also developed triploid

plants from rough and friable embryogenic calli derived from nucellar and endosperm tissue of pummelos and oranges on MT medium modified with different growth regulators.

Table 2 Days to induce callus and callus induction (%) on different media formulation in Citrus

Parameter	Cultivars	Callogenesis Medium				Cultivar Means
		CM_1	CM_2	CM_3	CM_4	
Days to callus induction	**Kinnow**	21.75a	26.5b	28.75b	-	**19.250**
	Succari	23 a	27.5 b	30.5 b	-	**20.250**
	Means	**22.38 C**	**27 B**	**29.63 A**	-	
Callus induction percentage	**Kinnow**	15.83 bc	11.66 cd	7.5 d	-	**8.74 B**
	Succari	25 a	17.91 b	13.5 bc	-	**14.16 A**
	Means	**20.14 A**	**14.78 B**	**10.62 C**	-	

*Means sharing the same letter s are statistically non-significant.
CM_1 = MT+BA (5mgL^{-1}) +2,4-D (2mgL^{-1}) +KIN (5mgL^{-1}) +ME (500mgL^{-1}) +CH (1000mgL^{-1})
CM_2 = MT+BA (5mgL^{-1}) +2,4-D (2mgL^{-1}) +KIN (5mgL^{-1}) +ME (500mgL^{-1})
CM_3 = MT+BA (5mgL^{-1}) +2,4-D (2mgL^{-1}) +KIN (5mgL^{-1})
CM_4 = MT+BA (5mgL^{-1}) +2,4-D (2mgL^{-1})

Table 3 Response of media for callus growth (%) in Citrus cultivars

Cultivars	Callus Proliferation Medium			Cultivar Means
	PM_1	PM_2	PM_3	
Kinnow	83 a	77 a	-	**53.3 A**
Succari	35.41 b	29.16 b	-	**21.25 B**
Means	**59.20**	**53**	-	

*Means sharing the same letter s are statistically non-significant.
PM_1 = MT+ ME (500mgL^{-1})
PM_2 = MT + Lactose (500mgL^{-1})
PM_3 = MS+ IBA (0.5mgL^{-1}) + BA (0.5mgL^{-1})

Table 4 Physiological characteristics of endosperm derived calli on different media

Medium	Kinnow			Succari		
	Growth	Color	Texture	Growth	Color	Texture
PM_1	+++	Creamy white	Rough & friable	++	White	Smooth & watery
PM_2	++	Creamy white	Rough & friable	+	Brownish	-
PM_3	-	Brownish	-	-	Brownish	-

(+++) excellent, (+) poor (-) no growth

Table: 5 No. of sprouted buds, days to sprout and shoot length of colchiploids

Cultivars	Av. Days to bud sprout		Bud sprouting %age		[z]Av. Shoot length (cm)	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
Kinnow	**5.7 c**	**38.0 b**	**100.0 a**	**83.3 b**	**57.1 a**	**15.4 c**
Feutrell's Early	**5.7 c**	**66.8 a**	**95.2 ab**	**75.1 bc**	**32.2 b**	**14.6 cd**
Musambi	**7.4 c**	**44.8 b**	**98.2 a**	**50.4 cd**	**47.2 a**	**16.4 c**
Succari	**10.2 c**	**67.3 a**	**99.1 a**	**58.9 c**	**38.7 b**	**11.1 d**
Means	**7.5 B**	**54.5 A**	**98.1 A**	**66.9 B**	**43.8 A**	**14.3 B**

[z]Average shoot length measured after 3 months.
Means sharing same letter are statistically non-significant.

Table 6: Effect of Colchicine on leaf size of *Citrus* Cultivars

Cultivars	Leaf lamina length (cm)		Leaf lamina width (cm)		Ratio leaf lamina (length/width)	
	Control	Treated	Control	Treated	Control	Treated
Kinnow	**4.6 d**	**6.7 c**	**2.7 d**	**3.6 c**	**1.70 bc**	**1.86 a**
Feutrell's Early	**5.5 cd**	**8.1 b**	**3.1 c**	**5.0 b**	**1.77 b**	**1.62 c**
Musambi	**5.2 cd**	**9.1 a**	**2.9 d**	**6.1 a**	**1.79 b**	**1.49 cd**
Succari	**5.6 cd**	**6.7 c**	**3.2 c**	**4.9 b**	**1.75 b**	**1.36 d**
Means	**5.2 b**	**7.6 a**	**2.9 b**	**4.9 a**	**1.75 a**	**1.58 b**

*Means sharing same letters are statistically non-significant

Table 7 Stomatal studies kinnow mandarin polyploids

Ploidy Level	No. of plants tested	No. of stomata	Width of stomata (µ)	Length of stomata (µ)
Diploids	50	7.5 a	15.3 b	19.8 b
Polyploids	50	5.2 b	19.3 a	21.9 a

*Means sharing the same letters are statistically non-significant

Table 8: Number of chloroplasts in colchiploids of *Citrus* cultivars

Culivars	Control	Treated
Kinnow	**7.86 b**	**9.28 bc**
Feutrell's Early	**5.11 d**	**9.03 b**
Musambi	**6.45 c**	**11.94 a**
Succari	**5.98 cd**	**6.56 c**
Means	**5.59 b**	**8.87 a**

Means sharing same letters are statistically non-significant

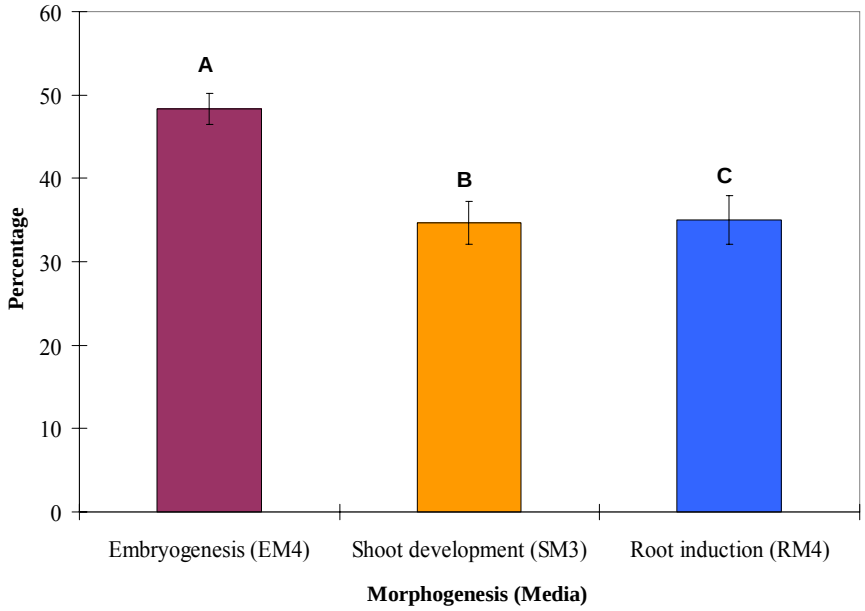

Fig. 1. Morphogenic response in endosperm induced calli of Kinnow mandarin on different media. Data are means of three reps. ± SE with 25-30 explants per rep.

EM4: Embryogenesis medium-MT medium + BA (10 mgL^{-1}) + NAA (2 mgL^{-1}),
SM3: Shoot development medium-MS medium + IBA + KIN (1 mgL^{-1}, each) and RM4: Root induction medium- MS medium + IAA + IBA (2 mgL^{-1}, each).

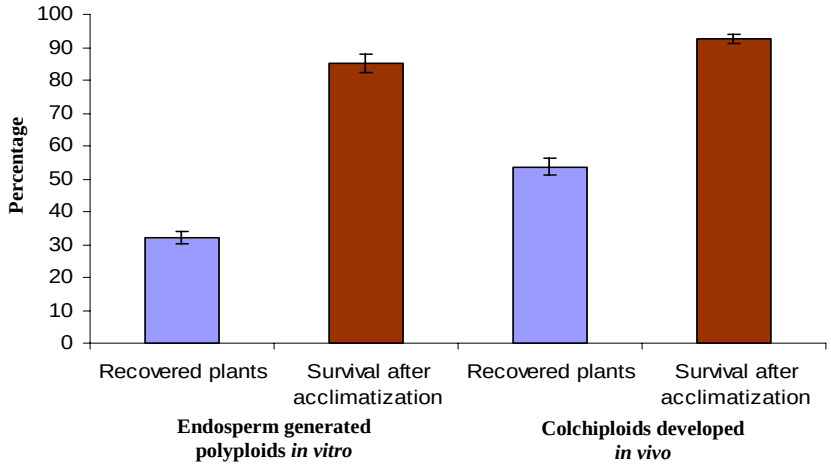

Fig. 2. Recovery and survival percentage of endosperm generated polyploids *in vitro* and colchiploids developed *in vivo* in Citrus cultivars

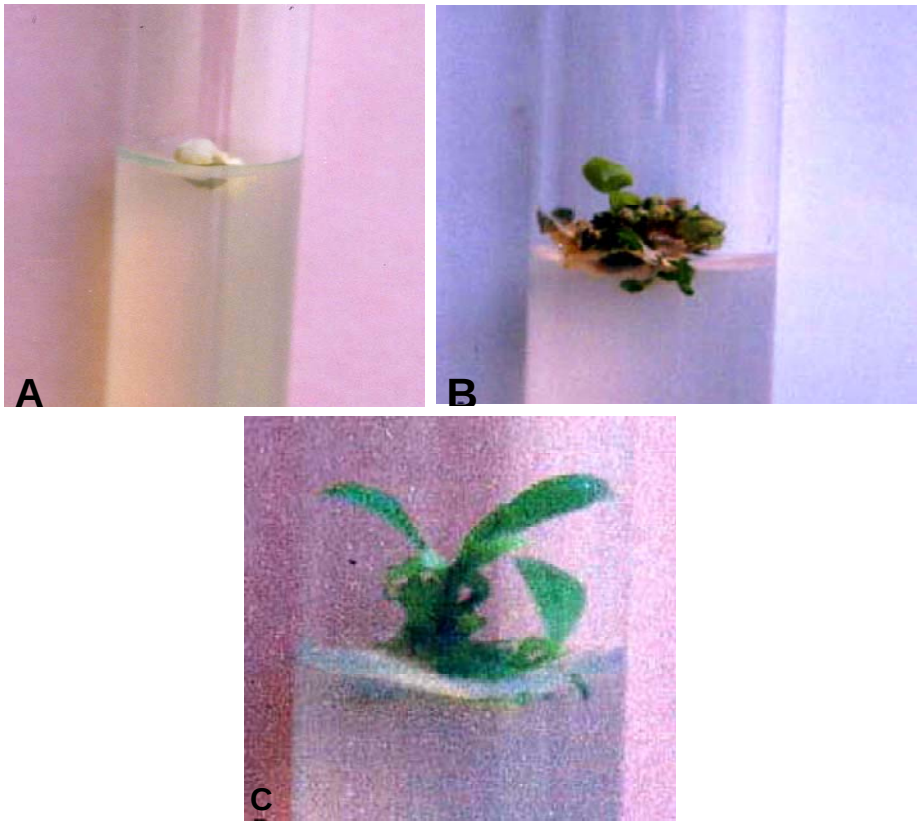

Fig. A-C. Callus induction (A) somatic embryogenesis (B) and shoot development (C) from kinnow mandarin endosperm derived calli on respective media.

Embryogenesis: For embryoid formation, calli derived from endosperm of Kinnow and Succari was sub-cultured on MT medium modified with different combinations of BA and NAA. Embryoids (45%) were developed only in Kinnow on medium (EM_4) containing BA and NAA (10 and 1 mgL^{-1}) after 8 weeks of sub-culture (Fig. B). Rest of the media formulations showed no response for embryogenesis (Fig. 1). While callus developed from Succari failed to give embryogenic response on all media rather it changed its color from white to yellow and then turned brown. Our findings are strengthened by the results of Bello et al. (2000) who reported similar results of embryoid formation with highest frequency on MT medium supplemented with NAA + BA. Somatic embryos were developed in citrus on transfer of embryogenic calli to MT medium with lactose (Starrantino, 1990, 1999; Mooney *et al.*, 1996) and MS medium containing IBA and GA (Altaf, 2006a). These findings vary with our results in cultivars used and medium modifications for callogenesis and subsequent embryogenesis.

Shoot development and rooting: Somatic embryos of kinnow developed shoots (65.5%; Fig. C) on MT medium supplemented with IBA and kinetin (1mgL^{-1} each, SM_3) while

rest of the media depicted no shoot growth (Fig. 1). Similar trend was found by Oh *et al.* (1991) for shoot development on sub-culturing embryoids giving best shoot initiation on medium supplemented with IBA + BA. Shoots obtained were excised and sub-cultured on MS medium supplemented with different auxins. Only MS medium supplemented with IAA and IBA @ $2mgL^{-1}$ each yielded better rooting (75.5%) while no response was depicted on other media formulations. There was no evidence of shoot regeneration found for the endosperm derived calli on any embryogenesis and shoot initiation media in both Kinnow and Succari and our findings are strengthened by the similar earlier reports in Prunus (Liu & Liu, 1980), coffea (Raghusamalu, 1989), Citrus (Gmitter *et al.,* 1990) and Acacia (Garg *et al.*, 1996). Further, we obtained efficient rooting in excised shoots on MS medium containing IBA and IAA ($2\ mgL^{-1}$ each) contrary to earlier reports of rooting in citrus shoots on half strength MS media containing IBA alone (Altaf, 2006b) The plants with roots were transplanted in plastic pots and were subjected to stomatal studies.

Colchicine application *in vivo:* Bud sprouting and shoot growth: Cultivars responded to the colchicine treatment variably which might be due to killing or retardation (Barret, 1974) of treated buds. Kinnow depicted significantly higher ($P \geq 0.001$) bud sprouting percentage (83.3%) followed by Feutrell's Early while sweet orange cultivars Musambi and Succari showed the lowest bud sprouting percentage (50.4%) in plants treated with colchicine. Treated buds took more number of days to sprout than control. Kinnow and Musambi buds were the earliest to sprout (37.5 and 45.8 days, respectively) whereas Succari and Feutrell's Early took almost double time (67.8 and 67.1 days, respectively) to sprout. Shoot length in the sprouted buds was significantly less than control (Table 5).

Leaf size: Significant ($P \geq 0.001$) differences were observed in leaf lamina length treated with colchicine than control. *Citrus* cultivar Musambi had the longest leaves (9.1 cm) in treated buds with colchicine followed by Feutrell's Early. Kinnow and Succari also depicted more leaf lamina length than control (4.6 cm); Similar trend was depicted regarding leaf lamina width as the highest width was found in Musambi (6.1 cm) followed by Feutrell's Early and Succari. The lowest leaf lamina width (3.6 cm) was observed in Kinnow mandarin (Table 6). The results summarized in Table 5 for the leaf lamina length and width show a significant increase in leaf size of colchicine treated plants of all varieties. Similar higher leaf lamina length after colchicine application was reported in cherry by Theiler-Hedtrich (1991). Khan *et al.,* (1992a; b); Jaskani *et al.,* (1996) and Jaskani & Khan (2000) also reported broader leaves in colchicine induced *Citrus* tetraploids than diploids suggesting that these plants are hyperploids.

Stomatal Studies

Stomatal density and size in endosperm generated polyploid Kinnow mandarin plants: Significant ($P > 0.05$) differences were observed among polyploids explored for number and size of stomata in Kinnow mandarin. Study of stomatal density revealed maximum number of stomata (7.5) in the per unit area of the leaves of diploid plants than polyploid plants (5.2)**.** However length and width of stomata was found directly proportional to increase in ploidy level. The polyploid plants were found to be significantly higher for stomatal size (21.9µ and 19.3µ) as compared to diploid seedlings (Table 7). The number, size and frequency of stomata have been useful for the

comparison of polyploids particularly diploids, triploids and tetraploids. Stomatal size was found directly related to ploidy level (Tan *et al.,* 1973). This study strengthens our findings which also show direct relationship of stomatal size with hyperploidy while stomatal number decreased with increase in ploidy level (Jia and Chen, 1985; Jaskani and Khan, 2000). The number of stomata are higher while size of stomata is lower in diploid than polyploid Kinnow plants and the findings are in line with Khan *et al.* (1992b), Khan (1996) and Jaskani & Khan (2000). On the basis of these stomatal parameters studied, almost all endosperm generated plants were found polyploids probably triploids, however, stomatal studies alone are not fully reliable because difference in these seems to be cultivar dependent and climatic conditions may also alter growth of plants and in adverse climatic conditions, wide differences can be observed in morphological parameters.

Stomatal chloroplast density in colchiploids of citrus cultivars: Significant differences were observed for chloroplast number (8.87) in stomata of leaves of treated plants compared to plants (5.59) under control conditions. Musambi showed about two-fold increase in chloroplasts in treated buds as compared to untreated buds (6.45 and 11.94, respectively) (Table 8). A slight increase in chloroplast number was observed in Succari and Kinnow. Mean number of chloroplast per pair of guard cells has been used to estimate the number of chromosomes of anther derived potato plants by Singsit & Eveilleux (1988). Colchicine application significantly delayed the bud sprouting decreased the bud sprouting percentage and shoot length. On the other hand it showed about two fold increase in the leaf lamina length, width and the chloroplast density. The findings are supported by Jaskani, *et al.,* (1996) who reported that the number of stomata was fewer but their size was greater in colchiploids of Valencia late, Jaffa, Blood red, Pineapple and Eureka lemon cvs. of *Citrus*. Our findings are also in line with Khan *et al.,* (2000) who concluded that number of the stomatal guard cells were directly proportional to the ploidy level of the plants.

Kinnow mandarin showed superior behavior than succari sweet orange for callus proliferation, embryogenesis and plant regeneration for development of polyploids from endosperm culture. Bud sprouting percentage and shoot length was found inversely proportional while leaf size was directly proportionate to the colchicine application. Recovery of polyploids from endosperm culture was significantly low (32%) compared to colchicine induced ploids (53.67%) as shown in Fig. 2. However, no significant differences were observed for survival percentage of these plants. After acclimatization, the plants were transferred to greenhouse for further ploidy confirmation and genetic analysis. Although the stomatal studies revealed recovered plants as hyperploids compared to diploids and helped in early screening of the germplasm, however, further studies are suggested for genetic characterization of these ploids using flow cytometery and marker assisted selection (MAS) for future breeding and biotechnology applications.

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