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EMBRYO CULTURE TO ENHANCE EFFICIENCY OF COLCHICINE INDUCED POLYPLOIDIZATION IN GRAPEFRUIT

MUHAMMAD USMAN*, BILQUEES FATIMA, MUHAMMAD USMAN, WAQAR ABDUS SAMAD AND KARIM BAKHSH

Institute of Horticultural Sciences, University of Agriculture, Faisalabad
**Corresponding author's e-mail: musman74@gmail.com*

Abstract

Different cultivars of grapefruit (*Citrus paradisi* L.) were explored for colchicine induced polyploidization using embryo culture on MS medium. Embryo germination was found to be highly genotype dependent on colchicine containing MS medium. The highest number of germinated embryos per seed was observed in cvs. Shamber and Frost Fresh 2.89 and 2.27, respectively, compared to other cultivars. Treatment of antimitotic agent colchicine arrested shoot growth in most of the cultivars whereas number of leaves was significantly higher in cv. Foster and Red Max Cane Foster (6.15 and 4.87, respectively) compared to control (2.17 each). Ratio leaf lamina was also higher in case of treated plants compared to control. Number of stomata decreased (11) while length and width of the stomata increased (2.9 and 2.4, respectively) in leaves of treated plants compared with control (2.3 and 1.7, respectively) plants in cv. Shamber. These putative hyperploid plants will be further confirmed for ploidy using chromosomal studies. The plants were shifted to greenhouse after acclimatization for further genetic characterization of ploidy and nucellar or zygotic origin of the plants.

Introduction

Citrus industry of Pakistan is contributing 1% of fresh fruit in total production from 2% area under *Citrus* cultivation round the world yielding merely 12.7 tons per hectare. *Citrus* provides major stake to the fruit industry of this country contributing 25% of total fruit production out of which 95% share is coming from Punjab (Anon., 2010). *Citrus* industry is mainly dependent upon Mandarins including Kinnow and Feutrell's Early covering 80% of total area under *Citrus* (Usman *et al*., 2002). Grapefruit is an important member of *Citrus* and offers plenty of vitamin C, higher amount of β-carotene that body converts to vitamin A and a handful amount of spermidine that prolongs cell ageing process. Progress in *Citrus* industry is handicapped by narrow genetic base, alternate bearing, high number of seeds, post harvest losses and a number of biotic and abiotic stress. Public likeliness towards seedless cultivars is rising and to be competitive in the global *Citrus* industry we need to develop our own seedless cultivars for instance in Grapefruit which is one of the least explored and underutilized *Citrus* member in Pakistan. Success stories of seedlessness in the world include Marsh seedless, Tahiti lime and many others.

Seedlessness through triploidy is commonly induced by interploid hybridization followed by embryo rescue *In vitro* (Soost & Cameron, 1985; Usman *et al*., 2002; Grosser & Chandler, 2004; Reforgiato *et al*., 2005; Zhang *et al*., 2007). For interploid crossing we need autotetraploids which are not available in our germplasm resources and less likely available in the world as well (Zeng *et al*., 2006; Usman *et al*., 2008). In Pakistan, narrow *Citrus* germplasm base is further confined to research stations only. There is no report of availability of autotetraploid grapefruit that is required for triploid grapefruit development and for further grapefruit crop improvement programs. These facts suggest dire need to exploit potential of seedless *Citrus* breeding (Rao *et al*., 1992) and to develop a genetically diverse hyperploid population of grapefruit germplasm.

Citrus polyploids are important for their horticulturally useful characteristics, source of variability and used as parents in breeding programs. Additionally hyperploids are vigorous and healthy plants. *In vitro* chromosome doubling is a popular source of producing tetraploid plants artificially in *Citrus* and other fruit trees (Predieri, 2001). Tetraploids often generate variants having favorable horticultural characteristics as increase in organ size like leaves, flowers, fruits leading to high production and by doubling genome tetraploids provide a wider germplasm base for breeding programs (Aranda *et al*., 1997; Thao *et al.,* 2003; Zhang *et al*., 2007).

Conventional methods of inducing tetraploidy include soaking roots or whole plants in colchicine solution, culturing shoots in colchicine-containing medium in greenhouse, injecting colchicine into secondary buds and applying colchicine-soaked cotton swab to auxiliary buds. These methods are less efficient in polyploid plant production with higher frequency of chimeras that need several propagation cycles to separate chimeras (Pei, 1985). Colchicine forms tubulin-colchicine complex preventing microtubule assembly and arrests spindle formation during mitosis resulting in duplicated chromosomes which stay within the same nucleus leading to tetraploidy (Khan & Rahman, 1994; Panda *et al*., 1995; Sarasan & Roberts, 2004; Caperta *et al*., 2006). Plant cells survive on colchicine and resulting colchiploids have played a significant role in variety development programs (Barrett, 1974). Non-chimeric autotetraploids using colchicine treatment *In vitro* has been developed in *Citrus* (Wu & Mooney, 2002; Zhang *et al*., 2007). Use of multicellular somatic tissues or organs has lead to chimeric polyploid plant development by colchicine (Shao *et al*., 2003; Thao *et al*., 2003) compared to embryo culture (Yang *et al*., 2006). Therefore embryo culture of grapefruit cultivars was preferably used to minimize the chance of development of chimerism in developed hyperploid germplasm. The study was initiated to exploit advantages of polyploids and to widen germplasm base for future crop improvement in grapefruit.

Materials and Methods

Plant material, sterilization and inoculation procedures: Fruit of grapefruit cultivars viz., Shamber,

Frost Fresh, Red Max Cane Faster, Foster, J BC 430 Marsh, and Reed were collected from Experimental Fruit Garden, Institute of Horticultural Sciences, University of Agriculture, Faisalabad. Fruits were cut in to two halves with a sharp knife, squeezed and seeds were extracted from fruit by twisting. Extracted seeds were washed by tap water to remove the pulp. Underdeveloped and immature seeds were discarded. Developed seeds were surface sterilized with 70% ethanol for 5 minutes and then with 10% sodium hypochlorite plus 1-2 drops of Tween 20 for 15-20 minutes followed by 3-4 rinses with sterilized water. Seed coat was removed and embryos were excised with sterile needle and forceps. These embryos were cultured on MS medium supplemented with various levels of colchicine (0.0, 0.001, 0.005, 0.01, 0.02, 0.03, 0.05 and 0.1mgL^{-1}).

Media sterilization and culture conditions: Media were sterilized in autoclave at a temperature of 121^{o}C and 15 Psi for 20 minutes. Cultures were placed in growth room facilitated with 2500 lux light intensity and maintained at temperature 25°C ± 2.

Morphological and cytological characterization of putative hyperploids: These putative hyperploid plants developed from embryo germination *In vitro* were identified and screened out on the basis of morphological and cytological characterization. Data were collected for leaf area, root and shoot length (cm) and survival (%) after transplanting.

Stomatal studies: This included determination of stomatal size, number and frequency of stomata in diploid (parent) and putative hyperploid plants.

Frequency of stomata: Number of stomata was counted by removing a thin layer from the lower epidermis. A drop of distilled water was put on a glass slide and mounted the epidermal layer on the drop of distilled water. The slide was covered with the fine slide cover very carefully to avoid any air bubbles under the slide cover. Fine tissue paper was used to absorb extra water by placing it aside the cover slip. The slide was then adjusted on the stage of microscope and was observed at different magnifying powers. Number of stomata was counted under magnification of 1250x.

Size of stomata (µ): The length and width of stomata were measured in microns (µ**)**. The stage micrometer was placed under appropriate magnification of microscope. The ocular micrometer has 50 small divisions in the scale from 0 to 1, while in the case of stage micrometer there were 100 small divisions in the scale from 0 to 1. The focus was adjusted in such a position that the zero of both micrometers coincided. Then the divisions of stage micrometer which coincided with the fifty of ocular micrometer were noted. The divisions of stage micrometer were 5 which were used to calculate value of each division of ocular piece in micron by putting values in the formula:

Size of Stomata = division of stage x 10 division of ocular
= 5/50 x 10, = 50/50 = 1

Each division of stage micrometer is equal to 1µ. To determine the length and width, divisions were multiplied with 1µ thus obtaining measurement of any particular stomatal dimension under the microscope (1250x magnification).

Length and width of stomata: Length and width of the stomata was measured by removing the stage micrometer and placing the slide on the stage of the microscope. Then the ocular micrometer was adjusted length/width wise and the divisions were counted and then multiplied with the factor 1µ.

Length/Width = Counts of ocular division x 1µ

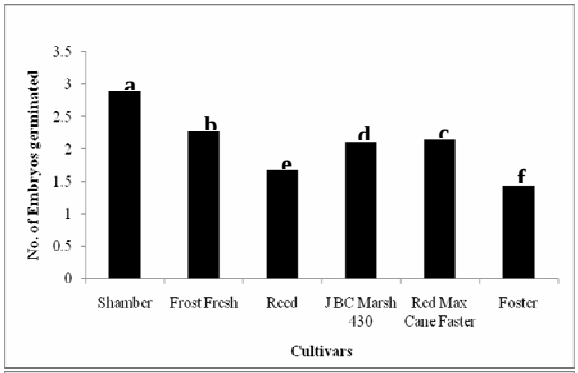
Size of stoma (µ): Size of stoma (Stomatal opening) was measured by revolving the eye tube that had the ocular micrometer and by adjusting the division in cross sectional position from the centre of stomata, the number of divisions were counted and then multiplied with the factor 1µ.

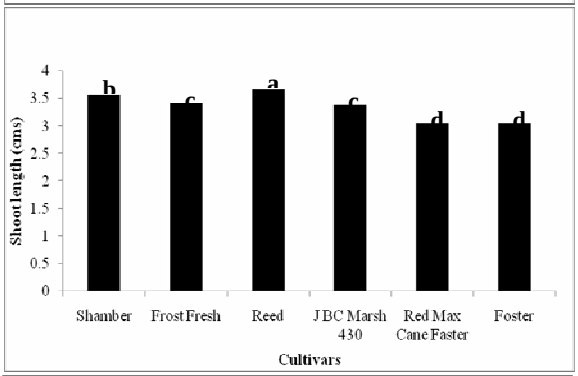
Aperture = Counts of ocular divisions x 1µ

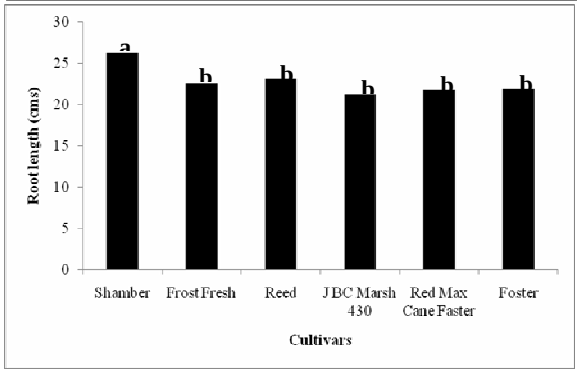
Results and Discussion

Genotypic response for embryo germination, shoot length and root length: Highly significant genotypic response was observed for embryo germination and shoot length compared to root length. In embryo germination, significantly ($p>0.005$) higher germination (2.9 embryos) was observed in grapefruit cultivar Shamber and the genotypic trend was observed as Shamber >Frost Fresh >Red Max Cane Faster > J BC Marsh 430 >Reed >Foster (Fig. 1). Regarding shoot length, cv. Reed showed significantly ($p>0.005$) more shoot growth (3.6 cm) and the rest of the cultivars showed shoot growth as Reed >Shamber >Frost Fresh, J BC Marsh 430 >Red Max Cane Faster > Foster (Fig. 2). Maximum root length was observed in cv. Shamber while rest of the cultivars showed non-significant differences for root length ranging from 20cms -25cms (Fig. 3).

Effect of *In vitro* Colchicine application on embryo germination, shoot length and root length: Embryo germination was found inversely proportional to the increase in colchicine level in the medium and embryo germination ranged from 2.06 to 2.39 from 0-0.02mgL^{-1} of colchicine (Fig. 4ab) and further increase in its concentration showed decline in the embryo germination suggesting the 0.03mgL^{-1} of colchicine as the lethal level for embryos (Table 1). Regarding shoot and root length both were found inversely proportional to the colchicine level in the medium and significantly higher shoot and root length was observed on control (4.08cm and 26.66cms, respectively) while the highest concentration of colchicine in the medium showed minimum shoot and root growth (2.67cms and 17.98cms, respectively). Leaf emergence and growth showed a variable response to colchicine levels as significantly higher number of leaves 6.15 were observed at 0.02mgL^{-1} while both lower and higher levels of colchicine showed decreased number of leaves per plant suggesting that colchicine treatment had markerd inhibitory effect on leaf development in shoots (Table 1). However, the variability observed might be attributed to the variation in development, health and age of embryos in a polyembryonic *Citrus* seed.







Figs. 1-3. Genotypic response for 1) embryo germination 2) shoot length and 3) root length on MS medium with colchicine in grapefruit.

Embryo germination percentage is an important parameter while applying colchicine to embryos for polyploid induction. Colchicine delayed germination with increasing levels and being an antimitotic agent caused death of embryos at higher levels inducing more stress. Results showed an inverse relationship between various colchicine concentrations and seed germination. These results agree with the work of Duren *et al.*, (1996) and Chakarborti *et al.*, (1998) who found negative effect of higher colchicine concentrations on germination of explants. Root and shoots arising from control treatments had higher growth rate than those from colchicine treated ones. Variable effect of colchicine treatment was observed on root and shoot length indicating an adverse and suppressing effect of colchicine on both root and shoot length of plants. Seedlings arising from treated embryos showed slow growth compared to control. Root and shoot length decreased with increase in colchicine concentration leading to more root and shoot length than control. The growth retardation in treated plants might be attributed to reduced rate of cell division caused by colchicine. Similar observations in which root and shoot length decreased due to growth retardation are also reported by Skidar & Jolly (1994). High concentration of colchicine coupled with long exposure time produced plants with stunted growth (Rubuluza *et al*., 2007; Zhang *et al.*, 2007) and recovery of triploids with reduced growth compared to diploids (Shao *et al.,* 2003).

Genotypic response for frequency and size of stomata (µ) in putative hyperploid grapefruit plants: Strong genotypic variability was observed for frequency of stomata and length and width of stomata (Fig. 5ab). Significantly ($p>0.005$) higher number of stomata were found in cultivars Frost Fresh and Foster (12.3-12.4) followed by Red Max Cane Faster, Reed and J BC Marsh 430 while the lowest number of stomata were observed in cv. Shamber (11.85; Fig. 6). Stomatal length was significantly higher in Shamber and Foster (2.8µ-2.9µ) while rest of the cultivars showed similar stomatal length (2.70µ-2.75µ; Fig. 7). Higher stomatal width was observed in cvs. Reed and Shamber (2.08µ-2.10µ) followed by J BC Marsh 430 and Foster while the lowest stomatal width was observed in Frost Fresh and Red Max Cane Faster (1.98µ-1.99µ; Fig. 8). The data suggested maximum size of stomata in cvs. Shamber and Foster with maximal length and width compared to other cultivars. Variable genotypic differences were observed for stomatal pore size. Aperture size was significantly higher (0.45µ) in cvs Shamber and Foster (0.43µ) followed by Red Max Can Faster (0.42µ) and J BC Marsh 430 (0.42µ) whereas minimum stomatal pore size was found in Frost Fresh (0.41µ) and Reed (0.40µ) as shown in (Fig. 9).

Table 1. Effect of *In vitro* colchicine application on embryo germination and grapefruit plant growth

MS medium + Colchicine (mgL^{-1})	No of embryos germinated	Shoot length (cm)	Root length (cm)	No. of leaves
0	2.06 D	4.08 A	26.66 A	5.711 B
0.001	2.06 D	3.87 B	25.54 AB	5.626 C
0.005	2.32 B	3.76 C	24.98 ABC	5.486 D
0.01	2.11 C	3.55 D	23.11 BCD	5.302 E
0.02	2.39 A	3.22 E	21.58 CD	6.158 A
0.03	1.91 F	2.852 F	20.26 DE	4.804 G
0.05	1.86 G	2.80 G	22.51 BCD	5.155 F
0.1	2.04 E	2.67 H	17.98 E	5.127 F

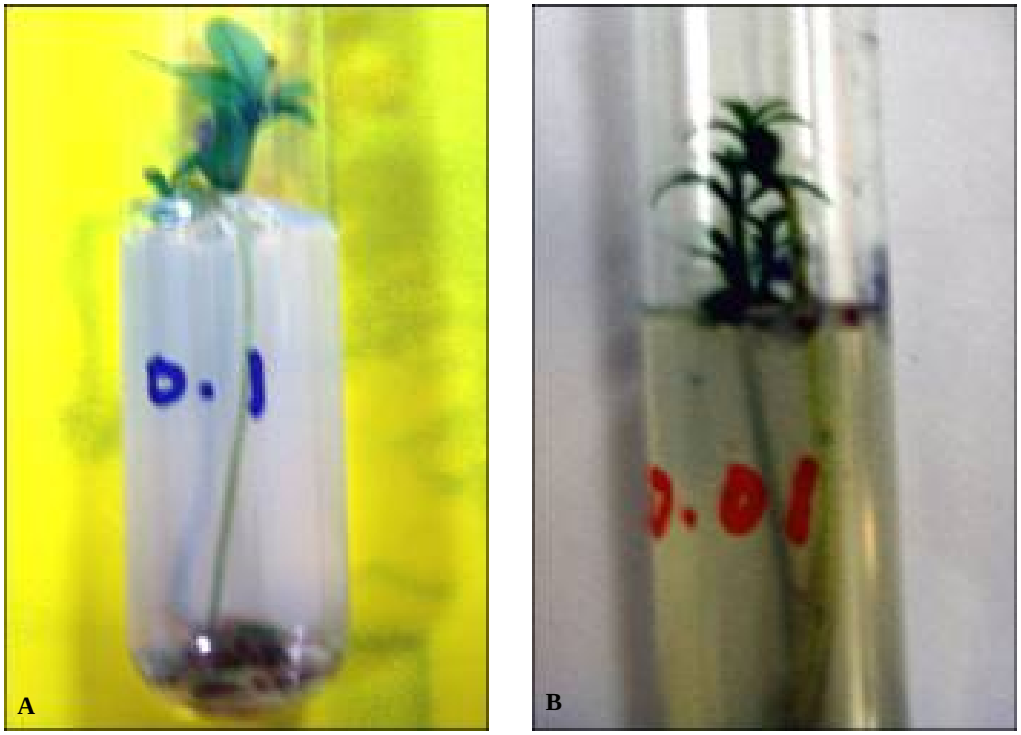


Fig. 4ab. Embryo germination of Grapefruit cv. Shamber on MS media with Colchicine 0.1% (A) and 0.01% (B).

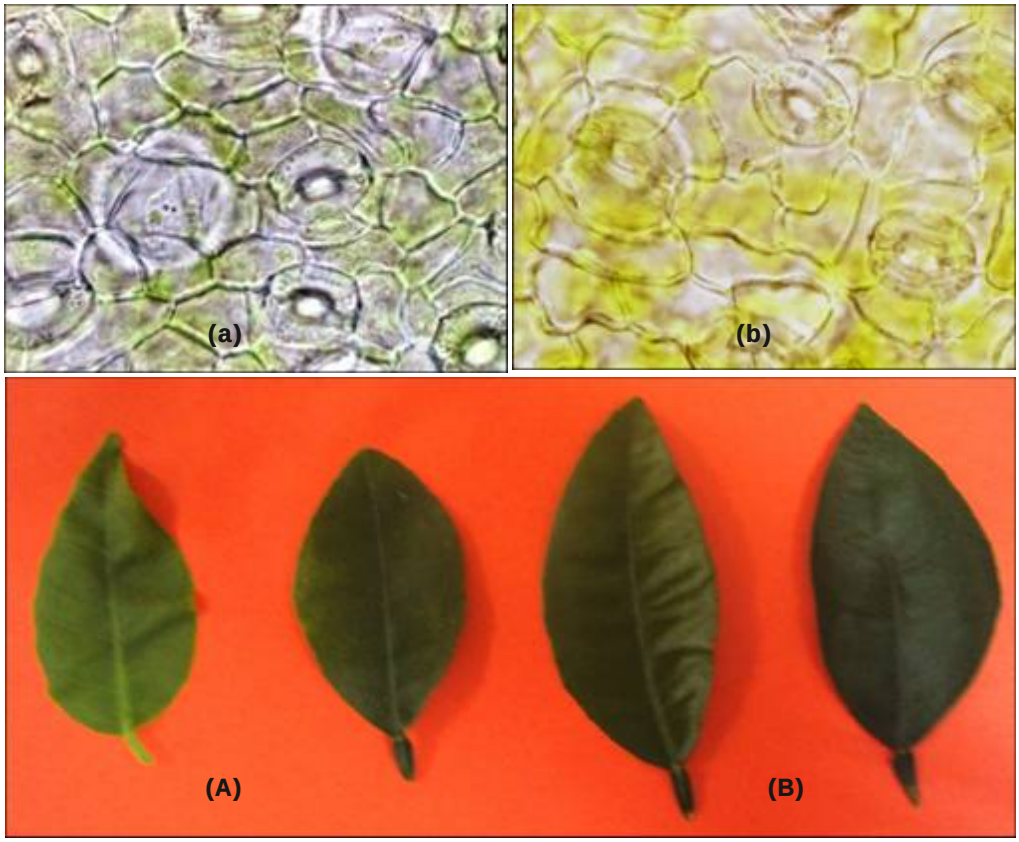
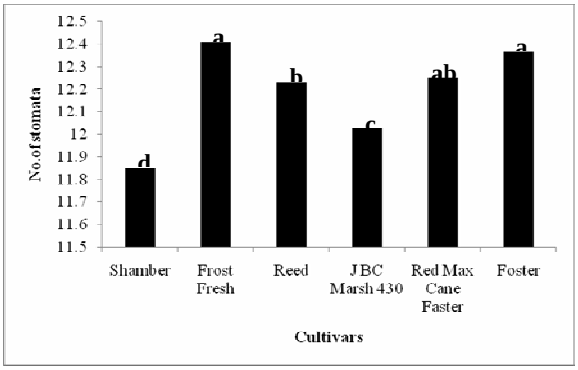
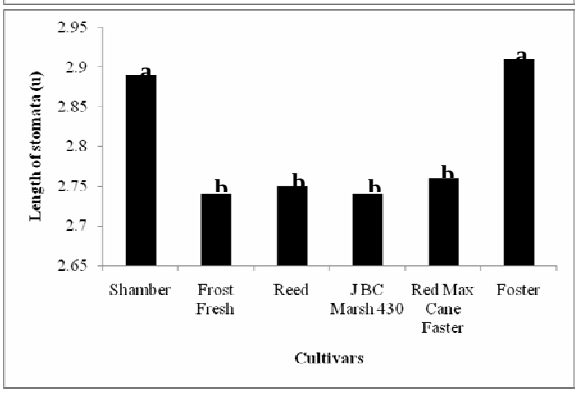
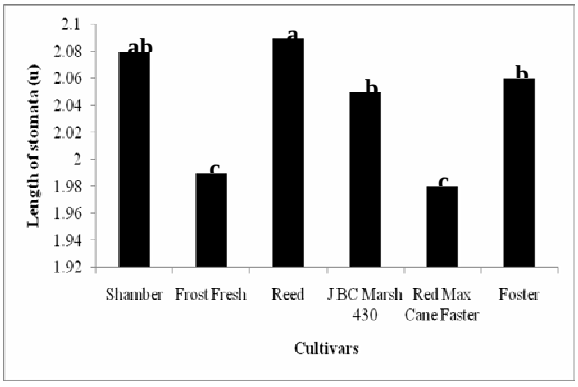


Fig., 5ab. Stomatal density in putatitive polyploid plants a) diploids and b) polyploids.







Figs. 6-8. Genotypic response in stomatal studies for 6) number, 7) length and 8) width of stomata (µ).

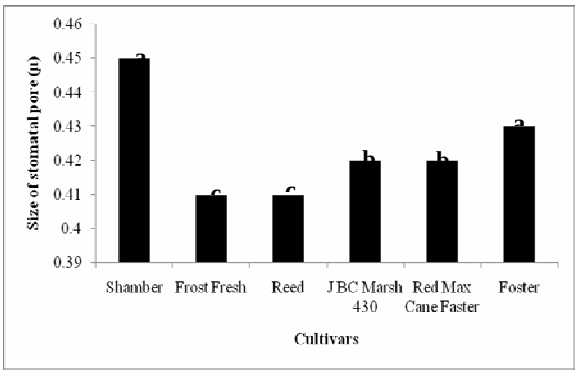


Fig. 9. Genotypic response for stomatal opening (µ).

Effect of *In vitro* Colchicine application on frequency and size of stomata (µ) in putative hyperploid grapefruit plants: Frequency of stomata was found inversely proportional to number of stomata and the number decreased (13.89 to 9.63) with increase in the colchicine concentration (0-0.1 mgL^{-1}) in the MS medium. Length and width of stomata were found directly proportional to the colchicine level in the medium and both length (2.47µ-3.18µ) and width (1.77µ-2.34µ) increased with increase in colchicine concentration (0-0.1 mgL^{-1}) in the medium (Table 2). These results suggest that colchicine application have a direct impact on stomatal density and size. Size of stoma (aperture) increased with increase in the level of colchicine in the medium and maximum opening (0.47-0.53µ) was found in all the cultivars on 0.10 mgL^{-1} of colchicine. Significantly higher stomatal size (0.51µ) was found on the higher level of colchicine 0.10 mgL^{-1} whereas it was less (0.35µ) in control medium (MS devoid colchicine).

Stomatal size has been directly proportional to ploidy and it increased with the increase in the ploidy level of plants (Cutter, 1986; Costa *et al.*, 2004). This trend has been reported in many fruit crops like *Citrus* cvs. mandarin (Khan *et al.*, 1992., Usman *et al.*, 2002, Usman *et al.*, 2008), *Vitis* (Yang *et al.*, 2006) and *Ziziphus* (Gu, 2005). Vandehout *et al.*, (1995) observed greater stomatal length of colchicine treated *Citrus* cultivars as well. Gu (2005) observed that the stomata size of tetraploid plants was significantly larger than those of diploid plants, while the frequency of stomata was reduced significantly in *Ziziphus*.

Our results suggested that both genotype and colchicine treatment has a significant impact on stomatal size in grapefruit cultivars. Higher concentration of colchicine has been more useful in inducing hyperploidy compared to low doses. Low concentration of the colchicine induces depolymerization of microtubular cytoskeleton during cell cycle and insufficient spindle disruption occurred leading to induction of polyploid cells with cell anomalies. Higher concentration was found more effective in formation of polyploid cells as it may lead to production of new tubulin structures allowing development of tetraploid nuclei (Caperta *et al.*, 2006). Zeng *et al.*, (2006) reported development of tetraploids on 0.1% of colchicine treatment for 4-8 days in grapefruit cultivar Frost cell lines. We also observed less stomatal density and more stomatal size on these levels of colchicine. However, we differ in use of explant (embryo) compared to protoplasts used by them. Stomatal studies proved to be a useful tool in initial screening of diploid and polyploids in grapefruit cultivars and are strengthened by similar reports of Carvalho *et al.*, (2005) in *Citrus* and Stanys *et al.*, (2006) in Japanese Quince. Similar results were reported by Saeed *et al.*, (2004) and Fatima *et al.*, (2010) who recovered spontaneous polyploids in mandarins, sweet oranges and grapefruit cultivar Foster. We differ, however, in induction of polyploidy in grapefruit cultivars by embryo culture *In vitro* on colchicine. This hyperploid germplasm has been transplanted in pots and shifted to green house after acclimatization (Fig. 10). In our studies, embryo culture of grapefruit cultivars on colchicine containing media proved to be an efficient method of polyploidy induction and successful recovery as reported in triploid orange (Minoru & Nario, 2006). Confirmation of hyperploid germplasm is suggested through chromosome counting or flow cytometery. Genetic characterization of the germplasm is suggested to screen mixoploids, chimeras and the origin of embryos (zygotic/nucellar). These autotetraploids could be useful in crop improvement programs in grapefruit.

Fig. 10. Putative grapefruit polyploids in greenhouse after acclimatization.

Table 2. Effect of *In vitro* colchicine application on stomatal density and size.

MS media + Colchicine (mgL^{-1})	No. of stomata	Length of stomata (µ)	Width of stomata (µ)	Stomatal pore size (µ)
0	13.89 A	2.47 H	1.77 H	0.35 E
0.001	13.61 B	2.53 G	1.79 G	0.35 E
0.005	13.28 C	2.63 F	1.91 F	0.39 D
0.01	12.71 D	2.74 E	2.02 E	0.41 CD
0.02	12.07 E	2.85 D	2.08 D	0.42 C
0.03	11.59 F	2.95 C	2.17 C	0.45 B
0.05	10.77 G	3.06 B	2.24 B	0.48 A
0.1	9.63 H	3.18 A	2.34 A	0.51 A

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