DOI : 10.5958/0974-0112.2022.00019.6

Indian J. Hortic. 79(2), June 2022: 136-144

Anatomical studies of *in vitro* raised haploid and diploid citrus plants generated through gamma irradiated pollen

Manoj Kundu*[1] and A. K. Dubey

Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi -110013, Delhi, India.

ABSTRACT

Histological studies of stem, root and leaf of ovule and embryo cultured haploid and diploid plantlets of *Citrus grandis* × *C. sinensis* and *Citrus grandis* × *C. limetta* crosses were carried out. The area of xylem and phloem in the stem increased gradually with the increasing age of explants in both cross combinations; however, it was measured minimum in haploid plantlets (1.95 and 1.62 mm^2, respectively) compared to diploid ones. A similar trend was also observed for the area occupied by the xylem and phloem in the root section of those plantlets. A negative relation was obtained between the ploidy level and per cent area of the xylem and phloem within the stem and root of those plantlets. No cuticular and epicuticular wax was observed on the leaf surface in any plantlets irrespective of ploidy level and age of cultured explants. However, the leaf thickness increased gradually with the increasing age of the explants with minimum in haploid ones. Although the area of all the parameters evaluated in stem, root and leaf under this experiment was reduced in haploid plantlets, they were well developed and similar to diploid ones, which confirmed that these haploid plantlets could also grow normally under open field conditions if intensive care is taken during acclimatization.

Keywords: Anatomy, Haploid citrus, Diploid citrus, Gamma irradiated pollen, Embryo culture.

INTRODUCTION

The importance of haploids for plant breeding and genetic research is well recognized, especially for woody perennials, which are generally characterized by a prolonged reproductive cycle, heterozygosity, large canopy size and often, self-incompatibility (Germana and Chiancone, 7). Haploids have significant potential in fruit breeding, because homozygous plants, which are very important for genetic analysis and breeding, are easily obtained by doubling their chromosome numbers. Regarding *Citrus*, interest on haploids lies on the possibility of using them as inbred line in controlled hybridization; for triploid seedless cultivar breeding by somatic hybridization of haploid and diploid protoplasts (Ollitrault *et al*., 14) induction of new forms of interspecific and intergeneric hybrids through the fusion of haploid protoplasts (Grosser *et al*., 8); overcoming incompatibility barrier (Pandey, 16); gene transformation (Pandey, 15) and nuclear substitution (Raquin *et al*., 18). Among different techniques, *in situ* parthenogenesis by pollination with irradiated pollen followed by pseudo-fertilized ovule culture is one of the most viable techniques for inducing haploids in citrus. But the major hindrance for haploid induction by this method is the involvement of *in vitro* ovule or embryo culture technique, resulting low plantlets regeneration capacity (Kundu *et al*., 10). Moreover, the survival percentage of those plants under field condition is also a vital issue, because i*n vitro* cultured plantlets grow in conditions,which is different from those grows in open field and frequently exhibit peculiarities in shoot and leaf development. In general, the shoots of *in vitro* cultured plants are thinner, fragile and have less collenchyma and sclerenchyma than plants grown in wild (Preece and Sutter, 17). Moreover, physiological disorders like hyperhydration can be induced under *in vitro* condition, and giving rise to organs and tissues with peculiar anatomy, morphology and physiology resulting greater abnormalities. Such abnormalities ultimately makes those *in vitro* raised haploid plantlets very weak with slow growth rate under field condition as compared to the diploid ones. However,very little efforts has been encountered till now concerning the effects of ploidy level on anatomical characters of *in vitro* raised plantlets of different ploidy levels. Therefore, looking into the importance of haploid plants in modern breeding programme and their survivability under open field condition in comparison to diploid one, the present investigation was carried out to study the anatomical feature of stem, root and leaf of haploid and diploid plantlets, regenerated from *in vitro* cultured embryo and ovules of different ages after pollination with irradiated pollen technique.

MATERIALS AND METHODS

In vitro raised diploid plantlets obtained from ovule culture at 20, 35 and 50 days after pollination

*Corresponding author: manojhorti18@gmail.com

[1]Department of Horticulture (Fruit & Fruit Technology), Bihar Agricultural University, Sabour, Bhagalpur– 813210, Bihar , India

(DAP) and embryo culture of mature seeds of *Citrus grandis* × *C. sinensis* and *C. grandis* × *C. limetta* crosses were used in the present experiment along with haploid plantlets, regenerated from ovule culture at 50 DAP in the same cross combinations.

After sufficient growth of *in vitro* raised ovule and embryo cultured plantlets, stem, root and leaf were collected from these plantlets under aseptic condition and samples were prepared. The stem and root segments(1.0 cm each) were collected separately from each *in vitro* raised plantlet at the height of 5.0 cm above and below the junction of stem and root respectively. Thereafter, these stem and root segments were fixed separately for 3 days in FAA (formaldehyde-acetic acid-alcohol) solution containing 95% ethanol (50 ml), glacial acetic acid (5 ml), formalin 40 v/v (10 ml) and water (35 ml). After 3 days, dehydration of these segments was carried out by putting the samples in increasing concentrations of ethanol (50%, 70%, 80%, 90% and 100%) for 5 min in each. Following that, transverse sections of these segments were prepared by cutting through the radial plane followed by rehydration in descending strength of ethanol (100%, 90%,80% and 70%) for 1 min in each. The cross sections were then stained with Safranin O, a biological stain (3% solution made up in 50% ethanol) for 1 hr followed by 5 min gentle washing in running tap water. They were then transferred to crystal violet solution (0.25% solution made up in 90% ethanol) for 2 min followed by a dip in distilled water for 1 min and several dips in 90% ethanol, until no more violet stain was removed. After that, they were dipped in fast green solution (1% solution made up in distilled water) for 10 min and then dipped into two separate isopropanol solutions one by one for 3 min in each. The stained stem and root sections were then put separately in small petri dish, containing distilled water. Then, the slides were prepared by placing one section on the slide mounting with a cover slip and anatomical characteristics of stem and root were studied under optical microscope (light microscope at 10× magnification). On the other hand, third leaf down from the terminal one was collected from each *in vitro* raised plantlet and a portion of about 10 mm × 5 mm leaf area was taken from the area mid-way between the leaf apex and base, and mid-way between the mid-rib and margin of the leaf lamina. These portions were then kept in separate and labeled vials containing FAA solution. All manipulations were carried out quickly to avoid wilting of leaves. The further procedures like dehydration, sectioning, rehydration, staining and mounting were done in a similar way described previously for stem and root sections. Thereafter, leaf sections were examined under a light microscope and was photographed with the camera attached to microscope at 10× magnification.

Quantification of anatomical characters of stem, root and leaves were done by using menu driven, image analysis software (Digimizer software package). The experiment was laid out in a complete randomized design with 5 replications. Statistical analysis was performed using statistical analysis software (SAS 9.3; SAS Institute, Cary, NC, USA) and the means were compared using Tukey's Honest Significant Difference (THSD) Test at $P \leq 0.05$.

RESULTS AND DISCUSSION

It is evident from the table 1 that the area of xylem and phloem within the stem cross section varied significantly with the age of cultured explants and ploidy level. Regardless of stage of explants for culture, significantly higher xylem (3.75 mm^2) and phloem (2.97 mm^2) area in stem were recorded in *C. grandis* × *C. limetta*. Irrespective of pollen parent, formation of xylem and phloem within the stem of *in vitro* raised plantlets also varied significantly due to the age of the explants (Fig. 1) and a direct relation was observed in the amount of xylem and phloem formation within the stem with age of the cultured explants and ploidy level. A perusal of the data (Table 1) clearly indicated that the embryo cultured diploid plantlets had maximum xylem (6.46 mm^2) and phloem area (4.89 mm^2) followed by ovule cultured diploid plantlets at 50 days after pollination (DAP) (4.19 and 3.43 mm^2, respectively). However, it was minimum in ovule cultured (50 DAP) haploid plantlets (1.95 mm^2 and 1.62 mm^2, respectively). Similarly, per cent of xylem and phloem within the stem was also increased gradually with the age of explants (Fig. 2). The data revealed that embryo cultured diploid plantlets had maximum xylem and phloem per cent (15.77% and 11.72%, respectively) followed by ovule cultured (50 DAP) diploid plantlets. However, it was depicted minimum in ovule cultured haploid plantlets (4.99% and 4.13%, respectively). Contrary to the area of xylem and phloem within the stem section of these citrus plantlets, the area of pith was measured maximum in haploid plantlets (8.64 mm^2) as compared to its diploid counterpart. Among diploids, the area of pith increased gradually with the increased age of cultured explants (Fig. 3). Data pertaining to interaction between age of explants and ploidy level revealed that haploid plantlets of *C. grandis* × *C. limetta* crosses had maximum pith area (8.82 mm^2) followed by haploid plantlets of *C. grandis* × *C. sinensis* crosses (8.47 mm^2). It decreased gradually with the decreased age of explants, being minimum in ovule cultured (20 DAP) diploid plantlets of *C. grandis* × *C. sinensis* crosses (4.04 mm^2)

Table 1. Effect of pollen parent, age of cultured explants and ploidy levels on xylem and phloem formation in stem of *in vitro* raised *Citrus grandis* plantlets.

Treatment combination	Xylem area (mm^2)	Phloem area (mm^2)	Xylem : Phloem ratio
	Pollen patent (P)		
C. sinensis	3.61^{b}	2.68^{b}	1.34^{a}
C. limetta	3.75^{a}	2.97^{a}	1.26^{b}
SEm (±)	0.03	0.01	0.01
LSD (P ≤ 0.05)	0.08	0.03	0.03
	Age of explants (A)		
Embryo cultured (2x)	6.46^{a}	4.89^{a}	1.32^{b}
50 DAP (2x)	4.19^{b}	3.43^{b}	1.22^{c}
35 DAP (2x)	3.46^{c}	2.49^{c}	1.39^{a}
20 DAP (2x)	2.33^{d}	1.70^{d}	1.37^{ab}
50 DAP (x)	1.95^{e}	1.62^{e}	1.20^{c}
SEm (±)	0.04	0.02	0.02
LSD (P ≤ 0.05)	0.17	0.08	0.07
	P × A Interaction		
	C. sinensis		
Embryo cultured (2x)	6.38^{a} ± 0.09	4.86^{a} ± 0.05	1.31^{bcd} ± 0.05
50 DAP (2x)	4.12^{b} ± 0.09	2.87^{c} ± 0.05	1.44^{a} ± 0.05
35 DAP (2x)	3.38^{c} ± 0.22	2.38^{e} ± 0.08	1.42^{ab} ± 0.05
20 DAP (2x)	2.24^{de} ± 0.09	1.71^{f} ± 0.05	1.31^{bcd} ± 0.05
50 DAP (x)	1.92^{f} ± 0.06	1.58^{g} ± 0.05	1.22^{cd} ± 0.07
	C. limetta		
Embryo cultured (2x)	6.54^{a} ± 0.07	4.92^{a} ± 0.06	1.33^{abc} ± 0.06
50 DAP (2x)	4.26^{b} ± 0.10	3.98^{b} ± 0.08	1.07^{e} ± 0.04
35 DAP (2x)	3.54^{c} ± 0.24	2.59^{d} ± 0.07	1.37^{ab} ± 0.05
20 DAP (2x)	2.41^{d} ± 0.10	1.69^{fg} ± 0.04	1.43^{a} ± 0.04
50 DAP (x)	1.99^{ef} ± 0.07	1.66^{fg} ± 0.04	1.20^{d} ± 0.05
SEm (±)	0.06	0.03	0.02
LSD (P ≤ 0.05)	0.28	0.13	0.11

Value indicates mean of five replicates and ± values indicates standard deviation. Different letters in the same column indicate significant differences at P ≤ 0.05 (Tukey's Honest Significant Difference Test); DAP: days after pollination; x: Haploid; 2x: Diploid

followed by ovule cultured (20 DAP) plantlets of *C. grandis* × *C. limetta* of same ploidy level (5.01 mm^2). Almost similar trend was also observed for the per cent area occupied by pith in the stem of those *in vitro* raised citrus plantlets of different ploidy levels. Lower xylem per cent in the stem of *C. grandis* × *C. limetta* crosses might be due to higher incidence of apical meristem deformity or loss of bipolarity of auxins (Alemanno, 1). However, normal development of apical meristem within the regenerated plantlets of *C. grandis* × *C. sinensis* favoured xylogenesis by greater transport of auxin and higher auxin to cytokinin ratio within the stem (Roberts, 19). These variations in apical meristem development and transportation of different plant growth regulators within the plant system might be due to genotypic variations of these *in vitro* raised plantlets which confirmed the earlier findings in different citrus rootstocks (Saeed *et al*., 20). On the other hand, among all the regenerated plantlets, pith, and phloem per cent in stem was significantly higher in *C. grandis* × *C. limetta* crosses which might be attributed to lower auxin to cytokinin ratio favouring differentiation of phloem tissue (Digby and Wareing, 5). Among all the *in vitro* raised diploid plantlets, area of xylem and phloem within the stem increased gradually with

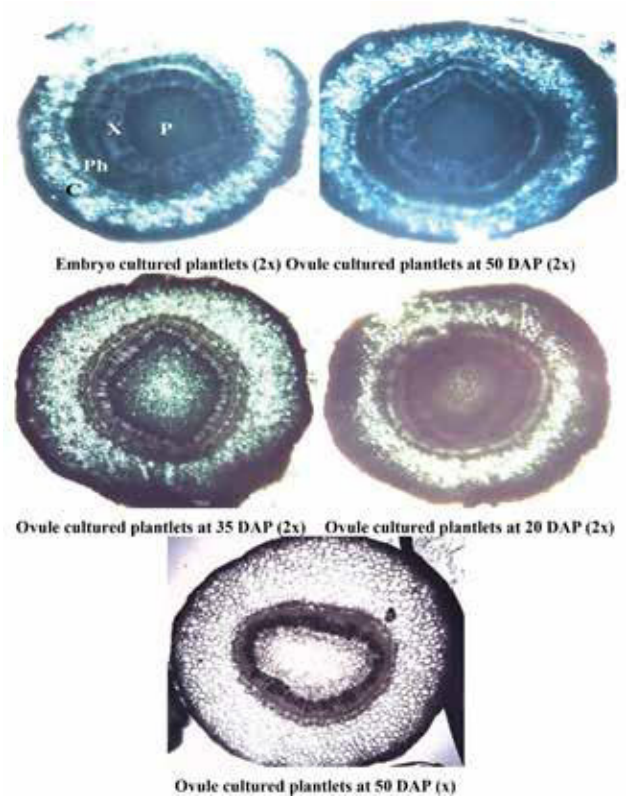


Fig. 1. Photomicrographs of stem cross-section showing proportion of pith, xylem, phloem and cortex of *in vitro* raised plantlets of *Citrus grandis* crossed with irradiated pollen of *C. limetta.*

C: Cortex; Ph: Phloem; X: Xylem; P: Pith; DAP: Days after pollination; x: Haploid; 2x: Diploid

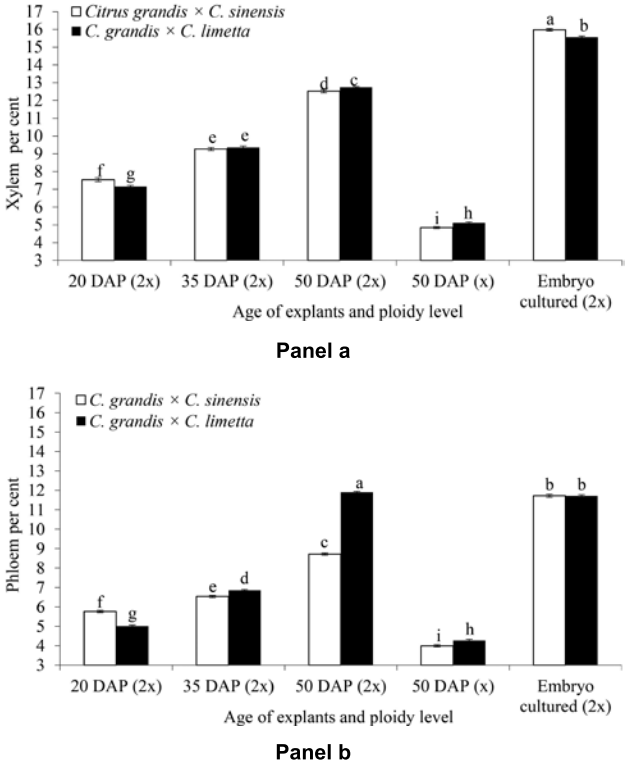
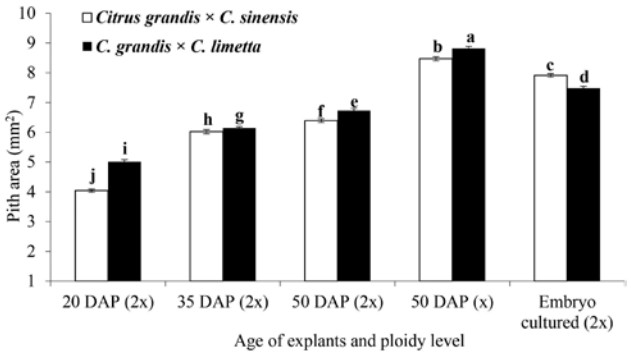


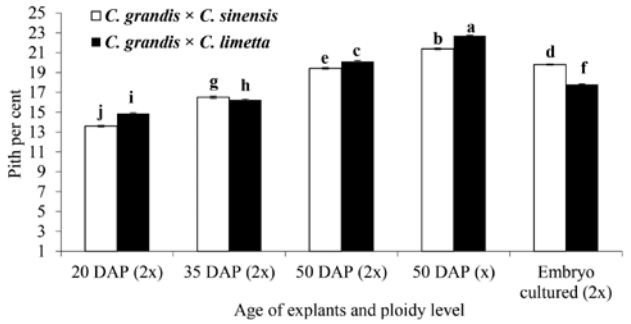
Fig. 2. Per cent area occupied by xylem (Panel a) and phloem (Panel b) in stem cross-section of embryo and ovule cultured plantlets of *Citrus grandis* crossed with irradiated pollen of *C. sinensis* and *C. limetta*. Vertical bars indicate the mean value±standard deviation. LSD at P ≤ 0.05 for P×A interaction of xylem per cent: 0.16 and for phloem per cent: 0.11, respectively. DAP: Days after pollination; x: Haploid; 2x: Diploid.

the increase in the age of explants with maximum in embryo cultured plantlets. Normal development of embryos within the mature seeds without any abnormalities might lead to the proper development of apical meristem within the *in vitro* raised embryo cultured plantlets; while in ovule culture, abnormality at embryo developmental stage might increase when young age of the ovules were used for culture. Our findings are in agreement with the previous findings in *Gossypium hirsutum* (Hussain *et al*., 9) and *Carica papaya* (Fernando *et al*., 6). From the experiment it was observed that although the xylem and phloem per cent in the stem of haploid plantlets were significantly reduced, but they were well organized as of its diploid complements. This might be due to the close relation between ploidy level and nuclear DNA content and cell size which has inter-related effects on structural, biochemical, and physiological elements (Nassar *et al*., 13). This phenomenon of increase of the number and size of vascular bundle, area of stem wall and vascular bundle with increase in ploidy level of the tested plants was known as 'gene dosage effect' (Liang *et al*., 11) resulting increased nuclear volume at higher ploidy level which in turn increase the cell volume in vascular tissues, resulting wider coverage by secondary xylem and phloem in plantlets having higher ploidy level as compared to its lower counterpart (Musial and Przywara, 12).

On the other hand, irrespective of pollen parent, area of xylem, phloem into the root of diploid plantlets was increased gradually with the increased age of explants, and significantly higher area of xylem (1.20 mm^2) and phloem (0.65 mm^2) into the root was recorded in embryo cultured diploid plantlets, while it was least in haploid plantlets (30.00 and 30.77% less than embryo cultured diploid ones) (Table 2). A perusal of data pertaining to interaction between pollen parent and age of cultured explantsindicates a minimum area of xylem and phloem of *C. grandis* × *C. sinensis* crosses in haploid ones (28.69% and 31.34% less than embryo cultured diploid plantlets of



Panel a



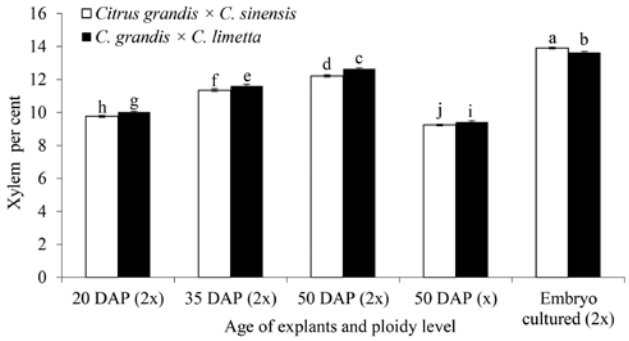
Panel b

Fig. 3. Area of pith (Panel a) and per cent area occupied by pith (Panel b) in stem cross-section of embryo and ovule cultured plantlets of *Citrus grandis* crossed with irradiated pollen of *C. sinensis* and *C. limetta*. Vertical bars indicate the mean value ± standard deviation. LSD at P ≤0.05 for P×A interaction of pith area: 0.11 and for pith per cent: 0.12, respectively. DAP: Days after pollination; x: Haploid; 2x: Diploid.

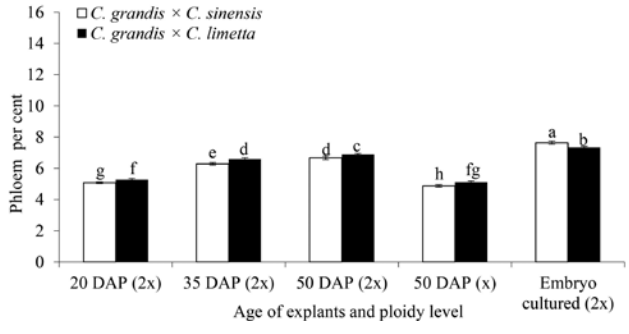
same crosses, respectively). A similar trend was also followed in *C. grandis* × *C. limetta* crosses. The xylem: phloem ratio in the root system of *in vitro* raised citrus plantlets differed significantly with the differences in the age of cultured explants. Irrespective of pollen parent, 50 days old ovule cultured diploid plantlets had significantly higher xylem: phloem ratio (1.92) followed by its haploid counterpart (1.87); however, it was recorded minimum in diploid plantlets obtained from ovule culture at 35 DAP (1.78) (Table 2).Similar trend was also observed for the per cent area occupied by xylem and phloem in the root of these *in vitro* raised citrus plantlets (Fig. 4). Significantly higher per cent of xylem (13.91%) and phloem (7.64%) was measured in embryo cultured diploid plantlets of *C. grandis* × *C. sinensis* crosses followed by embryo cultured diploid plantlets of *C. grandis* × *C. limetta* crosses. Whereas, it was recorded minimum in haploid plantlets of *C. grandis* × *C. sinensis* crosses (9.24% and 4.88%, respectively) followed by haploid plantlets of *C. grandis* × *C. limetta* crosses.

Since diploid plants have higher vascular tissues (phloem and xylem) in the stem as compared to haploid ones, hence, the per cent area coverage by xylem and phloem in root was much higher in diploid plantlets than haploid ones. This finding is in agreement with the earlier findings in a polyploid series of wheat (Dean and Leech, 4). From the experiment, it was also observed although thickness and/or per cent area coverage by xylem, phloem and cortex within root varied significantly among different variants, but the orientation of xylem, phloem and cortex within all the plantlets, regenerated under *in vitro* condition were same, irrespective of their ploidy level and age of the explants.

It is evident from table 3 and 4 that the anatomical parameters of leaf of these *in vitro* raised plantlets varied significantly with the differences in age of cultured explants and ploidy levels. Among the



Panel a



Panel b

Fig. 4. Per cent area occupied by xylem (Panel a) and phloem (Panel b) in root cross-section of embryo and ovule cultured plantlets of *Citrus grandis* crossed with irradiated pollen of *C. sinensis* and *C. limetta*. Vertical bars indicate the mean value ± standard deviation. LSD at P ≤ 0.05 for P×A interaction of xylem per cent: 0.13 and for phloem per cent: 0.15, respectively. DAP: Days after pollination; x: Haploid; 2x: Diploid.

diploids, total leaf thickness increased gradually as the age of the cultured explants increased with maximum in embryo cultured diploid plantlets (0.221 mm); however, it was minimum in haploid ones (0.186 mm). Similar pattern of positive relation with age of

Table 2. Effect of pollen parent, age of cultured explants and ploidy levels on xylem, pholem formation and their ratio in root of *in vitro* raised *Citrus grandis* plantlets.

Treatment combination	Xylem area (mm^2)	Phloem area (mm^2)	Xylem : Phloem ratio
Pollen patent (P)			
C. sinensis	1.03[a]	0.56[a]	1.84[a]
C. limetta	1.02[a]	0.55[a]	1.85[a]
SEm (±)	0.008	0.005	0.010
LSD (p ≤ 0.05)	0.02	0.01	0.02
Age of explants (A)			
Embryo cultured (2x)	1.20[a]	0.65[a]	1.85[b]
50 DAP (2x)	1.10[b]	0.60[b]	1.83[b]
35 DAP (2x)	1.03[c]	0.58[b]	1.78[c]
20 DAP (2x)	0.96[d]	0.50[c]	1.92[a]
50 DAP (x)	0.84[e]	0.45[d]	1.87[b]
SEm (±)	0.010	0.010	0.013
LSD (p ≤ 0.05)	0.05	0.03	0.05
P × A Interaction			
C. sinensis			
Embryo cultured (2x)	1.22[a]±0.04	0.67[a]±0.03	1.82[bcd]±0.06
50 DAP (2x)	1.08[cd]±0.04	0.59[bc]±0.02	1.83[bcd] ± 0.05
35 DAP (2x)	1.01[def]±0.03	0.56[c]±0.04	1.80[cd]±0.03
20 DAP (2x)	0.96[ef]±0.05	0.50[d]±0.03	1.92[a]±0.06
50 DAP (x)	0.87[gh]±0.04	0.46[de]±0.03	1.89[ab]±0.06
C. limetta			
Embryo cultured (2x)	1.17[ab]±0.04	0.63[ab]±0.03	1.86[abc]±0.07
50 DAP (2x)	1.12[bc]±0.04	0.61[bc]±0.03	1.84[abcd]±0.03
35 DAP (2x)	1.04[cde]±0.04	0.59[bc]±0.03	1.76[d]±0.05
20 DAP (2x)	0.95[fg]±0.04	0.50[d]±0.02	1.90[ab]±0.04
50 DAP (x)	0.81[h]±0.03	0.44[e]±0.04	1.84[abcd]±0.04
SEm (±)	0.02	0.01	0.02
LSD (p ≤ 0.05)	0.08	0.05	0.09

Value indicates mean of five replicates ± standard deviation. Different letters in the same column indicate significant differences at P ≤ 0.05 (Tukey's Honest Significant Difference Test); DAP: days after pollination; x: Haploid; 2x: Diploid

Table 3. Effect of pollen parent, age of cultured explants and ploidy levels on total leaf thickness and mesophyll, spongy parenchyma formation in leaf of *in vitro* raised *Citrus grandis* plantlets.

Treatment combination	Leaf thickness (mm)	Mesophyll thickness (mm)	Thickness of spongy parenchyma (mm)
Pollen patent (P)			
C. sinensis	0.203[a]	0.176[a]	0.144[a]
C. limetta	0.199[b]	0.173[b]	0.141[b]
SEm (±)	0.001	0.001	0.001
LSD (p ≤ 0.05)	0.002	0.002	0.002
Age of explants (A)			
Embryo cultured (2x)	0.221[a]	0.194[a]	0.159[a]
50 DAP (2x)	0.210[b]	0.184[b]	0.148[b]
35 DAP (2x)	0.198[c]	0.173[c]	0.142[c]
20 DAP (2x)	0.189[d]	0.163[d]	0.132[d]
50 DAP (x)	0.186[d]	0.162[d]	0.132[d]
SEm (±)	0.001	0.001	0.001
LSD (p ≤ 0.05)	0.005	0.005	0.006
P × A Interaction			
C. sinensis			
Embryo cultured (2x)	0.223[a]±0.004	0.195[a]±0.003	0.160[a]±0.005
50 DAP (2x)	0.212[bc]±0.003	0.185[bc]±0.003	0.148[bc]±0.005
35 DAP (2x)	0.199[d]±0.004	0.174[de]±0.004	0.143[cd]±0.004
20 DAP (2x)	0.191[de]±0.004	0.165[fg]±0.004	0.134[def]±0.004
50 DAP (x)	0.188[e]±0.004	0.163[g]±0.003	0.133[ef]±0.004
C. limetta			
Embryo cultured (2x)	0.219[ab]±0.003	0.192[ab]±0.005	0.157[ab]±0.004
50 DAP (2x)	0.208[c]±0.004	0.182[cd]±0.003	0.147[c]±0.004
35 DAP (2x)	0.197[d]±0.004	0.172[ef]±0.005	0.140[cde]±0.005
20 DAP (2x)	0.186[e]±0.004	0.161[g]±0.005	0.129[f]±0.005
50 DAP (x)	0.184[e]±0.003	0.160[g]±0.004	0.131[ef]±0.004
SEm (±)	0.002	0.002	0.002
LSD (p ≤ 0.05)	0.008	0.009	0.01

Value indicates mean of five replicates ± standard deviation. Different letters in the same column indicate significant differences at P ≤ 0.05 (Tukey's Honest Significant Difference Test); DAP: days after pollination; x: Haploid; 2x: Diploid

cultures explants was also observed in thickness of mesophyll tissue, spongy parenchyma and palisade parenchyma as well as per cent area of palisade parenchyma. Perusal of data pertaining to interaction between pollen parent and age of explants indicates that mesophyll thickness decreased at higher rate with the decrease of the explants age and in the haploid of *C. grandis* × *C. sinensis* cross 16.41% reduction of mesophyll thickness was recorded as compared to embryo cultured diploid plantlets of same cross. Similarly, in haploid of *C. grandis* × *C. limetta* cross, it was reduced by 16.67% as compared to the embryo cultured diploid plantlets of same cross.

Table 4. Effect of pollen parent, age of cultured explants and ploidy levels on thickness (mm) and per cent area of palisade parenchyma *in vitro* raised *Citrus grandis* plantlets.

Treatment combination	Thickness of palisade parenchyma (mm)	Per cent area of palisade parenchyma (%)
Pollen patent (P)		
C. sinensis	0.045^{a}	24.20^{a}
C. limetta	0.044^{a}	23.73^{b}
SEm (±)	0.001	0.09
LSD (≤ 0.05)	0.002	0.25
Age of explants (A)		
Embryo cultured (2x)	0.051^{a}	28.00^{a}
50 DAP (2x)	0.047^{b}	24.36^{b}
35 DAP (2x)	0.045^{b}	23.30^{c}
20 DAP (2x)	0.040^{c}	21.98^{d}
50 DAP (x)	0.040^{c}	22.20^{d}
SEm (±)	0.001	0.14
LSD (≤ 0.05)	0.004	0.57
P × A Interaction		
C. sinensis		
Embryo cultured (2x)	0.051^{a}±0.002	28.06^{a}±0.32
50 DAP (2x)	0.047^{ab}±0.003	24.48^{b}±0.60
35 DAP (2x)	0.045^{abc}±0.003	23.42^{cd}±0.72
20 DAP (2x)	0.041^{bc}±0.003	22.49^{de}±0.65
50 DAP (x)	0.040^{c}±0.003	22.56^{de}±0.48
C. limetta		
Embryo cultured (2x)	0.051^{a}±0.003	27.94^{a}±0.55
50 DAP (2x)	0.047^{ab}±0.002	24.24^{bc}±0.56
35 DAP (2x)	0.044^{bc}± 0.002	23.18^{d}±0.59
20 DAP (2x)	0.039^{c}± 0.003	21.46^{f}±0.50
50 DAP (x)	0.039^{c}±0.002	21.84^{ef}±0.30
SEm (±)	0.001	0.20
LSD (≤ 0.05)	0.006	0.94

Value indicates mean of five replicates and ± values indicates standard deviation. Different letters in the same column indicate significant differences at P ≤ 0.05 (Tukey's Honest Significant Difference Test); DAP: days after pollination; x: Haploid; 2x: Diploid

In each *in vitro* raised plantlet, the thickness of spongy parenchyma was recorded higher than the palisade parenchyma. From the interaction between pollen parent and age of cultured explants, it is evident that embryo cultured diploid plantlets of *C. grandis* × *C. sinensis* had maximum thickness of spongy parenchyma (0.160 mm) which was statistically at par with embryo cultured diploid plantlets of *C. grandis* × *C. limetta*. However, it was minimum in haploid plantlet of *C. grandis* × *C. limetta* (0.131 mm) having statistical similarity with haploid plantlet of *C. grandis* × *C. sinensis*. Similar pattern of thickness variation was also observed in the palisade parenchyma of all these *in vitro* raised plantlets as of spongy parenchyma. In the present experiment, the layer of cuticular and epicuticular waxes were absent in all the *in vitro* raised haploid and diploid plantlets of both the cross combinations. This non-existence of cuticular and epicuticular waxes on the leaves of those *in vitro* raised plantlets was apparently related to the fact that these plantlets grew under controlled environmental conditions, which did not demand the formation of a thick waxy layer as the humidity remained consistently inside the containers. These results are in agreement with the previous findings reported in peach palm seedlings (Batagin-Piotto *et al*., 3).

Moreover, under controlled environmental condition (80% RH, 25-27ºC temp.), regenerated plantlets with higher ploidy level grew faster than plantlets of lower ploidy level which might be attributed to faster cellular elongation in plantlets of higher ploidy level resulting larger volumes of leaf parenchyma cell and epidermal cell area in the plantlets having higher ploidy level (Allario *et al*., 2).

The experimental findings clearly indicates that among all the *in vitro* raised plantlets, thicker xylem and phloem tissues were formed in embryo cultured diploid plantlets, resulting better growth of those regenerants; however, the regenerated haploid plantlets had minimum vascular tissues per unit area of stem and roots. These haploid also had reduced thickness of mesophyll tissues in the leaf resulting weaker growth under *in vitro* condition. But the orientation of different tissues in stem, root and leaf of all those haploid plantlets were similar to that

of diploid ones in terms of their anatomical structure which confirms that these plantlets can grow normally under open field condition.

AUTHORS' CONTRIBUTION

Conceptualization of research (AKD); Designing of the experiments (AKD); Execution of field/lab experiments and data collection (MK); Analysis of data and interpretation (MK and AKD); Preparation of the manuscript (MK and AKD).

DECLARATION

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

Authors are highly grateful to Department of Science & Technology, Government of India for providing financial assistance for this study.

REFERENCES

1. Alemanno, L, Berthouly, M. and Michaux-Ferriere, N. 1996. Histology of somatic embryogenesis from floral tissues of cocoa. *Plant Cell Tissue Organ Cult.* **46**: 187-94.

2. Allario, T., Brumos, J., Manuel Colmenero-Flores, J., Tadeo, F., Froelicher, Y. and Talon, M. 2011. Large changes in anatomy and physiology between diploid Rangpur lime (*Citrus limonia*) and its autotetraploid are not associated with large changes in leaf gene expression. *J. Exp. Bot.* **62**: 2507-19.

3. Batagin-Piotto, K.D., De Almeida, C.V., Angelo-Piotto, F. and De Almeida, M. 2012. Anatomical analysis of peach palm (*Bactris gasipaes*) leaves cultivated *in vitro*, *ex vitro* and *in vivo*. *Braz. J. Bot*. **35**: 71-78.

4. Dean, C. and Leech, R.M. 1982. Genome expression during normal leaf development. 2. Direct correlation between ribulosebisphosphate carboxylase content and nuclear ploidy in a polyploid series of wheat. *Plant Physiol*. **70**: 1605-08.

5. Digby, J. and Wareing, P.F. 1966. The effect of applied growth hormone on cambial division and differentiation of the cambial derivatives. *Ann. Bot.* **30**: 539-48.

6. Fernando, J.A., Melo, M., Soares, M.K.M.and da Gloria, A.B. 2001. Anatomy of somatic embryogenesis in *Carica papaya* L. *Braz. Arch. Biol.Technol*. **44**: 247-55.

7. Germanà, M.A. and Chiancone, B. 2001. Gynogenetic haploids of Citrus after *in vitro* pollination with triploid pollen grains. *Plant Cell Tiss. Org.* **66**: 59-66.

8. Grosser, J.W., Ollitrault, P. and Olivares-Fuster, O. 2000. Somatic hybridization in citrus: an effective tool to facilitate variety improvement. *In Vitro Cell Dev-Pl*. **36**: 434-49.

9. Hussain, S.S., Rao, A.Q., Husnain, T. and Riazuddin, S. 2009. Cotton somatic embryo development affects its concersion to plant. *Biol. Plant*. **53**: 307-11.

10. Kundu, M., Dubey, A., Srivastav, M. and Mallik, S. 2017. Induction of haploid plants in citrus through gamma-irradiated pollen and ascertain of ovule age for maximum recovery of haploid plantlets. *Turk. J. Biol*. **41**: 469-83.

11. Liang, J., Zhong, X.Q., Fu, X.J.and Yin, Z.X. 2009. Comparative studies on stem anatomical structure of rice plants with various ploidy level. *J. Zhej. Univ*. **35**: 489-96.

12. Musial, K. and Przywara, L. 1998. Influence of irradiated pollen on embryo and endosperm development in kiwifruit. *Ann. Bot.* **82**: 747-56.

13. Nassar, N.M.A., Graciano-Ribeiro. D., Fernandes. S.D.C. and Araujo, P.C. 2008. Anatomical alterations due to polyploidy in cassava, *Manihotesculenta* Crantz. *Genet. Mol. Res*. **7**: 276-83.

14. Ollitrault, P., Dambier, D., Vanel, F. and Froelicher, Y. 2000. Creation of triploid Citrus hybrids by electrofusion of haploid and diploid protoplasts. *Acta Hortic*. **535**: 191-97.

15. Pandey, K.K. 1978. Gametic gene transfer in Nicotiana by means of irradiated pollen. *Genetica*, **49**: 53-69.

16. Pandey, K.K. 1974. Overcoming interspecific pollen incompatibility through the use of ionising radiation. *Heredity*, **33**: 279-87.

17. Preece, J.E.and Sutter, E.G. 1991. Acclimatization of micropropagation plants to the greenhouse and field. *In:* Debergh, P.C. and Zimmerman, R.H. editors. *Micropropagation Technology and Application*. Netherland (Kluwer Academic Publishers, Dorderecht), pp. 77-93.

18. Raquin, C., Cornu, A., Farcy, E., Maizonnier, D., Pelletier, G.1989. Nucleus substitution between Petunia species using gamma ray induced androgenesis. *Theor. Appl. Genet*. **78**: 337-341.

19. Roberts, L.W. 1969. The initiation of xylem differentiation. *Bot. Rev*. **35**: 201-50.

20. Saeed, M., Dodd, P.B. and Sohai, L. 2010. Anatomical studies of stems, roots and leaves of selected citrus rootstock varieties in relation to their vigour. *J. Hortic. Forest*, **2**: 87-94.

Received : April 2021; Revised : January 2022;
Accepted : May 2022