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MORPHOLOGICAL AND STOMATAL DIVERSITY IN COLCHIPLOID GERMPLASM OF GRAPEFRUIT

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Seeds and vegetative parts of two white and four pink flesh grapefruit (*Citrus paradisi* Macf.) varieties were exposed to colchicine treatment under *in vitro* and field conditions for the development of colchiploid germplasm for breeding programs. Genotype dependent plant growth responses were observed under both *in vitro* and field conditions. Embryo germination and plant growth were arrested at higher levels of colchicine. Cultivar Shamber had higher embryo germination (65.47%), number of leaves were more in Shamber and Red Blush (7.19) whereas long shoots and roots were developed in Red Blush and Red Mexican (4.07 cm and 9.18 cm, respectively). Under field conditions, Red Blush developed more axillary sprouts (2.78) and large sized leaves in colchicine treated branches. Shoot growth was more in Reed and Red Mexican (11.57 cm) while number of leaves were more in Shamber (8.79). Number of leaves were reduced from 12 (control) to 4 leaves at higher levels of colchicine (0.3%) under *in vitro* and field conditions. Stomata were maximum in Frost Fresh and Red Mexican (9.02) and their size was larger in Red Blush. Stomatal frequency was reduced (from 13.65 to 5.79) while size increased at higher levels of colchicine. These findings indicate existence of a strong genotypic variation in plant growth parameters and stomatal attributes in colchiploid germplasm. Further screening of the putative polyploids is suggested for the assessment of colchicine induced genetic diversity.

Keywords: Citrus, Colchicine, Germplasm, Stomata, Ploidy.

INTRODUCTION

Citrus especially mandarins are being widely cultivated in Pakistan on an area of 0.2 million hectares with annual production of 2.25 million tons (MINFAL, 2019). Agro-climatic conditions of Pakistan and particularly Indus plain supports production of citrus fruit in the region with best quality and quantity. Among various problems faced by the local citrus industry, narrow genetic base is one of the key handicaps (Fatima *et al.*, 2015; Zaman *et al*., 2019; Khan *et al*., 2020). Further, Pakistan is also facing climate change leading to enhanced abiotic stress elements. Hence, it is desired to develop citrus germplasm which could have better tolerance to abiotic stresses like salt, drought and temperature changes. Grapefruit (*Citrus paradisi* Macf.), member of citrus family, is a natural hybrid of *C. grandis* L. (pummelo) and *C. sinensis* L. (sweet orange) (Ouesleti *et al.*, 2017). It has the highest nutritional value including vitamins, mineral substances and flavonoids. It helps to prevent cancer, heart diseases and some birth defects. Grapefruit is estimated to be cultivated in Pakistan on about 5000 hectares (Usman *et al.*, 2020). Polyploid organisms have more than two sets of chromosomes and offer several advantages including gigantism, higher heterosis, gene redundancy, modified gene expression, seedlessness, better adoption to dry and cold conditions particularly in Citrus scion (Padoan *et al.*, 2013; Fatima *et al.*, 2015; Usman *et al.*, 2020) and rootstocks (Saleh *et al.*, 2008; Oustric *et al.*, 2019), a greater source of essential oil in citrus (Bhuvaneswari *et al.*, 2020) compared with their diploid progenitors and other fruit crop species. Hence, polyploids also showed better survival during the evolutionary process and have more potential towards climate change resilience (Oustric *et al.*, 2019; Khalid *et al*., 2020). Polyploids could be developed using different chemicals like colchicine, oryzalin and trifluralin. However, colchicine is being more widely used for somatic doubling of the chromosomes in citrus and other crops (Shao *et al*., 2003; Usman *et al.*, 2012; Rana *et al.*, 2020). Synthetic tetraploid plants were developed from somatic embryos of diploid cultivars in many crops including grapefruit and lime (Petersen *et al.*, 2003; Rego *et al.*, 2011). *In vitro* colchicine treatment has developed non-chimeric autotetraploids in citrus (Zhang *et al.*, 2007). The developed tetraploid plant material could be utilized as rootstock, better tolerant germplasm to abiotic stress factors (Oustric *et al.*, 2019; Khalid *et al.*, 2020) and parental material for interploid crossing (2x × 4x, 4x × 2x) to develop triploids for seedlessness (Usman *et al.*, 2002, 2012; Ollitrault *et al*., 2008). Hence, seeds and vegetative parts of grapefruit varieties were treated with colchicine under *in vitro* and field conditions for the development of polyploids and screened for

stomatal diversity for germplasm enhancement and developing parental material for interploid hybridization.

MATERIAL AND METHODS

Plant Material and Colchiploid Induction In vitro:

Explant Sterilization, Colchicine Treatment and Embryo Culture: Mature and disease-free fruit of two white flesh grapefruit varieties i.e., Frost Fresh (FF) Marsh and Reed (R) Marsh and four pink flesh varieties i.e., Shamber (SH), Foster (F), Red Blush (RB) and Red Mexican (RM) Foster were selected from Experimental Fruit Garden (EFG) No. 9, Institute of Horticultural Sciences (IHS), University of Agriculture, Faisalabad (UAF). Fruits were cut into two halves and the mature and developed seeds were extracted. Seeds were separated from the pulp and surface sterilized with 70% ethanol (C_2H_5OH) for 3 minutes followed by 5% sodium hypochlorite (NaOCl) for 10 minutes along with two drops of Tween 20 ($C_{26}H_{50}O_{10}$) which act as a surfactant. Seeds were washed 2-3 times with sterilized distilled water (dH_2O) to rinse the impurities of NaOCl. Murashige and Skoog (1962) media was prepared following standard procedure, 8% agar was added for media solidification and 3% sucrose was added in the media as carbon source. Media pH was adjusted to 5.8. The sterilized seeds were treated with different levels of colchicine i.e., 0%, 0.05%, 0.1%, 0.2% and 0.3% for 4 hours under continuous agitation (Abou Elyazid and El-Shereif, 2014). Embryos were excised from colchicine treated seeds and cultured on MS medium for germination and plant growth following Usman *et al.* (2012). Cultures were kept in the growth room where 2500 lux fluorescent light was provided for proper plant growth and temperature was adjusted at 25 ± 2 ºC. Data were collected after 16-18 weeks of culturing.

***Plant Material and Colchiploid Induction In vivo*:** Bearing trees of all six grapefruit varieties including white flesh (FF, R) and pink flesh (SH, F, RB and RM) varieties were selected for application of colchicine in the EFG No. 9, IHS, UAF. Shoot tip dip method was used for the application of colchicine (Yetisir and Sari, 2003). Five young and healthy branches per tree were selected in each variety and wrapped with cotton for maximum absorption of colchicine solution. Colchicine solution was prepared following Usman et al. (2012) at different concentrations (0, 0.05%, 0.1%, 0.2% and 0.3%) and applied drop by drop on cotton wrapped meristem region of the branches for seven days, consecutively. After seven days, the cotton swab was removed and branches were allowed to grow. The newly emerging colchicine treated shoots were evaluated for their morphological traits after 16-18 weeks of sprouting. Young leaves were collected and used for cytological screening for ploidy status.

***Morphological and cytological characterization*:** The colchicine induced shoots in field and *in vitro* developed plant material were screened for phenotypic variation in response to colchicine treatment. Morphological data were recorded for survival percentage, number of leaves, leaf length, root and shoot length at the time of hardening of plants raised *in vitro*. For field grown shoots, morphological characterization was done for parameters including number of leaves, leaf size and number of new/young sprouts. Stomatal studies were conducted with the help of Nikon Optiphot fluorescent microscope (Japan) for stomatal frequency, size of stomata and its opening (aperture).

Frequency of stomata: Lower thin epidermal layer of leaf was carefully removed with surgical blade (Usman *et al*., 2006) and spread on the slide. One small drop of water was added on sample placed on the glass slide. A cover slip was put on the sample and pressed gently to avoid air bubbles. Slide was put on stage of the microscope (Boso *et al*., 2016). Observations were made using fluorescent microscope at higher magnification (100x) and number of stomata were counted.

Size of stomatal guard cells (µm) and opening (aperture): An ocular micrometer was placed in eyepiece of the microscope to measure size of stomata. Slide was put on the stage of the microscope. First ocular micrometer was calibrated with stage micrometer following standard procedures. The ocular micrometer was used to measure stomatal size length and width wise and values were noted (Usman *et al.*, 2008). Ocular micrometer was also adjusted along the cross-section of center of the stomata to measure the stomatal opening (aperture) size. The stomatal area was calculated by multiplying length with the corresponding width values.

RESULTS

Genotypic responses for plant growth in colchicine treated grapefruit varieties

***In vitro*:** Embryo germination percentage was higher in Shamber (65.47%) followed by Foster Pink (58.33%) and minimum embryo germination was found in Red Mexican (52.38%). Number of leaves were greater in Shamber and Red Blush (7.19) while the lowest number of leaves were recorded in Reed (6.42). Maximum shoot and root lengths were observed in Red Blush (4.07 cm) and Red Mexican (9.18 cm) as shown in Table 1.

***In vivo*:** Grapefruit variety Red Blush developed maximum number of bud sprouts (2.78) from axillary buds of young colchicine treated shoots followed by Shamber (2.66) whereas bud sprouts were minimum in Reed (1.91). Maximum shoot growth length was noted in Red Mexican and Reed (11.57 cm each). Number of leaves recorded were maximum in Shamber (8.79) and the lowest in Red Blush (7.33). Maximum leaf length, width and area were recorded in Red Blush (4.98 cm, 2.85 cm 13.99 cm^2 respectively). However, Reed showed poor growth in leaf size (length, width) and area i.e., 4.48 cm, 2.43 cm and 11.36 cm^2, respectively (Table 1).

Table 1. Genotypic variability for embryo germination, shoot growth attributes, stomatal size and frequency in colchicine treated plant material of grapefruit under *in vitro* and *in vivo* (field) conditions.

Morphological Parameters	Varieties					
	White Flesh		Pink Flesh			
	Frost Fresh	Reed	Foster Pink	Red Mexican	Shamber	Red Blush
In vitro						
Germination %	57.14±0.18^{c}	52.43±0.18^{d}	58.33±0.25^{b}	52.38±0.17^{e}	65.47±0.20^{a}	53.57±0.20^{d}
Number of leaves	6.86±0.63^{d}	6.42±0.55^{e}	6.94±0.54^{c}	7.14±0.62^{b}	7.19±0.73^{a}	7.19±0.60^{a}
Shoot length	3.76±0.26^{d}	3.57±0.25^{e}	3.81±0.18^{c}	3.76±0.25^{d}	3.96±0.21^{b}	4.07±0.18^{a}
Root length	7.83±0.71^{e}	8.35±0.60^{c}	8.11±0.75^{d}	9.18±0.69^{a}	8.99±0.35^{bc}	9.03±0.61^{b}
In vivo						
Number of axillary sprouts	2.34±0.17^{d}	1.91±0.09^{f}	2.30±0.12^{e}	2.56±0.15^{c}	2.66±0.20^{b}	2.78±0.44^{a}
Branch length (cm)	10.72±0.61^{d}	11.57±0.69^{ab}	9.98±0.43^{e}	11.57±0.35^{a}	11.23±0.67^{b}	11.18±1.01^{c}
Number of new leaves	8.19±1.21^{b}	7.67±0.81^{d}	7.57±0.85^{e}	8.22±1.12^{c}	8.79±0.82^{a}	7.33±0.66^{f}
Leaf length	4.71±0.27^{c}	4.48±0.30^{e}	4.55±0.28^{d}	4.88±0.31^{b}	4.54±0.25^{de}	4.98±0.25^{a}
Leaf width	2.69±0.09^{b}	2.43±0.11^{d}	2.43±0.07^{d}	2.47±0.12^{c}	2.67±0.09^{bc}	2.85±0.10^{a}

Effect of colchicine treatment on plant growth in grapefruit varieties

***In vitro*:** Colchicine treatment adversely affected embryo germination and plant growth (Fig. 1a-d).

Figure 1. *In vitro* germination of colchicine treated embryos, acclimatization of transplanted seedlings and screening for variability in stomatal size and frequency. Figures are **a**) Shamber **b**) Red Blush **c)** Red Mexican Foster and d) Frost Fresh colchiploid seedling treated at different levels of colchicine **e**) stomatal frequency in leaves of untreated (control) seedlings, **f**) putative polyploids of Red Mexican Foster **g**) Red Blush and **h**) Shamber each at 0.2% colchicine, at 100X magnification of Nikon fluorescent microscope.

Maximum embryo germination was observed at untreated control (91.72%) which decreased with increase in concentration of colchicine and the lowest germination 34.72% was recorded at 0.3% (Fig. 2). Similarly, number of leaves were maximum at control (11.13) and was reduced to 4.09 at the highest level of colchicine (0.3%). Shoot and root growth were maximum at control (4.94 cm and 13.85 cm, respectively) which decreased with increase in colchicine concentration (3.11 cm and 5.57 cm, respectively) as depicted in Fig. 2.

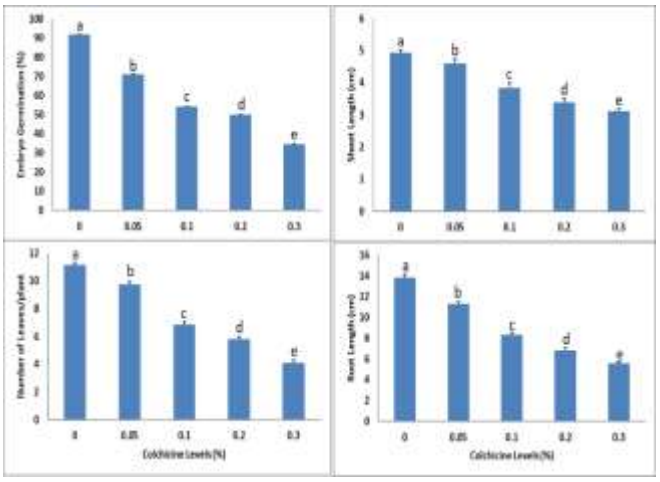

Figure 2. Effect of colchicine treatment on embryo germination (%), number of leaves/plant, shoot length and root length in grapefruit *in vitro*.

***In vivo*:** Bud sprouts and emergence of new shoots were maximum at untreated control (3.89) and sprouting was reduced to 1.78 at the higher level of colchicine (0.3%) as shown in Fig. 3. At control, the branches were longer (15.44 cm) whereas shoot growth was reduced to 8.47 cm at 0.3%. Similarly, number of leaves were significantly reduced from 12.59 (control) to 4.37 at the higher level of colchicine 0.3% (Fig. 3). Leaf length and width was increased in the treated shoots and maximum leaf size (5.56 cm and 3.08 cm, respectively) was observed at higher level of colchicine 0.3%

Table 2. Genotypic responses for stomatal size and frequency in young leaves collected from colchicine treated shoots of grapefruit

Stomatal Parameters	Varieties					
	White Flesh		Pink Flesh			
	Frost Fresh	Reed	Foster Pink	Shamber	Red Mexican	Red Blush
No. of stomata	9.02±0.82[a]	8.51±0.97[f]	8.59±0.76[d]	8.57±0.80[e]	8.96±0.65[b]	8.84±0.48[c]
Stomatal length (µm)	2.57±0.13[c]	2.35±0.14[e]	2.13±0.12[f]	2.44±0.09[d]	2.64±0.14[b]	2.70±0.15[a]
Stomatal width (µm)	2.17±0.14[c]	2.14±0.16[d]	1.84±0.13[f]	2.12±0.14[e]	2.34±0.12[b]	2.43±0.14[a]
Stomatal area (µm)	5.92±0.62[c]	5.24±0.71[e]	4.14±0.54[f]	5.42±0.51[d]	6.49±0.65[b]	6.92±0.73[a]
Stomatal aperture (µm)	0.31±0.02[c]	0.33±0.02[b]	0.33±0.02[b]	0.38±0.02[a]	0.33±0.02[b]	0.33±0.01[b]

Means sharing similar letters in a row are statistically non-significant (P<0.05)

compared with control treatment (3.55 cm and 1.85 cm, respectively).

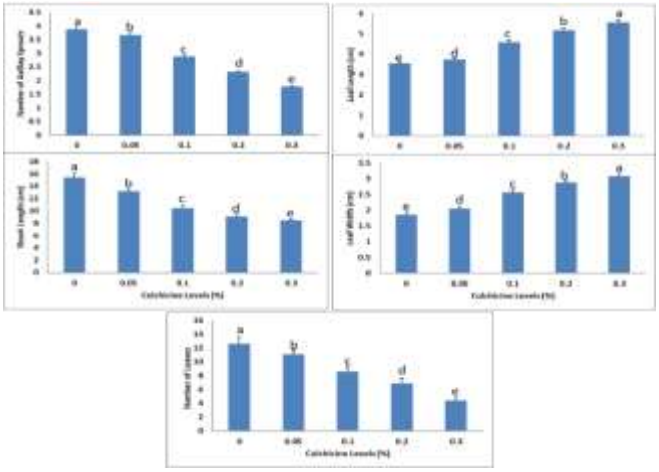

Figure 3. Effect of colchicine treatment on number of axillary sprouts, shoot length, leaf size and number of leaves in grapefruit *in vivo*.

Genotypic and colchicine induced variability in stomatal size and frequency: Number of stomata were maximum in Frost Fresh (9.02) followed by Red Mexican (8.96) whereas minimum number of stomata were noted in cultivars Shamber and Reed (8.51) in untreated shoots. Stomata of Red Blush were larger, wider and had maximum stomatal area (2.70 µm, 2.43 µm and 6.92 µm^2, respectively) compared with other varieties. The smallest stomata were observed in Foster Pink having 2.13 µm length, 1.84 µm width and 4.14 µm^2 stomatal area. Shamber showed wider aperture of stomata (0.38 µm) whereas Frost Fresh and Reed had the smallest stomatal opening (0.31 µm) as shown in Table 2. Number of stomata (13.65) were maximum in control leaves whereas stomatal frequency was reduced to half (5.79) at higher level of colchicine 0.3% (Fig. 4). Length and width of stomata were markedly increased at higher levels of colchicine (2.96 µm and 2.65 µm) application compared with control (Fig. 3). Stomatal area was also greater at the highest colchicine level 0.3% i.e., 7.92 µm². Aperture of stomata was found greater at 0.3% i.e., 0.39 µm and was almost double in size compared with control (0.22 µm) as shown in Fig. 4.

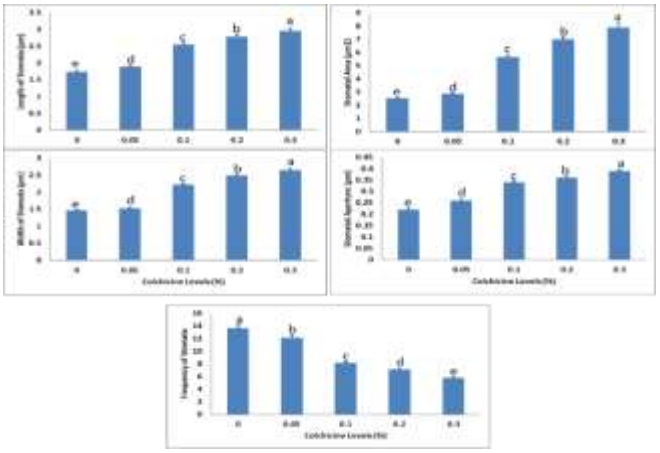

Figure 4. Effect of colchicine treatment on stomatal size, stomatal area, aperture of stomata and frequency of stomata in grapefruit.

DISCUSSION

Genotypic variability and impact of colchicine on plant growth attributes: The colchicine treated plant material showed great genotypic variation for embryo germination and plant growth (*in vitro*) and shoot growth attributes (*in vivo*). Embryo germination and number of leaves were higher in Shamber while shoots were longer in Red Blush under *in vitro* conditions. Similar findings were reported regarding higher embryo germination in grapefruit cv. Shamber whereas shoot growth was more in Reed (Usman *et al.*, 2012). Under field conditions, Red Blush developed a greater number of bud sprouts with larger leaves, shoots were longer in Red Mexican and number of leaves were more in Shamber. Colchicine treatment adversely affected germination and most of the plant growth attributes in both *in vitro* and field studies. Bud sprouting frequency, shoot growth and number of leaves were reduced whereas leaf size was increased at higher concentration of colchicine (0.3%) across grapefruit varieties. Similar effects of colchicine treatment on plant growth are reported previously (Liu *et al*., 2007). Colchicine application reduced seed germination when either exposure time to colchicine (Shao *et al*., 2003) or dose rate of colchicine was

increased (Pande and Khetmalas, 2012: Tiwari and Mishra, 2012). Similarly, number of branches and branch length decreased in colchicine treated explants *in vitro* whereas leaf size (length, width and area) was greatly increased in colchicine induced shoots (Liu *et al*., 2007). Bud sprouting and shoot length also decreased in colchicine treated shoots in different citrus species (Usman *et al*., 2008). Colchicine application greatly reduced number of newly emerging branches when applied in field at 0.025% to 0.05% (Abu-Qaoud and Munqez, 2014). Plant survival percentage also decreased whereas leaf area, chlorophyll content, stem and root diameter were greatly increased at higher levels of colchicine (Hosseini *et al*., 2018).

Genotypic variability and effect of colchicine on stomatal size and frequency: Diversity in frequency and size of stomata may occur due to variability in the genetic factors including ploidy and growth under diverse agro-climatic conditions. A negative correlation reported in these two stomatal traits is based on their developmental responses to the environmental changes or long-term evolutionary adaptation (Fanourakis *et al.*, 2015; de Boer *et al.*, 2016; Dittberner *et al.*, 2018). Colchicine treated shoots also showed great genotypic variability for stomatal frequency and size related attributes. Stomata were greater in Frost Fresh, larger in Red Blush and had wider stomatal opening in Shamber. In contrast, stomatal size was found greater in Shamber and Foster Pink (Usman *et al.*, 2012) which could be attributed to variation in the leaf size, plant age and growth stage. Reduced stomatal density may limit stomatal conductance (gs) and transpirational (E) losses thus shifting towards a reduced use of water. Colchicine application further influenced all the stomatal attributes across grapefruit varieties. Stomatal frequency was reduced while their size, area and stomatal opening greatly increased. Similar influence of colchicine on stomatal frequency and other attributes has been reported in other crops. Size of stomata and diameter of guard cells was enhanced by colchicine application compared to control cells and fewer stomata were observed in colchiploids (Tiwari and Mishra, 2012). The anomocytic type of stomata were found in different grapefruit varieties in the current study. This is contrary to the report of Obiremi and Oladele (2001) who found paracytic and hemiparacytic types of stomatal complex in grapefruit which may be attributed to the difference in varieties studied or the mutagen concentrations applied. Application of colchicine significantly increased plant ploidy level, diameter and length of stomata, and chloroplast number in guard cells whereas stomatal frequency was decreased (Usman *et al*., 2008, Hosseini *et al*., 2018). Stomatal size has been directly proportional to colchicine application whereas its frequency has been inversely proportional (Dittberner *et al.*, 2018) in other fruit crops including *Ziziphus* (Gu *et al.*, 2005), *Vitis* (Yang *et al.*, 2006) and citrus (Usman *et al.*, 2008; 2012).

Conclusion: Strong genotypic variability was observed in both white and pink flesh varieties for plant growth parameters in the *in vitro* raised seedlings and colchicine induced shoots in the field. Colchicine treatment effectively altered the stomatal size and frequency across genotypes. Further screening of the putative polyploids is in progress.

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