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Embryogenic responses in anther derived calli of white and pink flesh guava (*Psidium guajava* L.) cultivars

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Androgenesis is reported in a few tree crops such as citrus and papaya; however, there is little information available about guava. Different modified Chu (N_6) media and cold pretreatments of floral buds were tested to induce embryogenic calli in the anthers of two white flesh guava cultivars, 'Round' and 'Pyriform'. Four modified Chu (N_6) media formulations, i.e., M_1 (400 mgL^{-1} casein hydrolysate, 200 mgL^{-1} glutamine, 0.2 mgL^{-1}NAA, 1.0 mgL^{-1} KIN, 0.5 mgL^{-1} 6-BAP, 0.5 mgL^{-1} zeatin and 5% coconut water), M_2 (400 mgL^{-1} casein hydrolysate, 200 mgL^{-1} glutamine, 0.2 mgL^{-1}NAA, 1.0 mgL^{-1} KIN, 0.5 mgL^{-1} 6-BAP), M_{10} (0.5 mgL^{-1} 2,4-D), and M_{11} (0.5 mgL^{-1} 2,4-D and 50 mgL^{-1} PVP) enhanced anther swelling and callus formation. Genotypic variability was found in pollen viability, phenolic exudation, and anther tissue browning after cold pretreatment for 0-72 hrs. intervals. Anthers of 'Round' showed more pollen viability across cold pretreatment intervals with less phenolic exudations and tissue browning than 'Pyriform'. On both M_{10} and M_{11} media, anthers with uni-nucleate microspores developed embryogenic calli; however, anthers of 'Pyriform' resulted in more calli induction (69.58%) on M_{10} media, whereas anthers of 'Round' developed more calli (66.60%) on M_{11} media. The proliferating calli in white flesh 'Round' and 'Pyriform' cultivars and pink flesh 'Round' cultivar formed more embryogenic masses upon transfer to growth hormone-free Chu (N_6) media under light conditions; however, embryos showed delayed maturity with limited germination. More genotypes shall be explored to enhance embryo germination, and embryogenic media shall be further fine-tuned. This study provides the foundation for the generation of homozygous lines in guava using androgenesis.
Keywords: Androgenesis, homozygosity, inbreeding, Guava, Gola, Surahi

INTRODUCTION

Guava (*Psidium guajava* L.) is a heterozygous tree fruit crop, and the diversity present in the existing seedling populations is due to the variable frequency of cross-pollination (Dinesh and Vasugi, 2016). High genetic diversity, an epigynous flower (profuse stamens of different sizes), and more juvenility limit genetic improvement in guava by conventional breeding. Moreover, selfing is laborious, time-consuming, and expensive for producing homozygous parents (Usman *et al.,* 2015; Khan *et al.,* 2017) and requires multiple selfed generations (Germanà, 2011a). Even then, it results in incomplete homozygosity (<100%), and self-incompatibility may also cause several problems (Germanà, 2006). Production of the inbred lines is the primary requirement for the development of F1 hybrids and is laborious and time-consuming in perennial fruit crops (Germana, 2011a; Niazian and Shariatpanahi, 2020). Inbred lines may be produced more efficiently using androgenesis compared to other techniques (Heidari-Zefreh *et al.,* 2018).

In vitro, anther culture is a simple, efficient, and widely used approach for generating haploids and double haploids (Germanà, 2011a;Usman *et al.*,2015). Androgenesis produces homozygous lines, enhances breeding efficiency, and shortens the breeding cycle. Furthermore, haploid plants serve as suitable plant material for genomic and sequencing studies (Germanà, 2011b; Zhang *et al.,* 2011a). Substantial progress has been made user *in vitro* anther culture in fruit, ornamental, medicinal, woody, and vegetable species, though response remained low in a few cases (Germanà, 2006; Wedzony *et al.*, 2009; Zhang *et al.*, 2011b; Shi *et al.*, 2012; Usman *et al.*, 2015). Doubled haploids are reported in several other fruit crops, including citrus, mulberry, papaya, apple, and custard apple (Eng and Ho, 2019).

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Androgenesis has also resulted in spontaneous polyploids in fruit crops, including papaya (Germana *et al.*, 2005), apple (Hofer *et al.*, 2002), clementine (Germana 2007, 2009), and pear (Kadota and Niimi, 2004). Several factors affect the efficiency of androgenesis like donor plant's genotype, its vigor and physiological condition, culture medium compositions (Salma-Ayed *et al.*, 2010; Pencik *et al.*, 2015), combinations of plant growth regulators (PGRs), growing conditions (Bajaj, 1990; Kumar *et al.*, 2003; Qi *et al.*, 2011; Chen *et al.*, 2013), microspore development stage and pre-treatments of anthers or buds (Koleva-Guedeva *et al.*, 2007; Usman *et al.*, 2015). There is no success story of androgenesis in guava except a preliminary report about callogenesis in Indian guava genotypes (Babbar and Gupta, 1986). Hence, the current study was initiated to screen elite indigenous genotypes to establish androgenesis for the development of haploids for future breeding programs. Genotypic responses of anthers of guava cultivars to different cold shock pre-treatments, basal media composition, and PGRs are elaborated.

MATERIALS AND METHODS

A. Effect of Donor Plant, Cold Pretreatments, and Media Formulations on Androgenesis in White Flesh Guava Cultivars:

Plant Material and Media Formulations: The study was carried out during winter seasons in the Guava Germplasm Unit, Experimental Fruit Garden of the Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan (N 31° 25' 46.8048", E 73° 4' 14.3112"). The average growing conditions of donor plants were 25-27°C temperature and 60%-65% relative humidity. Five healthy plants each of two white flesh guava cultivars, viz., Round 'RW' (Gola) and Pyriform 'PW' (Surahi), were selected for androgenesis. The unopened mature and healthy floral buds (11-12 mm in length and 8-9 mm in diameter) were collected at the stage when buds swelled longer and petals appeared (Fleckinger, 1964). Most of the microspores inside the anthers are vacuolated during this phase, and it is considered the best stage for inducing gametic embryogenesis (Peixe *et al.*, 2004). Buds were exposed to 4°C temperature in the dark for inductive pre-treatment for 0, 24, 48, and 72 hours, and pollen viability was assessed. Chu (N_6) basal medium (Chu, 1978) was modified using different PGRs and vitamins (Nitsch and Nitsch, 1969) following Germana et al. (2011) and media pH was adjusted to 5.8 (Table 1). The media formulations were evaluated for callogenic and subsequent embryogenic responses.

Pollen Viability Assessment: Anthers were excised from cold pre-treated buds and pollens were isolated for the viability assessment. Pollen viability was determined by fine chopping of pollens in 1% acetocarmine staining dye for 3to5 minutes (Germana *et al.*, 2005; Abdelgadir *et al.*, 2012). Stained pollens were examined under the fluorescent microscope (CyScope Sysmex, UK) at 40X magnification and, pollen viability was calculated as given below:

$$Pollen\ viability\ (\%) = \frac{No.of\ viable\ pollens\ counted}{Total\ No.of\ pollens} \times 100$$

Explant Sterilization, Inoculation, and Growth Conditions: After the pollen viability test, pre-treated floral buds were sterilized by immersing them in 70% ethanol and 10% sodium hypochlorite solution containing a few drops of Tween-20 for 3 minutes, respectively, and finally rinsed in sterile distilled water thrice (Germana *et al.*, 2011; Usman *et al.*, 2015). In each petri plate, 25 anthers were inoculated on 10 ml of solid medium. Petri dishes were sealed with parafilm, incubated in the dark at 25±2 °C for the first 20 days, and then kept under fluorescent light (16/8 hrs. photoperiod).

Data was recorded for the following parameters:

Table 1. Modified Chu N_6 Medium Supplemented with Vitamins and PGRs for Androgenesis in Guava Cultivars.

Ingredients	Plant Development Media (1L)										
	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8	M_9	M_{10}	M_{11}
Chu N_6 with Vitamins	3.99g	3.99g	3.99g	3.99g	3.99g	3.99g	3.99g	3.99g	3.99g	3.99g	3.99g
2, 4-Dichlorophenoxyacetic acid (2,4-D)	--	--	--	1mg	1mg	0.5mg	0.5mg	1mg	1mg	0.5mg	0.5mg
Polyvinylpyrrolidone (PVP)	--	--	--	--	50mg	--	50mg	--	50mg	--	50mg
Casein hydrolysate	400mg	400mg	400mg	400mg	400mg	400mg	400mg	--	--	--	--
Glutamine	200mg	200mg	200mg	200mg	200mg	200mg	200mg	--	--	--	--
Naphthaleneacetic acid (NAA)	0.2mg	0.2mg	0.2mg	0.2mg	0.2mg	0.2mg	0.2mg	--	--	--	--
Kinetin	1.0mg	1.0mg	1.0mg	1.0mg	1.0mg	1.0mg	1.0mg	--	--	--	--
6-Benzylaminopurine (6-BAP)	0.5mg	0.5mg	0.5mg	0.5mg	0.5mg	0.5mg	0.5mg	--	--	--	--
Zeatin	0.5mg	--	0.5mg	0.5mg	0.5mg	0.5mg	0.5mg	--	--	--	--
Gibberellic Acid (GA_3)	--	--	0.5mg	0.5mg	0.5mg	0.5mg	0.5mg	--	--	--	--
Coconut Water	5%	--	5%	5%	5%	5%	5%	--	--	--	--
Sucrose	50g	50g	50g	50g	50g	50g	50g	50g	50g	50g	50g
Agar	8g	8g	8g	8g	8g	8g	8g	8g	8g	8g	8g

$$\text{Swollen yellow anthers}\ (\%) = \frac{\text{No. of Yellow Swollen Anthers}}{\text{Total No. of Swollen Anthers}} \times 100$$

$$\text{Swollen brown anthers}\ (\%) = \frac{\text{No. of Brown Swollen Anthers}}{\text{Total No. of Swollen Anthers}} \times 100$$

$$\text{Browning}\ (\%) = \frac{\text{No. of Brown Anthers}}{\text{Total No. of Anthers Cultured}} \times 100$$

$$\text{Callus induction}\ (\%) = \frac{\text{No. of Induced Calli}}{\text{Total No. of Anthers Cultured}} \times 100$$

$$\text{Embryogenesis}\ (\%) = \frac{\text{No. of Germinated Embryos}}{\text{Total No. of Anthers Cultured}} \times 100$$

***B. Effect of Cold Pretreatments and M_{10} media on Androgenesis in White and Pink Flesh Guava Cultivars*:** Based on the findings of the experimental study 'A', an extensive experiment was conducted including two white flesh (RW and PW) and one Round pink flesh (RP) guava cultivar. Anthers were collected from the promising medium-sized floral buds and pretreated for 0, 24, 48, and 72 hrs. at 4°C as discussed above. The pretreated anthers were cultured following explant sterilization conditions of experiment 'A' on promising media formulation M_{10} (Table 1) for callogenesis and embryogenic responses.

***Experimental Layout and Statistical Analysis*:** The experiment was laid under a completely randomized design (CRD) with a three-factor factorial arrangement. Differences among media, cultivars, and cold pre-treatments were tested by analysis of variance at $P < 0.05$ level. Differences among means were tested by the Least Significant Difference (LSD) test (Steel *et al.*, 1997) for both experiments. Correlations were determined in the variables of the first experiment using R software (Gogtay and Thatte, 2017) to identify the relationship between two variables and their distribution. The inter-correlated quantitative dependent variables were analyzed by Principal Component Analysis (PCA) and Cluster Analysis (CA) (Abdi and Williams, 2010; Saraçli *et al.*, 2013), which was conducted using Ward's clustering method for hierarchical clustering to identify the natural clusters among variables characterized by similar attributes (Murtagh and Legendre, 2014). XLSTAT statistical software was used for multivariate analysis (Vidal *et al.*, 2020).

RESULTS

***A. Effect of Donor Plant, Cold Pretreatments, and Modified N_6 Media Formulations on Anthers of White Flesh Guava Cultivars for Androgenesis*:** The floral buds were stored for up to one week at 4 °C to assess the storage longevity of the anthers. The pollen of both cultivars was triangular, and genotypic variability was observed among guava cultivars for pollen viability percentage. Pollens of white flesh Round 'RW' cultivar showed consistently higher viability (Fig. 1C) across cold pretreatment intervals (0-72 hrs.) compared with cv. Pyriform 'PW' (Fig. 2a). The pollen viability was greater (88%-94%) in freshly harvested pollens without cold treatment (0 hrs.) and viability was reduced in both Round (80%- 52%) and Pyriform (74%-44%) cultivars with the increasing interval of cold treatment from 24 to 72 hrs., respectively. The outer covering of floral buds turned blackish due to the higher phenolic contents and was slightly shrunk due to moisture loss.

After one week of culturing, both the brown (Fig. 2b) and yellow (Fig. 2c) colored anthers were swollen, and the increase in anther size continued for 5-6 weeks (Fig. 2e). Overall, browning was greater (37%-72%) in anthers cold pretreated for 0 hrs., and the browning percentage reduced with an increase in the pretreatment time intervals (24 to 72 hrs.). However, a higher percentage of yellow anthers turned brown on M_5 and M_9 media after 72-hour treatment (Fig. 2b, 3a). In contrast, the anthers maintained yellow color (no browning), and their percentage was higher in anthers treated for 24 and 48 hrs. compared with anthers treated for 0 and 72 hrs.

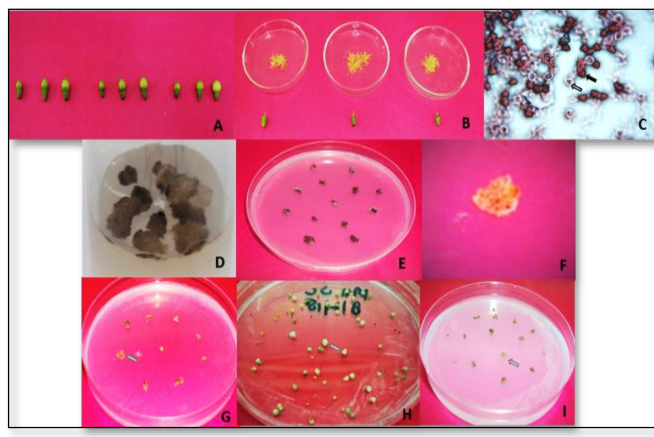

Figure 1. Androgenic responses in guava cultivars. (A) Bud selection from small, medium, and large size floral buds. (B) Anther collection from mature buds. (C) Pollen viability analysis black arrow shows viable pollens while the black lined arrow shows non-viable pollen. (D-E) Anther swelling, browning, and callus induction. (F, G) Proembryogenic calli development (grey arrows) in white flesh cv. Round anthers at M_{10} media. (H, I) Proembryogenic calli development (grey arrows) in cv. Pyriform at M_{11} media.

Among media treatments, the percentage of yellow anthers was maximum at M_{10} and M_{11} media in both Round and Pyriform cultivars after 24 hrs. of treatment, followed by M_1, M_2, M_{10}, and M_{11} media after 48 hrs. of cold treatment (Fig. 2c, 3c). Swelling was higher in browned anthers pretreated for 0 hrs. on M_2, M_{10}, and M_{11} media (Fig. 1D) and anthers treated for 24 hrs. on M_2 media (Fig. 2d, 3b). In contrast, the swelling was higher in yellow anthers pretreated for 24 and 48 hrs. on

M_{10} and M_{11} media (Fig. 2e, 3d). Overall, anther swelling percentage remained consistently higher (72%-80%) on M_{10} and M_{11} media at all four cold pretreatment intervals (Fig. 2f, 3e). Many swollen anthers later started callus initiation and proliferation (Fig. 1F-H).

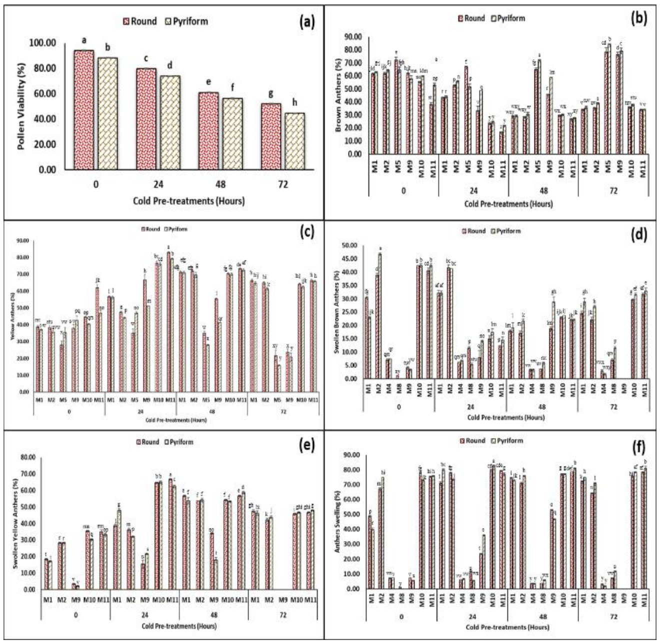

Figure 2. Effect of cold pre-treatments and modified N_6 Media on (a) Pollen viability%, (b) Brown anthers%, (c) Swollen brown anthers%, (d) Yellow anthers%, (e) swollen yellow anthers%, (f) Anthers swelling% in white flesh Guava cvs. i.e., Round and Pyriform.

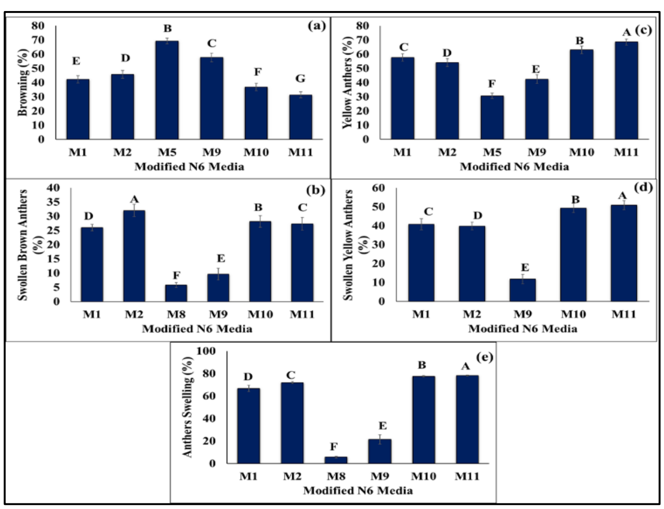

Figure 3. Overall effect of modified N_6 Media treatments on (a) Anther browning%, (b) Swollen brown anthers%, (c) Yellow anthers%, (d) Swollen yellow anthers%, (e) Anthers swelling% in white flesh Guava cvs.

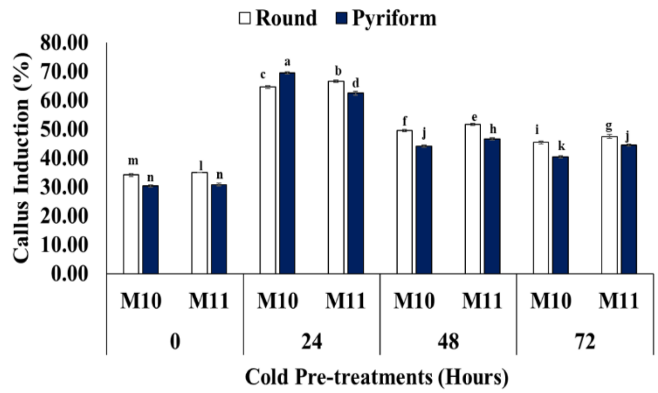

Figure 4. Embryogenic callus induction responses in cold pretreated (0, 24, 48, 72 hrs.) anthers on modified N_6 media treatments M_{10} and M_{11} in white flesh guava cvs. Round and Pyriform. Different alphabets show significant differences according to the LSD test at 5% probability.

Cold pretreatment of anthers for 24 hrs. significantly enhanced their potential to induce calli in both Round and Pyriform cultivars compared with control (0 hr.). The callus induction percentage was maximum in the anthers of cv. Pyriform pretreated for 24 hrs. on M_{10} media, which decreased with an increase in the pretreatment interval from 48 to 72 hrs. (Fig. 4). Overall, among genotypes, the callus induction response was greater in cv. Round at all treatment intervals compared with cv. Pyriform on M_{10} media. The developed calli proliferated well on the same media and grew into proembryogenic masses in anthers pretreated for 24 hrs. However, embryos showed delayed maturation and germination. Overall, anthers pre-treated at 4°C for 24 hrs. have shown a better response for callus induction on M_{10} media. The chunks of a few developed calli turned brown after 6-8 weeks of culturing, which could be attributed to the development of more phenolic contents.

A significant positive correlation of swollen browned anthers % was observed with yellow anthers % (0.67), swollen yellow anthers % (0.73), anthers swelling % (0.89), and callus induction% (0.42). Likewise, yellow anthers % showed a significant positive correlation between swollen yellow anthers % (0.91), anthers swelling % (0.88), and callus induction% (0.61) (Fig. 5). Whereas callus induction % was positively correlated to anthers swelling % (0.63). Moreover, a negative correlation of browning % was found between swollen brown anthers % (-0.67), yellow anthers % (-1.00), swollen yellow anthers % (-0.91), anthers swelling % (-0.88), and callus induction % (-0.61) (Fig. 5).

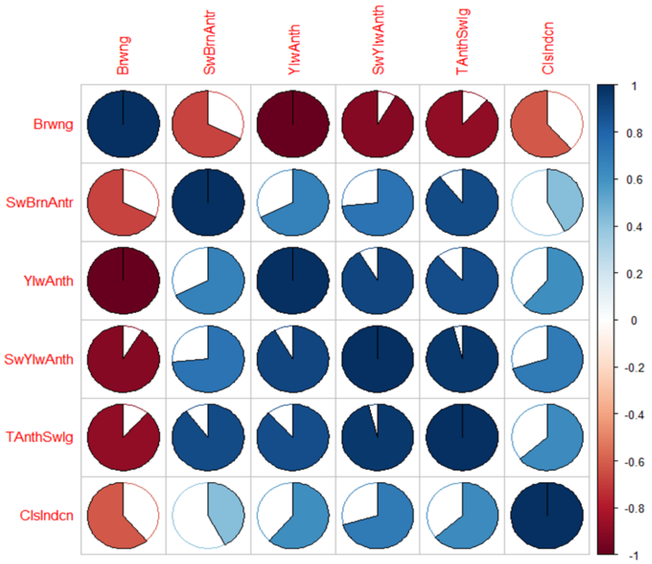

Figure 5. Correlation matrix of anther browning, swelling, callus induction responses on modified N_6 media in white flesh Guava cultivars. Negative correlations are shown in red, whereas positive correlations are shown in blue. The correlation coefficient determines the color intensity, therefore the darker the boxes are, the stronger the correlation is (i.e., the closer to -1 or 1). The correlation coefficients and the related colors are displayed in the color legend on the correlogram's right side.

PCA for callus induction rate on different media concentrations, guava cultivars, and cold pre-treatments showed great variation and was grouped into different clusters based on their characteristics. A PCA plot was developed based on first and second component factors, which showed great variation (81.55% and 10.23%) (Fig. 6a). The most divergent components and outliers in the current investigations were $M_9V_1T_1$, $M_9V_2T_2$, $M_1V_2T_o$, and $M_2V_2T_o$. A dendrogram developed based on divergence data showed the formation of two main groups (C1-C2) with nine sub-groups (A-I) and showed variation within different media concentrations, guava cultivars, and cold pre-treatments. Sub-group 'F' had a higher number of variables, while sub-groups C and I had only three variables (Fig. 6b).

***B. Effect of Cold Pretreatments and Promising Culture Media (M_{10}) on Anthers of White and Pink Flesh Guava Cultivars for Androgenesis*:** Based on the findings discussed above, cold-pretreated anthers of three different cultivars of guava, including white flesh Round 'RW', Pyriform 'PW', and pink flesh Round (RP), were cultured on M_{10} media for embryogenic callus induction, proliferation, and embryogenesis. The calli induced on M_{10} media proliferated well and developed proembryogenic masses in cultivars PW and RP (Fig. 7, 8). The developed embryos showed delayed maturity and less germination, indicating a need for further tuning fine-tuning of the embryogenic medium-medium.

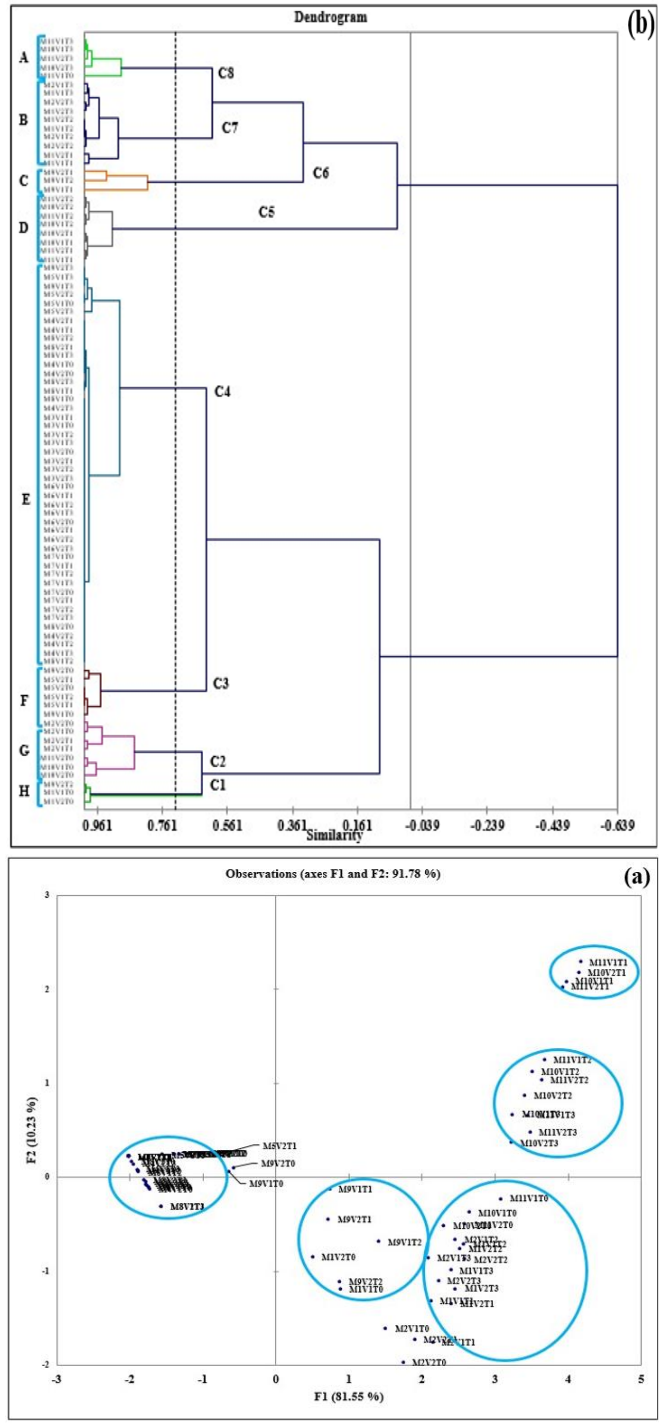

Figure 6. Principal Component Analysis (a) and Clustering Analysis (b) for different media formulations, cold pre-treatments in guava cultivars Round and Pyriform

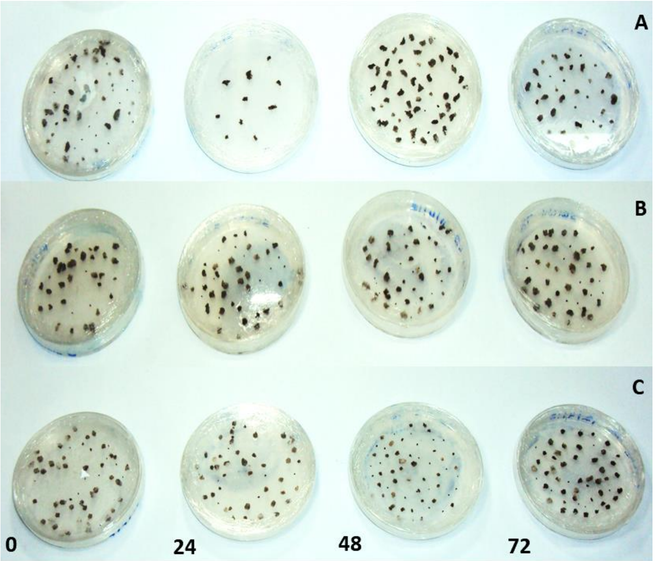

Figure7. Proembryogenic calli development and proliferation in different guava cultivars after cold pretreatment of anthers for 0, 24, 48, and 72 hrs. on M_{10} media (A) Round white, (B) Pyriform white, and (C) Round pink.

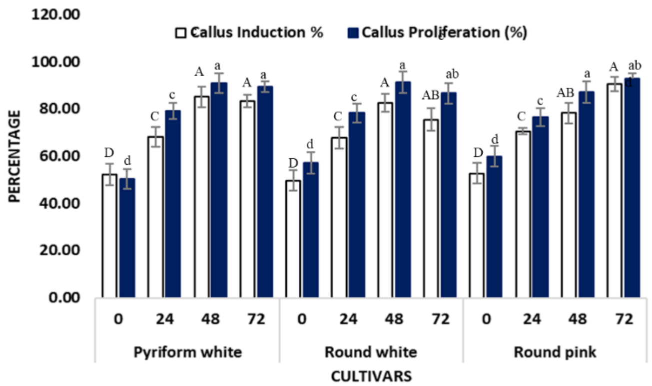

Figure 8. Embryogenic callus induction and proliferation responses in cold pretreated (0, 24, 48, 72 hrs.) anthers on modified N_6 media M_{10} in guava cvs. Pyriform white, Round white, and Round pink.

DISCUSSION

***Effect of cold treatment and genotype on pollen viability*:** Pollen viability determines the success rate of the reproductive process in a specie (Abdelgadir *et al.*, 2012) and is a vital requirement for androgenesis. Androgenesis has been reported in many fruit crops, however, little is known about interaction between the water content of pollen, cold pre-treatments, and androgenic responses in guava. Staining of pollen for viability assessment is affected by plant genotype, physiology, pollen maturity, and media composition (Ferri *et al.*, 2008). Pollen grain internal conditions like moisture content, air temperature, gametic maturity, reserve substances, and their interactions also play an important role (Pacini *et al.*, 2006). Acetocarmine has been widely used to stain pollen grains in fruit crops (Pham *et al.*, 2015; Wang *et al.*, 2015) and indicate the presence of enzymes or cytoplasm. Higher pollen variability was reported in roses (Pearson and Harney, 1984), pomegranate (Sharma and Gaur, 1984), jatropha (Lyra *et al.*, 2011), dwarf guava (Nongyai *et al.*, 2015), and litchi where 1% acetocarmine was considered more suitable for pollen viability assessment (Gupta *et al.*, 2018). In the current study, the use of 1% acetocarmine dye properly stained the pollens for viability assessment in guava cultivars and pollens of white flesh Round cultivar showed higher viability at all cold treatment time intervals. The average viability in fresh guava pollens was 91%, which is comparable to Nongyai et al. (2015), who reported 92% viability in dwarf ornamental guava. Pollen storage longevity varied greatly amongst cultivars, plant species, and storage conditions (Acar and Kakani, 2010; Mortazavi *et al.*, 2010). Pollen storage longevity ranged from several minutes to a few hours in cereals (Luna *et al.*, 2001) and 4 weeks in mangoes at room temperature (Dutta *et al.*, 2013). A reduction in pollen viability is also linked to moisture loss and the maintenance of a dehydrated state (Lisci *et al.*, 1994). Pollen viability in guava was reduced up to 50% after 72 hrs. pretreatment at 4°C. Pre-cold shock treatments of anthers induce callus formation, delay senescence of the anther wall, and increases the symmetric division of the pollen grains (Gueye and Ndir, 2010; Sen *et al.*, 2011). Essential substances like thermal shock proteins and amino acids for androgenesis are also released through cold treatments (Kiviharju and Pehu, 1998).

Effect of media, plant growth regulators, and sucrose on androgenic responses: Various factors like plant genotype, pre-culture treatments, culture medium, plant growth regulators, and culture conditions (Moubayidin *et al.*, 2009; Germanà, 2011; Żur *et al.*, 2015; Usman *et al.*, 2015) can influence *in vitro* androgenesis synergistically or alone. A significant impact of cold pretreatment was observed in enhancing callus induction and reducing phenolic exudation in guava cultivars. The regeneration ability of callus depends on endogenous and exogenous concentrations of plant growth regulators (Guo *et al.*, 2017). Anthers swelling indicates the initial anatomical shift associated with the morphogenic reaction (Germanà *et al.*, 2006). Media formulations played a significant role in anther swelling, and out of eleven different media, only four media (M_1, M_2, M_{10}, M_{11}) induced anther swelling. M_1 media was additionally supplemented with zeatin and coconut water, whereas both supplements were missing in the M_2 medium. In M_{10} and M_{11} media formulations, all additional supplements (casein hydrolysate, glutamine, and coconut water) and PGRs (NAA, Kin, BAP, Zeatin, and GA_3) available in M_1 and M_2 media were missing (Table 1). Further, M_{10} media was supplemented with 2,4-D, whereas M_{11} media had 2,4-D and PVP. The addition of 2,4-D to the anther culture medium increased the effectiveness of gametic embryogenesis in guava, as reported previously in other fruit crops (Nowaczyk *et al.*, 2016; Abdollahi and Rashidi, 2018). The lack of anther swelling in media M_3-M_9 could be attributed to the addition of GA_3 in these media, which was missing in M_1, M_2, M_{10}, and M_{11} media inducing swelling in anthers. Similar variable callus induction responses in guava varieties were reported by Hidano et al. (1994) and Tsukuni (2006). Genotypic variations could also be attributed to the growing conditions of the donor plant.

Cold pre-treatments and modified Chu N_6 media affected callus initiation in tomatoes (Motallebi-Azar, 2010), citrus (Aboshama, 2011), and strawberry cultivars (Shahvali-Kohshour *et al.*, 2013). Callus induction also depends on the excised anther's nutritional requirements. Exhaustive investigations on the androgenesis using anther culture have been carried out on the type and concentration of plant growth regulators (Perera *et al.*, 2009; Smykalova´ *et al.*, 2009). Regarding culture medium, Chu N_6 medium has been the most often used and effective medium for anther culture (Aboshama, 2011) and has induced gametic embryogenesis in various woody plant species like citrus, apricot, hazelnut, and loquat (Germanà and Chiancone, 2003; Karasawa *et al.*, 2016; Cardoso *et al.*, 2016). In the current study, Chu's N_6 salts and Nitsch vitamins, with or without additional supplements, PGRs, and PVP, were used for callogenesis and subsequent embryogenesis. Embryogenic callus was induced on M_{10} and M_{11} media having 2,4-D with or without PVP, devoid of supplements (casein hydrolysate, glutamine, and coconut water) and PGRs (NAA, Kin, BAP, Zeatin, and GA_3). In contrast, supplements of substances like casein, glutamine, coconut water, and polyvinylpyrrolidone have also been reported (Achar, 2002; Aboshama, 2011) to promote callogenesis. In fruit trees, callus induction from anthers was induced by the addition of various kinds of auxins, like NAA in *Carica papaya* (Rimberia *et al.*, 2007), and IAA in *Annona squamosa* (Nair *et al.*, 1983), 2,4-D in *Eriobotrya japonica* (Li *et al.*, 2008), 2,4-D and NAA in *Citrus clementina* (Germana` and Chiancone, 2003). Mishra and Rao (2016) stated that PGRs resulted in a high callus induction rate and reduced browning. Previously, 2,4-D has been used in anther culture medium (Abdollahi and Rashidi, 2018) and as an external pre-treatment in donor plants to increase the effectiveness of gametic embryogenesis (Nowaczyk *et al.*, 2016). Similarly, in the current study, anthers induced embryogenic calli on media (M_{10} and M_{11}) having 2,4-D. The carbon source acts as an osmoregulatory in anther culture and has been documented in many species (Burbulis *et al.*, 2005). Elevation of sucrose concentration increased the efficiency of callus induction in different fruit crops, including citrus. Similarly, a higher level of sucrose (5%) along with suitable growth regulators increased the efficiency of callogenesis in all three guava cultivars. The embryonic initials were observed on a few proliferating calli after 4-6 weeks of culture, whereas the rest of the calli did not develop embryogenic masses.

Conclusion: Anther culture at the uninucleate stage is found has been the most suitable stage for androgenesis. Development of embryogenic calli from anthers of guava cultivars was obtained on modified Chu N_6 media (M_{10} and M_{11}). This study provides the baseline for establishing androgenesis in guava, whereas more genotypes and media formulations may be explored for fine-tuning the regeneration process. Furthermore, the findings of this study could lead to the development of inbred lines for breeding programs and will create new opportunities for genetic improvement of guava.

Conflict of Interest: The authors declare that there is no conflict of interest.

Author's Contribution Statements: SAMB executed the research work in the lab and prepared the article draft, BF and MU supervised the work in tissue culture cell, whereas MU, MSK, and BR conceived the idea and supervised the research plan, MU provided funds for research. MU and MSK edited the article.

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