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Research Article

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# Androgenesis in tomato (*Solanum lycopersicum* L.) -Effect of genotypes, microspore development stage, pre-treatments and media composition on induction of haploids

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## ABSTRACT

Doubled haploid (DH) technology remarkably accelerates the crop breeding by obtaining homozygous lines in a single generation. The present study was targeted in generating haploid plants through androgenesis. Anthers from immature flower buds of six tomato genotypes *viz.*, LE-1230, LE-1236, LE-1256 TLCV 2, PKM 1 and TNAU tomato hybrid CO 3 were used for induction of haploids. A preliminary study based on callus induction frequency (CIF), more than 5% was helpful in short listing flower bud size, pre-treatments and growth regulator combinations. Subsequently, anthers from two different sized flower buds (4 and 6 mm length), dissected either from fresh or pre-treated flower buds (2 and 5 days in dark at 4 °C or gamma irradiated) were inoculated in MS medium fortified with different growth regulators for callus induction. Among the genotypes, TLCV 2 had recorded the maximum CIF (38.80%) from anthers of 4 mm long flower buds followed by TNAU tomato hybrid CO 3 (34.70%). Throughout the study, anthers from 4 mm long flower buds responded the best for callus induction. Among the pre-treatments, anthers from gamma irradiated flower buds recorded the highest CIF (31.90%) when compared to others. Cold shock (4 °C) in dark to flower buds for 2 days had improved the CIF of anthers when compared to fresh in LE 1230, LE 1238, TLCV2 and TNAU tomato hybrid CO 3, but when the cold shock was increased to 5 days, invariably there was a reduction in CIF in all the six genotypes. TA 8 (MS + 2iP (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$)) medium was found to be the best for maximum CIF in LE 1230 and PKM1, TA1 (MS + 2iP (1.0 mg $L^{-1}$) + IAA (2.0 mg $L^{-1}$)) in LE 1238, LE 1256 and TNAU tomato hybrid CO 3 and TA7 (MS + 2iP (0.5 mg $L^{-1}$) + Kinetin (1.5 mg $L^{-1}$) + NAA (1.0 mg $L^{-1}$)) for TLCV 2 genotypes. The callus induced was sub cultured at monthly intervals in the same medium for proliferation and later transferred to regeneration medium. A good number of shoots got regenerated only from anther calli of TNAU hybrid CO 3 that was sub cultured in MS medium fortified with Zeatin (0.5 mg $L^{-1}$). The clumps of shoots induced were separated and inoculated in MS medium supplemented with $GA_3$ (0.5 mg $L^{-1}$) for shoot elongation. After 4-6 weeks, the elongated shoots were transferred to half strength MS medium enhanced with IBA (1 mg $L^{-1}$). Profuse rooting from the base of the shoot was noticed in 4-5 weeks. The stomatal count with leaves from the diploid plants and *in vitro* plants observed were 3-4 and 1 respectively indicating the haploidy nature of *in vitro* plants.

**KEYWORDS:** Androgenesis, Tomato, Haploid, Callus, CIF, Cold shock

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## INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the second most consumed vegetable, next to potato. World production of tomatoes in 2020 was 186.821 million metric tonnes (mT) (FAOSTAT, 2020). Tomatoes are considered as an excellent source of folic acid and potassium, and are noteworthy for high levels of vitamin C, minerals like Ca, P, Na, Mg, K, Fe, Zn and Cu, which are essential nutrients for humans making tomatoes a substantial contributor (Kumar *et al.*, 2020). A short generation time, simple growing requirements, and several other properties make tomato currently one of the

most effective crop model species for basic and applied research.

Androgenesis is a powerful, fast and cheap method leading to the production of homozygous doubled haploid (DH) lines in just one *in vitro* generation compared to traditional breeding methods. The use of doubled haploids for breeding allows for time and cost savings in the production of pure lines, compared to conventional inbreeding and selection techniques (Mir *et al.*, 2021; Seguí-Simarro *et al.*, 2021a). Production of haploids shortens the time for inbreeding for superior hybrid genotypes. One key issue in androgenesis is the correct identification of the optimal stage of microspore for anther excision and culture. In species such as tobacco, rapeseed, wheat or barley, it is relatively easy to produce androgenic DHs in some cultivars (Seguí-Simarro & Nuez, 2008; Sood *et al.*, 2021; Eliby *et al.*, 2022; Patial *et al.*, 2022). The advantages have made androgenesis a method of choice in several economically interesting crops (Kašpar *et al.*, 2022). At present, refined protocols have been developed to produce DHs from tobacco anther and isolated microspore cultures on a routine basis and with an acceptable efficiency (Belogradova *et al.*, 2009; Weyen, 2021). Despite the fact that anther culture is routinely used in a number of economically important crops, this technology is poorly developed in tomato and there are still many drawbacks that prevent tomato breeders from adopting this technique, and improvements in methodology are required (Seguí-Simarro & Nuez, 2005; Karimi-Ashtiyani, 2021).

Stress application during the developmental period of pollen grains, such as cold shock, temperature shock, osmotic shock, starvation and irradiation are highly indispensable for the induction of androgenesis. A low or high temperature, osmotic or a physiological starvation could be applied for induction of response during androgenesis (Koleva-Gudeva *et al.*, 2008).

Cold pre-treatment assists in conversion of the gametophytic development of microspores into sporophyte during culture (Touraev *et al.*, 1997), delay of anther wall senescence, increase of symmetric division of pollen grains and release of substances necessary for androgenesis (amino acids and shock-thermic proteins) (Kiviharju & Pehu, 1998). High temperature pre-treatment disrupts the normal integrated development of somatic anther tissue and subsequently synchronizes the physiological states of the two tissues, thereby stimulating the induction process (Dunwell *et al.*, 1983). Osmotic stress or loss of cellular water content often disrupts the plasmodesmatal connections between the pre-embryonic cells, making the cells physiologically isolated and allowing a greater number of cells to differentiate (Wetherell, 1984). Sugar starvation is an effective inducer in androgenesis through the changes cytoplasmic and nuclear contents, protein kinase activity and plasma lemma phosphoprotein composition and synthesis of HSP (Shariatpanahi *et al.*, 2006).

Irradiation is known to deactivate nuclei which could lead to the death of some microspores thereby reducing the competition between the remaining viable microspores within the anther. Irradiation has also been reported to alter the auxin and cytokinin levels within plant tissues (Shariatpanahi *et al.*, 2006) and the combination of these actions could trigger the androgenesis result in higher levels of embryoid production during anther culture.

Although much research have been conducted on tomato (*Solanum lycopersicum* L.) through pollen or anther culture, the number of anthers producing calli and the number of plants regenerated per callus have been limited. Very few tomato genotypes have callus forming and regeneration potential from anthers and various media/methods have been used to produce plantlets of unknown ploidy (Hassan & Islam, 2021; Marin-Montes *et al.*, 2022). This paper explored the factors that need optimization or further study prior to routine haploid plantlet production *via* androgenesis.

## MATERIALS AND METHODS

### Genotypes

The tomato genotypes used in this study were LE-1230, LE-1236, LE-1256, and TLCV 2 (germplasm accessions), PKM1 (variety), and TNAU tomato hybrid CO 3 (hybrid). Seeds were germinated in pots, transplanted after 20 days and the plants established were maintained in the greenhouse for collection of flower buds.

### Explant Collection and Preparation

The unopened flower buds of different sizes (2, 4, 6 and 9 mm) were collected from the healthy plants maintained in green house (Figure 1A). Anthers were dissected out after surface sterilization from either fresh or pre-treated flower buds (cold shock or gamma irradiated) and used for inoculation. The unopened flower buds were sterilized with mercuric chloride (0.1%) for three min, followed by four rinses with sterile distilled water. The sterilized flower buds were then placed over an autoclaved petri-dish of 90 mm diameter with Whatman No. 1 filter paper for absorption of water droplets (Figure 1B). The sepals and petals were removed. First, the anther cone was dissected out (Figure 1C) and anthers were separated from cone and inoculated (Figure 1D).

### Pre-Treatments

Collected flower buds were used either fresh or cold pretreated (4 °C in dark for 2, 5, 7 and 9 days) or gamma irradiated (4 Gy for 2 min) to understand the influence of stress treatments on callus induction. The cold pretreatment was carried out in a BOD Incubator, whereas the gamma irradiation was carried out using the Gamma Chamber 1200. Pre- treated flower buds were surface sterilized as that of the fresh, anthers were separated and inoculated.

### Nutrient Medium

MS nutrient medium (Murashige & Skoog, 1962) of full strength was used for callus induction, regeneration and half strength

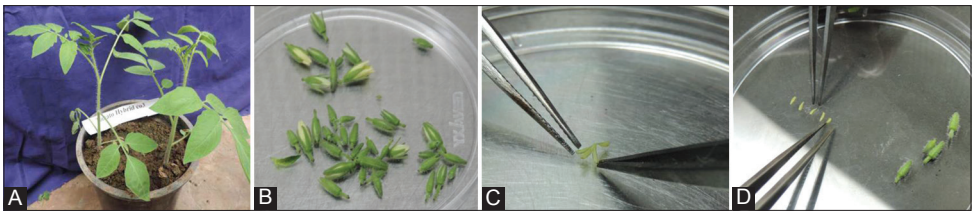


**Figure 1:** Explant collection and preparation. A) Mother plant; B) Surface sterilised flower buds; C) Anther cone; D) Dissected anthers

for *in vitro* rooting. Stock solutions of macro, micro, minor, iron stock, potassium iodide, vitamins and plant growth regulators (PGR) were prepared and stored until further use. The required volume of macro, micro, minor nutrients and vitamins stock were pipetted out according to the concentration of the basal medium and mixed to known quantity of distilled water. Sucrose (30 g/L) and myo-inositol (100 mg $L^{-1}$) were also added and mixed well. The growth regulators at required concentration were added and homogenized. The volume was made up to one liter after adjusting the pH of medium to 5.6-5.8 with 0.1N HCL or 0.1N NaOH. The agar, 8 g $L^{-1}$ was dissolved before autoclaving. Then, it was sterilized in an autoclave at a temperature of 121 °C at a pressure of 15 psi for 20 min. (Dodds & Roberts, 1985). The heat labile compounds such as Zeatin and $GA_3$ were included in the medium after autoclaving using 0.22-micron filters in the laminar air flow. The autoclaved medium was poured in sterile petri-dishes/ jams bottles under aseptic conditions inside the laminar airflow chamber and kept in the culture room at 25 °C until further use.

## Culture Conditions

For callus induction, anthers were kept in dark immediately after inoculation at 25±2 °C at a RH of 60-70 per cent for 30 days and later incubated under a light intensity of 2000- 4000 lux using white fluorescent tube lights with a photoperiod of 16/8 h light and dark cycle. For regeneration studies, the cultures were illuminated at 2000-4000 lux with 16/8 h light dark photoperiod.

## Preliminary Study

The developmental stages of flower buds were documented and categorized as 1-14 based on size (Figure 2A) and inoculation was carried out with different sized flower buds (2, 4, 6 and 9 mm) in 8 different medium combinations along with a control. Anthers from fresh flower buds as well as after stress treatment *viz*., cold shock (4 °C in dark) for different durations-2, 5, 7 and 9 days were given prior to inoculation. Eight growth regulator combinations tried in MS medium for callus induction is furnished in Table 1.

## Microspore Development Stages

Based on the results on callus induction frequency (CIF) of anthers from preliminary study, two different sized flower buds (4 and 6 mm) were chosen (Figure 2B). The microspore/ pollen developmental stages of these buds (4 mm and 6 mm length) with corresponding anther length of 2 mm and 4 mm respectively were analyzed by fixing the flower buds in Carnoy's fluid (ethanol (100%) and glacial acetic acid, 3:1 ratio) along with ferric chloride aqueous solution. Later, microspore stage was observed by squeezing the anthers, staining with acetocarmine (1%) and viewing under the light microscope (40 X) ultimately to establish a correlation between the flower bud length and microspore stage during collection of flower buds.

**Table 1: MS medium with growth regulator combinations**

| Code | Medium and growth regulators (mg $L^{-1}$) |
|---|---|
| TA0 | MS basal |
| TA1 | MS + 2iP (1.0) + IAA (2.0) |
| TA2 | MS + 2iP (1.5) + IAA (2.0) |
| TA3 | MS + 2iP (2.0) + IAA (2.0) |
| TA4 | MS + 2iP (1.0) + NAA (2.0) |
| TA5 | MS + 2iP (0.5) + BAP (0.5) + NAA (2.0) |
| TA6 | MS + 2iP (0.5) + BAP (2.0) + NAA (2.0) |
| TA7 | MS + 2iP (0.5) + Kinetin (1.5) + NAA (1.0) |
| TA8 | MS + 2iP (0.5) + NAA (0.5) |

## Callus Induction Studies

Based on the CIF (>5%) and morphology of callus from preliminary results, anthers from 2 and 4 mm flower buds, cold shock for 2 and 5 days at 4 °C in dark and five best growth regulator combinations were shortlisted for further experiments. The combinations were (i) TA1-MS + 2iP (1.0 mg $L^{-1}$) + IAA (2.0 mg $L^{-1}$),(ii)) TA4-MS + 2iP (1.0 mg $L^{-1}$) + NAA (2.0 mg $L^{-1}$), (iii) TA5-MS + 2iP (0.5 mg $L^{-1}$) + BAP (0.5 mg $L^{-1}$) +NAA (2.0 mg $L^{-1}$),(iv) TA7-MS + 2iP (0.5 mg $L^{-1}$) + NAA (1.0 mg $L^{-1}$) + Kinetin (1.5 mg $L^{-1}$) and (v) TA8-MS + 2iP (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$) along with MS basal medium as a control. The best treatment was decided based on the explants response to callus initiation after 30 days (expressed in percentage). CIF was calculated using the following formula:

Callus induction frequency (%) = No. of explants showing callus induction/Total no. of explants X 100

## Sub Culturing

For maintenance of callus, the callus initiated was transferred to fresh medium of same combination for every 30 days. During sub culturing, the residual pieces of inoculums and the brown part from the callus were removed before transferring to the fresh medium.

## Shoot Regeneration

The callus developed from anthers were transferred on to the regeneration medium, MS medium containing the cytokinins,

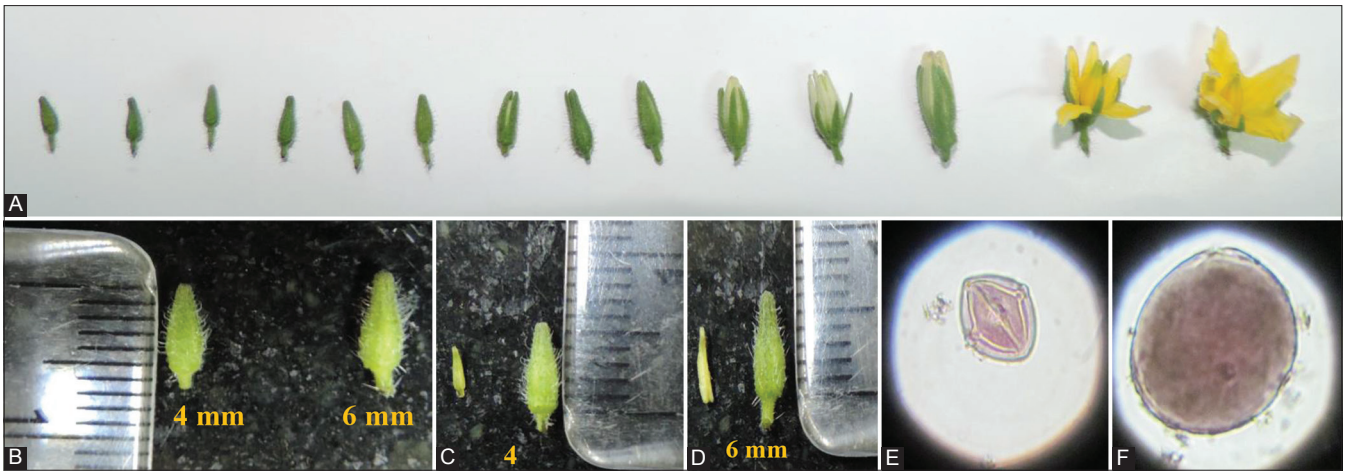


**Figure 2:** Developmental stages of flower buds and microspores. A) Developmental stages of flower buds (1 -youngest flower bud to 14 opened flower); B) Shortlisted flower bud size; C) 4 mm Flower bud; D) 6 mm Flower bud; E) Microspore-uninuleate stage; F) Microspore- vacuolated Accordingly, in Figure 2, 2F has to be renamed as 2E, photo shifted to the front and 2E to be renamed as 2F and photo to be placed in back

zeatin at three different concentrations (0.25, 0.50 and 2.0 mg $L^{-1}$) or BAP at single concentration (2.0 mg $L^{-1}$) to initiate shoot regeneration.

### Shoot Elongation and Rooting

The clumps of shoots induced were separated and inoculated in MS medium supplemented with GA3 (0.5 mg $L^{-1}$) for shoot elongation. After 4-6 weeks, the elongated shoots were transferred to half strength MS medium supplemented with IBA (1.0 mg $L^{-1}$) for *in vitro* rooting.

### Characterization of *in Vitro* Generated Haploids through Stomatal Count

To confirm ploidy level, stomatal count was done with leaves from greenhouse grown plants (diploid) and from the plants regenerated (haploid) from anther callus using nail polish blotting method. The leaves were cleaned the dust off with wet cotton wool and a layer of clear nail polish was evenly applied to the back of the leaf blade. After the nail polish completely got dried, the blotting film was peeled off to make a temporary slide. Observations on stomatal count were carried out under an optical microscope (Leica DM6000B, Monroe, LA, USA) under 100 X magnification.

### Statistical Analysis

The study was carried out in three replications with 36 explants per replication. The data generated from the various experiments were subjected to statistical analysis. All data on callused anthers were subjected to analysis of variance (ANOVA). Observations recorded from three replications as percentage were subjected to angular transformation. The CD values were worked out for five per cent (0.05) probability and the results were interpreted. Analysis was carried out with AGRIS software package and MS Excel® (2019) spreadsheet.

## RESULTS

The effect of different genotypes, LE-1230, LE-1236, LE-1256, TLCV2, PKM 1 and TNAU tomato hybrid CO 3, flower bud size (microspore stage), pre-treatments and plant growth regulator combinations on callus induction was studied by culturing anthers as explants on MS medium reinforced with different plant growth regulators in combinations. In the preliminary study, the flower bud size, pre treatment (cold shock) and growth regulator combinations were assessed based on CIF (more than 5%) and nature of callus. Among the different sized flower buds (2, 4, 6 and 9 mm long) tried in 8 different medium combinations, the genotypes, LE-1256, TLCV 2 and CO 3 had registered a callus induction frequency of more than 20 per cent with anthers from 4 mm size flower bud in TA7 (MS + 2iP (0.5 mg $L^{-1}$) + Kinetin (1.5 mg $L^{-1}$) + NAA (1.0 mg $L^{-1}$)) medium (Table 2). Anthers from 4 mm flower buds of LE-1256, TLCV 2, CO 3, PKM 1- and 6-mm flower buds of TLCV 2, CO 3, PKM 1 had recorded a callus induction frequency of 16-20%. Anthers from 2- and 9-mm flower buds either did not respond or if responded, the induction was very low (1-5%). Similarly, the MS medium with growth regulator combinations- TA2, TA3 and TA 6 (MS + 2iP (1.5 mg $L^{-1}$) + IAA (2.0 mg $L^{-1}$), MS + 2iP (2.0 mg $L^{-1}$) + IAA (2.0 mg $L^{-1}$) and MS + 2iP (0.5 mg $L^{-1}$) + BAP (2.0 mg $L^{-1}$) + NAA (2.0 mg $L^{-1}$) respectively) did not perform well, hence not included in further experiments. In cold pre-treatment (4 °C dark), for different durations (2, 5, 7 and 9 days) tried, two- and five-days exposure of flower buds to cold and dark at 4 °C had registered a callus induction frequency above 5 per cent, hence shortlisted for further experiments. Prolonged exposure of flower buds to cold shock (4 °C dark) for 7 and 9 days actually totally inhibited the anther response in most of the genotypes except a meager response (1-5%) with 4 and 6 mm sized buds (Table 2).

From the preliminary results, two different sized flower buds(4 and 6 mm), five growth regulator combinations and three pre treatments, (cold shock for 2 and 5 days at 4 °C) including gamma irradiation were finalized for further studies. The flower

Table 2: Effect of size of flower bud, pre-treatments and growth regulators on callus induction during androgenesis of different tomato genotypes

| Genotype | Treatments | CIF Fresh | | | | CIF Cold@4°C 2d | | | | CIF Cold@4°C 5d | | | | CIF Cold @4°C 7d | | | | CIF Cold@4°C 9d | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm |
| LE 1230 | TA0 | - | + | + | - | - | + | + | - | - | - | + | - | - | - | - | - | - | - | - | - |
| | TA1 | + | +++ | +++ | + | - | ++++ | +++ | + | - | ++ | ++ | - | - | + | + | - | - | + | - | - |
| | TA2 | - | ++ | + | - | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA3 | - | ++ | ++ | - | - | + | + | - | - | ++ | + | - | - | - | - | - | - | - | - | - |
| | TA4 | - | ++ | ++ | - | - | +++ | ++ | + | - | ++ | ++ | - | - | + | - | - | - | - | - | - |
| | TA5 | - | ++ | ++ | - | - | +++ | ++ | - | - | ++ | ++ | - | - | - | - | - | - | - | - | - |
| | TA6 | - | ++ | + | - | - | + | + | - | - | ++ | + | - | - | - | + | - | - | + | - | - |
| | TA7 | - | +++ | ++ | + | - | +++ | +++ | + | + | +++ | ++ | + | - | - | - | - | - | + | + | - |
| | TA8 | + | +++ | ++ | + | - | +++ | ++ | - | - | ++ | ++ | - | - | + | - | - | - | - | + | - |
| LE 1236 | TA0 | - | + | + | - | - | - | + | - | - | - | + | - | - | | - | - | - | - | - | - |
| | TA1 | + | +++ | ++ | - | - | ++++ | ++ | - | - | ++ | ++ | - | - | - | + | - | - | - | - | - |
| | TA2 | - | ++ | ++ | - | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA3 | - | ++ | ++ | - | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA4 | - | ++ | ++ | + | - | +++ | ++ | - | - | ++ | + | - | - | - | - | - | - | - | - | - |
| | TA5 | - | +++ | ++ | - | - | +++ | ++ | - | - | ++ | + | - | - | | - | - | - | | - | - |
| | TA6 | - | ++ | + | - | - | ++ | + | - | - | | | - | - | - | - | - | - | - | - | - |
| | TA7 | + | ++++ | +++ | + | - | +++ | ++ | - | - | ++ | ++ | - | - | - | + | - | - | + | - | - |
| | TA8 | - | +++ | +++ | - | - | +++ | ++ | - | - | ++ | ++ | - | - | - | - | - | - | - | - | - |
| LE 1256 | TA0 | - | + | + | - | - | + | + | - | - | + | ++ | - | - | - | - | - | - | - | - | - |
| | TA1 | - | ++++ | +++ | + | - | +++ | +++ | - | - | ++ | ++ | - | - | + | - | - | - | - | - | - |
| | TA2 | - | ++ | ++ | - | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA3 | - | | | - | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA4 | - | +++ | ++ | - | - | ++ | ++ | - | - | ++ | +++ | - | - | + | + | - | - | - | - | - |
| | TA5 | - | +++ | +++ | + | - | ++ | ++ | - | - | ++ | ++ | - | - | - | - | - | - | - | - | - |
| | TA6 | - | + | ++ | - | - | | | - | - | | | - | - | - | - | - | - | - | - | - |
| | TA7 | + | +++++ | +++ | + | - | +++ | +++ | - | - | +++ | ++ | - | - | - | - | - | - | + | + | - |
| | TA8 | - | +++ | ++ | - | - | +++ | ++ | - | - | ++ | ++ | - | - | - | - | - | - | - | - | - |

| Genotype | Treatments | CIF (%) Cold 2d (Flower buds) | | | | CIF (%) Cold 3d (Flower buds) | | | | CIF(%) Cold 5d (Flower buds) | | | | CIF(%) Cold 7d (Flower buds) | | | | CIF(%) Cold 9d (Flower buds) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm |
| TLCV02 | TA0 | - | + | + | - | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA1 | - | ++++ | +++ | + | - | ++++ | +++ | - | - | +++ | ++ | + | - | - | + | - | - | + | - | - |
| | TA2 | - | ++ | ++ | - | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA3 | - | ++ | + | - | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA4 | - | +++ | ++ | + | - | +++ | +++ | - | - | +++ | ++ | + | - | - | - | - | - | - | - | - |
| | TA5 | - | ++++ | +++ | + | - | +++ | ++ | - | - | ++ | ++ | - | - | - | - | - | - | - | - | - |
| | TA6 | - | ++ | ++ | - | - | + | + | - | - | - | + | - | - | - | - | - | - | - | - | - |
| | TA7 | + | +++++ | ++++ | + | - | ++++ | ++++ | - | - | +++ | +++ | + | + | + | - | - | - | - | + | - |
| | TA8 | - | +++ | +++ | + | - | ++++ | +++ | - | - | +++ | ++ | - | + | + | - | - | - | + | + | - |
| CO3 | TA0 | - | + | + | - | - | + | + | - | - | + | - | - | - | - | - | - | - | - | - | - |
| | TA1 | + | ++++ | +++ | + | - | ++++ | +++ | - | - | ++ | ++ | - | - | + | + | - | - | + | - | - |
| | TA2 | - | ++ | + | - | - | ++ | + | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA3 | - | ++ | ++ | - | - | | | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA4 | - | +++ | ++ | + | - | +++ | ++ | - | - | +++ | ++ | + | - | - | - | - | - | + | - | - |
| | TA5 | - | +++ | ++ | - | - | ++ | ++ | - | - | ++ | ++ | - | - | | - | - | - | - | - | - |
| | TA6 | - | ++ | ++ | - | - | - | + | - | - | | | - | - | - | - | - | - | - | + | - |
| | TA7 | + | +++++ | ++++ | + | - | +++ | ++ | - | - | +++ | ++ | - | - | + | - | - | - | + | - | - |
| | TA8 | - | ++ | ++ | - | - | +++ | +++ | - | - | ++ | ++ | - | - | + | - | - | - | - | - | - |

(*Contd...*)

Table 2: (*Continued*)

| Genotype | Treatments | CIF (%) Cold 2d (Flower buds) | | | | CIF (%) Cold 3d (Flower buds) | | | | CIF(%) Cold 5d (Flower buds) | | | | CIF(%) Cold 7d (Flower buds) | | | | CIF(%) Cold 9d (Flower buds) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm | 2 mm | 4 mm | 6 mm | 9 mm |
| PKM01 | TA0 | - | + | + | - | - | + | + | - | - | + | + | - | - | - | - | - | - | - | - | - |
| | TA1 | + | +++ | +++ | + | - | +++ | +++ | - | - | +++ | +++ | - | - | - | - | - | - | - | - | - |
| | TA2 | - | ++ | + | - | - | + | + | - | - | ++ | + | - | - | - | - | - | - | - | - | - |
| | TA3 | - | ++ | + | - | - | + | + | - | - | + | - | - | - | - | - | - | - | - | - | - |
| | TA4 | - | +++ | +++ | + | - | +++ | +++ | - | - | +++ | +++ | + | - | - | - | - | - | - | - | - |
| | TA5 | - | +++ | +++ | - | - | +++ | +++ | - | - | +++ | +++ | - | - | - | - | - | - | - | - | - |
| | TA6 | - | ++ | ++ | - | - | | | - | - | | | - | - | - | - | - | - | - | - | - |
| | TA7 | + | +++++ | +++++ | + | - | +++ | +++ | - | - | +++ | +++ | - | - | - | - | - | - | - | - | - |
| | TA8 | + | +++ | +++ | + | - | +++ | +++ | - | - | +++ | +++ | + | - | - | - | - | - | - | - | - |

Note: CIF- Callus induction frequency: + = 1 to 5%, ++ = 6 to 10%, +++ = 11 to 15%, ++++ = 16 to 20%.

bud sizes short listed namely 4 and 6 mm had a corresponding anther length of 2 and 4 mm respectively (Figure 2C & 2D). These anthers were squashed for microspore stage analysis and the appropriate stage identified were uninucleate (Figure 2E) and vacuolated (Figure 2F) stage of microspore for 4 and 6 mm flower bud size respectively. Henceforth, the flower buds were collected according to this correlation during collection and used for further experiments.

The effect of flower bud size, pre-treatments and growth regulators of LE-1230 had a significant influence on callus induction. Among the two different flower bud sizes, consistently anthers from 4 mm long flower buds recorded the maximum response. The best pre-treatment observed was gamma irradiation with the highest CIF of 21.22 per cent. The best medium with maximum CIF (28.25%) was TA8 (MS + 2iP (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$). The cold shock (4 °C in dark) for two days improved the CIF to 12.51 from 8.68 per cent for fresh buds but when the duration was increased to five days; it had actually reduced the CIF (7.42%). However, gamma irradiation to the flower buds had improved the frequency to more than two times higher (21.22%) as compared to the fresh buds. The best interaction in the experiment with the maximum CIF (29.15) for LE 1230 genotype was 4 mm long flower buds in TA 8 (MS + 2iP (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$) medium after gamma irradiation (Table 3 & Figure 3).

The results obtained during the study on androgenesis of LE-1236 confirmed the best choice for flower buds was 4 mm long in all the treatments, the best pre treatment was gamma irradiation (18.49% CIF) and the best medium (22.20% CIF) was TA1 (MS + 2iP (1.0 mg $L^{-1}$) + IAA (2.0 mg $L^{-1}$)). Cold shock at 4 °C for 2 days improved the CIF from 9.41 to 12.64 per cent. Extended period of cold shock from 2 to 5 days had actually reduced the response from 12.64 to 8.24 per cent. After gamma irradiation, the CIF got increased to 18.49 per cent. Interaction effects were non-significant for flower bud size and medium combinations after gamma irradiation. The best combination in the whole experiment was from gamma irradiated anthers from 4 mm long buds that had the highest callus induction frequency 25 per cent in TA1 (MS + 2iP (1.0 mg $L^{-1)}$ + IAA (2.0 mg $L^{-1}$)) medium, followed by 23.55 per cent in TA7 (2iP (0.5 mg $L^{-1}$) + NAA (1.0 mg $L^{-1}$) + KIN (1.5 mg $L^{-1)}$) and TA8 (MS + 2iP (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$)) medium (Table 4 & Figure 4).

With respect to anthers of LE-1256 genotype, among the two different sizes flower buds observed, anthers from 4 mm long size flower buds were the best in all the treatments and gamma irradiation was the best treatment with highest CIFs induction frequency 19.71 per cent. Cold shock to flower buds either for 2 or 5 days actually recorded a lower CIF (10.44 and 8.10% respectively) than fresh (11.94%) flower buds. Results from growth regulator combinations documented TA1 (MS+2iP (1.0 mg $L^{-1}$) + IAA (2.0 mg $L^{-1)}$) as the best combination (30.50% CIF).The callus induction frequency got improved from 22.20 to 33.30 per cent when the anthers from 4 mm flower buds (fresh) were gamma irradiated and cultured on TA1 (MS + 2iP (1.0 mg $L^{-1}$) + IAA (2.0 mg $L^{-1}$)) medium which was the

Table 3: Effect of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of LE 1230

| Genotype | Medium | CIF (%) (Fresh) | | | CIF (%) (DC-2 days at 4°C) | | | CIF (%) (DC-5 days at 4°C) | | | CIF (%) (Gamma-4 Gy for 2 min ) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean |
| LE-1230 | TA0 | 2.70g | 1.80h | 2.25 | 3.60f | 2.70g | 3.15 | 2.70h | 1.80h | 2.25 | 4.10g | 2.70h | 3.40 |
| | TA1 | 12.00b | 10.17c | 11.08 | 18.46a | 12.00d | 15.23 | 10.13b | 10.17b | 10.15 | 26.35bc | 22.15e | 24.25 |
| | TA4 | 8.30e | 7.36f | 7.83 | 16.60b | 8.30e | 12.45 | 7.36d | 7.39d | 7.37 | 25.00cd | 23.60de | 24.30 |
| | TA5 | 8.30e | 7.36f | 7.83 | 14.77c | 9.20e | 11.98 | 6.43e | 3.66f | 5.04 | 22.20e | 18.00f | 20.10 |
| | TA7 | 13.86a | 9.23d | 11.55 | 19.40a | 18.40a | 18.90 | 16.60a | 7.30d | 11.95 | 26.35bc | 27.70ab | 27.02 |
| | TA8 | 12.93a | 10.17c | 11.55 | 15.70bc | 11.07d | 13.38 | 9.20c | 6.40e | 7.80 | 29.15a | 27.35ab | 28.25 |
| Mean | | 9.68 | 7.68 | 8.68 | 14.75 | 10.27 | 12.51 | 8.73 | 6.12 | 7.42 | 22.19 | 20.25 | 21.22 |
| | | F | T | F X T | C2 | T | C2 X T | C5 | T | C5 X T | G | T | G X T |
| SEd | | 0.158 | 0.274 | 0.387 | 0.195 | 0.334 | 0.478 | 0.146 | 0.252 | 0.357 | 0.270 | 0.467 | 0.661 |
| CD (0.05) | | 0.326** | 0.565** | 0.799** | 0.402** | 0.697** | 0.986** | 0.301** | 0.521** | 0.737** | 0.557** | 0.964** | 1.364* |

CIF-Callus induction frequency, DC-Dark cold; *, ** Significant at 5% and 1% level, respectively; NS-Non significant

Table 4: Effect of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of LE 1236

| Genotype | Medium | CIF(%) (Fresh) | | | CIF(%) (DC-2 days at 4°C) | | | CIF(%) (DC-5 days at 4°C) | | | CIF(%) (Gamma-4 Gy for 2 min) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean |
| LE1236 | TA0 | 3.60h | 1.80i | 2.70 | 4.50g | 2.70h | 3.60 | 2.70g | 1.80h | 2.25 | 5.50 | 2.77 | 4.13 |
| | TA1 | 12.00bc | 9.23e | 10.61 | 21.26b | 12.00de | 16.63 | 8.30e | 9.24d | 8.77 | 25.00 | 19.40 | 22.20 |
| | TA4 | 7.36f | 6.40g | 6.88 | 15.70c | 8.15f | 11.92 | 10.13c | 7.37f | 8.75 | 21.00 | 18.00 | 19.50 |
| | TA5 | 10.17d | 9.20e | 9.68 | 12.00de | 11.70e | 11.85 | 7.30f | 8.60de | 7.95 | 22.20 | 20.80 | 21.50 |
| | TA7 | 18.46a | 12.90b | 15.68 | 23.13a | 12.90d | 18.01 | 12.90a | 10.17c | 11.53 | 23.55 | 19.40 | 21.47 |
| | TA8 | 11.70c | 10.17d | 10.93 | 12.93c | 14.73d | 13.83 | 11.23b | 9.23d | 10.23 | 23.55 | 20.80 | 22.17 |
| Mean | | 10.54 | 8.28 | 9.41 | 14.92 | 10.36 | 12.64 | 8.76 | 7.73 | 8.24 | 20.13 | 16.86 | 18.49 |
| | | F | T | F X T | C2 | T | C2 X T | C5 | T | C5 X T | G | T | G X T |
| SEd | | 0.166 | 0.287 | 0.406 | 0.197 | 0.340 | 0.481 | 0.153 | 0.266 | 0.376 | 0.246 | 0.426 | 0.602 |
| CD (0.05) | | 0.342** | 0.593** | 0.838** | 0.406** | 0.703** | 0.994** | 0.317** | 0.549** | 0.776** | 0.508** | 0.879** | 1.243NS |

CIF-Callus induction frequency, DC-Dark cold; *, ** Significant at 5% and 1% level, respectively; NS-Non significant

Table 5: Effect of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of LE 1256

| Genotype | Medium | CIF(%) (Fresh) | | | CIF(%) (DC-2 days at 4°C ) | | | CIF(%) (DC-5 days at 4°C ) | | | CIF(%) (Gamma-4 Gy for 2 min) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean |
| LE-1256 | TA0 | 4.50h | 2.70i | 3.60 | 3.60g | 1.80h | 2.70 | 2.70h | 1.80h | 2.25 | 5.50g | 5.10g | 5.30 |
| | TA1 | 22.20b | 10.17e | 16.18 | 14.77b | 11.93c | 13.35 | 12.93a | 10.13a | 11.53 | 33.30a | 27.70b | 30.50 |
| | TA4 | 11.94d | 6.45g | 9.19 | 8.30f | 9.30de | 8.80 | 6.40g | 6.43g | 6.41 | 22.20d | 16.60f | 19.40 |
| | TA5 | 14.77c | 11.55d | 13.16 | 9.20e | 8.30f | 8.75 | 7.36f | 8.30f | 7.83 | 23.60cd | 22.20d | 22.90 |
| | TA7 | 24.96a | 12.00d | 18.48 | 16.60a | 14.73b | 15.66 | 12.00b | 11.70b | 11.85 | 25.00c | 19.40e | 22.20 |
| | TA8 | 13.83c | 8.30f | 11.06 | 16.60a | 10.17d | 13.38 | 8.30e | 9.23e | 8.76 | 19.40e | 16.60f | 18.00 |
| Mean | | 15.37 | 8.52 | 11.94 | 11.51 | 9.37 | 10.44 | 8.28 | 7.93 | 8.10 | 21.50 | 17.93 | 19.71 |
| | | F | T | F X T | C2 | T | C2 X T | C5 | T | C5 X T | G | T | G X T |
| SEd | | 0.191 | 0.331 | 0.469 | 0.176 | 0.304 | 0.430 | 0.152 | 0.264 | 0.373 | 0.258 | 0.446 | 0.631 |
| CD (0.05) | | 0.395** | 0.684** | 0.967** | 0.363** | 0.628** | 0.888** | 0.3141* | 0.544** | 0.769** | 0.531** | 0.920** | 1.302* |

CIF-Callus induction frequency, DC-Dark cold; *, ** Significant at 5% and 1% level, respectively; NS-Non significant

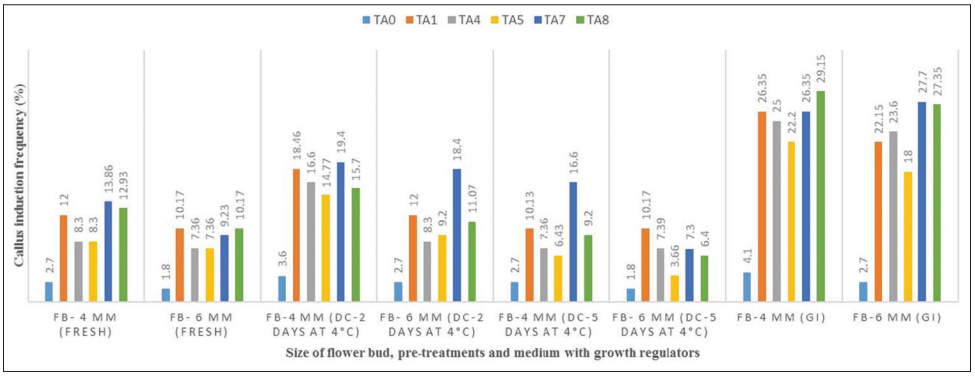


**Figure 3:** Response of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of LE 1230. TA0 - MS basal; TA1 - MS + 2iP (1.0) + IAA (2.0); TA4 - MS + 2iP (1.0) + NAA (2.0); TA5 - MS + 2iP (0.5) + BAP (0.5) + NAA (2.0); TA7 - MS + 2iP (0.5) + Kinetin (1.5) + NAA (1.0); TA8 - MS + 2iP (0.5) + NAA (0.5),PGR( mg $L^{-1}$)

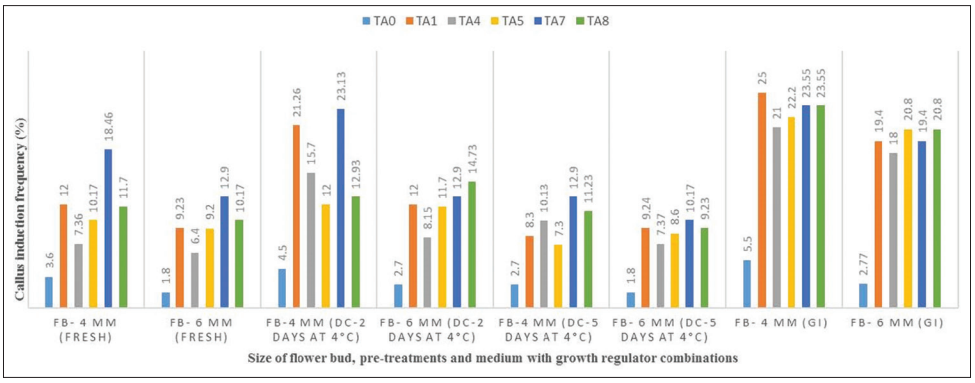


**Figure 4:** Response of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of LE 1236 TA0 - MS basal; TA1 - MS + 2iP (1.0) + IAA (2.0); TA4 - MS + 2iP (1.0) + NAA (2.0); TA5 - MS + 2iP (0.5) + BAP (0.5) + NAA (2.0); TA7 - MS + 2iP (0.5) + Kinetin (1.5) + NAA (1.0); TA8 - MS + 2iP (0.5) + NAA (0.5) ,PGR( mg $L^{-1}$)

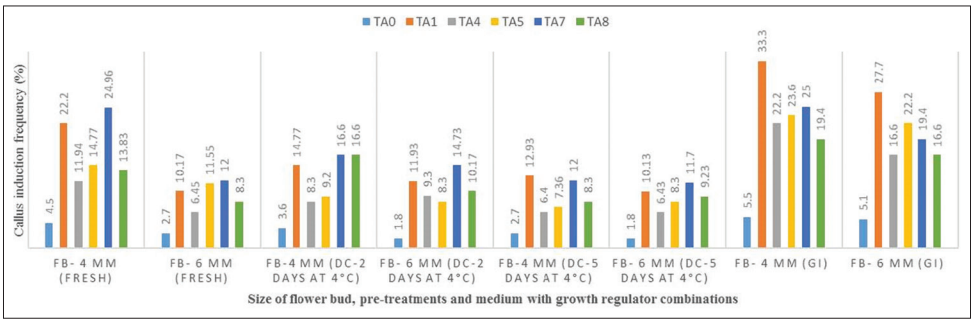


**Figure 5:** Response of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of LE 1256. TA0 - MS basal; TA1 - MS + 2iP (1.0) + IAA (2.0); TA4 - MS + 2iP (1.0) + NAA (2.0); TA5 - MS + 2iP (0.5) + BAP (0.5) + NAA (2.0); TA7 - MS + 2iP (0.5) + Kinetin (1.5) + NAA (1.0); TA8 - MS + 2iP (0.5) + NAA (0.5), PGR( mg $L^{-1}$)

best combination of treatments for the maximum CIF in the whole experiment. The next better performance was also in same medium (TA1) from anthers in 6 mm long flower buds (27.70%) (Table 5 & Figure 5).

Anthers of TLCV 2 genotype from 4 mm long flower bud were found to be the best in all treatments. Among the pre treatments, gamma irradiation was found to be the best (27.88%) followed by cold shock at 4 °C for 2 days (18.62%). Gamma irradiation had improved the CIF of anthers 2.5 times as that of anthers from fresh flower buds (12.87 %). The CIF from cold pre-treated flower buds at 4 °C for 5 days was almost same as that of anthers from fresh flower buds. MS medium supplemented with 2iP (0.5 mg $L^{-1}$) + NAA (1.0 mg $L^{-1}$) + KIN (1.5 mg $L^{-1}$) (TA7) was observed as the best medium combination for fresh, cold shock and gamma irradiated flowers (34.95% CIF). The best CIF (38.80%) observed was from anthers of gamma irradiated 4 mm long flower buds inoculated in TA7 medium (Table 6 & Figure 6).

In the androgenesis study with the genotype PKM 1, 4 mm size anthers were found the best and gamma irradiation to flower buds was the best stress treatment (22.75% CIF). Cold shock to flower buds at 4°C for 2 and 5 days actually reduced CIF for fresh from 12.05 to 9.05 and 8.21 per cent respectively. Gamma irradiation treatment uniformly improved the CIF from anthers irrespective of growth regulator combinations and 4 or 6 mm sized flower buds. The gamma irradiation to flower buds had improved the CIF to 22.75 per cent compared to the CIF (12.05%) of fresh flowers. TA8 (MS+ 2iP (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$)) was found the best medium for androgenesis. The maximum CIF (30.50%) in the experiment recorded was from anthers of 4 mm long gamma irradiated flower buds inoculated in TA8 medium (Table 7 & Figure 7).

In the genotype TNAU hybrid CO3, it was observed that anthers from 4 mm long flower buds were consistently performing better than anthers from 6 mm long flowers. Anthers from gamma irradiated flower buds were most responsive to callus induction (25.38%). The gamma irradiation had nullified the effect due to size of the flower buds on callus induction. In other words, anthers from 4 mm and 6 mm flower buds performed equally well when inoculated in medium combination with any of the growth regulators combinations tried after their irradiation. Among the medium combinations, TA1 (2iP (1.0 mg $L^{-1}$) + IAA (2.0 mg $L^{-1}$)) was the best as it has registered a maximum of 31.90 per cent CIF compared to other medium combinations.The CIF had improved from 9.91 for fresh to 14.79 per cent when anthers were collected from pre-treated flower buds (cold and dark for 2 days at 4 °C) but the same treatment if it was extended for five days, there was a significant reduction in CIF (8.72%) which was lesser (9.91%) than the fresh anthers. The best performance (34.70% CIF) was from anthers of 4mm long flower buds gamma irradiated at 4 Gy for 2 minutes when inoculated in TA 1 medium (Table 8 & Figure 8).

At the initial phase during the callus induction, the anthers curl, appeared to be swollen, and later anther wall got dried up after 30 days (Figure 9A). Then callus induction was observed at the

Table 6: Effect of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of TLCV 2

| Genotype | Medium | CIF (%) (Fresh) | | | CIF (%) (DC-2 days at 4°C) | | | CIF (%) (DC-5 days at 4°C) | | | CIF (%) (Gamma-4 Gy for 2 min ) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean |
| TLCV 2 | TA0 | 3.60h | 1.80i | 2.70 | 4.10f | 2.70g | 3.40 | 2.70f | 1.80g | 2.25 | 8.30f | 2.70g | 5.50 |
| | TA1 | 18.50b | 13.83cd | 16.16 | 26.80a | 19.40d | 23.10 | 12.00cd | 11.70d | 11.85 | 30.50cd | 29.10de | 29.80 |
| | TA4 | 10.13g | 9.20g | 9.66 | 22.20bc | 15.70e | 18.95 | 13.83b | 10.17e | 12.00 | 36.11a | 27.70e | 31.90 |
| | TA5 | 14.77c | 12.93de | 13.85 | 21.26c | 16.60e | 18.93 | 12.93bc | 12.90bc | 12.91 | 36.11a | 29.10de | 32.60 |
| | TA7 | 26.80a | 19.40b | 23.10 | 27.70a | 24.03b | 25.86 | 16.60a | 16.60a | 16.60 | 38.80a | 31.10bcd | 34.95 |
| | TA8 | 12.00ef | 11.50f | 11.75 | 26.35a | 16.60e | 21.47 | 12.45cd | 9.70e | 11.07 | 33.25b | 31.90bc | 32.57 |
| Mean | | 14.30 | 11.44 | 12.87 | 21.40 | 15.83 | 18.62 | 11.75 | 10.47 | 11.11 | 30.51 | 25.26 | 27.88 |
| | | F | T | F X T | C2 | T | C2 X T | C5 | T | C5 X T | G | T | G X T |
| SEd | | 0.120 | 0.346 | 0.489 | 0.249 | 0.430 | 0.609 | 0.182 | 0.315 | 0.445 | 0.326 | 0.564 | 0.797 |
| CD (0.05) | | 0.412** | 0.713** | 1.009** | 0.512** | 0.888** | 1.2563* | 0.375** | 0.649** | 0.918** | 0.672** | 1.164** | 1.646** |

CIF-Callus induction frequency, DC-Dark cold; *, ** Significant at 5% and 1% level, respectively; NS-Non significant

Table 7: Effect of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of PKM 1

| Genotype | Medium | CIF(%) (Fresh) | | | CIF(%) (DC-2 days at 4°C) | | | CIF(%) (DC-5 days at 4°C) | | | CIF(%) (Gamma-4 Gy for 2 min ) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean |
| PKM 1 | TA0 | 4.10g | 1.80h | 2.95 | 2.70g | 1.80h | 2.25 | 3.60h | 1.80i | 2.70 | 5.50 | 3.60 | 4.55 |
| | TA1 | 18.46b | 11.60d | 15.03 | 11.07b | 9.20d | 10.13 | 10.13c | 9.23d | 9.68 | 27.35 | 23.16 | 25.25 |
| | TA4 | 14.66c | 7.30f | 10.98 | 8.30e | 8.30e | 8.30 | 11.07b | 8.30e | 9.68 | 27.75 | 23.55 | 25.65 |
| | TA5 | 13.87c | 7.36f | 10.61 | 10.17cd | 9.23d | 9.70 | 7.37f | 6.43g | 6.90 | 27.70 | 24.95 | 26.32 |
| | TA7 | 24.03a | 13.83c | 18.93 | 18.46a | 7.30f | 12.88 | 14.77a | 8.30e | 11.53 | 27.75 | 22.20 | 24.97 |
| | TA8 | 17.53b | 10.17e | 13.85 | 11.96b | 10.17c | 11.06 | 10.17c | 7.36f | 8.76 | 30.50 | 29.10 | 29.80 |
| Mean | | 15.44 | 8.67 | 12.05 | 10.44 | 7.66 | 9.05 | 9.51 | 6.90 | 8.21 | 24.42 | 21.09 | 22.75 |
| | | F | T | F X T | C2 | T | C2 X T | C5 | T | C5 X T | G | T | G X T |
| SEd | | 0.192 | 0.332 | 0.470 | 0.162 | 0.281 | 0.397 | 0.153 | 0.265 | 0.375 | 0.282 | 0.488 | 0.690 |
| CD (0.05) | | 0.396** | 0.686** | 0.970* | 0.335** | 0.580** | 0.820** | 0.316** | 0.548** | 0.775** | 0.582** | 1.007** | 1.425NS |

CIF-Callus induction frequency, DC-Dark cold; *, ** Significant at 5% and 1% level, respectively; NS-Non significant

Table 8: Effect of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of TNAU hybrid CO 3

| Genotype | Medium | CIF(%) (Fresh) | | | CIF (%) (DC-2 days at 4°C) | | | CIF(%) (DC-5 days at 4°C ) | | | CIF (Gamma-4 Gy for 2 min ) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean | 4 mm | 6 mm | Mean |
| TNAU hybrid CO 3 | TA0 | 2.77g | 1.80h | 2.28 | 4.10 | 2.70 | 3.40 | 3.60f | 1.80g | 2.70 | 5.50 | 2.77 | 4.13 |
| | TA1 | 15.70b | 9.20d | 12.45 | 24.96 | 12.00 | 18.48 | 9.20d | 10.17c | 9.68 | 34.70 | 29.10 | 31.90 |
| | TA4 | 10.17c | 6.40f | 8.28 | 18.46 | 12.90 | 15.68 | 11.07b | 9.20d | 10.13 | 29.10 | 26.35 | 27.72 |
| | TA5 | 10.17c | 7.36e | 8.76 | 16.60 | 12.90 | 14.75 | 8.30e | 9.23d | 8.76 | 30.50 | 23.60 | 27.05 |
| | TA7 | 22.16a | 16.60b | 19.38 | 25.90 | 16.60 | 21.25 | 13.83a | 11.70b | 12.76 | 31.50 | 30.50 | 31.00 |
| | TA8 | 9.23d | 7.37e | 8.30 | 17.53 | 12.94 | 15.23 | 8.30e | 8.26e | 8.28 | 31.90 | 29.10 | 30.50 |
| Mean | | 11.70 | 8.12 | 9.91 | 17.92 | 11.67 | 14.79 | 9.05 | 8.39 | 8.72 | 27.20 | 23.57 | 25.38 |
| | | F | T | F X T | C2 | T | C2 X T | C5 | T | C5 X T | G | T | G X T |
| SEd | | 0.172 | 0.298 | 0.421 | 0.216 | 0.374 | 0.529 | 0.158 | 0.274 | 0.387 | 0.304 | 0.527 | 0.746 |
| CD (0.05) | | 0.355** | 0.615** | 0.869** | 0.445NS | 0.772** | 1.092 NS | 0.326** | 0.565** | 0.799** | 0.628** | 1.088** | 1.539NS |

CIF-Callus induction frequency, DC-Dark cold; *, ** Significant at 5% and 1% level respectively; NS-Non significant

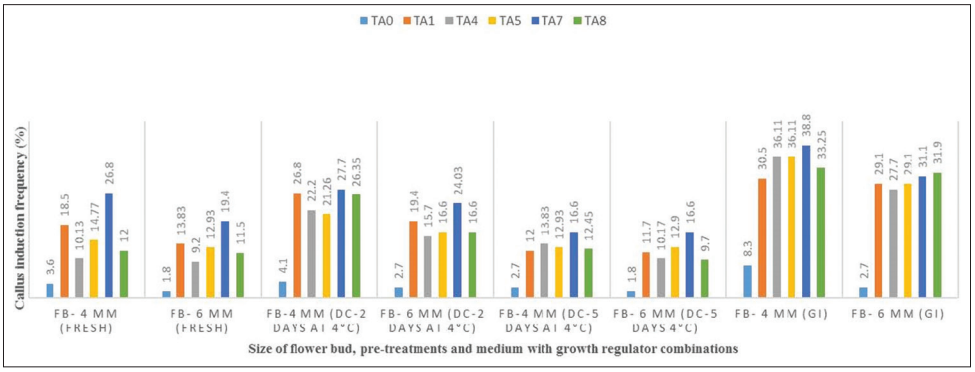


**Figure 6:** Response of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of TLCV 2. TA0 - MS basal; TA1 - MS + 2iP (1.0) + IAA (2.0); TA4 - MS + 2iP (1.0) + NAA (2.0); TA5 - MS + 2iP (0.5) + BAP (0.5) + NAA (2.0); TA7 - MS + 2iP (0.5) + Kinetin (1.5) + NAA (1.0); TA8 - MS + 2iP (0.5) + NAA (0.5) ,PGR( mg $L^{-1}$)

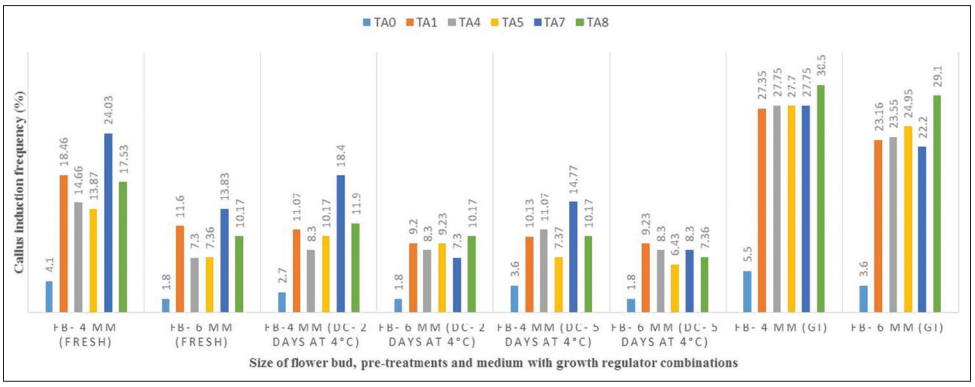


**Figure 7:** Response of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of PKM 1. TA0 - MS basal; TA1 - MS + 2iP (1.0) + IAA (2.0); TA4 - MS + 2iP (1.0) + NAA (2.0); TA5 - MS + 2iP (0.5) + BAP (0.5) + NAA (2.0); TA7 - MS + 2iP (0.5) + Kinetin (1.5) + NAA (1.0); TA8 - MS + 2iP (0.5) + NAA (0.5) ,PGR( mg $L^{-1}$)

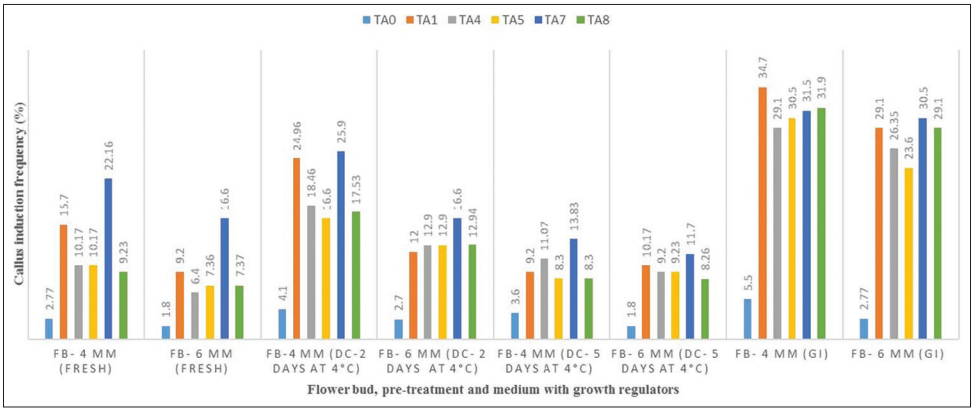


**Figure 8:** Response of size of flower bud, pre-treatments and growth regulator combinations on callus induction from anthers of TNAU hybrid CO 3. TA0 - MS basal; TA1 - MS + 2iP (1.0) + IAA (2.0); TA4 - MS + 2iP (1.0) + NAA (2.0); TA5 - MS + 2iP (0.5) + BAP (0.5) + NAA (2.0); TA7 - MS + 2iP (0.5) + Kinetin (1.5) + NAA (1.0); TA8 - MS + 2iP (0.5) + NAA (0.5), PGR( mg $L^{-1}$)

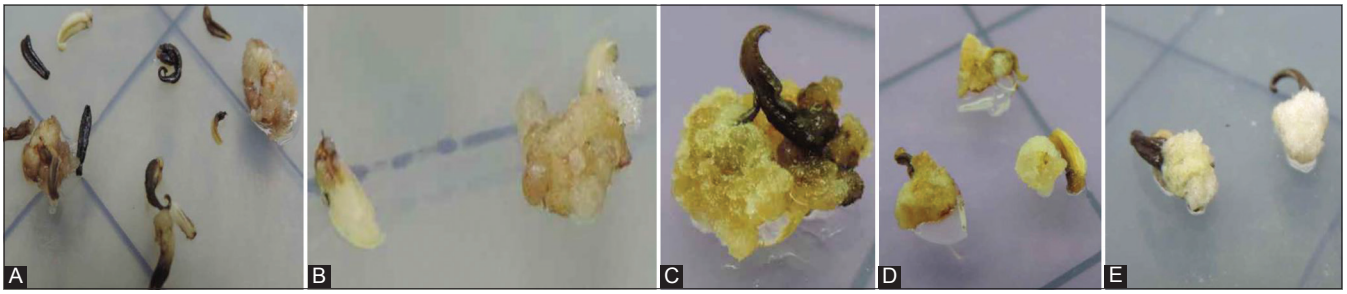


**Figure 9:** Callus induction from anthers of tomato genotypes. A) Drying of anther wall; B) Brown calli from anthers of fresh flower buds; C, D) Translucent light creamy calli from anthers of flower buds after cold shock; E) Amorphous white calli from anthers of irradiated flowers

posterior end in case of anthers from fresh and gamma irradiated flowers and from middle of the locule in case of cold shock. The callus induced was brown tinted, globulated type II Calli in case of fresh flowers (Figure 9B). The callus was translucent, light creamy in case of anthers from cold shock (Figure 9C & D). The callus induced was white amorphous in case of gamma irradiation (Figure 9E).

## Regeneration

The callus that was sub cultured in the callus induction medium (Figure 10A) was transferred to medium with different levels of either Zeatin or BAP (Figure 10B). There was greening of callus from all the genotypes both in Zeatin and BAP supplemented medium (Figure 10C). Initially, there were appearance of dark green globules indicating the meristemoids (Figure 10D) and soon these globules had initiated regeneration of shoot (Figure 10E & F), only callus induced from anthers of TNAU hybrid CO 3 that was sub cultured in Zeatin @ 0.5 mg $L^{-1}$ supplemented medium had initiated shoots (Figure 10G). The micro shoots induced were separated and inoculated in half MS medium supplemented with $GA_3$ (0.5 mg $L^{-1}$) for shoot elongation. After 4-6 weeks, the elongated shoots were transferred to half strength MS medium enhanced with IBA (1 mg $L^{-1}$). Profused rooting was noticed from the base of the shoot in 4-5 weeks.

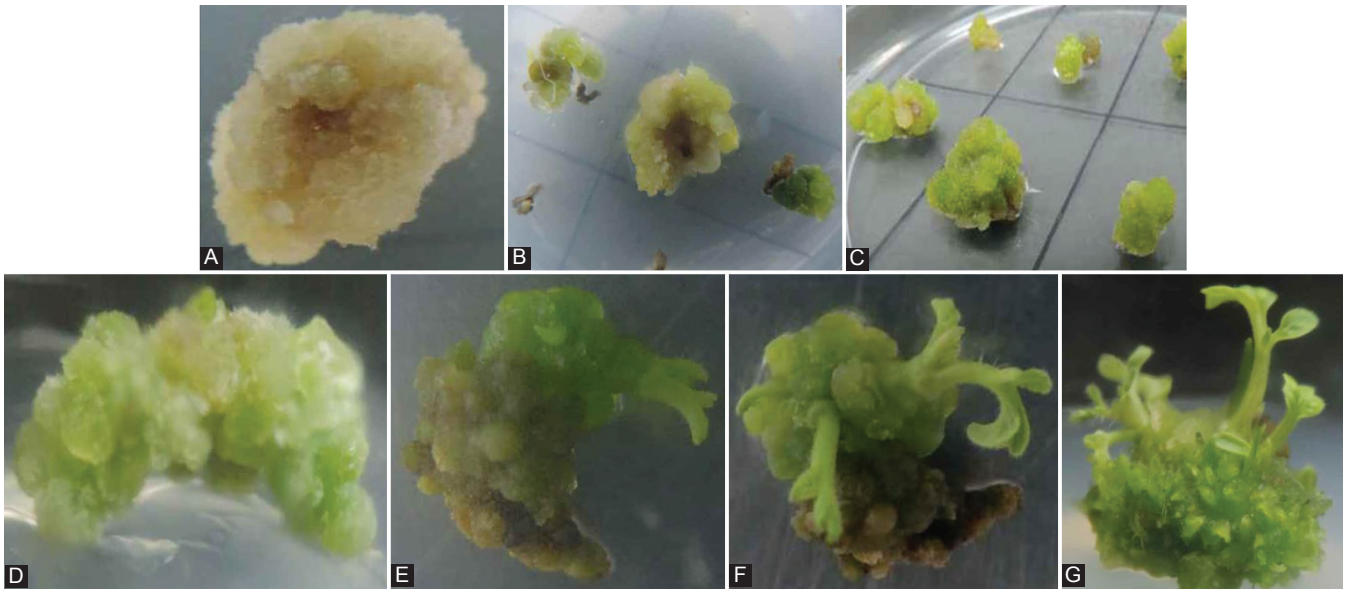


**Figure 10:** Shoot regeneration. A) Callus; B) Callus in shoot regeneration medium; C) greening of callus; D) Meristemoids; E, F) Shoot regeneration; G) Clump of shoots

### Characterization of *In Vitro* Generated Haploids through Stomatal Count

As a preliminary ploidy confirmation, for stomatal count with leaves was analyzed from the plants regenerated *in vitro* from anther callus along with greenhouse grown plants (diploid) for comparison. The count observed was a single stoma in leaves of TC plants and 3-4 in normal plants (Figure 11A & B).

Among the genotypes, TLCV2 was found to be the best performing genotype followed by TNAU tomato hybrid CO 3. Among the pre-treatments tried, gamma irradiation 4 Gy for 2 minutes was found to be best for the highest callus induction as it remarkably improved the callus induction when compared to anthers isolated from fresh and cold treated flower buds. The cold and dark pre-treatment at 4 °C had improved the callus induction frequency in all the genotypes compared to fresh except LE 1256 and PKM genotypes. But, when the duration was extended to five days, there was reduction in CIF invariably in all the genotypes. Among the medium combinations tried for callus induction, TA8 was found to be the best for anthers from LE1230 and PKM1 genotypes, TA1 for LE 1238, LE 1256 and TNAU Tomato hybrid CO3 and TA7 for the genotype TLCV 2. Though basal medium had responded, the performance was negligible. The callus after sub culturing was transferred to regeneration medium consisting of BAP or Zeatin, but only the callus from TNAU hybrid CO 3 genotype had induced shoot initials in the medium containing Zeatin (0.5 mg $L^{-1}$). These shoots were elongated in $GA_3$ medium and later elongated in IBA containing medium. The stomatal counts were 3-4 in diploids and single in *in vitro* regenerated plants.

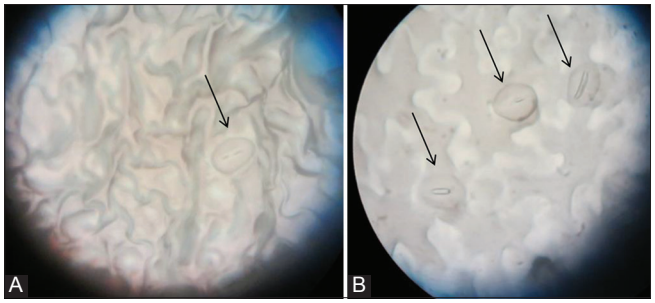


**Figure 11:** Characterization of ploidy with stomatal count. A) *In vitro* generated haploids; B) Plant generated through seeds (diploids). The arrow indicates the stomata

## DISCUSSION

Haploid plants can be obtained spontaneously through parthenogenesis, semigamy or polyembryony, but the frequency of haploid is very low (Dunwell, 2010; Asadi & Seguí-Simarro, 2021; Seguí-Simarro *et al.*, 2021b; Badulescu *et al.*, 2022). Traditional breeding efforts to improve the quality and yield in crops like tomatoes and other solanaceous crops are labour intensive, costly and time consuming since many generations of crossing and selection are routinely required for the cultivar development (Morrison *et al.*, 1986). Haploid production through androgenesis and gynogenesis are the most important *in vitro* breeding techniques used in modern breeding programmes (Don Palmer & Keller, 2005; Weyen, 2021). Pure lines (doubled haploid plants) can be obtained in a short time by using *in vitro* anther culture (Seguí-Simarro *et al.*, 2021b).

Anther culture had been successfully reported from crops like egg plant, tobacco, rice, maize (Alan *et al.*, 2021; Mir *et al.*, 2021) and in tomato (Marin-Montes *et al.*, 2022). A successful plant tissue culture protocol starts with effective explants sterilization. In the current study, a surface sterilization treatment with $HgCl_2$ (0.1%) for three min, followed by four rinses with sterile water was used for the disinfection of the flower buds and later anthers were dissected out for androgenesis experiments.

In the present study, initially the whole developmental stages of the flower bud were categorized from 1-14, 1 being unopened youngest flower bud and 14 was the fully opened flower bud. As the early stage flower buds had microspores at tetrad stage and later stages with bi nucleate stage, the four middle size flower buds were chosen *viz*., 2, 4, 6 and 9 mm for the preliminary study and the callus induction experiments were conducted in 8 different medium combinations with cold shock in dark at 4 °C for different durations (2, 4, 7, 9 days). Among these four sized flower buds, based on the performance, anthers from those flower buds (4 and 6 mm) that had registered a CIF of more than 5 per cent were shortlisted for further experiments. Among the pre treatments, cold shock at 4 °C for 2 and 4 days alone were shortlisted based on the CIF (more than 5%). The size and morphology of flower buds could be used as an indirect indication for determining microspore stage development (Adhikari & Kang, 2017). A proper correlation between microspore stage and morphology and physical parameters related to flower buds had to be established prior to inoculation which could bring forth a better selection of appropriate flower buds from green house without resorting to cytological study each time. In the current study, 4 mm flower buds containing uninucleate stage microspores were more responsive in all the genotypes. In line with this, in most of species, it has been reported that uninucleate stage of anther was most responsive for androgenesis for different varieties of tomato (Bal & Abak, 2007; Niazian *et al*., 2019). In different plant species, it was recommended that uninucleate microspores were most appropriate for induction of embryo formation during anther culture (Eliby *et al*., 2022). According to Supena *et al*. (2006) and İlhan and Kurtar (2022), the determining factor for successful anther culture is the selection of flower buds containing over 50 per cent microspores in the late-uninucleate phase. However, the early binucleate microspore stage is also amenable to androgenesis induction (Kim *et al*., 2004, 2008; González-Melendi *et al*., 2005). Kotyal *et al*. (2022) reported that anthers containing over 75 per cent microspores in early binucleate phase were optimal for embryo production in isolated microspore cultures. In *Brassica oleracea*, the callus induction frequencies of all stages of flower buds were found to be increasing (Alan *et al*., 2021).

In the current study, among the genotypes *viz*., (LE-1230, LE-1236, LE-1256, TLCV 2, PKM 1 and TNAU tomato hybrid CO 3) investigated for their performance on callus induction during androgenesis, all genotypes studied had the ability to form callus when anthers were used for the *in vitro* culture but TLCV 2 was found to be the best performing genotype. The genotype is the most important and often limiting factor in the pepper androgenesis (Irikova *et al*., 2011). Haploid production is greatly influenced by the genotypes induced by irradiated pollen (Seguí-Simarro *et al*., 2021a). A similar influence of genotype in support of present results was published by Sivachandran *et al*. (2017) in cocoa.

Stress pretreatments had a greater influence on induction of microspores towards callus induction and cold shock/irradiation divert most of the microspores from gametophytic to sporophytic pathway. In the present study during androgenesis in tomato, three different pre-treatments were given to the flower buds *viz*., dark cold pre-treatment at 4 °C for two and five days and gamma irradiation to investigate the effect of pre-treatments on callus induction along with fresh flower buds for comparison. Anthers dissected from whole flower buds that were kept in dark and cold pre-treatment at 4 °C for two days responded effectively as compared to the anthers from fresh flower buds in four of the genotypes (LE 1230, LE 1236, TLCV2 and TNAU tomato hybrid CO 3). However, if the pre-treatment (dark cold pre-treatment at 4 °C) period was extended to five days, there was a reduction in CIF of all the genotypes. The reduction was not only lesser than two days dark cold pre-treatment at 4 °C but also lesser than that of fresh flower buds. Flower buds subjected to heat shock or cold or in combination were found to enhance androgenesis in brinjal (Thriveni *et al*., 2020). The beneficial effect of cold treatment to flower buds prior to anther culture was first reported by Nitsch and Norreel (1973) with pollen culture of *Datura innoxia*. Cold pre-treatment of flower buds from 24 to 100 h before excising anthers for culture stimulated the androgenic response in pepper (Morrison *et al*., 1986; Supena *et al*., 2006). Similar results were observed in pepper (*Capsicum annuum* L.) where embryogenesis was improved and one to three plants per 100 anthers were obtained when 4°C temperature for 48 h was applied as a pre-treatment to flower buds (Thriveni *et al*., 2020). However, a different kind of observation was also documented in pepper and there were no significant effects of cold pre-treatment on flower buds (Vagera & Havranek, 1985; Kim *et al*., 2004; Jha *et al*., 2021). Özkum Çiner and Tipirdamaz (2002) and Ramasamy *et al*. (2022) also found a reduced embryo formation after cold pre-treatment due to callus induction on the explants and explained that the pretreatment effect could also vary based on the culture condition as well as genotypes. It was observed that among the pre-treatments used, gamma irradiation treatment was found to be best response to callus induction *viz*., 21.22%, 18.49%, 19.71%, 27.88%, 29.80% and 31.90% in LE-1230, LE-1236, LE-1256, TLCV 2, PKM-1 and TNAU tomato hybrid CO 3 genotypes respectively. Shtereva *et al*. (1998) found out similar results in tomato, that the cold shock and gamma rays treatments of anthers enhanced callus production, shoot formation and plant regeneration. Treatments at 4 °C (48 h) and 10 °C (9 days) stimulated these processes and combined treatment of anthers with 4 Gy and 10 °C for nine days was the most efficient.

The results suggested that various genotypes had different requirement of nutrients and growth regulators. The highest callus induction frequency for anthers from different size of fresh flower buds for different genotypes were TA8(MS + 2iP (0.5 mg $L^{-1}$) + NAA (0.5 mg $L^{-1}$)) for LE 1230 and PKM1, TA1(MS + 2iP (1.0 mg $L^{-1}$) + IAA (2.0 mg $L^{-1}$)) for CO 3, LE 1238 and LE 1256 and TA7 (MS + 2iP (0.5 mg $L^{-1}$) + Kinetin (1.5 mg $L^{-1}$) + NAA (1.0 mg $L^{-1}$)) for TLCV2 and the least was always recorded in TA0 (MS basal). The above results were in line with findings of Hassan and Islam (2021) and Marin-Montes *et al*. (2022) in tomato, Simeonova *et al*. (1990) and Phuong (2021) in chillies, Alan *et al*. (2021) in rice. Gulshan and Sharma (1981) verified that the medium containing 2.0 mg $L^{-1}$ NAA and 1.0 mg $L^{-1}$ kinetin was the most efficient for callus formation in tomato. Shtereva *et al*. (1998) considered NAA (2 mg $L^{-1}$) + 2iP

(1 mg $L^{-1}$) as the better growth regulators combination during their study on factors affecting induction of androgenesis in tomato. During androgenesis studies in tomato, genotype, pre-treatment and growth regulator combination had a significant effect on induction frequency of callus induction and type of callus. All the genotypes induced callus and subsequently the callus was transferred to fresh medium of same composition at monthly intervals and then transferred to regeneration medium containing two different cytokinins, zeatin and BAP at different concentrations. There were green growing points and greening of calli in MS medium at all levels of zeatin. But, only the calli that was sub cultured from anthers of TNAU tomato hybrid CO 3 could ultimately initiate large number of green globules and later green shoot initials in MS medium supplemented with Zeatin (0.5 mg $L^{-1}$). The above results were similar with Seguí-Simarro and Nuez (2005, 2007) and Corral-Martínez and Seguí-Simarro (2012). They used zeatin riboside at a concentration of 0.25 mg $L^{-1}$ in with MS medium for regeneration. But in the present study, the requirement of zeatin for shoot initiation was higher. In other genotypes, the calli did not respond for regeneration. Similar observations were made by earlier workers. Callus was initiated but the induction of somatic embryos did not occur, which was similar with the results reported by Binzel *et al.* (1996). Not all embryos emerging from anthers were able to regenerate in fully developed plants. Similar results in pepper (*Capsicum annuum* L.) were obtained by Koleva-Gudeva *et al.* (2008). They investigated 21 different pepper genotypes and reported that 12 possessed potential for direct somatic embryo formation. Genotype not only had an influence on callus induction, induction of embryogenesis and subsequent development of embryos to plant regenerants but also influenced the correlation between obtained haploid and spontaneously diploidized regenerants as reported by Dolcet-Sanjuan *et al.* (1997) and Koleva Gudeva and Trajkova (2012). The stomatal counts recorded were 3-4 in diploids and single in *in vitro* regenerated plants. Similarly, an easy method was developed to identify haploids by comparing the number of chloroplasts in stomatal guard cells, size of stomatal guard cells in haploid and diploid *A. thaliana* (Watts *et al.*, 2023).

## CONCLUSION

Results of the present study indicate that induction of haploids is a dependent process on several factors. Effect of size of flower bud, pre-treatments and medium with growth regulator combinations on callus induction from anthers of tomato genotypes were analyzed for production of haploid cultures. Haploid cultures can be used for production of homozygous plants and can be used directly as parental lines in the production of $F_1$ lines in breeding program. Reducing the ploidy level of breeding material via androgenesis might accelerate plant improvement efforts by recovery of recessive mutations and unique genetic recombination's and chromosome doubling of homozygotes can be used directly in hybrid production.

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