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# The effects of cold preculture and heat shock treatment on the *in vitro* Androgenesis of brinjal (*Solanum melongena* L.)

**V Thriveni, D Sharma and Zenu Jha**

**Abstract**
The study was conducted to investigate the effect of pre-treatments, incubation temperature and culture media in different eggplant genotypes through anther culture. The eggplants were given with different 1-4 hours of pre-treatments in combination with two different incubation temperatures 25 ºC for 3 weeks and 35 ºC for 8 days under dark condition. Anthers were implanted in two different C medium MS Media and $N_6$ supplemented with 2,4-D 2mg/l. Among different treatment combinations the treatment MS media +3 hours pre-treatment + incubation at 25 ºC for 3weeks showed maximum embryogenic calli induction with response frequency 19.55 per cent and took least number of days for callus initiation (12 days) and lowest calli responding frequency with 1h pretreatment interaction with N6 medium supplemented with 2,4-D @ 2ml/l (0.33%). A preculture treatment of -3h at 5 ºC exhibited the maximum calli induction frequency (12.34%) fallowed by 2.30h preculture (8.55) and minimum frequency was obtained in A1-1h preculture (0.39).

**Keywords:** preculture, media, incubation temperature, antherculture, brinjal

## Introduction
Brinjal (*Solanum melongena*. L) is the most commonly cultivated fruit vegetable crop of India belongs to the family Solanaceae. Vavilov (1928) [1] mentioned that Indo-Burma region is the centre of origin of egg plants. The term 'eggplant' applies to a large number of species of the genus *Solanum* (cultivated, semi wild, or wild) that bear fleshy berries (Daunay *et al.*, 2001) [2]. However Indo-Burma region is the primary centre of diversity because Solanum genus has vast diversification in various ecological conditions. Still we can find the most of the wild species of brinjal throughout the India. According to Food and Agriculture Organization Corporate statistical Database 2017, world brinjal production was 52,309,119 MT which was raised by 2.2% from 51,192,811 MT in 2016. However, India is accounting 12,510,000 MT production on an area of 733,000 ha in 2017 worldwide (http://faostat.fao.org). The highest eggplant production in the world is in China, which meets 62% of the world's production. India ranks second in production fallowed by the Egypt, Turkey and Iran.

The main method of obtaining haploid plants in eggplant is androgenesis. *in vitro* androgenesis in *S. melongena* has been commonly employed for the last 40 years (Dumas de Vaulx and Chambonnet, 1982; Rotino, 1996, 2005; Segui Simarro *et al.*, 2011) [3, 4, 5]. It is possible to produce haploid (n) plants, which re-establish their normal ploidy level (2n) either spontaneously or after colchicine treatment. With this technique the time required to obtain homozygous material from the initial heterozygous material has been reduced (Daunay *et al.*, 2001) [2]. Studies with anther culture have mostly been conducted for cultivated eggplant (S. melongena) with goal of obtaining double haploid parents for conventional breeding (Rotino, 1996) [20]. The double haploid plants have been successfully used in conventional breeding programs to obtain pure lines faster than selfed inbreds. Double haploid plants are homozygous at all loci, and this may help to study the genetic basis of quantitative traits by overcoming the problems associated with the envirobmental cariations. Raina and Iyer (1973) [17] were first to report plant regeneration from anther culture in eggplant. They regenerated homozygous diploid (double haploids) plants through callus developed from anthers cultured at uninucleate pollen stage that were previously treated with colchicines. Haploid plantlets were also obtained from the Research group of Haploid breeding (1978) and Isouard *et al.* (1979) [12] a year later. Dumas De Vaulx and Chambonnet (1982) [5] did an extensive work to improve the development of androgenic haploids. They showed that high temperature (35 ± 2

ºC) incubation of anthers under dark conditions for the first 7-8 days improved the efficiency of haploid plant formation. A combination of both auxin and cytokinin was essential during early stages of anther culture. Similarly, Rotino *et al.* (1987) [19] showed that haploid plant regeneration was affected by genotype, temperature, culture conditions, hormones and anther stage. A high temperature governs the shift of the microspores from gametophytic stage to sporophytic stage.

The technique of *in vitro* induction of anther-derived embryos and embryonic calli *via* anther culture in selected brinjal genotypes is the most ideal method for the production of plants from microspore through direct embryogenesis or regeneration from callus. Androgenis has been employed since 1980s in brinjal for the production of double-haploid plants from microspore derived embryos or embryogenic calli. Over the time period it has been refined and widely applied at both commercial levels for a fast generation double-haploid parental lines of F1 hybrids, as well as for experimental studies as the complete homozygosis of the microspore-derived plants make more simply the genetic analysis. In this, a step-by-step procedure is reported, taking into consideration all the aspects of the technique, including the growth condition of the pre-treatment, incubation temperature, anther donor plant and media composition, the *in vitro* regeneration of the androgenetic plantlets, briefly with a main aim on *in vitro* induction of anther-derived calli and plantlet formation in selected brinjal genotypes (*Solanum melongena* L.).

## Materials and Methods

The popular genotype IGB-17 × Kasi Taru (KT) was planted at Research Farm of the Department of Vegetable Science, IGKV, Raipur. Throughout the growing period, the normal cultivation practices were implemented to raise healthy plants. Plants were maintained free from insect-pests and diseases. To stimulate development of fresh flower buds, the young set fruits were removed periodically. During flowering period of plants, flower buds which were in the proper stage of anther development were collected and their anthers were cultured in different media.

Flower buds were collected at 12-15 mm size ie at petals reaches to sepal separation level at late uninucleate stage to early binucleate stages of microspore development. Later the size and morphology of flower buds can be used as an indirect indication for determining the stage of microspore development. Anthers from flower buds of different stages were subjected to cytological examination by staining with 2 per cent acetocarmine dye after squashing and observed under microscope at 100x magnification. These buds were washed with tap water and surface sterilized for one minute in 70 per cent ethanol followed by 30 sec-1 minute in a filter paper and washed with distilled water later kept in the refrigerator at 4-5°C chilling temperature for 1-3 hours duration. Then surface sterilized with $HgCl_2$ for 1-3 min and rinsed 3 to 4 times with sterile distilled water to remove the traces of $HgCl_2$. The anthers were aseptically removed from the buds carefully and were placed on Petri-plates containing C medium - MS media and $N_6$ media supplemented with 2,4-D2mg/l at 30 g/l sucrose along with control. About 220-225 anthers from 30 plants were cultured for each treatment. The cultured anthers were given heat shock treatment by keeping the inoculated Petri-plates in incubator at 35ºC under dark condition for 8 days as well as at 25°C for 3 weeks and then shifted the cultures to culture room at 25±2ºC under photoperiod of 16 h light and 8 h dark. After three to four weeks, anthers were sub-cultured on same treatment for callus maintenance. Calli from anthers with size 5-7 mm were transferred to R medium Cultures were then incubated in culture room at 25±2 ºC temperature, under photoperiod of 16 hours light and 8 hours dark.

The number of days taken to show callo genesis from the date of inoculation, no of calli induced and Per cent response frequency of anthers to callus initiation in various treatments of C medium, at two different incubation temperatures and three preculture were recorded. The mean and standard errors were worked out from triplicate data obtained from various experiments. The per cent data transformed using angular transformation and analyzed following factorial Completely Randomized Design (CRD).The anther derived plantlets were hardened for 15-20 days in primary hardening room in small pots composed with garden soil, Cocopeat at 1:1 ratio and then shifted in big poly bags in the green house for 30-45 days.

## Results & discussion

The effects of cold-preculture or pretreatment of *Solanum melongena* L. buds at 5°C for 1 or 3h, inoculated in two different media and the addition of heat shock treatment during incubation of culture in dark at different durations on anther derived calli induction frequency (%) were examined on different C media by using Murashige and Skoog (MS) basal nutrient medium and N6 chu media were supplemented with various hormones, 0.8% (w/v) agar and 3% (w/v) sucrose.

### Calli induction frequency (%)

The effects of cold-preculture or pretreatment of *Solanum melongena* L. buds at 5°C for 1 or 4h, inoculated in two different media and the addition of heat shock treatment during incubation of culture in dark at different durations on embryo/calli formation were examined. Murashige and Skoog (MS) basal nutrient medium and N6 Chu media were supplemented with 2mg l-1 2, 4-D, 0.8% (w/v) agar and 3% (w/v) sucrose.

According to the results of the analysis of variance the interaction of preculture and media showed that the percentage of anther derived calli induction frequency was highly significant at 0.05 probability level and it ranges from 0.033 to 19.55 per cent. Significant differences were observed in the calli induction frequency (Table 1) due to pre - treatment durations, media levels and their interactions on cultured anthers in *in vitro condition*. However the highest percentage of anther derived calli induced frequency was obtained by the interaction between preculture duration of 3h at 5°C + MS media supplemented with 2.0 mg/l 2,4-D (19.55%) and also by the interaction of-2.30 h pretreatment + MS medium (10.28%) and lowest calli responding frequency with 1h pretreatment interaction with N6 medium supplemented with 2,4-D @ 2ml/l (0.33%).

Pretreatment at 5ºC for 3h duration has recorded significantly the highest number of calli (13.48) on par with 2h preculture (13.28). Among two different Media supplemented with 2, 4-D @ 2mg/l the MS media recorded the highest frequency ($7.38^a$ %) than the B2 – N6 media ($4.41^b$ %). It is evident from the results that the highest frequency was noticed in the B1 compared to B2. This might be due to media composition favoring the enhancement of the survival of anthers and provides pressure /strength to burst open the pollen grains from the anther to induce calli or embryos. A preculture

treatment or pretreatment of -3h at 5℃ exhibited the maximum calli induction frequency (12.34%) fallowed by A4-2.30hpreculture (8.55) and minimum frequency was obtained in A1-1h preculture (0.39). According to calli induction frequency preculture were ranked in descending order 3h,2.30 h,4h,2h, 1.30h and 1h and their means 12.34 [a], 8.55[b], 5.46[c], 5.02[d], 3.63[e] and 0.39[f], respectively.

However the heat stress incubation temperatures were not significantly differed in calli induction frequency at 5% level of significance that means the temperature and its duration were not showing any significant effect on calli induction. Therefore due to the above mentioned reason the combined interaction of media x pretreatment x heat stress induction temperatures were found to be non-significant but interaction of temperature with media as well as pre-treatment duration showed significant impact on induction of calli derived from anther. Anther pretreatment is one of the crucial factors to switch on androgensis induction. Low temperature pretreatment was effective in enhancing anther derived calli brinjal Sanjeev Kumar *et al.* 2003 [23], as well as in 2 day preculture found best in the same crop pin Chen *et al.* 2008 and in other species of solanceous family Datura, tomato (Debergh and Nitsch, 1973) [6] and tobacco. In the present study on brinjal, the anthers were treated with low (5℃ for 1-4 h duration) and high temperatures (25 for 21 days and 35 for 8 days). Under dark condition only the low temperature treatment of 3h was effective in callusing whereas heat shock treatment had no significant effect on anther derived calli induction but noticed embyoid production and induced considerable percent of calli was observed. However the combination of preculture of both heat stress temperatures regenerated 11 of 21 days darkness form indirect androgensis and in 8 days darkness about 3 plants produced from 150 anthers 3 plants from direct embryogenesis and 11 plants derived from calli derived plants in eggplant.

The period of treatments also differed among the studies. The period of preculture is short as compared with two days preculture in other studies ( pin chen *et al.* 2008), same in one of the study by Sanjeev Kumar *et al.* 2003) [23] and one heat stress condition duration same with haploid production studies of brinjal (Dumas de valux 1981) [8]. And other is longer period of 21 days. The importance of preculture and the need for specific pretreatment to trigger microspore development from gametophytic to sporophytic pathways has been highlighted in some research studies (Zheng 2003; Parra-Vega *et al.* 2013, Eshaghi *et al.* 2015) [26, 21, 9].

**Table 3.1:** The influence the preculture × media on callus induction frequency of *in vitro* androgensis in brinjal

| Pretreatment duration/media | MS media | N6 media | Mean |
|---|---|---|---|
| 1h | 0.448 | 0.333 | 0.391f |
| 1.30h | 3.903 | 3.352 | 3.628e |
| 2h | 5.112 | 4.918 | 5.015d |
| 2.30 h | 10.278 | 6.817 | 8.548b |
| 3h | 19.548 | 5.128 | 12.338a |
| 4h | 5.017 | 5.912 | 5.464c |
| MEAN-B | 7.384a | 4.41b | |

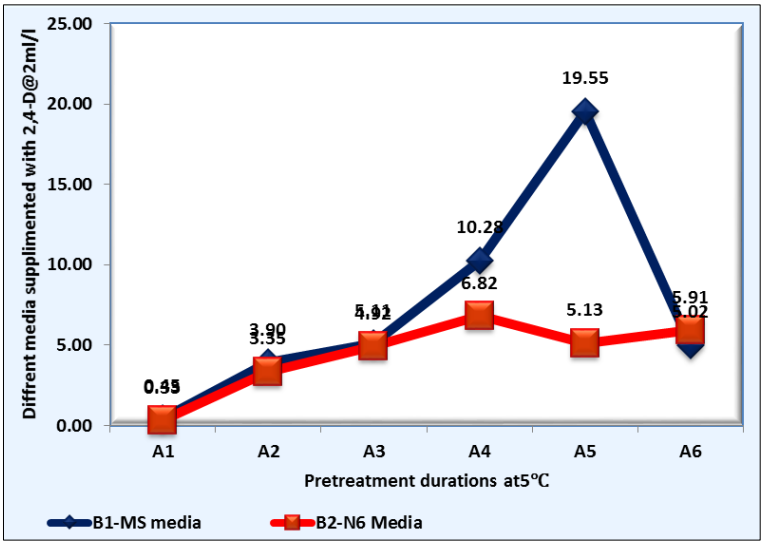


**Fig 3.1:** Influence of different pretreatment duration and media on anther derived calli induction frequency (%)

Therefore concluding the MS media supplemented with 2,4-D @2mg/l anthers which were subject to cold treatment 3hbest combination for optimize the calli production.

The regeneration from the callus was initially observed as green spot in the callus. Shoot initiation from the callus was observed on the MS medium containing 3 mg/l BAP+1mg/l Kin. These shoots were multiplied on MS media supplemented with BAP 3mg/ l+ Kin 1mg/l +0.2mg/l $GA_3$ +0.2 mg/l NAA medium. The explants bearing several shoot buds were transferred to GR free MS medium for elongation. The individual elongated shoots were cultured on GR free full strength MS medium to induce rooting. The putative haploid plants were acclimatized under conditions of high humidity and transferred to field (Figure 1 and Figure 2). The regenerated plants were found to be sterile with stunted morphology as compared to normal fertile plants. The anther derived haploid plants has reduced growth habit, pollen colour, pollen size, leaf size, leaf thickness as well as flowers in reduced size, color, more no of short styled flowers, huge flower drop and didn't observed fruit set. The haploids also

confirmed by histological studies in root tips. The variability observed in the callus induction of anthers of two hybrids in seven different suggests that it is better to consider behaviour of each genotype on different induction medium (Table.1 and Table. 2).

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