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The effect of cold preculture, media and incubation temperatures on callus induction via Antherculture in Brinjal (*Solanum melongena* L.)

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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 3 different 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). One cross (IGB-17 X Kasi Taru) was implanted in C medium supplemented with different hormones. Among different treatment combinations the treatment C_5 media +3 hours pre-treatment + incubation at 25℃ for 3weeks showed maximum embryogenic calli induction with response frequency 74.05 per cent and took least number of days for callus initiation (10 days) fallowed by C2+2h+25 ℃had % callus induction frequency with minimum days of 10.71, respectively.

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 and rogenesis. In vitro and rogenesis 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 another culture have mostly been conducted for cultivated eggplant (S. melongena) with goal of obtaining double haploid parents for conventional breeding (Rotino, 1996). 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 environmental cariations. Raina and Iyer (1973) [9] were first to report plant regeneration from another 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) [6] and Isouard *et al*. (1979) 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 another culture. Similarly, Rotino *et al.* (1987) 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. And rogenis has been employed since 1980s in brinjal for the production of double-haploid plants from microspore derived embryos or embryo genic 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 and rogenetic 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, and 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 12-13.7 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℃ 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 panthers were aseptically removed from the buds carefully and were placed on Petri-plates containing C medium - MS media with different concentrations and combinations of hormones at 30 g/l sucrose Vize control, C0, C1, C2, C3, C4, C5 and N6 media. 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 ℃ 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 ℃ 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 and Discussion

The effects of cold-preculture or pre-treatment 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 (%)

Regeneration of haploids through cultures of anthers fallowed by doubling of chromosomes is an effective method for generation homozygous lines in short span of time compared to conventional methods. Induction of callus from anthers has been revealed by, Dumas de Valux and Chambonnet, 1982 [3]. However early callus proliferation was observed but production was poor in this case and calli mortality also observed.in this investigation calli regenerated from and rogensis. Detection of ideal stage of pollen development was the most critical factor for developing homozygous lines. After 5-10 days of culture anthers starts to show brown colour and gradually turns dark brown and swelled from 20-25 days, depending on the genotype this durations vary. Finally anthers burst to produce callus from anther as well as from the wall. Among three pre-treatments durations, 3 hour pre-treatment duration had calli induction both in two induction temperature fallowed by 2 hour preculture given in Table.2, and least calli frequency 3.13 per cent as well as maximum number of days taken (85.17 days in IGB17 x KT) in treatment combination of 1hr pre-treatment with N_6 media supplemented with 24-D at 25 ℃. No response at 35 ℃ incubation period with same preculture and media in case of genotype IGB-17 x KT.

Table 1: Influence of different media, pre-treatment duration and incubation culture condition on callus induction frequency % in brinjal

S.l.no	Treatments	Total Callus induction frequency (%)			Mean
		1 hour	2 hour	3 hour	
1	A0a- control+25 ℃	0.00	0.00	0.00	0.00
2	A0b-control+35 ℃	0.00	0.00	0.00	0.00
3	A_1 -C_O+25 ℃	2.67	18.67	37.92	21.95
4	A_2 -C_O+35 ℃	0.89	12.50	22.17	13.03
5	A_3 -C_1+ 25 ℃	5.26	23.56	33.34	23.38
6	A_4 -C_1+35 ℃	6.56	27.55	25.93	22.72
7	A_5 -C_2 +25 ℃	6.84	55.33	64.22	44.88
8	A_6 -C_2 + 35 ℃	9.95	46.64	56.40	40.47
9	A_7 -C_3 +25 ℃	6.36	25.06	19.78	19.77
10	A_8 -C_3 +35 ℃	9.77	36.00	24.89	26.36
11	A_9 -C_4+ 25 ℃	8.56	48.19	59.69	41.26
12	A_{10} -C_4+35 ℃	12.77	52.11	64.56	45.85
13	A_{11} –C_5 +25 ℃	10.64	57.06	74.05	49.80
14	A_{12} -C_5+35 ℃	12.22	52.25	68.39	47.01
15	A_{13} -N_6+ 25 ℃	0.89	3.51	10.28	5.64
16	A_{14} -N_6+35 ℃	0.00	12.74	6.86	6.53
Mean		6.67 (13.36)	33.66	40.55	29.19

According to the results of the analysis of variance the interaction of cold pretreatment, media and incubation temperatures of specific period of darkness showed that the percentage of anther derived calli induction frequency was highly significant at 0.01 probability levels and it ranges from 0.00 to 74.05 per cent represented in interaction table no.1. The maximum anther derived calli induction frequency obtained in treatment combination of cold pretreatment 3h with C5 C media and at incubation temperature of 25 ℃ for 21 days ie three weeks 74.05 per cent which is on par with treatment combination of 2h + C2 media+25 ℃ (69.33%), 3h+C5+35 ℃ (68.39%) fallowed by 3h+C2 media+25 ℃(69.33%) and lowest percent observed inN6 +1h+35 and 0.89% in 1h+C0+35 ℃, respectively. However in Control treatment in three preculture in two temperatures doesn't had no response of callus.

Significant differences were observed in the calli induction frequency (Table 2) due to duration of cold pretreatment, 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 ℃ + MS media supplemented with 2.0 mg/l 2,4-D+0.5mg/l Kin (71.22%) and also by the interaction of-2 h pretreatment + C2 media (62.89%) and lowest calli responding frequency with 1h+ N6 combination, however in control treatment didn't observed the calli initiation response.

Table 2: Media X pretreatment duration at 5 ℃ Mean Table

	1h	2h	3h	Mean of media
Control	0.000	0.000	0.000	0.000
Co	1.777	15.583	30.042	15.801^{f}
C1	5.912	25.555	29.633	20.367^{d}
C2	8.395	62.888	48.278	39.854^{b}
C3	8.067	30.527	22.335	20.309^{e}
C4	10.165	28.123	46.152	28.147^{c}
C5	11.430	49.653	71.217	44.100^{a}
N6	0.445	8.128	8.568	5.714^{g}
Mean pretreatment	5.774^{c}	27.557^{b}	32.028^{a}	

Mean comparison of treatment combinations preculture and media indicated that pretreatment at 5 ^{0}C for 3h duration exhibited the maximum calli induction frequency (32.03) fallowed by 2h preculture (27.56%) and minimum frequency was obtained with 1h preculture. According to calli induction frequency preculture were ranked in descending order 3h >2h>1h. Among the different Media supplemented with phtyohormone the C5 media supplemented 2,4-D@2ml/l + Kn 0.5ml/L was recorded the highest frequency (44.10%) fallowed by C2 and minimum calli induced in the N6 media supplemented with 2, 4-D @2ml/l (5.72%). It is evident from the results that the highest frequency was noticed in the C5 compared with the other media combinations. This might be due to media composition favouring the enhancement of the survival of anthers and provides sufficient availability of sugars, macro, micro, vitamins and amino acids create pressure /strength to burst open the pollen grains from the anther to induce calli or embryos. In addition to it cold preculture favours to enhances the anther responding to calli induction frequency.

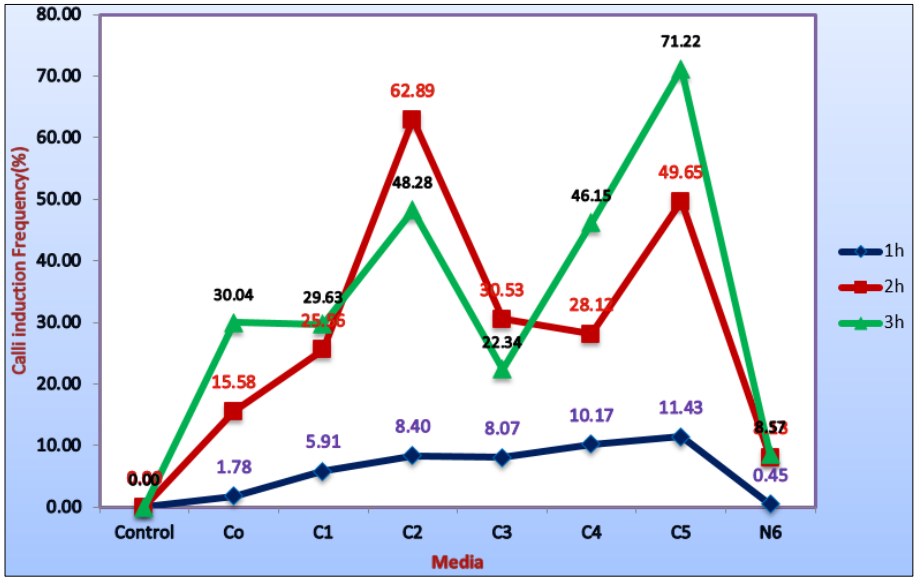

Fig 1: Interaction of different media and pretreatment duration on callus initiation frequency from anthers of brinjal cross IGB-17 X Kasi Taru (%)

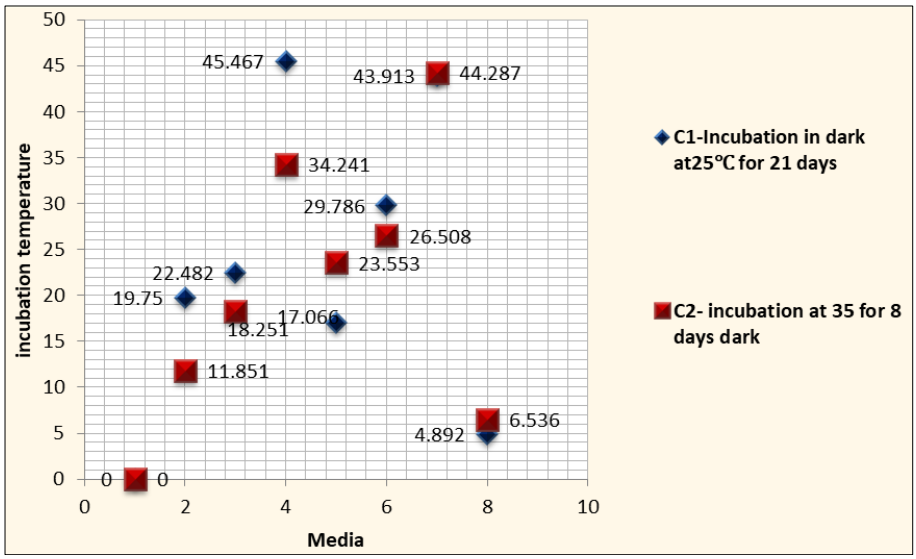

Fig 2: Interaction of different media and incubation heat shock temperature on callus initiation frequency from anthers of brinjal

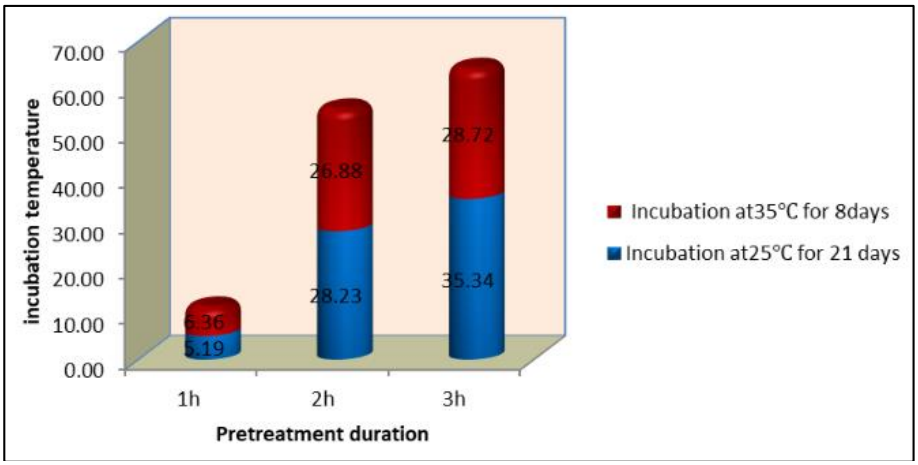

Fig 3: Interaction of incubation temperature and pretreatment duration on callus initiation frequency from anthers of brinjal cross IGB-17 X Kasi Taru (%)

Another pretreatment is one of the crucial factors to switch on and rogensis induction (Germana *et al*. 2011). Low temperature pretreatment was effective in enhancing anther derived calli brinjal Sanjeev kumar *et al*. 2003, as well as in 2 day preculture found best in the same crop pin chen *et al* 2018 and in other species of solanceous family Datura (Nitsch and Norrel,1973), tomato (Debergh and Nitsch, 1973) and tobacco (Radish and Reinert,1981). In the present study on brinjal, the

anthers were treated with low (5 ℃ for 1-3h 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 at 25 ℃ heat shock treatment had significant maximum effect on anther derived calli induction compared to 35 ℃ heat shock treatment for 8 days darkness represented in Fig.3 but noticed embyoid production (Mastubara *et al*. 1992).

Table 3: Influence of different media, pretreatment durations and incubation culture condition on days taken for callus induction

Sl.no		Days taken for callus induction			Mean
		1hour	2 hours	3 hours	
1	A0-control+25 ℃	0.00	0.00	0.00	0.00
2	A0-control+35 ℃	0.00	0.00	0.00	0.00
3	A_1 -C_O+25 ℃	56.78	18.23	16.63	30.55
4	A_2 -C_O+35 ℃	45.77	14.75	11.17	23.90
5	A_3 -C_1+ 25 ℃	49.30	11.33	13.00	24.54
6	A_4 -C_1+35 ℃	40.70	16.45	17.97	25.04
7	A_5 -C_2 +25 ℃	60.91	11.85	13.62	28.79
8	A_6 -C_2 + 35 ℃	36.07	14.04	11.40	20.50
9	A_7 -C_3 +25 ℃	44.70	10.05	10.67	21.81
10	A_8 -C_3 +35 ℃	42.05	10.74	13.56	22.12
11	A_9 -C_4+ 25 ℃	49.33	10.00	10.07	23.11
12	A_{10} -C_4+35 ℃	31.38	9.92	9.80	17.03
13	A_{11} –C_5 +25 ℃	56.40	12.96	10.00	26.43
14	A_{12} -C_5+35 ℃	37.13	12.08	9.93	19.76
15	A_{13} -N_6+ 25 ℃	85.17	60.00	49.93	65.03
16	A_{14} -N_6+35 ℃	0.00	29.12	26.08	18.40
	Mean	45.41	17.25	15.99	26.22

These results supported with the findings of Dumas de Vaulx and Chambonnet 1982 [5]; Sanjeev Kumar *et al.* 2003 in brinjal. The pretreatment of flower buds before excising the anthers for culture has been described as a means of increasing the and rogenetic response (Maheswari *et al*., 1982; Morrisson *et al*., 1986) [12, 13]. However, Vagera and Havranek (1985) [11]. Pre-treating flower buds with cold at 4 ºC for 48 or 96 hours resulted in a lower number of responding anthers and reduced embryo production, mainly by causing callus induction from the anthers (CINER and TIPIRDAMAZ 2002) of Capsicum. Hence, the anthers of IGB-17 × KT have induced maximum embryogenic callus induction frequency (74.0.5%) than the Khaira Singhi (70.04%) in treatment combination of 3h preculture+ C_5 media + incubation temperature 25 ℃ in 10.07 days and 10.81 days respectively. Among different medias calli formation frequency observed exclusively in MS media containing both auxins and cytokinins (C_5 – 2,4-D 2mg/l+0.5 kn, C_2- 2,4-D 5mg/l+ kn 5 mg/l and C_4 –BAP 1mg/l +NAA 1mg/l).For some members of solanaceous family, the inclusion of auxin in the culture media either alone or in combination with a cytokinins, frequently induces the formation of the pollen calli (Canthoto *et al*. 1990) [8]. Similarly Gulshan *et al.* 1981 [8] verified in tomato that medium containing 2.0 mg/l auxin and 1mg/l Cytokinins was most efficient for callus formation. There were three types of androgenic response observed in some anthers induced embryo genic callus, few developed embryos formation, embryo genic calli and direct and rogensis also observed with IAA instead of 2,4-D in the first culture medium. In Solanaceae family, as in *Datura metel* (Iyer and Raina, 1972) [9], both kind of and rogensis, direct (embryo formation-in this first rhizogensis happens later shootlets will developed) or indirect (embryogenic calli or embryos) observed. The colour of embryogenic calli globular creamy white were again multiplied on same medium and transferred to different media having varying concentration of NAA (0.1, 0.2, 0.5, 1.0, 2.0 and 2.5 mg/l) but had poor regeneration response. Therefore the embryogenic calli were transferred on different medium supplemented with varying concentrations of BAP, Kinetin, NAA and IAA, as well as combinations, respectively. The embryogenic calli induction was observed from three hours pre-treated anthers on MS medium with 2,4-D- 2mg/l+ Kinetin- 0.5 mg/l. (Matsubara *et al*.,1992, Tang *et al*., 2005) [6, 7] A chilling treatment of anthers at 5 ℃ about 2.30 - 3 hours had maximum callus initiation (Sanjeev Kumar *et al*. 2003). The low temperature or cold treatment/heat treatment has been revealed to be favourable for enhanced callus response of androgensis (Dumas de Valux and Chambonnet, 1982) [1]. Earlier Valiux and Chambonnet (1982) [3] used heat treatment at 35 ℃ for eggplant anthers. 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).

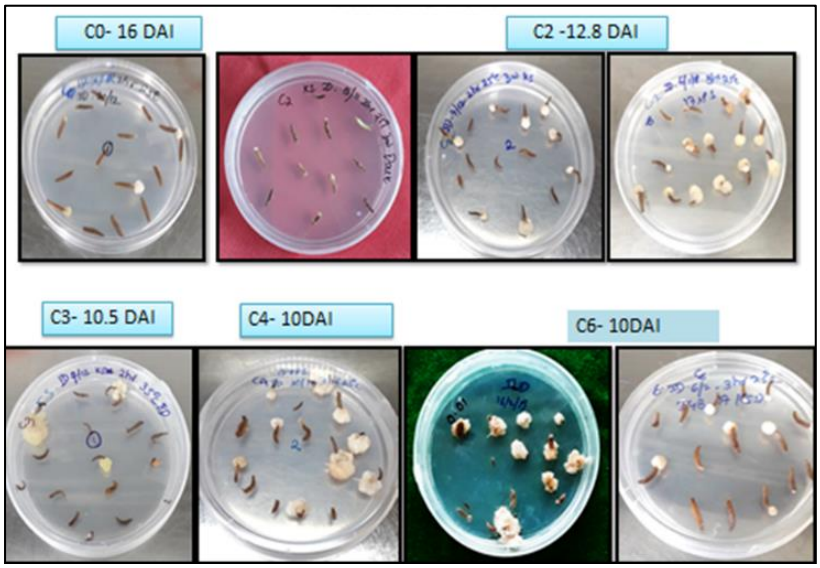

Fig 4: Days taken for callus initiation and induction response in different at 3 hour pre-treatment c- media

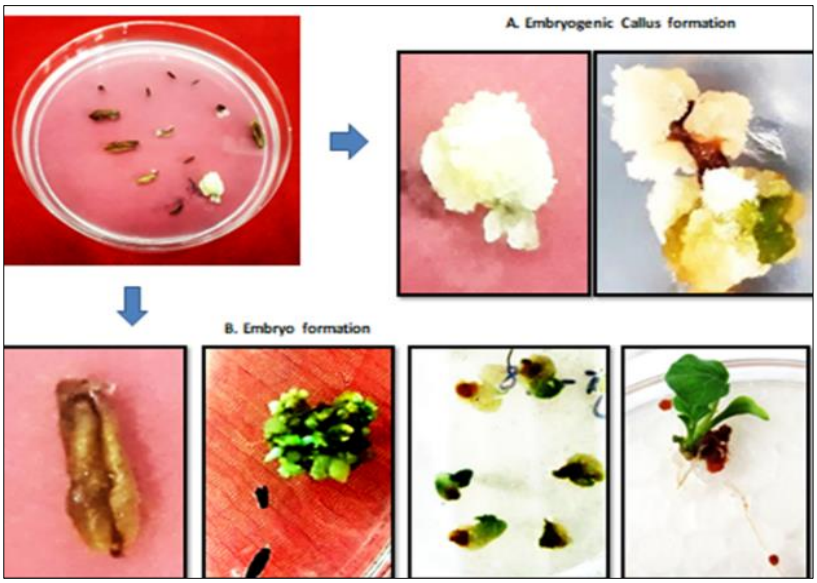

Fig 5: Indirect organogenesis

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