The Pharma Innovation Journal 2021; 10(8): 691-702

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(8): 691-702

www.thepharmajournal.com
Received: 12-06-2021
Accepted: 13-07-2021

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Optimization, regeneration and comparison of mature embryo with immature embryos of sunflower elite cultivars

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Abstract
Regeneration ability of plant cells or tissues in explant culture is one of the key factors affecting success of genetic transformation. In experiments, the effect of explant type (whole embryo, scutellum, embryonic axis, meristematic/central zone of embryonic axis) and plant growth regulators (BAP or TDZ) on mature embryo regeneration was determined. Explant type significantly affected regeneration efficiency. While no regenerants were observed using mature scutella, whole embryos or embryonic axes produced the highest number of regenerants. Using embryonic axes with discarded apical and basal parts, regeneration efficiency dramatically decreased. No statistical differences in regeneration were observed between BAP and TDZ added to the regeneration medium in concentration 0.1 or 1 mg l-1. At last, regeneration ability of mature embryos of nine Slovak spring barley cultivars (Donaris, Ezer, Levan, Ludan, Nitran, Pribina, ladar, Orbit, Pax) and Golden Promise as a model cultivar was examined and compared with regeneration ability of immature embryos which have been usually used for genetic transformation of barley. Although the regeneration from mature embryos was very weak, the same cultivars Golden Promise, Pribina and Levan showed the best regeneration ability by using both, immature and mature embryos. On the other hand, cultivars Ezer and Pax belonged to the weakest ones in both experiments.

Keywords: BAP, barley regeneration, explant type, immature embryo, mature embryo, thidiazuron (TDZ)

1. Introduction
The cultivated sunflower (*Helianthus annuus* L.) is one of the three important edible oilseed crop grown in the world after soybean and groundnut. Sunflower is probably originated in Southern United States and Mexico from where it was introduced into Europe and later into former USSR. Sunflower (*Helianthus annuus* L.) belongs to family Asteraceae (formerly Compositae). The genus *Helianthus* is named from the Greek word *Helio* meaning sun and *Anthos* meaning flower. It is an annual, diploid (2n=34) species. Sunflower is essentially a cross-pollinated plant, besides showing varying degrees of self-incompatibility. The commercial sunflower has a narrow genetic base and as a consequence only a few introductions constitute the base material for the development of new cultivars or hybrids. (Sujatha *et al*. 2006) [21].
The major reasons for the low productivity of the sunflower crop were, on the one hand, the highly fluctuating pedoclimatic conditions that were becoming more and more severe in the recent years and the different biotic stress factors, which are the bottleneck for increasing the productivity of sunflower, susceptibility to fungal diseases is a major limiting factor for increasing sunflower production in world. As all the currently grown varieties and hybrids are susceptible to Alternaria leaf spot, which is widespread in the world, it is necessary to identify durable resistance and this has to be a major thrust area in resistant breeding programmes. As a first step in this direction, efforts are needed to identify the resistant sources. Only in some perennial tetraploid and hexaploid species particularly in *H. tuberosus* there are indications of existence of some resistance. Due to the limited natural variation, the genetic improvement of the cultivated sunflower for such agronomic traits has been broadly based on the ability to transfer the desired genes from wild relatives through conventional breeding methods, but unfortunately the utilization of many wild species has been limited by natural barriers to the reproductive process such as embryo abortion.
Nowadays, SSRs marker became a choice of molecular markers for assessment of population genetic analyses including DNA fingerprinting, genetic mapping, and molecular breeding in

crop plants (Powell *et al*. 1996) [12]. In sunflower, several marker systems have been employed for assessment of genetic diversity in cultivated germplasm and wild sunflowers. Biochemical and molecular polymorphism were sufficient for distinguishing between closely or distantly related germplasm accessions in sunflower (Rieseberg and Seiler, 1990; Rieseberg, *et al*, 1993; Arias and Rieseberg, 1995) [13]. The choice of a molecular marker technique depends on its reproducibility and simplicity. The best markers for genome mapping, marker assisted selection, phylogenic studies, and crop conservation have low cost and labour requirements and high reliability, microsatellites or simple sequence repeats (SSRs), are short DNA sequences harboring motifs of 1-6bp that are tandemly repeated (Tautz and Renz, 1984) [17].

Genetic improvement of this crop through biotechnological tools is limited by the difficulties to establish an efficient system of plant regeneration which is a prerequisite for genetic transformation studies. Sunflower tissue has typically been very easy to culture. Alternatively, somatic embryogenesis has been highly preferred over organogenesis as the transformants recovered through this system are uniform and homogeneous with less variation among clones owing to their single cell origin (Jimenez, 2001) [18]. Sunflower has been regenerated from a number of explants and genotypes (Pelissier *et al*. 1990) [22]. Immature hybrid zygotic embryos are used for initiation of somatic embryogenesis and found that somatic embryos arose directly from the cotyledons of the zygotic embryo without the formation of callus intermediate. Embryos were initially observed as early as 6 days after culture.

Immature zygotic embryos often possess high morphogenic potential and are frequently used for plant regeneration in species where regeneration from other explants is difficult, such as various cereals, legumes and conifers. Regeneration was either through a callus intermediate or the frequency of induction of somatic embryos was low. Hence M. Sujatha & A. J. Prabakaran (2008) [19] carried out investigation to assess the influence of various factors facilitating high frequency of regeneration in cultured zygotic embryos of sunflower.

Fertile plants can be regenerated from immature zygotic embryos of sunflower through either somatic embryogenesis or organogenesis. Both morphogenic pathways can occur directly from the explants. (Alibert *et al*. 1994: Hahne 1995) [16, 23], mainly differing in hormonal concentrations of media (Brooner *et al.* 1994) [24]. Thus sugar plays a crucial role in this experimental system. Most recently, direct somatic embryogenesis (to the tune of 55 percent) followed by successful plant recovery has been reported from immature embryonic shoot tips (Loganathan *et al.* 2010) [20].

So far, no number of successful reports of regeneration and further transformation of Indian sunflower cultivars through somatic embryogenesis has been made available. In this context, the present investigation aims at,

1. Regeneration of three elite sunflower cultivars, LSF-10, DMLT-1Y and GMU-479 through direct somatic embryogenesis.
2. Evaluation of embryogenic potential at two steps, induction and maturation stage of somatic embryogenesis using immature zygotic embryos as explants.

2. Materials and methods

2.1 Plant material

The cultivars of Sunflower (*Helianthus annuus* L.) used in this study were LSF-10, DMLT-1Y and GMU-479 (Figure 3.1). These lines are belonging to different maturity durations (Late, Early and Early respectively). Seeds of LSF-10, DMLT-1Y and GMU-479 were obtained from Oilseed Research Station (O.R.S.), Latur of Marathwada Krishi Vidyapeeth, Parbhani. Seeds of the above cultivars were grown in a greenhouse under natural light conditions with staggered planting dates to ensure continuous availability of explants.

2.2 Induction of globular somatic embryos

Immature zygotic embryo explants were inoculated on SEI medium and maintained at 25±1 ºC and 16 hrs. photoperiod and sub-cultured onto a fresh SEI medium at 20 to 30 days interval. The primary somatic embryos were visually scored after 60 days and 90 days (M. Sujatha and Prabakaran, A.J., 2001). Inoculation using stereoscopic microscope (Labomed CXR2) and 1800x1200 megapixels camera for evaluating the somatic embryo induction frequency. Somatic embryo induction frequency was calculated using the given formula.

$$\text{Somatic embryo induction frequency} = \frac{\text{Number of explants showing somatic embryo induction}}{\text{Number of explants cultured}} \times 100$$

The frequency of somatic embryogenesis was evaluated after 60 and 90 days of culture initiation. Data from three replications (20 explants/replication) were used for calculating the mean induction frequency. Globular somatic embryo induction frequencies were calculated by analysis of variance following a completely randomized design. Treatment means were separated using (CRD test; $\alpha = 0.05$).

2.2.3 Proliferation of globular somatic embryos

Cultures with globular somatic embryos were transferred onto MSD20 medium (Wright *et al.,* 1991) for the secondary embryogenesis of primary embryos. Cultures were maintained at 25±1 ºC and 16 hrs. photoperiod for a month without subculture.

2.2.4 Embryo maturation

Globular stage somatic embryo clusters showing active proliferation were cultured onto MSM6AC maturation medium (Bailey *et al.,* 1993a) [1] for differentiation into cotyledonary stage somatic embryos. The plates were maintained at 25 ± 1 ºC and 16 h photoperiod (Table 3.0). One month after plating, individual embryos were transferred to fresh MSM6AC medium, and cultured for another four weeks. After 7 weeks of maturation on MSM6AC medium, mature somatic embryos were segregated into different morphological classes as described by Buchheim *et al.* (1989). Efficiency of somatic embryo maturation was calculated using the following formula.

$$\text{Maturation frequency} = \frac{\text{Number of well differentiated embryos}}{\text{Total number of embryos}} \times 100$$

Maturation frequency was compared among cultivars taking three replications each containing 37±40 somatic embryos. Means of somatic embryo maturation frequency of above cultivars were analyzed by analysis of variance following a completely randomized design. Treatment means were separated using (CRD test; α = 0.05).

2.2.5 Desiccation of mature somatic embryos and plantlet conversion

After 1 to 2 months, mature somatic embryos were desiccated in sterile empty Petri dishes at 25±1 ºC and 80% relative humidity. The partially dehydrated somatic embryos were transferred to MSB5 regeneration medium (Table 3.0). After attaining 2 to 3cm length, the rooted plantlets were transferred to test tubes containing the same medium.

Table 3: Composition of the culture media used in somatic embryogenesis from Sunflower (*Helianthus annuus* L.) immature zygotic embryos

Culture Medium	Composition
SEI medium	MS salts (Murashige and Skoog, 1962; Appendix 1a), B5 vitamins (Gamborg *et al.*, 1968; Appendix 1b), 6% sucrose, 5 mM asparagine (660 mg/L), 684 mM glutamine (100 mg/L), 181 µM (40 mg/L) of 2,4-dichlorophenoxy acetic acid (2,4-D; Duchefa), 0.25% gellan gum (Sigma), pH 5.8 (before autoclaving). (Loganathan *et al.*, 2010) [20].
MSD20 proliferation medium	MS basal salts (Murashige and Skoog, 1962; Appendix 1a), B5 vitamins (Gamborg *et al.*, 1968; Appendix 1b), 90.5 µM (20 mg/L), 2,4-D, 87.6 mM (3 % w/v) sucrose, pH 5.8 (Wright *et al.*,1991).
MSM6AC maturation medium	MS basal salts (Murashige and Skoog, 1962; Appendix 1a), B5 vitamins (Gamborg *et al.*, 1968; Appendix 1b), 175.3 mM (6% w/v) maltose, pH 5.8, 0.5 % Activated Charcoal, (Bailey *et al.*, 1993a) [1].
MSB5 regeneration medium	MS basal salts (Murashige and Skoog, 1962; Appendix 1a), B5 vitamins (Gamborg *et al.*, 1968; Appendix 1b), 87.6 mM (3% w/v) sucrose, pH 5.8.

2.2.6 Effect of desiccation period on rooting and germination

After 1 to 2 months of maturation in MSM6AC medium, mature somatic embryos were desiccated on sterile empty petri dishes or desiccation of callus cultures as well as somatic embryo was achieved on 1.6% (w/v) agar and thereafter desiccated calli and somatic embryo were cultured on a single shoot regeneration medium. At 25±1 ºC and 80 percent RH. The partially dehydrated somatic embryos were transferred to MSB5 regeneration medium (Table 3.0). After attaining 2 to 3 cm length, the rooted plantlets were transferred to test tubes containing the same medium described by Kaur, Ajinder and S.S. Gosal, (2009). After desiccation treatments, the somatic embryos were rehydrated with sterile, distilled water for 1 h before transfer to MSB5 regeneration medium. Loss of viability was generally associated with the embryo becoming pure white and vitrified. When an embryo had this appearance and did not develop a radicle or apex, it was considered to be nonviable. Each treatment mean was calculated from three replications each containing 20 randomly chosen mature somatic embryos from a plate. Treatment means were separated using (CRD test; α = 0.05).

2.3 Regeneration studies

For regeneration studies, apart from salts, vitamins and agar, two auxins namely 2,4-D and Dicamba in varying concentrations were added to fortify MS medium. Different concentrations and combinations of growth regulators used during the investigation are presented in Table. 3.1. The plant growth regulator types and their concentrations were selected based on preliminary work conducted in this laboratory and work conducted by various scientists throughout the world in the past. The results of preliminary experiments are presented in Table 3.2. For growth regulators large variations have been found in preliminary experiments where some combinations led to number of somatic embryos from cultured explants, while other produced few numbers of somatic embryos. It was observed that sucrose as well as a growth regulator alone is not adequate for inducing somatic embryogenesis in higher frequencies. So, as for final experiment, culture medium was fortified with combinations of standardized auxins and sucrose in varying concentrations.

Table 3.1: Culture media used during the preliminary experiment with different concentrations of plant growth regulator and sucrose used for optimization of somatic embryogenesis

Treatment		Frequency of embryogenesis (%)
Sucrose (g. l^{-1})	2, 4-D (mg. l^{-1})	
30	0.33	7.7^{i}
30	0.67	7.0^{i}
30	1.0	11.5^{hi}
60	0.33	19.3^{fgh}
60	0.67	24.6^{efg}
60	1.0	14.8^{ghi}
90	0.33	22.4^{efg}
90	0.67	27.2^{def}
90	1.0	22.7^{efg}
120	0.33	46.8^{ab}
120	0.67	45.6^{ed}
120	1.0	25.9^{def}
150	0.33	32.9^{edc}
150	0.67	41.6^{bc}
150	1.0	51.6^{a}

2.3.1 Histological Study

The cultures were mechanically and biochemically stabilized to frozen specimen embedding medium, *i.e.* 10% polyvinyl alcohol: 4% polyethyl alcohol, Shandon Chryomatri$^{TM.}$ Embedding was accomplished in freezing chamber of cryotome. Liquid embedding medium was freezed on transfer to cryochamber. Approximately 60 µm sections were cut. Sections were then placed on a glass slide for staining. Cotton blue and light green staining was used for histological study. The slides were then observed under the microscope (Figure 4.5, 4.6 and 4.7). Means followed by same letters are not significantly different according to DMRT at α=0.05. Embryos of categories 1-3 were used for the experiment.

Table 3.2: *In vitro* responses of immature embryos cultured on MS medium supplemented with different concentrations of auxins and sucrose in varying concentrations during the preliminary experiment

Zygotic embryos forming somatic embryo ± S.E.		Auxin
6% Sucrose	12% Sucrose	
3.8±3.4	21.5±10.1	3.3mg/Dicamba
12.5±6.3	8.3±14.4	10 mg/Dicamba
16.7±14.4	12.5±16.5	33 mg/Dicamba
8.3±7.2	15.0±18.0	0.33mg/2,4-D
10.4±9.5	31.2±11.7	1mg/2,4-D
0	6.3±4.4	3.3mg/2,4-D

2.3.3 Experimental plan and design
The factorial completely randomized design (CRD) was used to study the effect of culture medium and genotypes and their interactions on somatic embryogenesis of sunflower cultivars from cultured immature embryos. A total 05 culture bottles/ Petri dishes were cultured with twenty explants for each separate treatment and computation of the effect of all factors was carried out separately.

2.3.4 Observations Recorded
For all explants, observations were recorded at 3 stages;

Stage 1	-	After 25-30 days of initial culturing.
Stage 2	-	After 25-30 days from reculturing of embryogenic calli and somatic embryo on media.
Stage 3	-	After 25-30 days from reculturing of embryogenic calli and somatic embryo on media.
Stage 4	-	Desiccation of isolated somatic embryos for 72 hrs.
Stage 5	-	When the complete plants were obtained. All observations were based on initial culture media, irrespective of regeneration medium or germination medium.

2.4 Statistical analysis of data
The data were analyzed in completely randomized factorial design (CRD) to find out the significance of different culture media combination, genotypic effects and their interactions with five replications. The analysis was carried out as per method suggested by MAUSTAT 3.0 software to study the single as well as interactive effects. Since all the data were recorded in percentage, the arc-sine transformation was made before analysis.

3. Results and discussion
3.1 *In vitro* morphogenesis studies:
An experiment was conducted with single explant of sunflower *viz*. immature embryo. Explants of three accessions *viz.* LSF-10, DMLY-1Y and GMU-479 were cultured on different combinations of MS media. The media were selected on the bases of preliminary experiments conducted to screen better suitable plant growth regulators and there combinations for *in vitro* response. The basal MS medium was fortified with different combinations of Sucrose, Dicamba and 2, 4-D in varying concentrations. Five different combinations of culture media were used for each explant isolated from three cultivars. During present investigation observations were recorded for somatic embryo induction, proliferation, maturation, and germination formation abilities. Analysis of variance showed that the mean sum of square due to different culture media combinations and accessions were highly significant at 5% probability level.

3.2 Morphogenesis in cultured explants
Single explants immature embryo was cultured on five different MS medium fortified with different concentrations and combinations of plant growth regulators. The first response of cultured explants was similar after 7 days and mostly independent of culture media combinations and accessions. During the first week, explants became swollen and no callus proliferation was evident. After seven days of cotyledons in culture, somatic embryo proliferation started from the edges. From cultured embryonic axis, somatic embryo formation usually started from full length of embryonic axis. Later, somatic embryo proliferation was observed from most of the explants. First set of observations were recorded after 4 weeks of culture somatic embryo-initiating explants were counted.
After initial culture of the explants, somatic embryo tissue developed distinct characteristics such as dense, rough, soft and sometimes glossy. *In vitro* morphogenesis is the way in which a somatic embryo forms a new plant *in vitro* was variable during the present investigation plant regeneration from the explant cultures appeared to be direct as well as via callus phase. Culture media played an imperative role in the formation of somatic embryo (Figure 4.4, 4.5, 4.6 and 4.7).

3.3 Immature embryo culture (Somatic embryo induction)
The mean callus induction frequencies from immature embryo cultures varied from 70.00% to 50.00%. Maximum somatic embryo induction was evident from GMU-479 (95.0%) and minimum by DMLT-1Y (30.0%). Among different culture media, MS+3.3mgD (95.0%) and MS+0.4mg 2, 4-D (80.0%) was found the highest. The minimum response was exposed by inoculation media MS+1.2D (30.0%).

3.4 Induction of globular somatic embryos
One month from inoculation on the somatic embryo induction (SEI) medium, tiny globular somatic embryos were observed in the cultured explants (Figure 4.0d). Globular embryos (one to four in number) first appeared in a few explants after 30 to 45 d of culture on SEI medium. Globular embryos were also observed previously non responsive explants after 60 d (Figure 4.1b). In the process of induction, the basal tissue turned brown gradually and greenish yellow somatic embryos were induced either singly or in clusters (Figure 4.8c) without a dominant callus phase. On continuous culturing on the SEI medium for four months with a 30 d subculture period, secondary somatic embryogenesis could be well documented (Figure 4.8d). The presence of two intact embryonic leaves on the explants affected the induction process as the explants without them did not produce somatic embryos (Figure 4.2a, 4.3b). Further, the orientation of the explants seemed to be crucial for induction, as no somatic embryos could be noticed when the adaxial surface of the explants faced the medium. The somatic embryo induction frequency was found to vary with genotype when analyzed at 60 and 90 d after inoculation. Among the three cultivars tested, LSF-10, DMLT-1Y and GMU-479 performed equally with 56.00%, 65.00% and 57% induction frequencies respectively after 30 d of inoculation (Table 4.2). In contrast, DMLT-1Y exhibited a poor induction frequency of 30.00% (Table 4.1).

3.5 Somatic embryo proliferation
The responding explants from SEI medium, when placed onto MSD20 medium progressively showed lobed and elongated structures at the embryonic leaf tip and formed compact clusters of newly embryos (Figure 4.8a & 4.8c). At the end of proliferation (after 3.5 weeks) origin of newly formed secondary globular embryos could be clearly traced to primary globular embryos in few explants and branching out basally in a rosette manner from adjacent (Figure 5.0f). Apart from apical proliferation, the embryos also multiplied in large numbers by or the existing embryo initials of proembryonic cell masses (Figure 5.0A & 5.0B).
The overall proliferation of somatic embryo from explant varied from 88.33% to 74.43% (Table 4.3). Most proliferated somatic embryos were generated from DMLT-1Y (95.0%) and least by LSF-10 (56.66%). The performance of culture MS + 1.2mg, 2, 4-D was better. The low responsive inoculation media MS + 1.2mg 2, 4-D.

3.6 Embryo maturation

The mean embryo maturation from immature embryo cultures varied from 89.99% to 59.99% (Table 4.6). Higher embryo maturation was observed from LSF-10 (100%) and lowest by DMLT-1Y (40%). Among culture media response to *in vitro* culture, the performance of culture media MS + 1mg, 2, 4-D and MS + 0.4mg, 2, 4-D was found to be higher. Results of maturation stage of somatic embryos are analyzed with respect to media which is used in previous stage of somatic embryos viz., proliferation stage. The low response was exposed by inoculation media MS + 1.2mg Dicamba and MS + 3.3mg, Dicamba.

Well proliferated clumps of somatic embryos from the MSD20 medium when further transferred to MSM6AC maturation medium, suffer a flow of development stages through heart and torpedo types (Figure 5.1 and 5.0c).

Monocotyledonous (Figure 5.0a), polycotyledonous (5.0b), embryos showed normal development. However, a range of other morphotypes was also noticed. Abnormal, long hypocotyls with vestigial cotyledon (Figure 5.0E) and a few other unclassified embryo types were considered as abnormal. The maturation frequency when compared among the cultivars, LSF-10 cultivar performed better with a maturation frequency of 88 percent, than DMLT-1Y with a maturation frequency of 66.99% (Table 4.2).

3.7 Embryo germination

Partial dehydration of the mature embryos for different durations was carried out in an empty Petri dish for 72 hrs. (Figure 4.9) in order to evaluate the effect of desiccation on rooting, germination and plantlet conversion. The somatic embryos subjected to long desiccation treatments for 96hrs showed more rooting.

In the process of recovery of plantlets well differentiated somatic embryos belonging to monocotyledonous, dicotyledonous, tricotyledonous and polycotyledonous morphotypes showed normal development in MSB5 regeneration medium (Figure 5.2a) with well-developed shoots (Figure 5.2b) and roots (Figure 5.3c).

The mean embryo germination from immature embryo cultures varied from 50.00% to 64.44% (Table 4.7). Higher number of germination was observed from GMU-479 (64%) and lowest by DMLY-1Y (49.99%). Results of germination stage of somatic embryos are analyzed with respect to media which is used in previous stage of somatic embryos viz., maturation stage. Among culture media response to *in vitro* culture, the performance of culture media MS + 1mg, 2, 4-D was found to be higher. The low response was exposed by inoculation media MS + 1.2mg 2, 4-D.

4. Conclusions

Higher number of germination was observed from GMU-479 (64%) and lowest by DMLY-1Y (49.99%). Among culture media response to *in vitro* culture, the performance of culture media MS+1mg, 2, 4-D was found to be higher. The low response was exposed by inoculation media MS+1.2mg 2,4-D.

Maximum somatic embryo induction was evident from GMU-479 (95.0%) and minimum by DMLT-1Y (30.0%). Among different culture media, MS+3.3mgD (95.0%) and MS+0.4mg 2, 4-D (80.0%) was found the highest. The minimum response was exposed by inoculation media MS+1.2D (30.0%).

Higher embryo maturation was observed from LSF-10 (100%) and lowest by DMLT-1Y (40%). Among culture media response to *in vitro* culture, the performance of culture media MS + 1mg 2, 4-D and MS + 0.4mg, 2, 4-D was found to be higher. The low response was exposed by inoculation media MS + 1.2mg Dicamba and MS + 3.3mg, Dicamba.

Table 4.1: Somatic embryo induction from immature embryo of three cultivars of sunflower cultured on five different fortification of MS media

Cultivars/Culture media (120gm/lit)	DMLT-1Y	GMU-479	LSF-10	Mean
MS.0.4mg 2,4-D	75 (63.71)	80 (56.86)	55 (33.71)	70.00 (51.42)
MS.1mg 2,4-D	75 (53.14)	60 (37.43)	55 (33.71)	63.33 (41.43)
MS.1.2mg 2,4-D	55 (33.71)	55 (34.32)	50 (30.61)	53.33 (32.88)
MS.3.3mg Dicamba	45 (27.50)	95 (81.70)	40 (23.78)	60.00 (44.33)
MS.1.2mg Dicamba	30 (17.58)	35 (20.68)	85 (65.14)	50.00 (34.47)
Mean	56.00 (39.13)	65.00 (46.20)	57.00 (37.39)	
CD: 0.05. **Genotypes:** NS. **Concentration:** 12.15. **GXC:** 21.04.				
[Values are means of five replications, each with 20 explants; within a column, means followed by a common letter are not significantly different (*P = 0.05*) by CRD. Values within the parentheses are square root transformed values of arcs in values. SEd (genotypes)=3.40, SEd (concentration)=4.39 and SEd (gxc)=7.60, CD (genotypes)=9.41, CD (concentration)=1.21 and CD (gxc)=2.10, CV (%)=4.15]				

Table 4.2: Frequency of somatic embryo induction in immature zygotic embryo explants of three sunflower cultivars

Cultivar	Induction frequency after 30 d (% ± SE)
DMLT-1Y	56.00 (39.13)
GMU-479	65.00 (46.20)
LSF-10	57.00 (37.39)
[Values are means of five replications 30 d after inoculation, each with approx. 20 explants; within a column, means followed by a common letter are not significantly different (*P = 0.05*) by CRD. Values within the parentheses are arc sin transformed values. SEd (genotypes)=3.40, SEd (concentration)=4.39, and SEd (gxc)=7.60, CD (genotypes)=9.41, CD (concentration)=1.21, and CD (gxc)=2.10, CV (%)=4.15]	

Table 4.3: Proliferation from immature embryo of three cultivars of sunflower cultured on five different fortification of MS media

Cultivars/Culture media (60gm sucrose/lit)	DMLT-1Y	GMU-479	LSF-10	Mean
MS.0.2mg 2,4-D	95 (81.17)	86.64 (70.69)	76.66 (63.88)	86.10 (72.09)
MS.0.5mg 2,4-D	90 (77.99)	93.32 (80.34)	73.32 (56.34)	85.55 (71.55)
MS.0.6mg 2,4-D	80 (65.99)	86.64 (70.69)	56.66 (39.88)	74.43 (58.85)
MS.1.6mg 2,4-D	73.32 (56.34)	83.32 (63.78)	80 (65.99)	78.88 (62.03)
MS.0.6mg Dicamba	90 (77.99)	80 (65.99)	95 (81.70)	88.33 (75.23)
Mean	85.66 (72.00)	85.98 (70.33)	76.33 (61.56)	
CD: 0.05 **Genotypes:** 15.54 **Concentration:** 20.07 **GXC:** 34.76				
[Values are means of five replications, each with 20 explants; within a column, means followed by a common letter are not significantly different (*P = 0.05*) by CRD. Values within the parentheses are square root transformed values of arcsin values. SEd (g)=5.61, SEd (c)=7.25, and SEd (gxc)=1.25, CD (g)=1.55, CD (c)=2.00, and CD (gxc)=3.47, CV (%)=4.13]				

Table 4.4: Frequency of proliferation in immature zygotic embryo explants of three sunflower cultivars

Cultivar	Proliferation frequency after 30 d (% ± SE)
DMLT-1Y	85.66 (72.00)
GMU-479	85.98 (70.33)
LSF-10	76.33 (61.56)
[Values are means of five replications 30 d after inoculation, each with approx. 20 explants; within a column, means followed by a common letter are not significantly different (*P = 0.05*) by CRD. Values within the parentheses are arc sin transformed values. SEd (g)=5.61, SEd (c)=7.25, and SEd (gxc)=1.25, CD (g)=1.55, CD (c)=2.00, and CD (gxc)=3.47, CV (%)=4.13]	

Table 4.5: Maturation from immature embryo of three cultivars of sunflower cultured on five different fortification of MS media

Cultivars/Culture media	DMLT-1Y	GMU-479	LSF-10	Mean
MS. B5. Activated charcoal. 3% Maltose	85 (65.14)	76.64 (58.69)	90 (77.99)	83.88 (67.27)
MS. B5. Activated charcoal. 3% Maltose	76.65 (58.69)	93.32 (80.34)	100 (89.98)	89.99 (76.34)
MS.B5. Activated charcoal. 3% Maltose	83.32 (68.34)	90 (77.99)	70 (59.99)	81.11 (68.77)
MS. B5. Activated charcoal. 3% Maltose	50 (41.99)	85 (74.88)	100 (89.98)	78.33 (68.95)
MS. B5. Activated charcoal.3%Maltose	40 (35.99)	60 (53.99)	79.98 (66.23)	59.99 (52.07)
Mean	66.99 (54.03)	80.99 (69.18)	88.00 (76.83)	
CD: 0.05 **Genotypes:** 18.06 **Concentration:** NS **GXC:** 40.39				
[Values are means of five replications, each with 20 explants; within a column, means followed by a common letter are not significantly different (*P = 0.05*) by CRD. Values within the parentheses are square root transformed values of arcsin values. SEd (g)=6.52, SEd (c)=8.42, and SEd (gxc)=4.45, CD (g)=1.80, CD (c)=2.33, and CD (gxc)=4.03, CV (%)=4.89]				

Table 4.6: Frequency of maturation and germination in immature zygotic embryo explants of three sunflower cultivars

Cultivar	Maturation frequency after 30 d (% ± SE)	Cultivar	Germination frequency after 30 d (% ± SE)
DMLT-1Y	66.99 (54.03)	DMLT-1Y	49.99 (41.05)
GMU-479	80.99 (69.18)	GMU-479	64.00 (51.71)
LSF-10	88.00 (76.83)	LSF-10	55.20 (46.29)
[Values are means of five replications 30 d after inoculation, each with approx. 20 explants; within a column, means followed by a common letter are not significantly different (P = 0.05) by CRD. Values within the parentheses are arc sin transformed values. SEd (g)=6.52, SEd (c)=8.42, and SEd (gxc)=4.45, CD (g)=1.80, CD (c)=2.33 and CD (gxc)=4.03, CV (%)=4.89]		[Values are means of five replications 30 d after inoculation, each with approx. 20 explants; within a column, means followed by a common letter are not significantly different (P = 0.05) by CRD. Values within the parentheses are arc sin transformed values. SEd (g)=1.30, SEd (c)=1.68, and SEd (gxc)=2.91, CD (g)=3.60, CD (c)=4.66 and CD (gxc)=8.07, CV (%)=1.60]	

Table 4.7: Germination from immature embryo of three cultivars of sunflower cultured on five different fortification of MS media

Cultivars/Culture media (120gm/lit)	DMLT-1Y	GMU-479	LSF-10	Mean
MS+B_5vitamins +3% sucrose	66.62 (46.94)	70 (53.99)	36 (27.48)	57.54 (42.80)
MS+3% sucrose	73.32 (62.34)	50 (35.99)	70 (59.99)	64.44 (52.77)
MS+ B_5vitamins	70 (59.99)	60 (47.99)	20 (17.99)	50.00 (41.99)
MS+33mg 2,4-D+3% sucrose	20 (17.99)	80 (66.60)	70 (59.99)	56.67 (48.19)
MS+1.2mg Dicamba+3% sucrose	20 (17.99)	60 (53.99)	80 (59.99)	53.33 (45.99)
Mean	49.99 (41.05)	64.00 (51.71)	55.20 (46.29)	

CD: 0.05 **Genotypes:** 21.39 **Concentration:** NS **GXC:** NS
[Values are means of five replications, each with 20 explants; within a column, means followed by a common letter are not significantly different (*P = 0.05*) by CRD. Values within the parentheses are square root transformed values of arcsin values. SEd (g)=7.73, SEd (c)=9.98, and SEd (gxc)=1.72, CD (g)=2.13, CD (c)=2.76, and CD (gxc)=4.78, CV (%)=8.34]

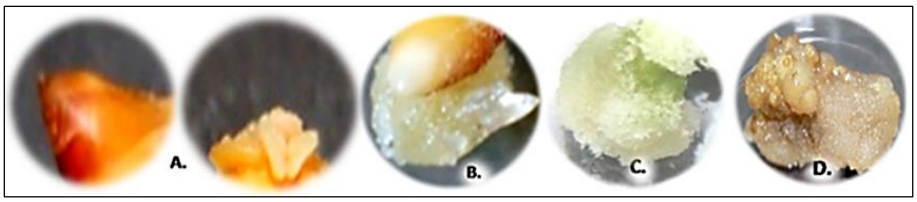

Fig 4.0: Induction at different stages on SE1 medium (Sucrose-120gm/lit. 2. 4-1) 0.40mg/lit)

A. Swelling were in immature embryo after 14 -25 days
B. The initiation of callus at 25 days
C. The initiation of callus at 30 days
D. Development of yellowish white friable callus at 45 days

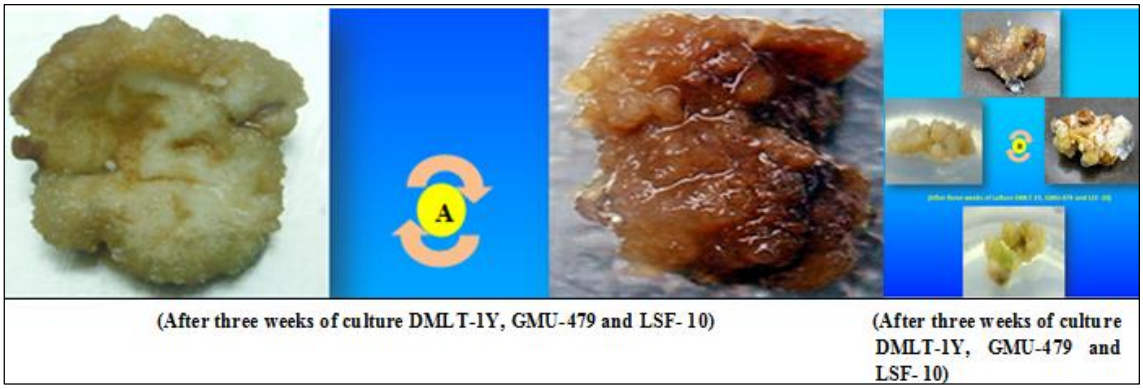

Fig 4.1: Globular stage somatic embryo formed from primodium of immature zygotic embryos of three elite genotypes on SEI medium (After three to eight weeks of culture)

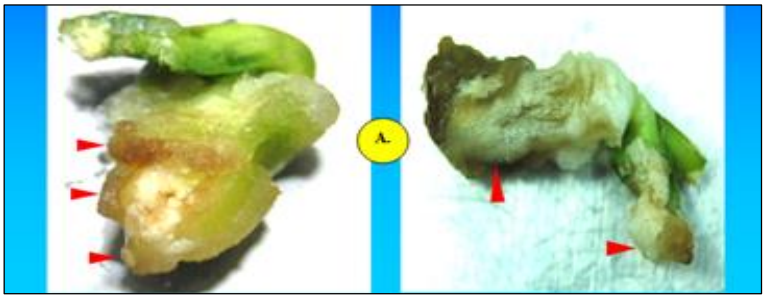

Fig 4.2: Failure of somatic embryo induction of immature zygotic embryos with leaf primodia on SEI medium of three elite sunflower genotypes

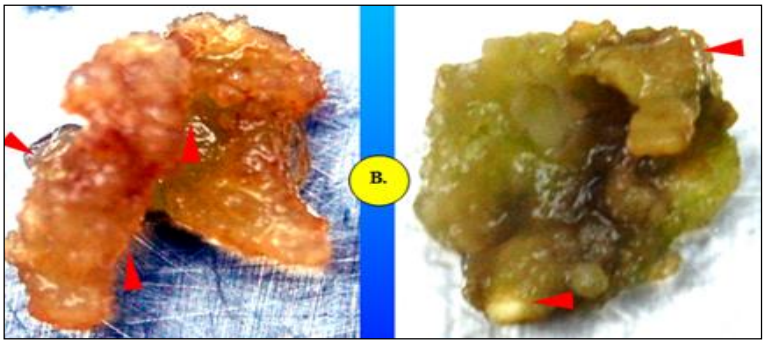

Fig 4.3 Failure of somatic embryo induction of immature zygotic embryos lacking leaf puimodia on SEI medium of three elite sunflower genotypes

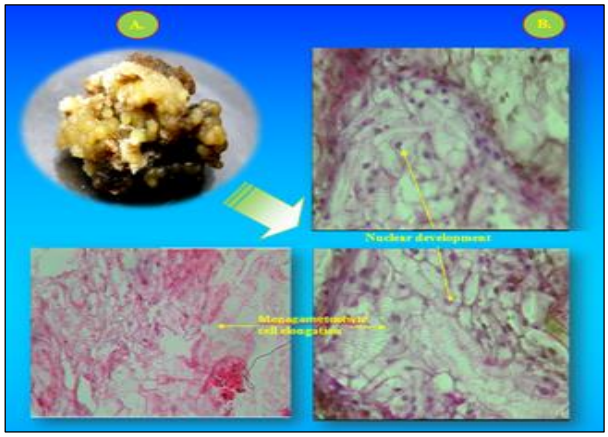

Fig 4.4: A. The morphology of embryogenic callus B. The microscopy of embryogenic callus

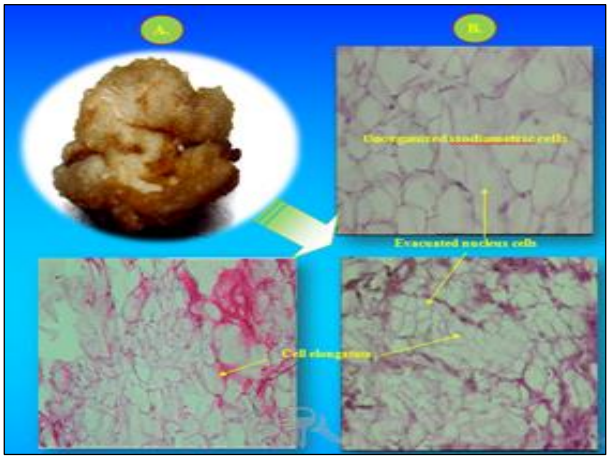

Fig 4.5: A. The morphology of embryogenic and nonembryogenic callus B. The microscopy of embryogenic and nonembryogenic callus.

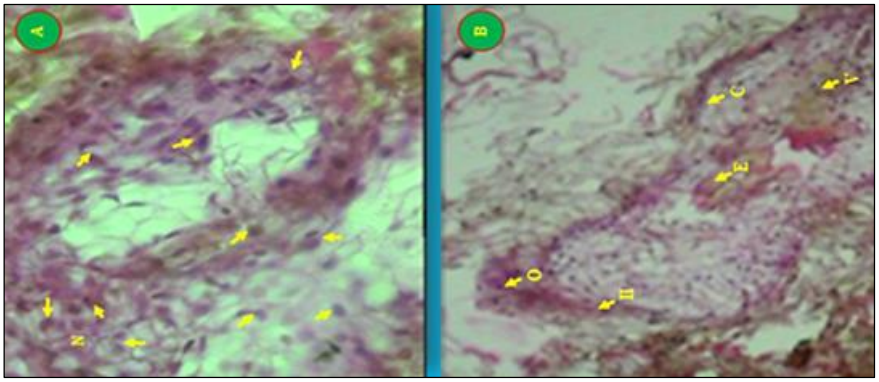

Fig 4.6: A. Section of part of the nucleus showing embryo initials (arrows) with thick wall B. Section of an ovule showing an embryogenic mass in the chalazal part of the ovule connected by a vascular network. C=chalazal end, E=embryo, II=inner integument, N=nucleus, OI=outer integument, V=vascular network

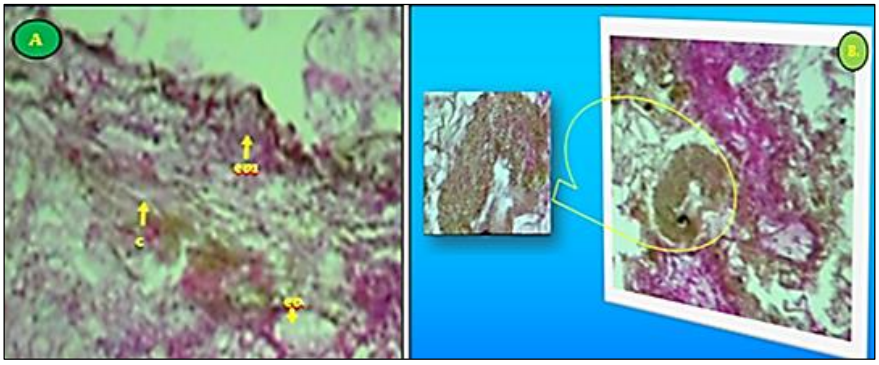

Fig 4.7: A. Thin section of an epidermal strip showing the initiation sites of so mafic embryos (em) in the hgtentiaggpart of the strip in relation to the epidermis (gp). The inner cells of the callus (c) B. Part on a somatic embryo at the heart-shaped stage

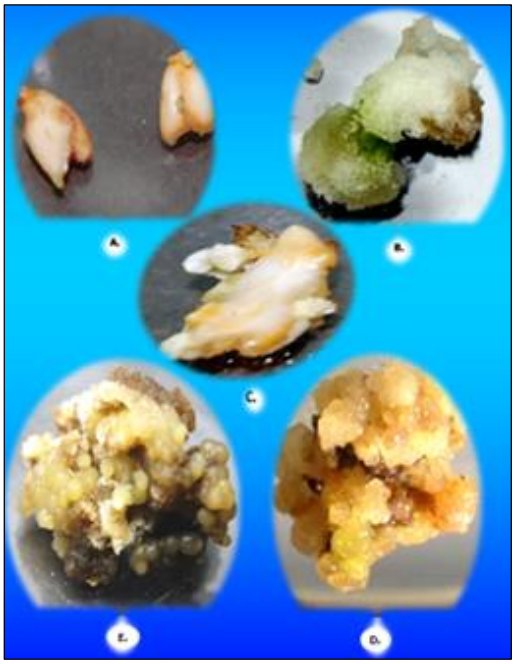

Fig 4.8: Proliferation at different stages

A. Swelling were in immature embryo after 14 days
B. The proliferation of callus at 21 days
C. The proliferation of callus at 28 days
D. Development of yellowish white friable $_{a}t_{m}$ at 45 days
E. Some somatic embryos were visible at 55 days the surfaces of the cotyledons were covered with globular embryos

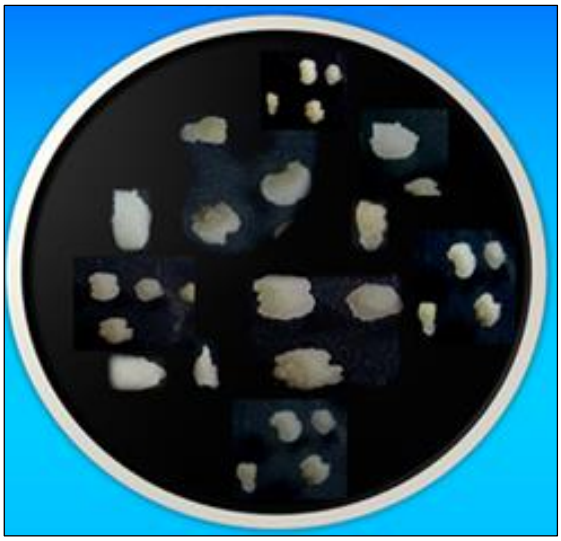

Fig 4.9: Somatic embryos collected from proliferation stage for maturation

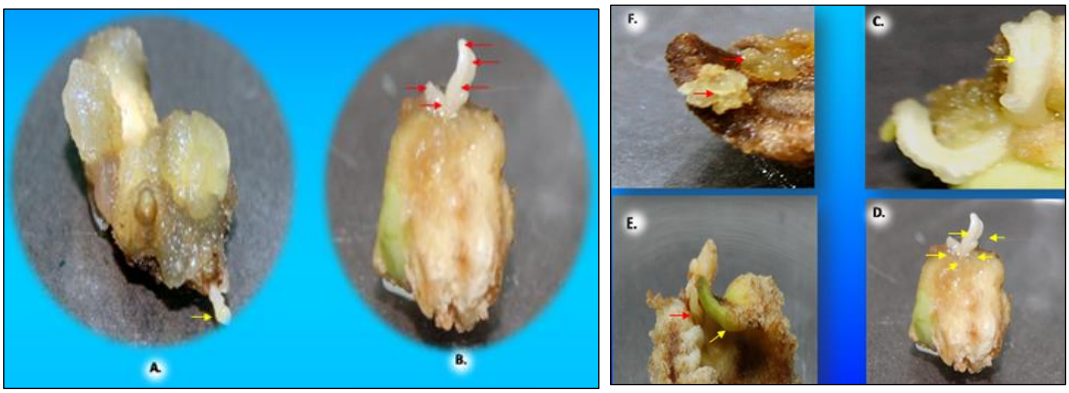

Fig 5.0: Maturation at different stages on SEM medium (Sucrose-120gm/lit Dicamba-3.3mg/lit,)

A. Development of embryo at 65 days
B. Development of embryo at 75 days
C. Early heart shape somatic embryo showing a notch
D. Polycotyledonous development of somatic embryos
E. Abnormal development (Long hypocotyls; vestigeal cotyledon) of somatic embryos
F. Cluster of globular stage embryo at 30 days

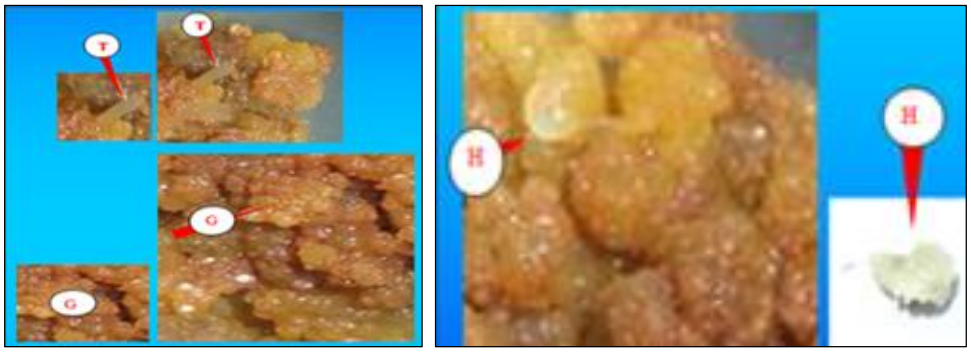

Fig 5.1: Somatic embryogenesis showing different stages. (T-Torpedo, G-Globular and H-Heart shape)

Fig 5.2: (A) Regeneration/germination from immature zygotic embryos derived somatic embryos at different stages 9,11GM medium. (Sucrose-30gm/lit)

Fig 5.2: (B) Regeneration/ germination from immature zygotic embryos derived somatic embryos at different stages on GNI medium (Sucrose-30gm/lit)

Fig 5.3: (a) an example of germinatin somatic embryos without a root

Fig 5.3: (b) an example of germinating somatic embryos without hypocotyl

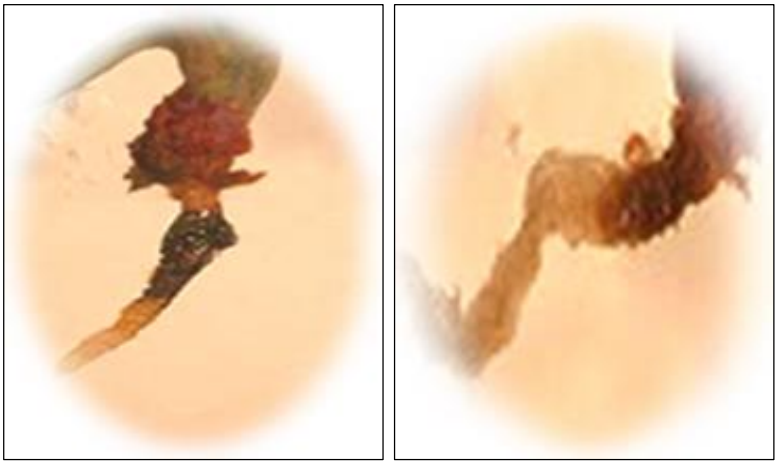

Fig 53: (c) An example of germinating somatic embrpas with well- developed *root*

5. Acknowledgements
This work was supported by Department of Biotechnology (DBT), New Delhi and Associate Dean and principal, V.D.C.O.A.B., Latur (MS). We thank to D.O.R., Hyderabad for providing seeds of sunflower cultivars/Hybrids.

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