In vitro Plant Regeneration through Organogenesis and Somatic Embryogenesis of *Punica granatum Linn*. (Lythraceae)

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Abstract - Reliable protocol was developed to obtain healthy plants of imported (Indian) *Punica granatum* Linn. using *in vitro* germinated seedlings and juvenile explants. *In vitro* seed germination of imported seeds was induced on MS medium supplemented with different concentrations of GA3. The best medium was found to be the MS medium supplemented with 5.0 mg/L GA3 and seed stratification induced the seed germination percentage and seedling height. Cotyledon, hypocotyle, internode and leaf explants were excised from *in vitro* germinated seedlings for calli induction. MS medium supplemented with 2.5 mg/L NAA and 3.0 mg/L BAP was found to be best medium for callus induction from cotyledons, 2.5 mg/L NAA and 4.0 mg/L BAP from hypocotyle, 1.5 mg/L NAA and 2.0 mg/L BAP from internode and 1.5 mg/L NAA and 3.0 mg/L BAP from leaf explants. Cotyledon explants showed the highest percentage of callus induction followed by internode, leaf and hypocotyle explants. MS medium supplemented with 0.5 mg/L NAA and 3.0 mg/L BAP was found to be the best medium for shoot regeneration from calli obtained from all types of explants. Highest shoot regeneration percentage was showed in cotyledon derived callus followed by hypocotyle, internode and leaf derived calli. For somatic embryogenesis leaf disc explants were taken from 45 days old *P. granatum* Linn. plantlets. MS medium supplemented with 500.0 mg/L L-Glutamine, 2.0 mg/L 2,4-D and 2.0 mg/L BAP was found to be the best medium for embryonic callus induction, MS medium with 500.0 mg/L L-Glutamine, 1.0 mg/L 2,4-D was found to be the most effective medium for embryonic callus formation. The treatment with 0.25 mg/L BAP, 500.0 mg/L Casein hydrolysate, 500.0 mg/L L-Glutamine and 30.0 g/L sucrose under dark conditions gave the highest percentage of matured embryonic callus.

Index Terms - *Punica granatum* Linn., seed culture, callus induction, shoot regeneration, somatic embryogenesis, embryonic callus.

1 Introduction

P. granatum Linn. (Family Lythraceae) is one of the most important fruit species in the tropical and subtropical regions of the world with immense properties of importance not only in indigenous medicine but also in western medicine [5].

Delicious edible fruit is popularly consumed as a fresh fruit or a beverage (as juice and wine) and as processed forms such as jam, jelly, syrup and sauce [17] [15]. *P. granatum* Linn. has been used for thousands of years to cure a wide range of diseases as it has great nutritional values and numerous health benefits [2].

P. granatum Linn. varieties from India are larger in size and deep red in color. The inner aril also deep red in color with flesh and soft seeds [3]. Consumer preference is higher for those varieties than Sri Lankan due to the attractive nature of imported variety although the fruits are very expensive in the market.

Conventional vegetative propagation methods, seed production and seed germination have limitations in mass propagation [5], thus tissue culture could be the attractive alternative in producing phenotypically superior and genetically improved plants of *P. granatum* Linn.

Protocols have been developed for regeneration of *P. granatum* Linn. *in vitro* through either indirect organogenesis through callus derived from leaf segments [12], cotyledons, or through somatic embryogenesis from various seedling explants and immature zygotic embryos [7].

In vitro germinated seedlings are the most commonly used source of explants for indirect organogenesis. Callus induction from *P. granatum* Linn. explants has been achieved in MS medium supplemented with different concentrations of growth regulators.

2 Materials and Methods

P. granatum Linn. (Indian variety) fruits were collected and the juicy pulp was removed from the seeds then the seeds were air dried.

The seeds were kept under running tap water for one hour followed by washing in a 5% (v/v) teepol solution and rinsed 5-6 times with distilled water before surface sterilization. Seeds were then surface sterilized by treating with 0.2% Carbendazim® for 10 minutes then in 15% sodium hypochlorite (Clorox) for 20 minutes and finally in 70% ethanol for 30 seconds each followed by three successive washings in sterile distilled water.

After that the seeds were soaked in sterilized distilled water for 24 hours to soften the seed coat, and the seed coat was excised in half of the seed lot by making a horizontal cut on the broad part of the seed (stratified) and other lot of the seeds were not treated (non stratified). Both stratified and non-stratified seeds were placed on the MS [11] medium supplemented with different concentrations (3.0, 4.0, 5.0 mg/L) of Gibberalic acid (GA3) for *in vitro* seed germination. Growth regulators free MS medium was used as the control. The PH of the medium was adjusted to 5.8 and cultures were incubated at 25 ±2 °C with 50% humidity for germination. Percentage seed germination and mean height of seedlings were determined after 30 days of incubation.

2.1 Initiation of callus cultures through *in vitro* germinated seedlings

Cotyledons without proximal ends were transversely cut into two halves (0.5cm); hypocotyl segments (0.5 cm); internodes segments (0.5 cm) and leaf parts (0.5 cm) were excised from thirty day-old *in vitro* germinated seedlings. Explants were treated with 0.2% Carbendazim for 5 minutes and cultured on MS medium supplemented with NAA (Napthalene Acetic Acid) (1.5-3.5 mg/L) in combination with BAP (Benzyl Amino Purine) (2.0 - 4.0 mg/L) for the induction of callus. Percentage of callus-forming explants and diameter of callus were recorded over a period of 7 weeks.

2.2 Shoot regeneration through indirect organogenesis

Callus was subcultured on MS medium supplemented with lowered concentrations of NAA (0.1-0.5 mg/L) and increased levels of BAP (3.0-5.0 mg/L). Percentages of callus showing shoot bud induction and mean number of shoots per callus were recorded.

2.3 Induction of embryonic callus from leaf explants of *in vitro* seedlings

About 45 days old *in vitro* germinated healthy seedlings were selected and 0.5×1.0 cm leaf discs were carefully excised including the midrib of the leaf. They were cultured on three different culture media.

Medium A: Different combination of NAA (2.0–6.0 mg/L) and Kin (Kinetin) (1.0–2.0 mg/L) with 15% coconut water and cultures were incubated under 16 hours photo period.

Medium B: Different combination of 2,4-D (2,4-Dichlorophenoxy acetic acid) (0.5–12.0 mg/L) and BAP (0.5–2.5 mg/L) with 500.0 mg/L L-Glutamine and cultures were incubated under dark condition.

Medium C: Different combination of 2,4-D (2.5-7.5 mg/L) and BAP (3.0–6.0 mg/L) 500.0 mg/L Casein hydrolysate and cultures were incubated under dark condition.

Five leaf discs were cultured in each culture vessel and after 4 weeks, induced calli were subcultured into same medium. Diameter of the embryonic callus and frequency of embryonic callus were observed after 14 days.

2.4 Maturation of somatic embryos

Induced embryonic calli were transferred into culture vessels containing MS medium with 100.0 mg/L myo-inositol with different combinations and concentrations of 2,4-D (0.0- 1.0 mg/L) BAP (0.0- 0.25 mg/L) and different amino acids Casein hydrolysate (0.0-500.0 mg/L); Glutamin (0.0- 500.0 mg/L) with 30.0- 90.0 g/L sugar. Culture vessels were kept under dark condition. Percentage of calli with globular shaped cells and mean number of somatic embryos with cotyledonary shape was determined.

3 Results and Discussion

3.1 *In vitro* seed germination

Germination was observed in stratified seeds after 8 days of incubation and non-stratified seeds took longer period (13 days). There was a combined effect of GA3 and stratification on seed germination and seedling height. Seeds cultured in growth regulations free medium also germinated however seedling height was significantly lower than other treatments (Fig 1). Stratification enhances the percentage seed germination significantly yet seedling height of stratified seeds was higher but not significantly different (Table 1).

Among treatments stratified seeds grown on MS medium supplemented with 5.00 mg/L GA3 found to be the best treatment for *in vitro* seed germination (75.00 %), which gave the highest seedling height after 30 days (4.53 ± 0.12 cm). MS medium supplemented with 4.00 mg/L GA3 could be considered as the second best medium which was significantly lower than the best treatment. Growth regulator free MS medium and non-stratified seeds showed the lowest response (40.00 %) with lowest seedling height (1.43 ± 0.13 cm) after 30 days.

TABLE 1: PERCENTAGE SEED GERMINATION AND MEAN HEIGHT OF

Stratified or Non Stratified	GA3 (mg/L)	Germination percentage (%)	Mean height of seedlings (cm) ± SD
Non Stratified	0.00	40.0	1.42 ± 0.13
	3.00	50.5	2.45 ± 0.11
	4.00	60.0	2.81 ± 0.14
	5.00	55.0	3.43 ± 0.13
Stratified	0.00	45.0	1.49 ± 0.11
	3.00	60.0	3.48 ± 0.10
	4.00	70.0	3.51 ± 0.12
	5.00	75.0	4.53 ± 0.12
LSD 5%		0.328	0.000

THE SEEDLINGS AFTER 30 DAYS IN THE PRESENCE OF GA3.

Seeds showed an increased percentage germination and seedling height with increased concentration of GA3 whether the seeds were stratified or not. According to the results stratified seeds showed significantly higher germination percentage and seedling height compared to non-stratified seeds.

The results of the present study also indicated that GA3 breaks dormancy by acting in the gene silencing involved in the maintenance of dormancy and also in the progression of embryonic prolongation. GA3 promote the enzyme synthesis involved in reserve mobilization of dormancy [9]. These observations showed that GA3 is a main agent involved in the dormancy breaking and stem elongation [14].

Results of the present study confirmed that stratification has a significant influence in seed germination. The seed coat acts as a mechanical barrier to the germination of seeds. A decline in this mechanical resistance of the seed coat appears to be a prerequisite for radical protrusion during seed germination. It is associated with an additional increase in seed water content in the water uptake [10]. Thus increased percentage germination and seedling height observed in the study could be considered as a combine effect of stratification and GA3.

FIG 1: SEED GERMINATION AFTER 30 DAYS OF INCUBATION; NON STRATIFIED A) GA3 FREE, B) 3.0 M/L GA3, C) 4.0 MG/L GA3, D) 5.0 MG/L GA3, STRATIFIED E) GA3 FREE, F) 4.0 M/L GA3, G) 4.0 M/L GA3, H) 5.0 M/L GA3.

3.2 Initiation of callus cultures through *in vitro* germinated seedlings

Initiation of callus in explants was observed along cut surface after three weeks of incubation. The whole surface of the explants was covered with the vegetative callus within 6-7 weeks of incubation. Results indicated that the plant growth regulators used have a significant effect on percent callus formation from different explants (fig 2). Explants cultured on growth regulator free MS medium (control) did not show any callus formation (Table 2).

TABLE 2: EFFECT OF DIFFERENT CONCENTRATIONS OF NAA AND BAP IN MS MEDIUM ON CALLUS INDUCTION FROM COTYLEDON, HYPOCOTYLE, INTERNODE AND LEAF EXPLANTS AFTER 7 WEEKS OF CULTURE.

Treatment code	NAA (mg/L)	BAP (mg/L)	Cotyledon		Hypocotyle		Internode		Leaf	
			Callus diameter (cm) ± SD	Callus induction (%)	Callus diameter (cm) ± SD	Callus induction (%)	Callus diameter (cm) ± SD	Callus induction (%)	Callus diameter (cm) ± SD	Callus induction (%)
Con.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
S1	1.50	2.00	0.90 ± 0.07	62.5	0.70 ± 0.00	40.0	1.13 ± 0.07	80.0	0.80 ± 0.07	62.5
S2	2.50	2.00	1.13 ± 0.11	77.8	0.67 ± 0.05	50.0	1.09 ± 0.07	77.8	0.80 ± 0.10	60.0
S3	3.50	2.00	1.00 ± 0.09	66.7	0.70 ± 0.00	42.9	0.73 ± 0.05	57.1	0.67 ± 0.06	50.0
S4	1.50	3.00	1.06 ± 0.05	71.4	0.77 ± 0.06	60.0	0.96 ± 0.05	71.4	0.90 ± 0.09	75.0
S5	2.50	3.00	1.23 ± 0.10	85.7	0.68 ± 0.10	66.7	0.83 ± 0.05	66.7	0.85 ± 0.06	66.7
S6	3.50	3.00	1.08 ± 0.15	83.3	0.65 ± 0.10	40.0	0.80 ± 0.10	60.0	0.75 ± 0.06	57.1
S7	1.50	4.00	0.67 ± 0.16	60.0	0.80 ± 0.00	60.0	0.98 ± 0.08	71.4	0.70 ± 0.08	57.1
S8	2.50	4.00	1.15 ± 0.06	80.0	0.87 ± 0.05	75.0	0.85 ± 0.05	66.7	0.80 ± 0.09	60.0
S9	3.50	4.00	0.83 ± 0.06	60.0	0.84 ± 0.05	71.4	0.70 ± 0.08	0.50	0.50 ± 0.00	42.9
Mean			0.98	72.11	0.74	55.61	0.89	65.84	0.75	59.47
LSD 5%			0.01	0.025	0.01	0.394	0.01	0.088	0.01	0.391

MS medium supplemented with 2.5 mg/L NAA and 3.0 mg/L BAP found to be the best treatment for callus induction (85.70 %) in cotyledons, which gave the highest mean callus diameter (1.23 ± 0.10 cm). The best medium for callus induction from hypocotyl explant was MS medium supplemented with 2.5 mg/L NAA and 4.0 mg/L BAP which gave 75.0% callus induction and 0.87 ± 0.05 cm callus diameter.

Highest callus induction (80.00 %) and callus diameter (1.13 ± 0.07 cm) from internode explants was achieved in MS medium supplemented with 1.5 mg/L NAA and 2.0 mg/L BAP. On the other hand, the highest percent callus induction (75.00%) and callus diameter (0.90 ± 0.09 cm) from leaf explants was obtained in MS medium supplemented with 1.5 mg/L NAA and 3.0 mg/L BAP.

In general, the cotyledon explants yielded a significantly higher callus induction percentage (72.11%) and mean callus diameter (0.98 cm) compared to hypocotyle (55.61 %, 0.74 cm), internode (65.84 %, 0.89 cm) and leaf (59.47 %, 0.75 cm) explants, irrespective of different media combination.

3.3 Shoot regeneration through indirect organogenesis

Shoot regeneration from callus pieces was observed after 3 weeks of incubation. Results indicated that the plant growth regulators used had a significant effect on percentage of calli producing shoots and number of shoots per callus.

Among tested treatments, the best medium for the shoot regeneration from calli was MS medium supplemented with 0.5 mg/L NAA and 3.0 mg/L BAP. Shoot regeneration was observed in 20 to 80 % of calli (Table 3). Highest percentage shoot regeneration was observed in callus derived from cotyledons (80.00%) followed by hypocotyle (75.00%), internode (75.00%) and leaf discs (66.66 %). There was a significant difference on shoot regeneration and explants type which generate callus.

TABLE 3: EFFECT OF DIFFERENT CONCENTRATIONS OF NAA AND BAP SUPPLEMENTED IN MS MEDIUM ON PERCENT SHOOT REGENERATION AND NUMBER OF SHOOTS PER CALLUS FROM COTYLEDON, HYPOCOTYLE, INTERNODE AND LEAF EXPLANTS AFTER 8 WEEKS OF CULTURE.

Treatment code Control	NAA (mg/L)	BAP (mg/L)	Cotyledon		Hypocotyle		Internode		Leaf	
			Treatment code	Treatment code	Treatment code	Treatment code	Treatment code	Treatment code	Treatment code	Treatment code
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
S1	0.10	3.00	4.0 ± 0.00	40.0	3.5 ± 0.71	40.0	4.00 ± 0.00	40.0	4.0 ± 0.00	50.0
S2	0.30	3.00	4.7 ± 0.58	75.0	4.3 ± 0.58	60.0	4.5 ± 0.71	66.7	5.0 ± 0.00	40.0
S3	0.50	3.00	5.8 ± 0.50	80.0	5.3 ± 0.58	75.0	5.3 ± 0.58	75.0	5.5 ± 0.71	66.7
S4	0.10	5.00	4.7 ± 0.58	75.0	5.0 ± 0.00	66.7	4.5 ± 0.71	50.0	4.5 ± 0.71	50.0
S5	0.30	5.00	4.3 ± 0.58	60.0	4.5 ± 0.71	50.0	4.3 ± 0.58	60.0	4.3 ± 0.58	60.0
S6	0.50	5.00	3.0 ± 0.00	50.0	3.0 ± 0.00	40.0	2.5 ± 0.71	50.0	2.5 ± 0.71	40.0
S7	0.10	7.00	1.0 ± 0.00	25.0	1.0 ± 0.00	33.3	1.0 ± 0.00	20.0	1.0 ± 0.00	25.0
S8	0.30	7.00	1.0 ± 0.00	33.3	2.0 ± 0.00	33.3	1.0 ± 0.00	25.0	1.0 ± 0.00	25.0
S9	0.50	7.00	1.0 ± 0.00	50.0	1.0 ± 0.00	25.0	1.0 ± 0.00	20.0	1.0 ± 0.00	20.
LSD 5%			0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Highest number of shoots (6.00 shoots) per callus was formed on MS medium supplemented with 0.50 mg/L NAA and 3.00 mg/L BAP. The maximum mean number of shoots per callus was observed from cotyledon derived callus (5.8 ± 0.50 shoots) followed by hypocotyle (5.3 ± 0.58

shoots), leaf (5.3 ± 0.58 shoots) and internode (5.5 ± 0.71 shoots) derived calli.

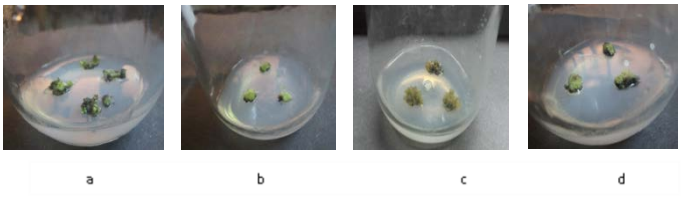


FIG 2: CALLUS INDUCTION THROUGH DIFFERENT EXPLANTS. A) COTYLEDON DERIVED CALLUS; B) HYPOCOTYLE DERIVED CALLUS; C) INTERNODE DERIVED CALLUS; D) LEAF DERIVED CALLUS;

3.4 Induction of embryonic callus from leaf explants from *in vitro* seedlings

MS medium supplemented with different concentrations of NAA, Kinetin and 15% coconut water not only induced the vegetative callus under 16 hrs photoperiod but also shoot induction and shoot multiplication. The highest shoot formation was observed in the MS medium supplemented with 2.0 mg/L NAA and 2.0 mg/L Kin. Control showed very low callus induction and no shoot formation, indicating that there is a significant effect of plant growth regulators on embryonic callus formation .

Al-Khayri, (1992) [1] showed that, 15% (v/v) coconut water (CW) to the culture medium significantly improved callus growth, shoot-regenerative capacity, and shoot growth in leaf disk cultures of spinach (*Spinacia oleracea* L.). Patil and Borkar (2015) [13] said it is very difficult to induce roots in callus when *P. granatum* Linn. micro propagation and as a solution application of Agrobacterium rhizogenes can be done.

MS medium supplemented with different combinations of 2,4-D and BAP with 500.0 mg/L L-Glutamine induced callus formation within the first 4 weeks after the establishment in dark condition. The highest percentage callus induction was observed in the presence of either 1.0 mg/L or 2.0 mg/L 2,4-D in combination with BAP at the range of 1.5 -2.5 mg/L (Fig 3a). Lower levels or too high levels of 2,4-D and BAP also induced calli however the percentages were very low. 2,4-D or BAP along also did not produce callus and 1.0 mg/L 2,4-D produced the highest amount of embryonic callus (Table 4).

No callus induction was observed in the presence of high levels of 2,4-D alone. This indicates that elevated concentrations of 2,4-D or Casein hydrolysate inhibit the formation of embryonic calli from leaf disc explants.

Bhansali (1990) [4] reported that, when half strength MS medium supplemented with 30% sucrose, 500.0 mg/L Glutamine and 500.0 mg/L Casein hydrolysate with no PGRs and same basal medium with 5.0 mg/L 2,4-D, 2.0 mg/L BAP and 1.0 mg/L Kinetin used for embryonic callus formation, 1.0 mg/L 2,4-D with low BAP concentration has increased the formation of embryonic callus and its growth. The results of the present study also confirmed that 1.0 or 2.0 mg/L 2,4-D with low concentrations of BAP increased the embryonic callus induction.

3.5 Maturation of somatic embryos

Embryonic calli started to mature when they were kept in the induction medium for long. However, for the efficient embryonic callus maturation, it was observed that, the concentration of 2,4-D has to be reduced as it may acts as a developmental blocker of the maturation of somatic embryos [4]. Induced embryonic calli undergo various characteristic morphological changes such as globular-shape, heart-shape, torpedo-shape and cotyledonary stage [6] when transferred into media with low or no 2,4-D and amino acid supplements, together with increased levels of sucrose to stimulate stress conditions. Out of all tested treatments it was observed that (0.0 mg/L 2,4-D, 0.25mg/L BAP, 500.0 mg/L Casein hydrolysate, 500.0 mg/L L-Glutamine, 30.0 g/L sucrose in MS medium showed the highest maturation rate. Increased levels of sucrose (60.0 or 90.0 g/L) retarded the somatic embryo formation (Table 5). In fact, those calli turned into dark brown in color and degraded. When globular shaped cells (Fig 3b) were subcultured into the same fresh culture medium and subcultured thrice within 20 days intervals, it was observed that after the second subculture, somatic embryos with heart shaped cells (Fig 3c) were formed but the frequency of obtaining heart shaped cells was very low.

TABLE 4: EFFECT OF 2,4-D AND BAP CONCENTRATIONS WITH MS BASAL MEDIUM WITH 500.0 MG/L L-GLUTAMINE FOR EMBRYONIC CALLUS FORMATION AFTER 4 WEEKS.

Treatment Code	2,4-D (mg/L)	BAP (mg/L)	Diameter of the embryonic callus (cm) ± SD	Percentage embryonic callus formation
C1	0.0	0.0	0.0±0.00	0.0
C2	0.5	0.0	0.6±0.08	40.0
C3	1.0	0.0	1.1±0.07	70.0
C4	1.0	0.5	0.8±0.09	60.0
C5	1.0	1.0	0.8±0.06	30.0
C6	1.0	1.5	0.4±0.05	20.0
C7	1.0	2.0	0.4±0.04	10.0
C8	1.0	2.5	1.0±0.09	10.0
C9	2.0	0.0	0.8±0.07	60.0
C10	2.0	0.5	0.4±0.04	40.0
C11	2.0	1.0	0.3±0.05	30.0
C12	2.0	1.5	0.3±0.06	10.0
C13	2.0	2.0	0.2±0.07	10.0
C14	2.0	2.5	0.1±0.02	10.0
C15	4.0	0.0	0.0±0.00	0.0
C16	6.0	0.0	0.0±0.00	0.0
C17	8.0	0.0	0.0±0.00	0.0
C18	10.0	0.0	0.0±0.00	0.0
C19	12.0	0.0	0.0±0.00	0.0

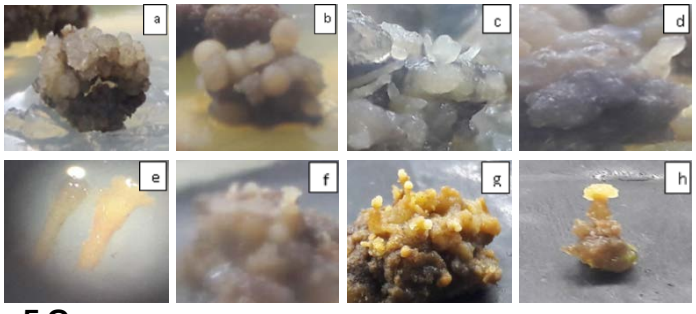
Two weeks after the second subculturing, the torpedo stage like cells (Fig 3d) were induced from the somatic embryonic callus. After the third subculture by transferring the maturing embryonic callus into the fresh medium, some of the torpedo stage cells were converted into the cotyledonary stage (Fig 3e, f, g, h). Exclusion or reduced

level of 2,4-D from the culture medium triggers the maturation of somatic embryogenesis [6] which is confirmed by the observations of the present study. Although some of the previous studies reported the lower concentration of 2,4-D enhanced the number of mature somatic embryos in *Saccharum officinarum* [8] and in *Scirpus robustus* [16].

Treat. Code	2,4-D (mg/L)	BAP (mg/L)	Casein hydrolysate (mg/L)	L-Glutamine (mg/L)	Sucrose (g/L)	Percentage of calli with globular shaped cells ± SD	Mean number of somatic embryos cotyledonary shaped cells ± SD
Cont.	0.0	0.0	0.0	0.0	0.0	0.0	0.0
E1	0.0	0.0	0.0	0.0	30.0	55.00±5.78	0.85±0.24
E2	0.0	0.0	500.0	500.0	30.0	65.50±6.37	0.90±0.19
E3	0.0	0.0	0.0	500.0	30.0	57.00±7.00	0.55±0.17
E4	0.0	0.0	500.0	0.0	30.0	48.00±5.11	0.45±0.17
E5	0.5	0.0	0.0	500.0	30.0	19.00±4.91	0.10±0.07
E6	0.5	0.25	0.0	500.0	30.0	28.00±6.05	0.60±0.23
E7	1.0	0.25	0.0	500.0	30.0	4.00±1.83	0.10±0.07
E8	0.0	0.25	0.0	500.0	30.0	81.00±5.32	0.95±0.21
E9	0.0	0.25	0.00	500.0	60.0	0.00±0.00	0.00±0.00
E10	0.0	0.25	0.00	500.0	90.0	0.00±0.00	0.00±0.00

TABLE 5: EFFECT OF DIFFERENT COMBINATION OF PGRS IN MS MEDIUM FOR FORMATION OF SOMATIC EMBRYOS WITH GLOBULAR CELLS (AFTER 4 WEEKS IN MATURATION MEDIA).

FIG 3: A) FORMATION OF EMBRYONIC CALLUS IN TREATMENT WITH 1.0 MG/L 2,4-D; B) FORMATION OF SOMATIC EMBRYOS WITH GLOBULAR CELLS (0.25 MG/L BAP, 500.0 MG/L L-GLUTAMINE, 30.0 G SUGAR); C) FORMATION HEART SHAPED CELLS IN SOMATIC EMBRYOS ; D) FORMATION TORPEDO SHAPED SOMATIC EMBRYOS ; E) TORPEDO AND EARLY COTYLEDONARY STAGE OF SOMATIC EMBRYO UNDER THE LIGHT MICROSCOPE. (10×10×1); F) FORMATION COTYLEDONARY SHAPED CELLS UNDER DARK CONDITIONS. G AND H) COTYLEDONARY STAGE CELLS OBSERVED AFTER THREE MONTH.



5 CONCLUSION

The results of tissue culture practices revealed that highest germination percentage and seedling height of *P. granatum* Linn. seeds can be obtain *in vitro* using MS medium supplemented with 5.0 mg/L GA3. Also the germination percentage and seedling height was increased when seeds were stratified before culturing.

The highest percentage of callus induction was obtained from cotyledon explants followed by internode, leaf and hypocotyle explants. Cotyledon derived callus showed the highest shoot regeneration percentage.

Treatments given by using different combinations of 2,4-D and BAP with basal MS medium supplemented with 500.0 mg/L L-Glutamine is the most effective medium for the callus induction from the leaf disc explant, embryonic callus formation and embryonic callus growth in the imported variety of *P. granatum* L. The MS medium with 2.0 mg/L 2,4-D, 2.0 mg/L BAP and 500.0 mg/L L-Glutamine is the most effective medium for the callus induction and MS medium with 1.0 mg/L 2,4-D, 0.0 mg/L BAP and 500.0 mg/L L-Glutamine is the most effective medium for the embryonic callus formation and embryonic callus growth.

The MS medium with 0.0 mg/L 2,4-D, 0.25 mg/L BAP, 500.0 mg/L Casein hydrolysate, 500.0 mg/L L-Glutamine and 30.0 g/L sucrose was the most effective medium for the formation of globular stage cells and formation of cotyledonary stage cells. It concludes that, the MS medium with 0.0 mg/L 2,4-D, 0.25 mg/L BAP, 500.0 mg/L Casein hydrolysate, 500.0 mg/L L-Glutamine and 30.0 g/L sucrose is the best medium for the embryonic callus maturation in somatic embryogenesis of imported variety *P. granatum* L.

6 REFERENCES

[1] J. M. Al-Khayri, F. H. Huang, T. F. Morelock, and T. A. Busharar, "Spinach Tissue Culture Improved with Coconut Water", *HORTSCIENCE*, 27 (4): 357–358, 1992.

[2] N. S. Al-Zoreky, "Antimicrobial activity of pomegranate (*Punica granatum* L.) fruit peels", *Int. J. Food Microbiol,* 134: 244-248, 2009.

[3] R. Ashton, B. Baer, and D. Silverstein, "The incredible pomegranate" Plant & fruit. 1stedn. Third Millennium Publishing, 1931, East Libra Drive, Tempe, AZ 85283, 2006.

[4] R. R. Bhansali, R. R. "Somatic Embryogenesis and Regeneration of Plantlets in Pomegranate", *Annals of Botany*, 66: 249–253, 1990.

[5] R. J. Chauhan, and K. Kanwar, K., "Biotechnological advances in Pomegranate (*Punica granatum* L.)", *In Vitro Cell. Dev. Biol. – Plant,* 48: 579–594, 2012.

[6] V. M. Jimenez, "Regulation of *in vitro* somatic embryogenesis with emphasis on the role of endogenous hormones", *R. Bras. Fisiol. Veg*, 13 (2): 196-223, 2001.

[7] K. Kanwar J. Joseph, and R. Deepika, "Comparison of *in vitro* regeneration pathways in *Punica granatum* L.", *Plant Cell Tissue Organ Cult*. 100: 199–207, 2010.

[8] I. Khan, and A. Khatri, "Plant regeneration via somatic organogenesis in sugarcane: histological studies", *Pakistan J. Bot,* 38: 631-636, 2006.

[9] M. Koornneef, L. Bentsink, and H. W. M. Hilhorst, "Seed dormancy and germination", *Current Opinion in Plant Biology*, 5: 33-36, 2002.

[10] B. Manz, K. Müller, B. Kucera, F. Volke, and G. L. Metzger, "Water uptake and distribution in germinating tobacco seeds investigated *in vivo* by nuclear magnetic resonance imaging", *Plant Physiology*, 138: 1538–1551, 2005.

[11] T. Murashige, and F. Skoog, "A revised medium for rapid growth and bio assays with tobacco tissue cultures", *Physiol Plant*, 15: 473–497, 1962.

[12] A. A. Murkute, S. Patil, B. N. Patil, and M. Kumari, " Micropropagation in Pomegranate, callus induction and differentiation", *South Indian Hortic*, 50 (1, 3): 49–55, 2002.

[13] N. M. Patil, and S. G. Borkar, "*In vitro* Callus induction and Root regeneration through the mediation of *Agrobacterium rhizogenes* in *Punica granatum*", *International Journal of Advanced Research*. 3: 162-165, 2015.

[14] J. Peng, and N. P. Harberd, "The role of GA-mediated signaling in the control of seed germination", *Current Opinion in Plant Biology,* 5: 376-381, 2002.

[15] J. Vidhan, A. D. Marderosian, and R. P. John, "Anthocyanins and polyphenol oxidase from dried arils of pomegranate (*Punica granatum* L.)", *Journal of Food Chemistry*, 118: 11-16, 2010.

[16] J. B. Wang, D. M. Seliskar, and J. L. Gallagher, "Plant regeneration via somatic embryogenesis in the brackish wetland monocot Scirpus robustus", *Aqua. Bot*. 79: 163-174, 2004.

[17] Q. Wenjuan, P. B. Andrew, P. Zhongli, and M. Haile, "Quantitative determination of major polyphenol constituents", *Journal of Food Chemistry,* 132: 1585-1591, 2010.

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