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COMBINED SUPPLEMENTATION OF 2, 4-D AND KINETIN ELEVATES CALLUS INDUCTION FROM COTYLEDON SEGMENTS OF DIFFERENT SOYBEAN CULTIVARS [*GLYCINE MAX* (*L.*) MERRIL]

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ABSTRACT

Callus induction and embryogenesis were tested in Soybean (*Glycine max* (*L.*) Merril) using cotyledon segments of different cultivars. In this study, embryogenic calli have been produced from cotyledon segments of various soybean cultivars like CO1, CO2, and CO3. To assess the callus induction and embryogenic potential of cotyledon explants, Murashige and Skoog media were added with different combinations of auxin and cytokinin. Among the treatments, medium added through 2,4-D (5µM) + Kinetin (10µM) exhibited a good size of profuse greenish callus (90.5±0.1g) with highest fresh weight of 2.5±0.1g and higher proliferation of embryogenic calli from cotyledons of CO3 variety. Pretreatment of cotyledonary explants with 0.5% polyvinypyrollidone overcome the limitations in generating callus from cotyledon segment cut ends. The present study implies the use of combined supplementation of 2,4-D (5µM) + Kinetin (10µM) on callus induction and the possibility of micro propagation in soybean cultivars using the cotyledonary explants that will be useful for genetic improvement studies in soybean cultivars .

Keywords: *Glycine max*, Callus induction, MS media, Auxin, Cytokinin.

Introduction

Soybean [*Glycine max* (*L.*) Merril] is being the vital food component in different part of the world as it contains high protein (40%), oils (20%) and carbohydrates (30%). Soybean is a member of the Fabaceae family and is commonly grown in Asian nations. In India, soybean cultivation was negligible until 1970 and thereafter it has increased manifold as compared to other oil seed crop plants. Soybean is the most important agricultural commodity in the world as well as in India. Its production rate must be increased to fulfill the food requirement for the upcoming decades (Tilman *et al*., 2011). Soybean is subjected to drought and other abiotic stresses in all stages which subsequently reduces crop yield. In the early decades, researchers were followed traditional plant breeding techniques through which stable productivity has been achieved by consuming more time with low input cost and lower synthetic chemical inputs (Manavalan *et al*., 2009). Plant tissue culture procedures can be utilized as an alternative to established plant breeding approaches. The utilization of medium ingredients and plant growth regulators is critical for successful callus development and fast micro propagation. (Hoesen *et al*., 2008, Jahan *et al*., 2009 & Shirin *et al*., 2007) which is often manipulated by optimizing culture conditions. Auxin and Cytokinin are considered the most important and inevitable plant growth regulators in plant tissue and organ cultures and *in vitro* regenerations (Evans *et al*., 1981 and Vasil *et al*., 1994). Auxin and cytokinin are required to stimulate *in vitro* cell division, cell elongation as well as callus formation (Yusnita, 2003). Development of callus depends mainly on the determination of an adequate balance of these plant growth regulators along with basal salts. However, this imbalance largely correlates to explants types and plant species. Further, many oil seed plants have been subjected to several explorations towards genetic improvement, *in vitro* regeneration and callus induction with limited success. In such explorations, callus induction is being the vital part of the technology which determines the success of such genetic improvement and optimization of regeneration potential of the transgenic with improved qualities. Hence, the objective of this study is to device the suitable medium with PGR combination for improved embryogenic callus induction using cotyledon segments of different soybean cultivars (*Glycine max* (*L.*) Merril) as explants.

Materials and Methods

Plant Material and Growth Conditions

Soybean seed variants CO1, CO2, and CO3 were obtained from the Tamil Nadu Agricultural University (TNAU) in Coimbatore, India. Seeds were well cleansed before being seeded and grew in earthen pots with a 1:2:1

soil combination (red soil, sand, and compost). Germinated seedlings were maintained in natural condition and utilized for preparation of explants at the time of inoculation and further seeds were stored.

Surface Sterilization of Explants

Cotyledon, hypocotyl, and epicotyl segments were prepared from one to two week old soybean seedlings. Washed with tap water first, then with 1 or 2 drops of tween 20 and water.

The explants were then rinsed with 0.5% sodium hypochlorite solution for five minutes, followed by 0.1% $HgCl_2$ for two minutes. Explants were surface sterilized (under aseptic conditions), washed twice with sterile distilled water, cut into short segments (0.5-1.0 cm), and injected into the appropriate sterile solid MS medium.

Medium Preparations

Murashige and Skoog (1962) media were used, with a new mix of plant growth regulators supplemented: 1) 2, 4 – D (5 µM) + TDZ (10 µM), 2) 2,4 – D (5 µM) + BAP (10 µM), 3) GA3 (5 µM) + Zeatin (1 µM), 4) 2,4-D (5 µM) + Kinetin (10 µM), 5) NAA (10 µM) + BAP (1 µM) and 6) 2,4 –D (5 µM) + Picloram (1 µM). Sucrose (3%) was used as a carbon source, while 0.8% agar was used as a solidifying agent. pH of the medium was adjusted to 5.8 before adding agar. After melting the agar, the prepared medium was distributed into culture vials and autoclaved for 20 minutes at 121°C and 15psi. Before inoculation, the sterilized medium were incubated at room temperature for two days.

Culture conditions

Surface sterilized explants were inoculated in MS medium with different PGRs in laminar airflow chamber. Cotyledons were sliced with a cut abaxial side, and the area was somewhat damaged on both edges. Inoculated culture vials were kept in a tissue culture chamber with a temperature of 23°C 25°C and a photoperiod of 16 hours for light circumstances and 8 hours for dark conditions. Table 1 shows the concentrations of plant growth regulators utilized for callus germination.

Callus Morphology and induction Frequency

The callus texture and color were observed in visually in the fully developed callus from each treatment. Sub culture was done at 15 days interval into the freshly prepared, sterile MS medium with suitable PGRs. Emergence of callus and further proliferation was recorded in different day's interval. The fresh weight of the callus was determined by weighing it without any medium or water droplets and expressing it in grams (g). Callus induction efficiency (CIE) was calculated according to the method prescribed by Devi *et al*. (2008). In brief, the starting and ultimate weights of the callus were observed after 15, 30, and 45 days following inoculation, and CIE (%) was determined using the method below.

Callus Induction Efficiency (CIE) (%) = Final Weight – Initial weight/ Initial weight × Culture time in days × 100

Statistical Data

The statistical data were reported as mean SD. Data were reported as means of three replicates (n= 3) with standard deviation (SD). Means followed by different letters within a column differ substantially at p 0.05.

Results

Callus Induction from Cotyledonary Segments of Soybean CO3 variety

The current study investigated the effect of different plant growth regulators to improve callus induction from soybean cultivars, particularly from cotyledon segments MS medium supplemented with six different combined combinations of appropriate hormone concentration were tested in the CO1, CO2, and CO3 varieties. Callus growth was observed in soybean cotyledons CO1, CO2, and CO3 cultured in all six combinations of plant hormones, and callus tissue proliferation was initiated from both sides. Initially, soybean seeds were grown in 0.7% agar (Fig 1A) and the cotyledon segments were used as explants (Fig 1B). Callus induction was observed on both cut edges of cotyledon (Fig 1C) that were proliferated profusely in different treatments. In CO3 variety, after 8 days of inoculation, the callus growth and development were found to be effective in all the treatments (Figs. 2, A, B, C, D, E, and F). Better callus proliferation was obtained in the treatment 2,4-D (5µM) + Kinetin (10µM) (Fig 3 – A) after 20-30 days of inoculation. Initiation of callus was started from the 8th day after inoculation. A proliferated portion of explants was excised carefully and subcultured into the same sterile medium after 14 days of inoculation. A greenish-white color callus mass was found to occupy the entire surface of the medium within 10-14 days of sub-culture (Fig 3A). In this treatment, the callus proliferation rate was found to be better (90±.5%) in CO3, when compared to the other two varieties CO1 & CO2. Further establishment of the soybean calli was proliferated to a higher level with maximum fresh weight observed (2.5±0.1g) (Table 2). In addition to the increment in the proliferated mass of calli, this PGR combination facilitated the conversion of calli into embryogenic mass (Fig 3B). Based on the establishment and callus induction, 2,4-D (5µM) + Kinetin (10µM) was found to effectively induce the greenish white compact type embryo genic callus after 30 - 35 days of sub-culture. On the whole, within 75-85 days from inoculation, the embryogenic mass of calli was obtained with high regeneration potential. To confirm the embryogenic nature of the calli, a small portion of the calli was crushed and mounted in a microscopic slide after staining with safranin under the compound light microscope. The cells were found to be compact and prominent cell wall and storage food materials found inside the cells (Fig. 3C) which was the initial indication of the conversion of calli into embryogenic mass and whereas non embryogenic callus cells were observed in individual callus cells (Fig. 3D).

Induction of Callus from Cotyledon Segments of Soybean CO2 variety

Cotyledon segments from the CO2 variety of soybean also showed callus induction in MS media supplemented with six various combinations of PGRs. The callus tissue development and proliferation in CO2 variety of soybean also were observed in the soybean CO2 variety after 8 days of inoculation in all six different PGR combinations (Figs. 2, G, H, I, J, K, and L) (Table 1). Among six different combinations, 2,4-D (5µM) + Kinetin (10µM) was found to enhance the callus proliferation better than other concentrations (83±5%). The fresh weight of the proliferated calli from the CO2 soybean variety was found to be maximum (2.1± 0.1g) at the hormone concentration 2,4-D (5µM) and Kinetin (10µM) (Table 2). Though all the treatments showed significant size in callus proliferation, MS medium supplemented with 2,4-D (5µM) + Kinetin (10µM) showed distinct characteristic features such as compact and embryogenic tissue. But the callus mass was less (2.1± 0.1g) when compared to the CO3 variety.

Callus induction from Cotyledonary segments of CO1 Soybean variety

In CO1 variety, the callus tissue development and proliferation were observed in all six different PGR combinations tested (Fig 2. M, N, O, P, Q, and R). Among the different responses studied, the same 2,4-D and Kinetin at the concentration of 5-10µM were found to be optimum (82±5%). The combined effect of both 2,4-D and Kinetin was found to be better with varied color, morphology, and size of the callus. The calli from the CO1 soybean variety was found to be maximum (82±5%) with a fresh weight of (1.85±0.9g) (Table 1 & 2). Even after several subcultures, the establishment and conversion of callus into embryogenic mass was found to be more complex in CO1soybean. But in the case of CO3, such conversion was found to be better in the same medium which was supplemented with 2,4-D and Kinetin. Hence further attempts were being centered on CO3 variety.

Discussion

Differential supplementation of plant growth regulators and adjuvants in the culture medium induced callus development in different types of soybean CO1, CO2, and CO3, followed by differentiation and organogenesis. In tissue culture, it is more favourable to maintain either a callus or a cell suspension, which may be stimulated to undergo somatic embryogenesis or adventitious shoot production at the appropriate time phase to generate the necessary number of plantlets. In actual practice, however, this approach is not applicable to many plant systems. Moreover, the morphogenetic potential of several unorganized tissue cultures declines when grown under *in vitro* conditions for an extended period of time and may give rise to genetic instability (Tsugawa and Suzuki, 2000). Cell division and tissue differentiation might be stimulated by stimulating endogenous growth factors or adding exogenous growth regulators to the nutritional medium. The callus developed in this investigation was embryogenic by nature.

Auxin supplementation at a suitable level is generally established as a basic need for every explant to generate callus. 2,4-D induced friable callus from cotyledon explants after seven days of culture. From the eighth day of inoculation, callus was produced from the cut surfaces of cotyledon explants. During 15 to 25 days, callus growth had completely covered the explants.

As a result, the findings in this study confirm earlier publications on the function of distinct PGRs in callus induction and establishment. (Soorni *et al*., 2015; Mishra *et al*., 2000). Supplementation of single plant growth regulator couldn't facilitate the callus establishment at maximum level. Combined supplementation of Kinetin and 2,4-D lead towards efficient and profuse proliferation of callus induction after 25-35 days of inoculation of cotyledon explants. Combination of 2,4-D and kinetin has been employed in the ratio of 1:5 and 1:10 in the concentration range from 1-25 µM. Among various range of PGRs supplemented, MS medium supplemented with 5µM 2,4-D and 10µM Kinetin have induced callus at the maximum level (90.5%) in case of cotyledon explants excised from three varieties of soybean *viz* CO1, CO2, and CO3.Various types of explants and different plant growth regulators concentrations were already tested in legume plants species like *Vigna radiata* (*L*.) (Rao *et al*., 2005), *Vigna mungo* (*L*.) (Mony *et al*., 2007), *Phaseolus vulgaris* (*L*.) (Mahamune *et al*., 2011) and *Cicer arietinum* (*L*.) (Khan *et al.,* 2011). The limitations in developing callus from oilseed plants such as recalcitrant in nature has also been experienced in our attempts. Hence, the cotyledon has been treated with 0.5% Polyvinylpyrollidone (PVP) for 5 minutes before surface sterilization and trimming the explants for inoculation. In this present study, addition of 0.5% PVP was found to eliminate such limitations by facilitating the nutrient uptake by the cells in the cut ends of the cotyledons. The results of the present study corroborate with the earlier report of Kiong *et al*. (2008) and Cai *et al*. (2020). Plant growth regulators are well-known for regulating the dedifferentiation and re-differentiation of plant cells, notably in the formation of callus inductions (Paris *et al*., 2004). Hong *et al*. (2007) established an organogenic callus from cotyledonary node explants using MS media and TDZ concentrations ranging from 1.35 to 2.25µM. Co-supplementation of TDZ (0.45µM) along with BAP (0.38µM) was reported to be a better medium for producing compact callus from cotyledon node explants of soybean. Radhakrishnan *et al*. (2007) reported callus induction from half seed explants using BAP (13.3µM) + 2, 4-D (13.5µM) supplemented MS media. Islam *et al*., (2017) reported high frequency of callus induction (82.40%) from cotyledonary node explants using in the combination of 1mg/l (2, 4–D + BAP). In this work, we devised a simple and low-cost strategy for directly inducing callus from cotyledon explants of the soybean CO3 variety. The particular treatment of 2,4-D (5µM) + Kinetin (10µM) resulted in the highest callus induction frequency (90.5%) and callus fresh weight (2.5g) (Table 1). Moreover, improved friability with pale white compact embryogenic callus was seen in MS medium supplemented with 2,4-D (5µM) + Kinetin (10µM) (Fig 3, A). This result showed that combinations of 1:5 and 1:10 ratio were efficient in production of compact, friable, and proliferating callus. Higher concentration of Kinetin (10µM) and lower concentration of 2,4-D (5µM) can effectively produce the embryo genic callus form cotyledon explants after 75-85 days of inoculation. Here, our present study suggests that the treatment of cotyledons with PVP might facilitate the callus induction by overcoming the recalcitrant nature. Further, it was observed that the combined supplementation of auxin (2,4-D (5µM) and cytokinin (Kinetin-10µM) could further improve callus induction and establishment of embryo genic calli from cotyledon explants of CO3 verities of soybean (Fig. 3, A). Somatic embryogenesis is an *in vitro* pathway where a group of somatic cells/tissues differentiate into a mass of embryo genic cells and ultimately resulting in the formation of embryo like organs as that of seeds and germinate in to whole plants. Plant regeneration through somatic embryogenesis from single cells and various types of explants has been demonstrated in many medicinal and rare, endangered species such as *Podophyllum hexandrum* (Arumuagam and Bojwani, 1990), *Asparagus cooperi* (Ghosh and Sen, 1991), *Madhuca longifolia* (Rout and Das., 1993), *Acanthopanax koreanum* (Choi *et al.,* 1997) *Typhonium trilobatum* (Das palai, *et al.,* 1999), *Cuminum cyminum* (Tawfik and Noga, 2002), *Holostemma ada-kodien* (Martin, 2003) and *Hevea brasiliensis* (Venkatachalam *et al.,* 2007). Differentiation of somatic embryogenic mass of cells and cell aggregates was successful when the medium was constantly altered and sub cultured at frequent intervals. The current study's findings are consistent with previous publications (Sugimoto *et al*., 2011). (Vanishree *et al.*, 2004).

The embryo genic cells had dense cytoplasm uniform in size and shape, and rich starch grains indicated the presence of uniform embryo genic cells as that of normal embryo with dense cytoplasm when stained with safforanin. This embryogenic cell mass was sub cultured onto freshly produced sterile MS medium enriched with 2,4-D for somatic embryo development and germination.

Conclusion

The current study confirms the combined effects of plant growth regulators (PGRs) such as 2,4-D and Kinetin on callus induction from diverse Indian soybean cultivars CO1, CO2, CO3 utilizing cotyledon segments. The maximum nominal PGR mixture used for inducing callus from cotyledon explants of soybean CO3 was found to be 2,4-D (5µM) and Kinetin (10µM).This PGR combination was also used for the further conversion of embryogenic calli. Pretreatment of cotyledon explants with 0.5% polyvinypyrollidone was reported to overcome recalcitrant constrains on callus initiation at the cut ends of cotyledon segments. The findings of this study suggest the usage of cotyledon and the combined effects of PGRs on *in vitro* callus induction will be a vital part in future to promote the genetic transformation studies in soybean for cultivar improvement.

Table 1 : Shows the percentage of callus induction in MS media with varying Auxin and Cytokinin concentrations.

S.No.	Plant Growth Regulator (mg l^{-1})	Explants of Cotyledon Segments		
		Soybean CO1	Soybean CO2	Soybean CO3
		Callus induction (%)	Callus induction (%)	Callus induction (%)
1	2,4-D (5µM) + TDZ (10µM)	68±3	70±8 ^{a*}	74±3 ^{b*c*}
2	2,4-D (5µM) + BAP (10µM)	60±0	64±0 ^{a*}	80±0 ^{b*c*}
3	GA3(5µM) + Zeatin(1µM)	47±7	56±7 ^{a*}	67±7 ^{b*c*}
4	2,4-D (5µM) + Kinetin(10µM)	82±5	83±5 ^{a*}	90±5 ^{b*c*}
5	NAA (10µM) + IBA (1µM)	74±2	66±2 ^{a*}	88±2 ^{b*c*}
6	2,4-D (5µM) + Picloram (1µM)	76±4	74±2 ^{a*}	82±3 ^{b*c*}

CO1 vs CO2 – a** (P value 0.01)
CO1 vs CO3 - **** b*(P value 0.0001)
CO2 vs CO3 - **** c * (P value 0.0001)

The values are presented as mean ± SD standard deviation, with P< 0.05 considered highly statistically significant. Graph pad Prism was used for statistical analysis. Multiple group comparisons were performed using two-way ANOVA, followed by a post hoc Tukey test. a* denotes a comparison of CO1 vs CO2, b* denotes a comparison of CO1 vs CO3, and c* denotes a comparison of CO2 vs CO3.

Table 2 : Shows the fresh weight callus on MS medium with varying Auxin and Cytokinin concentrations

S.No.	Plant Growth Regulator (mg l^{-1})	Explants of Cotyledon Segments		
		Soybean CO1	Soybean CO2	Soybean CO3
		Fresh weight (g)	Fresh weight (g)	Fresh weight (g)
1	2,4-D (5µM) + TDZ (10µM)	1.40±0.7	1.73±0.8	1.33±0.6 ^{*a*b}
2	2,4-D (5µM) + BAP (10µM)	0.80±0.4	1.12±0.5	1.45±0.7 ^{*a*b}
3	GA3(5µM) + Zeatin(1µM)	1.10±0.5	0.45±0.2	0.64±0.3 ^{*a*b}
4	2,4-D (5µM) + Kinetin(10µM)	1.85±0.9	2.1±0.1	2.5± 0.1 ^{*a*b}
5	NAA (10µM) + IBA (1µM)	1.67±0.8	1.60±0.8	1.80±0.9 ^{*a*b}
6	2,4-D (5µM) + Picloram (1µM)	1.48±0.7	0.94±0.4	1.12±0.5 ^{*a*b}

CO1 vs CO2 – NS
CO1 vs CO3 - *a (P value 0.05)
CO2 vs CO3 - **** *b (P value 0.0001)

The values are presented as mean ± SD standard deviation, with p<0.05 considered highly statistically significant. Graph pad Prism was used for statistical analysis. Multiple group comparisons were performed using two-way ANOVA, followed by a post hoc Tukey test. a* indicates comparison of CO1 vs CO3, & b* indicates comparison of CO2 vs CO3.

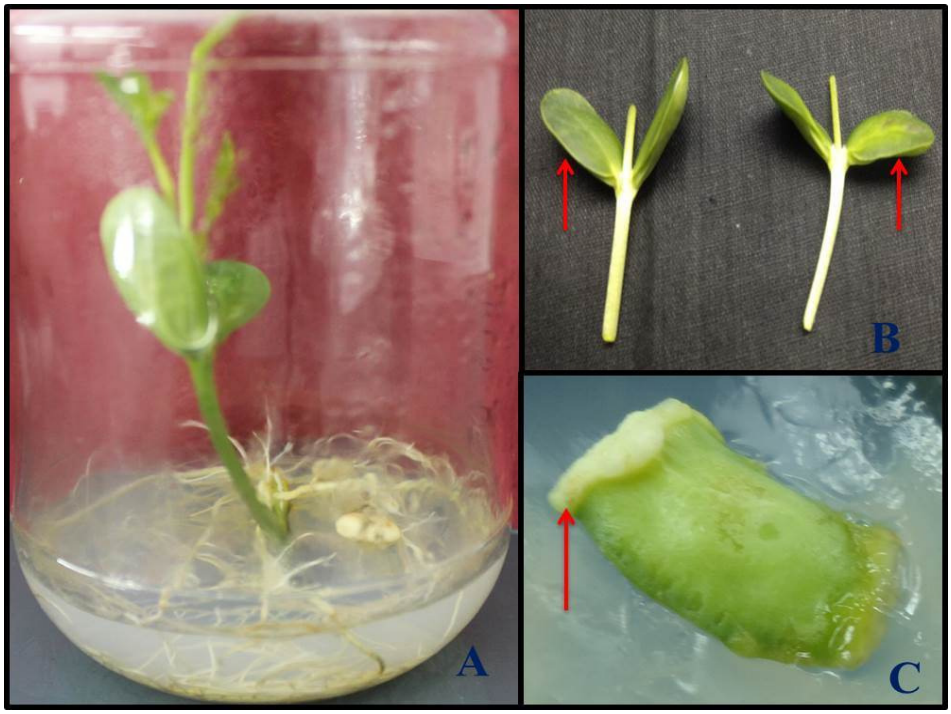

Fig. 1 : Soybean grown in bottle with the parts used for explant. **A –** *In vitro* seed grown as plant having cotyledons. **B** - Cotyledon segments of excised from the soybean plant & **C** – Explant from soybean cotyledon showing the callus induction on the two sides.

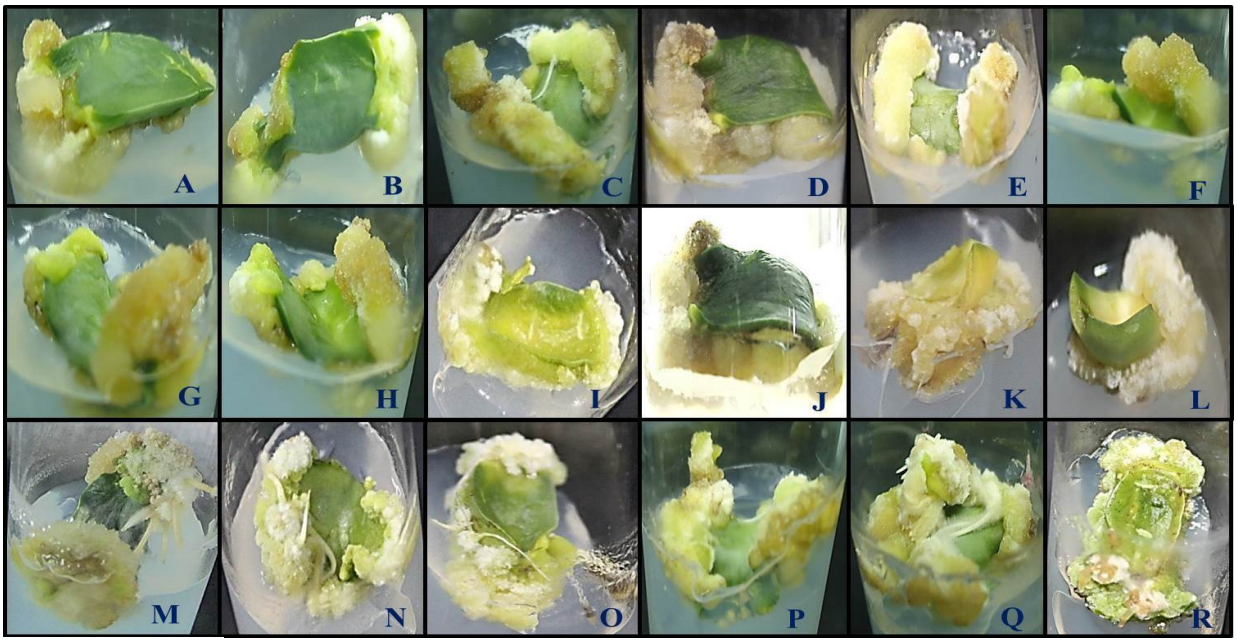

Fig. 2 : Callus initiation of soybean varieties after 8 days of inoculations - **A, B, C, D, E, F** are the callus initiation from six different combined combination of CO3 variety – **G, H, I, J, K, L** are the callus initiation from six different combined combination of CO2 variety and **M, N, O, P, Q, R** are the callus initiation from six different combined combination of CO1 variety

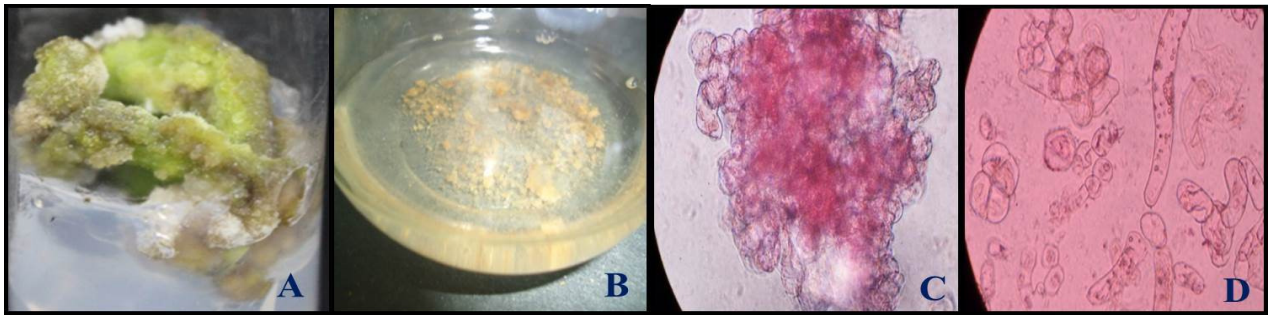

Fig. 3 : Confirmation of embryogenic callus tissue from soybean (CO3) observed under light microscope. **A -** Embroyogenic callus tissue grown in MS medium having 2,4-D (5µM) + Kinetin (10µM). **B** – Cell suspension culture in MS medium having same 2,4-D (5µM) + Kinetin (10µM) in the medium. **C** - Undifferentiated callus mass stained with safranin showing compact and prominent cell wall and storage food materials in the cell. **D**–Non-embryogenic callus showing no cell wall thickening and starch deposits in the cells.

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Conflict of the interest

The authors claim that they have no known conflicting financial interests or personal affiliations that may seem to have impacted the work revealed in this study.

Author's contributions

Study design: RM and KS; Data acquisition: RM, GS, Data analysis and interpretation: T K, JB, RM and GD, RM and KS to write the manuscript. The final manuscript was reviewed and approved by all authors.

Abbreviations

2, 4-D	**–**	Dichlorophenoxyacetic acid
NAA	**–**	Naphthaleneacetic acid
TDZ	**–**	Thidiazuron
AP	**-**	Benzylaminopurine
CI	**–**	Callus Induction
PVP	–	Polyvinypyrollidone
PGRs	**–**	Plant Growth Regulators

References

Arumugam, N. and Bhojwani, S.S. (1990). Somatic embryogenesis in tissue cultures of *Podophyllum hexandrum*. *Canadian Journal of Botany*, 68 (3): 487-491.

Cai, X., Wei, H., Liu, C., Ren, X. and Jeong, B.R. (2020). Synergistic effect of NaCl pretreatment and PVP on browning suppression and callus induction from petal explants of *Paeonia lacti* flora Pall. 'Festival Maxima'. *Plants,* 9(3): 346.

Choi, Y.E., Kim, J.W. and Soh, W.Y. (1997). Somatic embryogenesis and plant regeneration from suspension cultures of *Acanthopanax koreanum* Nakai. *Plant Cell Reports*, 17(2): 84-88.

Das, P., Palai, S.K., Patra, A., Samantaray, S. and Rout, G.R. (1999). In vitro somatic embryogenesis in *Typhonium trilobatum* Schott. *Plant Growth Regulation*, 27: 195-199.

Devi, R., Dhaliwal, M.S., Kaur, A. and Gosal, S.S. (2008). Effect of growth regulators on in vitro morphogenic response of tomato. *Indian Journal of Biotechnology*. *7:* 226 -230.

Evans, D.A., Sharp, W.R., Flick, C.E. (1981). Growth and behavior of cell cultures: embryogenesis and organogenesis. In: Thorpe TA (ed.) Plant Cell Culture: Methods and Applications in Agriculture, New York: Academic Press, 45-113.

Ghosh, B. and Sen, S. (1991). Plant regeneration through somatic embryogenesis from spear callus culture of *Asparagus cooperi* Baker. *Plant cell reports*, 9(12): 667-670.

Hoesen, D.S.H. and Witjaksono, S.L.A. (2008). Induksi Kalusdan Organogenesis KulturIn Vitro *Dendrobium lineale* Rolfe. *Berita, Biologia,* 9(3): 333-341.

Hong, H.P., Zhang, H., Olhoft, P., Hill, S., Wiley, H., Toren, E. and Cheng, M. (2007). Organogenic callus as the target for plant regeneration and transformation via *Agrobacterium* in soybean (*Glycine max* (*L.*) Merr.). In Vitro Cellular & Developmental Biology-*Plant,* 43(6): 558-568.

Islam, N., Islam, T., Hossain, M.M., Bhattacharjee, B., Hossain, M.M. and Islam, S.S. (2017). Embryogenic callus induction and efficient plant regeneration in three varieties of soybean (*Glycine max*). *Plant Tissue Culture and Biotechnology*, 27(1): 41-50.

Jahan, M.T., Islam, M.R., Khan, R., Mamun, A.N.K., Ahmed, G. and Hakim, L. (2009). In Vitro Clonal Propagation of Anthurium (*Anthurium andraeanum* L.) using Callus Culture. *Plant Tissue Culture and Biotechnology*, 19(1): 61-69.

Khan, S., Ahmad, F., Ali, F., Khan, H., Khan, A. and Swati, Z.A. (2011) Callus induction via different growth regulators from cotyledon explants of indigenous chick pea (*Cicer arietinum* L.) cultivars KK-1 and Hassan-2K. *African Journal of Biotechnology,* 10 (40): 7825-7830.

Kiong, A.L.P., Thing, Y.S., Gansau, J.A. and Hussein, S. (2008) Induction and multiplication of callus from endosperm of *Cycas revoluta*. *African Journal of Biotechnology,* 7(23): 4279-4284.

Mahamune, S.E., Bansode, R.P., Sangle, S.M., Waghmare, V.A., Pandhure, N.B. and Kothekar, V.S. (2011). Callus induction from various explants of French bean (*Phaseolus vulgaris L.*). *Journal of Experimental Sciences*, 2(4): 15-16.

Manavalan, L.P., Guttikonda, S.K., Tran, L.S. and Nguyen, H.T. (2009). Physiological and molecular approaches to improve drought resistance in soybean. *Plant Cell Physiol*., 50(7): 1260-1276.

Martin, K.P. (2003). Plant regeneration through somatic embryogenesis on *Holostemma ada-kodien,* a rare medicinal plant. *Plant Cell, Tissue and Organ Culture*, 72(1): 79-82.

Mishra, R., Wang, H.Y., Yadav, N.R. and Wilkins, T.A. (2003). Development of a highly regenerable elite Acala cotton (*Gossypium hirsutum* cv. Maxxa)–a step towards genotype-independent regeneration. *Plant Cell, Tissue and Organ Culture*, 73(1): 21-35.

Mony, S.A., Haque, M.S., Karim, M.A. and Roy, S.K. (2008). Callus Induction and Plantlet Regeneration in Blackgram (*Vigna mungo L.* Hepper). *Progressive Agriculture*, 19(2): 27-35.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15(3): 473-497.

Paris, R., Pratesi, D. and Negri, P. (2004). *In vitro* morphogenic ability of mature or embryonic apricot tissues. *Acta Horticultura*, 663: 487-490.

Radhakrishnan, R. and Ranjithakumari, B.D. (2007). Callus induction and plant regeneration of Indian soybean (*Glycine max* (L.) Merr. cv. CO3) via half seed explant culture. *Journal of Agricultural Technology*, 3(2): 287-297.

Rao, S., Patil, P. and Kaviraj, C.P. (2005). Callus induction and organogenesis from various explants in *Vigna radiata* (*L.*) Wilczek. *Indian Journal of Experimental Biology*, 4: 556-560.

Rout, G.R. and Das, P. (1993). Micropropagation of *Madhuca longifolia* (Koenig) MacBride var. latifolia Roxb. *Plant Cell Reports*, 12(9): 513-516.

Shirin, F., Hossain, M., Kabir, M.F., Roy, M. and Sarker, S.R. (2007). Callus Induction and Plant Regeneration from Internodal and Leaf Explant of Four Potato (*Solanum tuberosum* L.) Cultivars. *World Journal of Agricultural Sciences*, 3(1): 1-6.

Soorni, J. and Kahrizi, D. (2015). Effect of genotype, explant type and 2, 4-D on cell dedifferentiation and callus induction in cumin (*Cuminum cyminum L.*) medicinal plant. *Journal of Applied Biotechnology Reports,* 2(3): 265-270.

Sugimoto, K., Gordon, S.P. and Meyerowitz, E.M. (2011). Regeneration in plants and animals: de-differentiation, trans-differentiation, or just differentiation? *Trends in Cell Biology*, 21(4): 212-218.

Tawfik, A.A. and Noga, G. (2002). Cumin regeneration from seedling derived embryogenic callus in response to amended kinetin. *Plant Cell, Tissue and Organ Culture*, 69(1): 35-40.

Tilman, D., Balzer, C., Hill, J. and Befort, B.L. (2011). Global food demand and the sustainable intensification of agriculture. *Proc Natl Acad Sci.,* 108: 20260–20264.

Tsugawa, H. and Suzuki, M. (2000). A low-temperature method for maintaining plant regeneration activity in embryogenic callus of rice (*Oryza sativa L.*). *Plant Cell Reports*, 19(4): 371-375.

Vanisree, M., Lee, C.Y., Lo, S.F., Nalawade, S.M., Lin, C.Y. and Tsay, H.S. (2004). Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures. *Bot. Bull. Acad. Sin*, 45(1): 1-22.

Vasil, I.K. and Thorpe, T.A. (1994). *Plant cell and tissue culture*. Dordrecht: *Kluwer Academic Publishers*.

Venkatachalam, P., Thulaseedharan, A. and Raghothama, K. (2007). Identification of expression profiles of tapping panel dryness (TPD) associated genes from the latex of rubber tree (*Hevea brasiliensis* Muell. Arg.). *Planta,* 226(2): 499-515.

Yusnita (2003) *Tissue Culture: How to Increase Plant Efficiently* (*Jakarta: Agromedia Book*).