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Regeneration of tea [*Camellia sinensis* (L.) Kuntze] cv. TRI 5001 plants through somatic embryogenesis in a liquid dynamic medium

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ABSTRACT

Somatic embryogenesis is an efficient micro-propagation technique, which can lead to mass multiplication of tea with a tap root. Hence, the development of a viable somatic embryogenesis and multiplication protocol through liquid culture conditions is needed to increase the efficiency of the protocol. Cotyledon-derived somatic embryos, leaf and stem callus of TRI 5001 cultivar, were tested with different liquid MS media. A significantly higher cotyledon-derived somatic embryo multiplication rate was observed in MS +3 mg/L Thidiazuron (TDZ) medium. Callus proliferation and compact and embryonic callus formation were comparatively higher in MS + 1.1 mg/L TDZ, 1.86 mg/L NAA medium, and subsequent somatic embryo formation was observed in MS + 0.1 mg/L NAA,1 mg/L BAP and 0.2 mg/L GA3 medium on callus produced on leaf explants. Higher nodal callus proliferation and compact callus formation were recorded in MS+0.11 mg/L TDZ 0.1 mg/L IBA and higher embryonic callus was observed in MS+0.1 mg/L TDZ, 1.86 mg/L NAA media. Plant regeneration was resulted in MS+3 mg/LBAP 0.1 mg/L NAA medium. Identified treatment combinations can be used as a foundation to establish somatic embryogenesis protocol through different explants of tea.

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INTRODUCTION

The tea industry makes a vital contribution to the socioeconomic development of the country. Over one million Sri Lankans are employed in the tea industry, directly and indirectly. Over 200,000 hectares or approximately 4% of the country's land area are covered by tea plantations (Anon, 2020).

Most of the existing tea plantations are over 50 years old, with the current productivity far below the optimum. Thus, there is an urgent need for rejuvenation through the adoption of superior tea cultivars. To maintain the position of "Ceylon tea" in the world and to serve the local tea industry, it is imperative to focus on efficient tea crop improvement strategies to develop grower-acceptable tea cultivars (Gunasekare, 2012). Accordingly, the Tea Research Institute of Sri Lanka (TRI) focuses on the development of new cultivars with high yield, high-made tea quality, resistance to major pests and diseases, and drought tolerance (Ranatunga, 2019).

There is a high demand for new elite cultivars developed through plant breeding. However, there is a huge gap between production and the demand for planting materials. Approximately, 12500 plants/ha is needed for plantation establishment, and the TRI has recommended adopting a 2% replanting rate to maintain the productivity levels leading to a requirement of nearly 70 million cuttings per year. However, only about 7 million planting materials can be generated from the available mother bushes. Moreover, to fulfill the planting material requirement, a large area of land has to be maintained as mother bush areas. With the existing land scarcity, it is difficult to find suitable lands in tea-growing areas (Anon, 2018).

Planting materials can be obtained through vegetative cuttings or using seeds. Propagation by single node cuttings is the major propagation method of tea adopted on a commercial scale in Sri Lanka. Vegetative propagation is more popular over seedling tea because seed propagated plants display high heterogeneity causing difficulties in field management practices. The use of vegetative cuttings as planting materials leads to challenges in the nursery management field due to unfavorable weather conditions, poor survival rate due to poor root formation of some cultivars, the season-dependent rooting ability of cuttings, and high incidence of pests and diseases. These factors would ultimately contribute to poor-quality planting materials and lead to higher nursery casualties (Mondal, 2014).

Micropropagation technology appears to be a viable alternative to overcome the aforesaid problems related to the conventional propagation program of tea. It is a useful method to produce a higher number of uniform planting materials within a limited space and comparatively a short period on a year-round basis (Gunasekara, 2008).

Recently, research on micropropagation has been focused on exploring the potential of somatic embryogenesis as a more efficient means of uniform plant multiplication and regeneration in mass propagation (Akula *et al.*, 2000).

In vitro, culture systems based on liquid culture medium are considered to be more effective than solid culture medium systems due to better accessibility of medium components for the plant tissue, ease of handling, and the possibility to scale and automate (Edward *et al*, 2017). Hence, the study aimed at optimizing liquid culture conditions as the main objective and identifying the ideal media composition and the explant type as the specific objectives to increase the efficiency of somatic embryo (SE) induction, multiplication, and regeneration of different tea cultivar TRI 5001.

METHODOLOGY

The research was conducted at the Tissue Culture Laboratory of the Plant Breeding Division, at the Tea Research Institute (TRI), Talawakele, Sri Lanka at Latitude 6° 54' 54'' N, longitude 80° 42' 18'' E, Agro-Ecological Region WU2a, Elevation of 1371 m above Mean Sea Level (Panabokke and Kannangara, 1975).

Plant Materials

Leaf and nodal explants of TRI 5001 were collected from the mother plants maintained in the greenhouse in the Plant Breeding Nursery TRI, Talawakele. Plants were maintained in the greenhouse in the conditions described by Ranaweera *et al* (2020). Fruits in late developmental stages were harvested from the mother bushes, and cotyledons of late mature seeds of the same cultivar identified by visual observation of seed coat colour were used as the explant to induce somatic embryos.

Media preparation and initial material production

Murashige and Skoog (1962) (MS) basal solid medium supplemented with 30g/L sucrose and different growth regulator combinations at pH 5.8 with agar, the solidifying agent at 8g/L were used for the material production.

The media preparation and the surface sterilization procedure described by Ranaweera *et al*, (2020) were followed for different explants. The explants; leaf segments (15 x 10 mm^2), zygotic embryo-dissected mature cotyledons, and nodal cuttings with partial removal of the epidermal layer were inoculated in the prepared culture medium inside the laminar flow. Culture vessels were maintained in the culture room under controlled environmental conditions (25±2 ^oC, 30 µmole $m^{-2}s^{-1}$ light intensity, and 16-hour photoperiod). Three successive subcultures
were done in 12 weeks intervals to obtain a sufficient number of uniform initial materials for the study. Further, the materials were also maintained in the same culture conditions while subculturing.

Induction of somatic embryogenesis and callogenesis

a) Cotyledons
The MS medium supplemented with 3 mg/L BAP and 0.1 mg/L NAA was used for somatic embryo induction through mature cotyledons (Abeywardana *et al*, 2015).

b) Leaf
The MS medium supplemented with 0.11 mg /L TDZ and 1.86 mg/ L NAA and 0.0044 mg /L TDZ, 0.1 mg/ L IBA and 3 mg / L GA_3 combination was used for friable embryonic callus induction through leaf segments (Gunathilake and Ranaweera, 2020).

c) Stem nodal cuttings
The MS medium supplemented with 1.86 mg/ L NAA and 0.0044 mg /L TDZ, 0.1 mg/ L IBA and 3 mg / L GA_3 combination was used for friable embryonic callus induction through stem nodal explants (Gunathilaka and Ranaweera, 2020).

Multiplication of somatic embryos and friable calli

a) Multiplication of somatic embryos (SEs) derived from cotyledons
Seven different liquid media were used for the experiment while TC8 medium supplemented with 0.2 mg/L NAA, 0.2 mg/L BAP (Solid) was used as the control (Table 1).

Previously generated SEs of TRI 5001 cultivar were subcultured into the above media in 5 replicates, each culture vessel containing 75 ml of the medium and varying numbers of initial SEs. The cultures were maintained by shaking at 60 rpm under the same culture conditions mentioned above. The liquid media was prepared with the filter paper bridge as a temporary immersion system (TIS) to increase the aeration for SEs. Twelve weeks after subculturing, the average number of SEs was recorded, and the multiplication rate was calculated as follows.

$$\text{Multiplication rate} = \frac{\text{No of final SEs} - \text{No of initial SEs}}{\text{No of initial SEs}}$$

.

Table 1: Growth regulators and nutrient components on multiplication of somatic embryos of cotyledon explants.

Treatment	TDZ mg/L	IBA mg/L	NAA mg/L	BAP mg/L	B5 Vitamins	NH_4NO_3	Glutamine mg/L
TC1	-	-	0.2	0.2	-	-	-
TC2	-	0.1	-	2	-	-	1000
TC3	-	-	-	-	-	-	-
TC4	-	-	-	-	B5 Vit	Free	-
TC5	3	-	-	-	-	-	-
TC6	3	-	0.1	-	-	-	-
TC7	3	-	1	-	-	-	-
TC8 (Solid)	-	-	0.2	0.2	-	-	-

b) Multiplication and somatic embryo induction from leaf calli

Callus was initiated from leaf pieces of TRI 5001 cultivar as described above. The initiated calli were used as the inoculum for liquid cultures. The prepared liquid media consisted of major and minor components of MS with various growth regulator combinations for the somatic embryogenesis as described in Table 2. Embryonic calli were sub-cultured into the prepared media (five culture bottles for treatment and five calli masses per bottle) and the cultures were maintained under controlled environmental conditions (25 ± 2^0C, 30 µmole $m^{-2}s^{-1}$ light intensity, and 16-hour photoperiod) on a rotary shaker set at 60 rpm. Growing calli were sub-cultured at six weeks intervals, and the percentage growth of callus proliferation, compact callus formation, and embryonic callus formation was recorded while maintaining in the same media.

c) Multiplication and somatic embryo induction from stem nodal

Small pieces of friable uniform nodal callus of the above-selected cultivar obtained at previous steps were subcultured to the prepared liquid MS media as given in Table 3. Embryonic calli were sub-cultured into the prepared media (five culture bottles for treatment and five calli masses per bottle) and the cultures were maintained under controlled environmental conditions (25 ± 2^0C, 30 µmole $m^{-2}s^{-1}$ light intensity, and 16-hour photoperiod) on a rotary shaker set at 60 rpm. Subculturing was done at six weeks intervals by transferring to the same media and maintaining the same cultural conditions. Percentage growth of callus proliferation, compact callus formation, and embryonic callus formation was recorded while maintaining in the same media.

Table 2: Plant growth regulators and other components of the medium on induction of somatic embryos from leaf callus (mg/L).

Treatment	TDZ mg/L	IBA mg/L	NAA mg/L	BAP mg/L	GA_3 mg/L	$AgNO_3$ mg/L
TL1	-	-	0.1	2	-	-
TL2	-	-	3.5	2	-	8.6
TL3	-	-	0.1	3	-	-
TL4	1.1	0.1	-		3	8.6
TL5	-	-	0.1	1	0.2	
TL6	0.044	-	0.1	-	3	-
TL7	0.044	0.1	-	-	3	-
TL8	0.044	0.1	-	-	-	-
TL9	1.1	-	1.86	-	-	-
TL10(Solid)	-	-	0.1	2	-	-

Table 3: Growth regulator combinations and media constituents used for somatic embryo induction from nodal explants (mg/L).

Treatment	TDZ mg/L	IBA mg/L	NAA mg/L	BAP mg/L	GA_3 mg/L	$AgNO_3$ mg/L
TN1	-	-	0.1	2	-	-
TN2	0.11	0.1	-	-	-	-
TN3	-	0.1	-	0.5	3	-
TN4	-	0.1	-	0.5	3	8.6
TN5	-	-	0.2	2	-	-
TN6	0.044	0.1	-	-	3	-
TN7	0.044	0.1	-	-	3	8.6
TN8	0.44	0.1	-	-	-	-
TN9	0.1	-	1.86	-	-	-
TN10(Solid)	-	-	0.1	2	-	-

Effect of culture conditions for somatic embryo regeneration

The MS with 3 mg/L BAP + 0.1 mg/L NAA liquid medium (Sivapriyadharshini *et al*, 2018), including 1mg/L, activated charcoal was prepared as the SE regeneration medium as described in above step and 1/3 volume of 30 ml culture bottles were filled with medium followed by setting up of filter paper bridges in the bottles for aeration. Twenty bottles were maintained as replicates for each of the seven treatments. The same composition with a solid medium was used as the control treatment. Generated SEs, were sub-cultured to the prepared culture bottles, and those were maintained under the same culture conditions mentioned in previous steps. Subculture was continued at eight weeks intervals, and the number of regenerated plants was recorded while evaluating the performance of the plants.

Data analysis

Experiments were laid in complete randomized design (CRD) with 5 replicates per treatment. Multiplication rates of somatic embryos of each treatment were analyzed by the Chi-Square analysis method. Embryonic callus, compact callus, and callus proliferation percentage values were analyzed through the probit analysis method. Mean separation was done with the Duncan Multiple Ranges Test at a 5% significant level using SAS statistical analysis software.

RESULTS AND DISCUSSION

After 12 to 16 weeks of *in vitro* cotyledon culture in SE induction medium, enough somatic embryos were generated, and friable callus masses were observed in the inoculation media of nodal and leaf cultures (Figure 1).

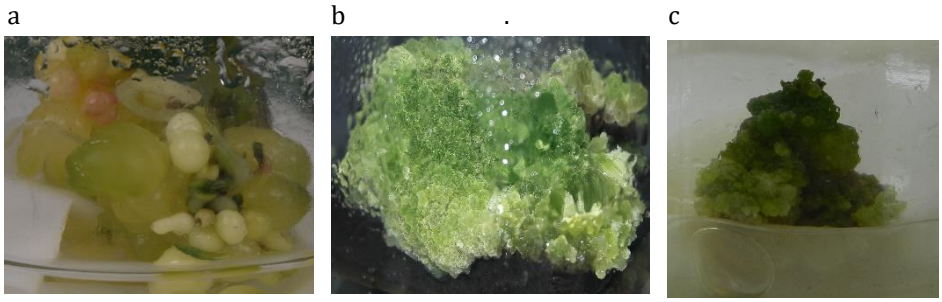

Figure 1: Initial materials generated for the experiments. a) cotyledon-derived SEs b) Nodal-derived friable calli c) Leaf-derived friable calli

Cotyledonous somatic embryo multiplication in liquid dynamic medium

The inoculated cotyledonary SE was slightly enlarged and appeared light green in colour in culture, and secondary SEs were observed in all the treatment combinations at different rates of multiplication. A significantly ($P<0.05$) higher SE multiplication rate was recorded in medium composition with MS liquid+3mg/L TDZ (TC5) (Figures 2, and 3). Thidiazuron (TDZ) has been used in the range of 0.5-10 µM to stimulate somatic embryogenesis from cotyledons of white ash (Huetteman and Preece, 1993; Bates *et al*., 1992). Paul *et al.* (2011) observed a higher SE generation frequency in the embryonic axis of Murraya *koenigii* aromatic plant with basal media containing 1-2 mg/L TDZ.

Somatic embryo induction from leaf callus in liquid medium

Treatment combinations 1.1 mg/L TDZ 1.86 mg/L NAA (TL9) showed comparatively higher (65%) callus proliferation (CPR), 65% light green colour compact callus formation (CPC) and 71% embryonic callus formation (EMC) (Figure 4 and Table 4). After 12-16 weeks of the subculturing process, somatic embryo formation was observed in 0.1mg/L NAA 1mg/L BAP 0.2mg/L GA3 (TL5) medium during the period of 6 weeks subculture interval.

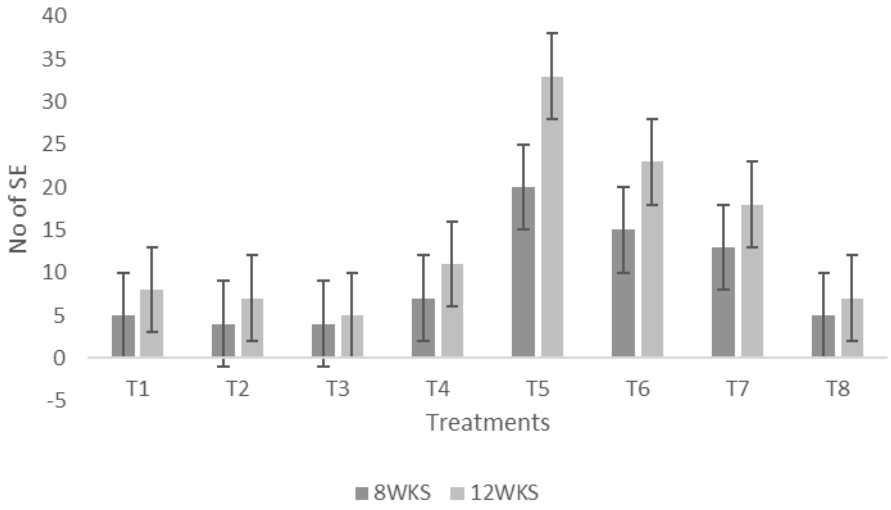

Figure 2: Mean number of SE in different media at 8 and 12 weeks after subculturing.

Figure 3: Performance of Cotyledonous somatic embryo multiplication in TC5 medium after 12 weeks.

Table 4: The influence of different liquid media on Callus proliferation (CPR), Compact callus formation (CPC), embryonic callus formation (EMC) and somatic embryo induction (SEI) through the leaf calli.

Treatment	CPR%	CPC%	EMC%	SEI %
TL1	50±4	46±5	40±4	-
TL2	59±5	55±4	60±4	-
TL3	55±5	50±5	50±5	-
TL4	50±5	55±5	50±5	-
TL5	46±4	46±7	50±5	5.6
TL6	55±5	50±5	55±5	-
TL7	59±5	55±4	50±5	-
TL8	59±5	55±4	60±4	-
TL9	65±6	65±6	71±6	-
TL10 (Solid)	36±7	37±4	40±6	-

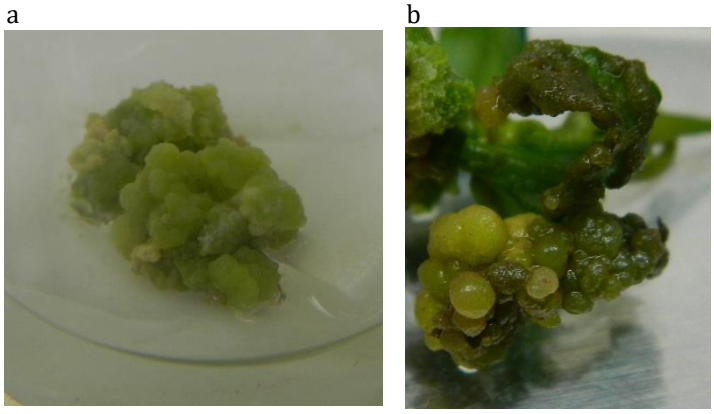

Figure 4: Performance of embryonic leaf callus formation a) Leaf embryonic callus b) Somatic embryo formation through embryonic leaf callus

TDZ at 3 mg/L resulted in the best response of proliferation rate of fresh callus mass from leaf explants of *Phalaenopsis amabilis* recording 13.8 mean number of embryos per explant (Chang *et al*, 2010). At concentrations higher than 1 µM, TDZ can stimulate the formation of callus and somatic embryos (Huetteman and Preece, 1993). Ghosh *et al*. (2018) reported that the plant growth regulator TDZ possesses the potential for somatic embryo formation when added to a nutrient medium at high concentrations (9µM). TDZ has also been reported to be enhancing embryo germination at low concentrations (2.3µM), in which Chang and Chen (2006) observed small and compact cell formation of embryogenic calli through cytological studies. Ibraheem *et al*. (2013) revealed that a liquid medium consisting of 0.1 NAA+ activated charcoal is suitable for the induction of somatic embryos of date plants. The liquid medium showed higher embryogenic potential than the solid medium at the 16th week of incubation of leaf explants of tea (Charles & Vincent, 2007). Total single cells, cell clusters, single embryogenic cells, and embryogenic cell clusters were comparatively higher in the liquid medium than in the solid medium. Further, the mean number of total single cells and single embryogenic cells in 1.0 mL was significantly higher in liquid than in the solid medium (Seran, 2006).

Induction of somatic embryo from nodal callus in liquid medium

Six weeks after initial subculturing, the highest percentage of light green colour callus proliferation (CPR) was recorded (76%) in

MS+0.11mg/L TDZ 0.1mg/L IBA (TN2) °treatment. Comparatively higher yellowish green compact callus (CPC) formation percentage (65%) was also observed in the same medium at the 2nd subculturing. Furthermore, 0.1 mg/L TDZ 1.86 mg/L NAA (TN9) medium were identified to have a higher (76%) embryonic callus (EMC) formation medium at the 3rd subculture period (Figure 5 and Table 5). However, 12-16 weeks after 1st subculturing, plantlets (DRP) were observed in a few culture bottles in the 0.044 mg/L TDZ 0.1mg/L IBA (TN8) treatment combined with a lower percentage of 5.4%. Choun-Sea Lin (2003) reported that TDZ and NAA promoted callus growth, but NAA reduced embryo germination, while Mohajer *et al*. (2012) observed higher direct plant regeneration with combinations of IBA + BAP. Comparing liquid and solid media with similar growth regulator combinations (TN1 and TN10), higher performances were observed in the liquid medium through all the steps of the embryogenesis process. Seran *et al*. (2007) also stated that total single cells, cell clusters, single embryogenic cells, and embryogenic cell clusters are comparatively higher in the liquid medium compared to the solid medium.

Table 5: The influence of different liquid media on Callus proliferation (CPR), Compact callus formation (CPC), embryonic callus formation (EMC) and Direct plant regeneration (DPR) through nodal calli.

Treatment	CPR%	CPC%	EMC%	DRP %
TN1	59±6	46±6	41±0	-
TN2	**76**±3	**65**±3	**59**±3	**-**
TN3	41±4	41±4	41±0	-
TN4	59±3	50±4	50±4	-
TN5	41±0	37±3	46±3	-
TN6	42±7	50±4	46±3	-
TN7	64±0	55±4	50±4	-
TN8	37±3	59±3	59±3	5.4
TN9	**64**±3	**59**±3	**76**±0	**-**
TN10 (Solid)	41±0	37±3	41±4	-

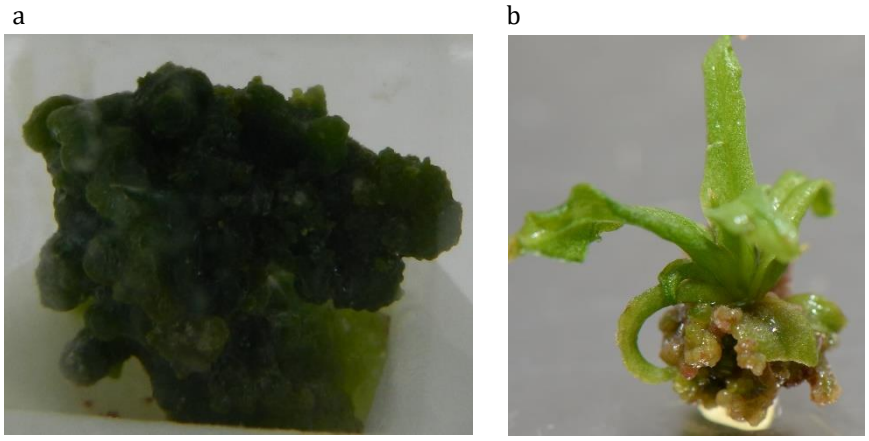

Figure 5: Performance of embryonic nodal callus formation. a) Nodal embryonic calli. b) Regenerated plants through embryonic nodal callus.

Zhang *et al*. (2016) observed friable embryogenic callus and yellow colour direct somatic embryos from stem nodal segments of *Santalum albu* plants with different concentrations of TDZ through hard callus/ shoots and embryos, somatic embryos, and yellowish green granular callus with different concentrations; 0.5mg/L, 1mg/L, and 2mg/L of TDZ.

The concentrations of TDZ used in driver and kuniyaki walnut (DKW) salts-based primary callus growth medium significantly affected the rate of callus growth, the frequency of embryogenesis, and the number of somatic embryos produced from each responsive explant (Li *et al*., 1998). A TDZ concentration of 22.7 nM was the optimal concentration for the effective induction of somatic embryos from various cacao genotypes (Li *et al*., 1998).

Effect of culture conditions in somatic embryo regeneration

Germination of cotyledon-derived somatic embryos follows four stages namely, globular, heart, torpedo, and cotyledonary. At the stage of subculturing, the majority of the cotyledonous SEs were identified as globular and heart shape embryos, and simultaneous SE regeneration and germination were observed while maintaining in the regeneration medium. Four weeks after subculturing, tap root formation was observed first and subsequently shoot growth started, where 50 % germination was observed in the liquid medium compared with 30% germination in the control solid medium after 12-16 weeks (Figure 6). However, only two stages (globular, and heart) were identified after three weeks of somatic embryo induction in the same cotyledons without transferring them into the germination medium. Confirming the above, Vieitez *et al.* (1991) have reported that somatic embryo development did not require two-phase culture procedures, whereas the development and maturation of embryos occurred in the induction medium itself. Further, early researches state that somatic embryos cultured on a solid medium usually contain all four stages of development at any one time because of repetitive (secondary) embryogenesis (Akula and Dodd, 1998; Akula *et al*., 2000). Lu *et al*. (2013) also observed tested growth regulator combinations to be suitable because higher morphogenesis response (76%) of somatic embryos could be germinated directly and develop into plantlets of *Camellia nitidissima* on medium supplemented with 0.9µM BAP and 0.1µM NAA.

Figure 6: Steps of the regeneration from cotyledon-derived SEs in the liquid medium.

CONCLUSIONS

The MS liquid medium with different concentrations of the growth regulators is suitable for somatic embryo induction through different explants of the TRI 5001 cultivar. TDZ at 3 mg/L was suitable for somatic embryo multiplication through cotyledons, and 1.1 mg/L TDZ, 1.86 mg/L NAA combination was observed as the suitable medium for callus proliferation, compact callus formation, and embryonic callus formation. The concentrations of 0.1 mg/L NAA 1 mg/L BAP 0.2 mg/L GA3 display a certain degree of potential to induce somatic embryos through the leaf callus. Furthermore, 0.11mg/L TDZ 0.1mg/L IBA was identified as the suitable medium for callus proliferation and compact callus formation, and 0.1 mg/L TDZ 1.86 mg/L NAA was suitable for embryonic callus formation from nodal callus. Further, it was observed that liquid MS with 3 mg/L BAP + 0.1 mg/L NAA is the suitable medium for somatic embryo regeneration.

ABBREVIATIONS

Indole-3-Butaric Acid (IBA), 6-Benzyl –Amino Purine (BAP), Thiadiazuron (TDZ), Naphthalene acetic acid (NAA), Gibberellic acid (GA3), Driver and Kuniyaki Walnut (DKW), Somatic embryo (SE), revolution per minute (rpm)

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