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Somatic Embryogenesis Protocol for Tea (*Camellia Sinensis* (L.) O Kuntze)

Abeywardana DASR.[1], Ranaweera KK[2], Ranatunga MAB[2*], Warnasooriya WMRSK[1]

Department of Plant Sciences, Faculty of Agriculture, Rajarata University of Sri Lanka, Puliyankulama, Anuradhapura[1]

Plant Breeding Division, Tea Research Institute of Sri Lanka, Thalawakelle[2]

ABSTRACT

Somatic embryogenesis is an efficient micropropagation technique, which can be adapted to mass multiplication of tea. Hence, the development of a viable somatic embryogenesis protocol for tea is a timely need. Four factor factorial experiment with two growth stages of two ex-plants namely; ex-vitro leaves (2nd and 3rd leaf) and cotyledons (immature and mature) of TRI 2024 and 2043 cultivars with MS medium, containing two growth regulator combinations were designed ((I)3 mg l^{-1} BAP+0.1 mg l^{-1} NAA, 3 mg l^{-1} BAP+0.1 mg l^{-1} NAA, 3 mg l^{-1} BAP+0.1 mg l^{-1} NAA /(II)2 mg l^{-1} BAP+3 mg l^{-1} NAA, 3 mg l^{-1} BAP+0.1 mg l^{-1} NAA, 1 mg l^{-1} BAP+0.1 mg l^{-1} NAA for ex-vitro leaves for callusing, embryo induction and germination and (I) 3 mg l^{-1} BAP+0.1 mg l^{-1} NAA, 3 mg l^{-1} BAP+0. 1 mg l^{-1} NAA (II)2 mg l^{-1} BAP+0.2 mg l^{-1} NAA, 3 mg l^{-1} BAP+0.5 mg l^{-1} NAA for cotyledons for embryo induction and germination). Somatic embryos were developed by direct pathway from cotyledons and indirect pathway from ex-vitro leaves. Somatic embryos were developed from cotyledons, while ex-vitro leaves developed up to the callus stage. Somatic embryo development was significantly different among cultivars, growth stage of explants and the media. MS medium with 3 mg l^{-1}BAP and 0.1 mg l^{-1} NAA is the best medium for embryo induction of mature cotyledons with both cultivars. The highest somatic embryogenesis (40%) was observed in mature cotyledons of TRI 2043. Embryo germination stages namely; globular and heart shapes were observed in cotyledons. High callus induction of ex-vitro leaves were observed in MS medium with 2 mg l^{-1}BAP and 3 mg l^{-1}NAA, irrespective of growth stage. TRI 2024 recorded a higher proliferation rate in the above medium than TRI 2043. Protocol developed can be optimized by repeating with more cultivars.

Keywords: *Callus, Cotyledon, Ex-vitro leaves, Growth regulators, Somatic embryogenesis*

1. Introduction

Conventionally tea (*Camellia sinensis* (L.) O. Kuntze) is propagated through vegetative propagation with single nodal cuttings, which is adopted at commercial scale in Sri Lanka and elsewhere. However, the planting material requirement of the nation cannot be solely fulfilled by this method. Thus, micropropagation technology appears to be an alternative choice for mass multiplication of tea. However, it has made little progress due to tea being a recalcitrant, high level of contaminations at *in-vitro* conditions and lack of reproducibility in the developed protocols for multiplication, rooting and acclimatization (Gunasekara, 2008). While extensive research has focused on micropropagation of tea, review and overviews of tea micropropagation revealed that it has not become a commercial reality (Gunasekare, 2008). Thus, it is of paramount importance to develop a micropropagation technique with increased multiplication rate.

Somatic embryogenesis emerged as the best alternative to overcome poor multiplication rate. Somatic embryogenesis is the process of developing somatic cells to form new plants through specific stages of embryonic development without going through the fusion of gametes and without vascular connection with the original tissue (Von Arnold *et al,*, 2002). It is considered to be the most efficient regeneration system of tea (Jain and Newton, 1999) and considered profitable over other *in-vitro* propagation systems as it reduces the proliferation time and maintain high genetic integrity (Kothari *et al.*, 2010).

2. Statement of Problem

Plenty of researches have focused on production of viable somatic embryos on tea by using different ex-plant and different growth medium compositions. Seran *et al.*, (2006) reported that somatic embryogenesis could be initiated from leaf callus. In a similar study, direct somatic embryogenesis of selected tea cultivar *i.e.* TRI 2025 from nodal segment has been reported (Akula & Dodd, 1998). However, plantlet regeneration from somatic embryos has not been attempted much. A recent study conducted at Tea Research Institute of Sri Lanka (TRI) has produced somatic embryos from cotyledon axis and plantlets were regenerated successfully (Unpublished).

Success of the somatic embryogenesis highly depends on source and type of ex-plants and growth regulators. In tea, immature and mature cotyledons have been used as starting materials for somatic embryogenesis directly or indirectly (Kato, 1986). Further, de-embryonated cotyledons were used as ex-plants to produce somatic embryos (Mondal *et al.*, 2000) and somatic embryos were also obtained from non embryonic tissue *i.e.* stem and leaf ex-plants (Sarachchandra *et al.*, 1988). Immature embryos produced somatic embryos whereas mature embryos showed pronounced zygotic embyo growth (Sarachchandra *et al.*, 2001). Hence, selection of ex-plants at a particular physiological stage is an important criterion in somatic embryogenesis.

Further, the type, concentration, and time of application of different growth regulators in culture media extensively affect on somatic embryogenesis. In general, a high cytokinin to low auxin or low cytokinin alone was found to be necessary for the induction of somatic embryos in *Camellia*, but cytokinins has often been reduced or omitted in subsequent sub culturing (Mondal, 2014). MS (Murashige and Skoog, 1962) was the most commonly used medium for induction of somatic embryos. In tea, efficient production of somatic embryos has been achieved in the presence of cytokinins (BAP or Kinetin) in combination with NAA or IBA (Kato, 1986; Mondal *et al.*, 2000; Srachchandra *et al.*, 2001). Therefore, selection of appropriate combinations and concentrations of growth regulators is vital on direct or indirect somatic embryogenesis. Thus, selection of ex-plants at a particular physiological stage and selection of appropriate combinations and concentrations of growth regulators is vital in developing a commercially viable somatic embryogenesis protocol to use in mass multiplication of tea.

3. Objectives of the Study

The general objective of the study was to develop plants through economically viable somatic embryogenesis protocol to enhance mass multiplication of tea. The specific objectives were to identify suitable ex-plants with ideal maturity stage and growth regulator combination for producing viable somatic embryos.

4. Review of Literature

Tea can be propagated either by seeds or by cuttings. Seeds were the only source of planting material used until economically viable vegetative propagation method was discovered and established by the Tea Research Institute of Sri Lanka in 1938 (Kehl, 1950). Tea is completely self incompatible and requires cross pollination, thus seedlings show a wide variability in the attributes such as yield, quality *etc.* (Mondal, 2014). In this scenario, vegetative propagation offers a better solution by producing genetically similar offsprings. Vegetative propagation with single nodal cuttings is the conventional propagation technique of tea, which is adopted at commercial scale in Sri Lanka. Single nodal cutting consisting of a nodal leaf with an auxiliary bud and node of 2.5-3.5 cm length is used as the most appropriate cuttings (Gunasekara and Trixie, 2012). However, slower rate of propagation, unavailability of suitable planting material due to winter dormancy, drought stress, poor survival rate at nursery and season dependent rooting ability of the cutting (Mondal, 2009) are considered as the limitations in conventional tea breeding.

In the last decade, significant progress had been made in the *in-vitro* propagation of tea cultivars directly and indirectly through organogenesis (Gunesekara and Evan, 2000) or somatic embryogenesis (Mondal *et al.*, 2000). In Sri Lanka, micropropagation has been used to enhance the efficiency of the conventional breeding programme which has a great impact on shortening the tea breeding cycle (Ranaweera, 2012). Tea being a woody perennial, it takes 20-25 years to release a new tea cultivar. However, with tissue culture techniques, TRI has cut down 6-7 years from the conventional breeding programme (Ranaweera, 2012). Yet, micropropagation of tea has not been commercially exploited. Phenolic exudation from ex-plant and microbial contamination in tissue culture medium are the major limiting factors (Akula and Akula, 1999), while higher cost of production, loss of juvenility of *in-vitro* culture and absence of tap root system that make micropropagated tea more susceptible to drought are other factors that prevent micropropagation becoming a commercially viable propagation technique in tea.

Somatic embryogenesis is considered to be the most efficient regeneration system of tea (Jain and Newton, 1990) and considered profitable over other *in-vitro* propagation systems as it reduces the proliferation time and maintain high genetic integrity (Kothari *et al.*, 2010). Somatic embryogenesis can be initiated by two mechanisms, *i.e.* directly on ex-plant tissues, where plants are genetically identical (clonation), and indirectly from unorganized tissues (callus). Direct somatic embryogenesis has low probability of genetic variation than other propagation methods (Merkle, 1997) and it is a desirable approach because of genetic stability of regenerated plantlets (Pedroso and Pais, 1995). Propagation by indirect embryogenesis carries the risk of producing plants that may differ genetically from each other and from the parental plant. Seed of somatic embryo is a bipolar structure resembling a zygotic embryo. Therefore, plant propagation through somatic embryo formation is more favorable than using organogenesis, which produces uni-polar seeds (Ibrahim, 2013).

Embryogenesis has been successfully exploited for higher regeneration rates than with organogenesis providing an alternative approach to conventional micropropagation for large scale clonal propagation of elite cultivars (inter specific or intra specific hybrids) (Deo *et al.*, 2010), artificial seed production, cryopreservation for the storage of elite cultivar with low risk of genetic variation, embryo rescue and most importantly *in-vitro* manipulations through genetic transformation (Mondal *et al.*, 2001). Further it offers higher multiplication rates of cells or embryos, simultaneous root and shoot formation to eliminate the need of root induction phase as with conventional micropropagation methods (Deo *et al.*, 2010), synchronized embryo formation and germination to maximize plant output while minimizing labour cost (Von Arnold *et al.*, 2002), possibility of automation in cell suspension to improving the efficiency of embryo production in a short period of time (Ibaraki and Kurata, 2001).

5. Methodology

The research was carried out at the Plant Tissue Culture Laboratory, Plant Breeding Division, Tea Research Institute of Sri Lanka. *Ex-vitro* leaves and cotyledons of TRI 2043 and TRI 2024 cultivars

were used as ex-plants. Two maturity stages of each ex-plant were selected for the experiment namely; 2nd and 3rd *ex-vitro* leaf and immature (06 months old whitish cream colour cotyledon) and mature (07 months old pale yellow colour cotyledon) cotyledons.

4.1 Procedure for Cotyledon Culture

Seeds, extracted from the undamaged fruits using a knife were put into a beaker and soaked overnight in distilled water, while floating seeds were discarded. Soaked seeds were washed with teepol, followed by 20% Clorox solution with tween 20 for 10 minutes. They were rinsed 03 times with distilled water. This process was repeated once and seeds were washed with autoclaved distilled water, before culturing.

Table 1: Growth regulator combinations used for somatic embryo induction from cotyledons

Growth Regulator Combination	Somatic Embryo Induction	Embryo Germination
H1	3 mg l^{-1} BAP + 0.1 mg l^{-1} NAA	3 mg l^{-1} BAP + 0.1 mg l^{-1} NAA
H2	2 mg l^{-1} BAP + 0.2 mg l^{-1} NAA	3 mg/l BAP + 0.5 mg l^{-1} NAA

After removing seed coat with a knife, a single cotyledon was isolated from each seed at different development stages and embryogenic axes were dissected carefully. Approximately 07 mm wide, 04 mm long, 1.5-02 mm thick small cotyledon pieces were excised from sterilized cotyledon using scalper and forcep. These were then inserted into the media (Table 1), leaving abaxial side down inside the laminar flow.

4.2 Procedure for Leaf Culture

The 2nd and 3rd leaf of TRI 2043 and TRI 2024 cultivars maintained in the poly house were selected for the experiment. Leaves were placed in beakers with distilled water immediately after collection. Same surface sterilization procedure was followed for extracted leaves before culturing. Leaf segments (10 x 5 mm^2) were excised from the sterilized leaves by using scalper and forcep. They were cultured on MS basal medium (Table 2) in 125 ml culture vessels inside the laminar flow.

Table 2: Growth regulator combinations used for somatic embryo induction from leaf ex-plants

Growth Regulator Combination	Embyogenic Callus Induction	Somatic Embryo induction	Embryo Germination
H3	3 mg l^{-1} BAP + 0.1 mg l^{-1} NAA	3 mg l^{-1} BAP + 0.1 mg l^{-1} NAA	3 mg l^{-1} BAP + 0.1 mg l^{-1} NAA
H4	2 mg l^{-1} BAP + 3 mg l^{-1} NAA	3 mg l^{-1} BAP + 0.1 mg l^{-1} NAA	1 mg l^{-1} BAP + 0.1 mg l^{-1} NAA

Both cultures were maintained in a controlled environment having 25±2 ^{0}C temperature and light intensity of 2000 lux for 16 hours of photoperiod.

4.3 Data Collection

Morphological changes of the leaf ex-plants were observed weekly. Number of culture vessels which had callus initiated leaves were taken as a percentage. Friable and compact calli were observed in each callus initiated culture vessel by visual observation as a qualitative measurement. Quantity of callus was measured according to the scale (Plate 1), where a rate was given by observing callusing percentage of leaf margins as; 1 for 05%, 2 for 10%, 3 for 25%, 4 for 75% and 5 for 100%.

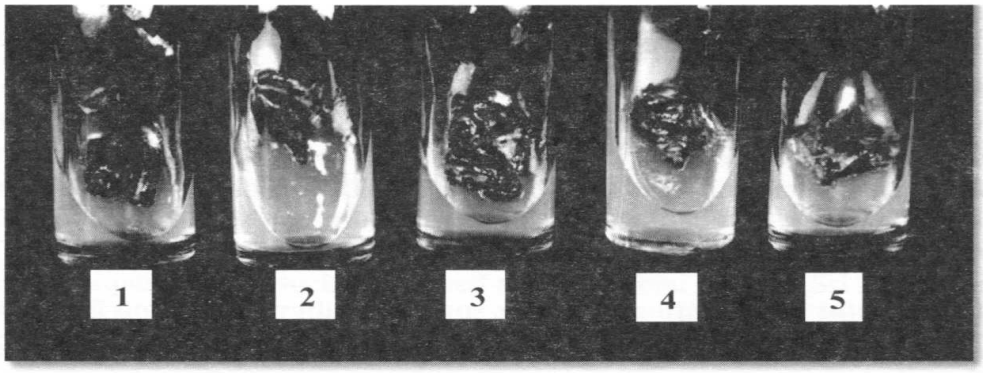


Plate 1: Scale used for quantifying callus induction from leaves

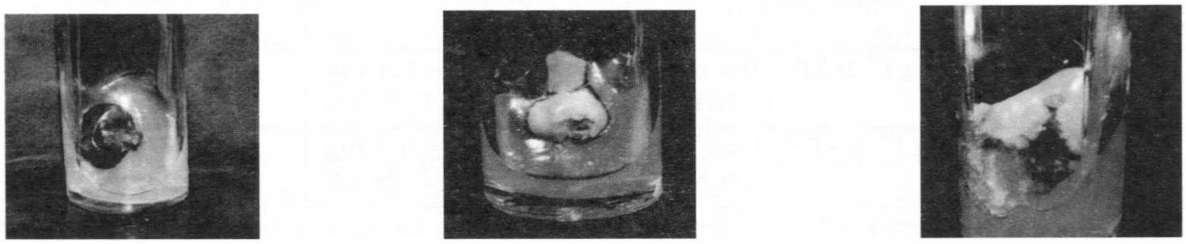
Morphological changes of cotyledon ex-plants were observed weekly. Number of culture vessels which had somatic embryos was taken as a percentage and also numbers of somatic embryos per cotyledon were taken as a quantitative measurement. In addition, different stages of somatic embryos were identified.

3.4 Experimental Design and Data Analysis

Experiment was designed as a four factor factorial CRD (Complete Randomized Design) with cultivar, ex-plants, growth stages and hormone combination as factors. Embryo induction percentage and callus induction percentage values were transformed using Arcsine transformations before statistical analysis. Both qualitative and quantitative data were subjected to ANOVA and mean separation procedures using SAS and results were interpreted at 95% confidence level.

4. Results and Discussion

Morphological changes were observed in both cotyledon types within 02-03 weeks after culturing, where white colour cotyledon pieces were turned into dark green after the 02nd week of culturing (Plate 2). Yellowish protuberant tissues were seen only on adaxial side of the cotyledon ex-plants. The embryos were visible to the naked eye after 04th week even before transferring them to a fresh medium. Seran *et al.* (2006) reported similar morphological changes to the naked eye after the 06th week on cotyledons. Responses of ex-plants varied among different media. Morphological changes were first observed in the MS media, supplemented with 3 mg l^{-1} BAP and 0.1mg l^{-1} NAA. Somatic embryos were first observed in mature cotyledon of TRI 2043 in the MS media supplemented with 3 mg l^{-1}BAP and 0.1mg l^{-1} NAA after 04th week of culturing.



(a) 02nd week **(b) 03rd week** **(c) 04th week**

Plate 2: Morphological changes observed in cotyledon pieces after culturing

Highest percentage of somatic embryos (40%) was produced with mature cotyledon of TRI 2043 in 3 mg l^{-1} BAP and 0.1mg l^{-1} NAA medium (Figure 01). Mondal *et al.* (2000) reported that somatic embryos were formed directly on the mature and immature cotyledon ex-plants in MS medium with BAP in combination with NAA. Further, Bano *et al.* (1991) observed that the potential of embryogenesis in immature cotyledon segments were more responsive than with a mature ones. However, in the present study, mature cotyledons gave more response than immature cotyledons, may

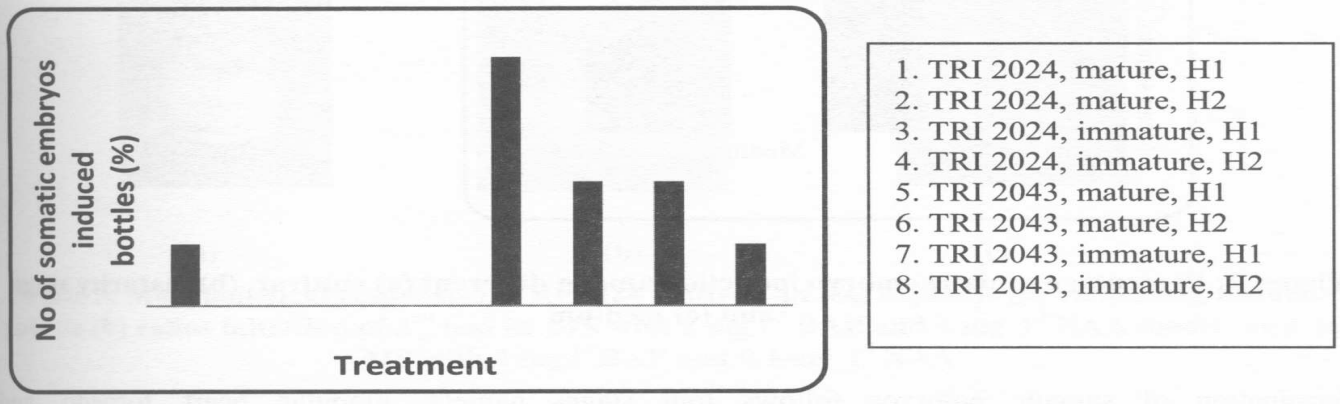


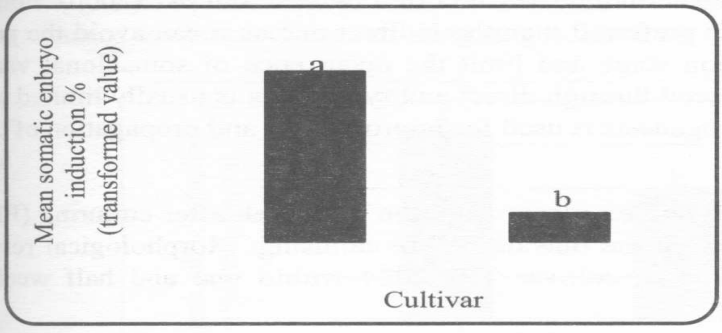
Figure 1: Percentage somatic embryo induction among different treatments

A significant difference was observed among cultivars, maturity stage of ex-plants and growth regulator combinations used (Figure 2). TRI 2043 reported higher somatic embryogenesis (mean value: 0.48) than TRI 2024 (mean value: 0.08) (Figure 2 (a)). Results revealed that efficiency of somatic embryogenesis varied among different tea cultivars and it is suggested to establish somatic embryogenesis rates with more cultivars.

As illustrated in figure 2 (b), mature cotyledons produced higher somatic embryos (mean value: 0.38) than immature cotyledons (mean value: 0.20) irrespective of the cultivar. Thus, it is important to identify the correct maturity stage of cotyledons, which may vary with cultivars. In the present study, maturity stage of cotyledon was identified by visual assessment of colour of fruit coat and seed coat. Therefore, it is suggested to introduce a tagging system of floral buds after pollination to collect cotyledons with known maturity in order to produce consistent results.

The 3 mg l^{-1} BAP and 0.1mg l^{-1} NAA (mean value: 0.38) medium was found to be more effective than 2 mg l^{-1} BAP and 0.2 mg l^{-1} NAA (mean value: 0.20) medium for somatic embryogenesis. Results are in agreement with previous studies, where high rate of somatic embryos obtained with 3 mg l^{-1} BAP and 0.1mg l^{-1} NAA, when cotyledons of TRI 2043 cultivar used as ex-plants (Unpublished). According to the observations, highest numbers of somatic embryos were observed with mature cotyledons of TRI 2043 cultivar in 3 mg l^{-1} BAP and 0.1mg l^{-1} NAA medium.

(a)



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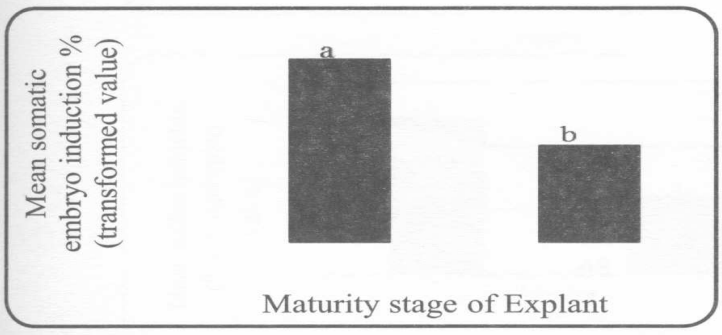


(c)

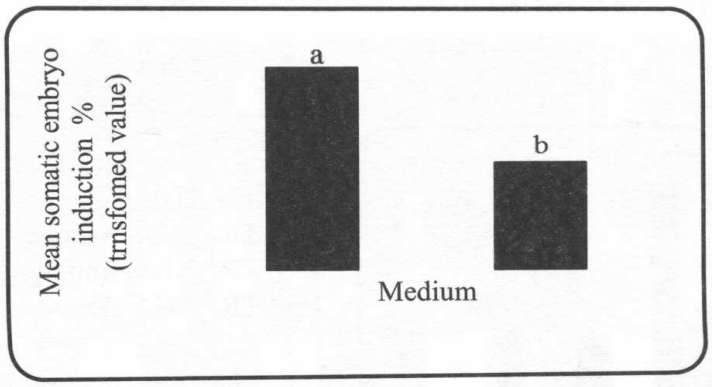


Figure 2: Percentage somatic embryo induction among different (a) cultivar, (b) maturity stage and (c) medium

Germination of somatic embryos follows four stages namely; globular, heart, torpedo, and cotyledonary. However, only two stages could be identified after the 03rd week of somatic embryo induction in the same cotyledons, before transferring them into the germination medium (Plate 3). Confirming above, Vieitez (1995) has reported that somatic embryo development did not require two phase culture procedures where, 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 & Dodd, 1998; Akula *et al.*, 2000).

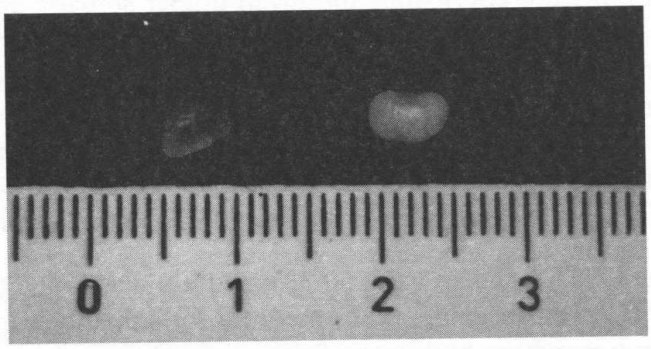
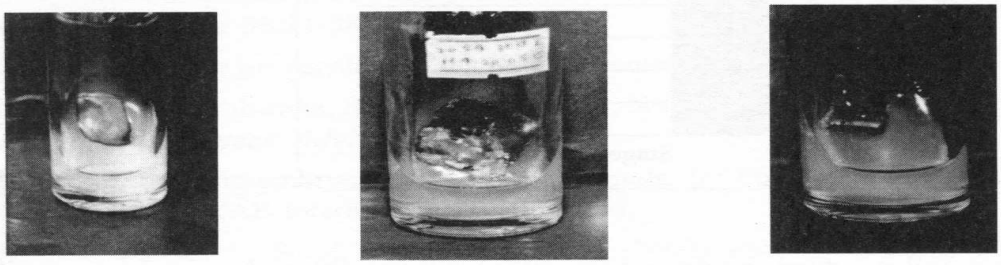


Plate 3: Stage of embryo development

Morphology studies revealed that somatic embryos developed directly from the cotyledon without intervening them into callus stage and similar finding was reported by Seren *et al.,* (2006). Formation of embryo through direct embryogenesis is preferred than the indirect one as it can avoid the problem of forming seed in the somatic germination stage and limit the occurrence of somaclonal variation. However, the number of somaclones produced through direct embryogenesis is usually limited and not uniform. Therefore indirect somatic embryogenesis is used for improvement and propagation of crops.

Morphological responses were observed in leaf ex-plants from the 01st week after culturing (Plate 4). Leaf ex-plants were slightly embossed toward the outside before callusing. Morphological responses for callusing media were initially observed in cultivar TRI 2024 within one and half week after culturing.



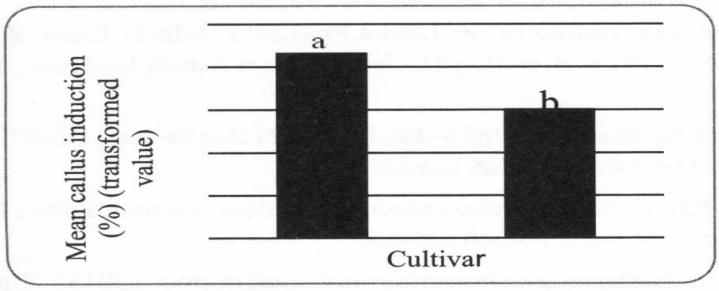
(a) (b) (c)

Plate 5: Morphological response of TRI 2024 cultivar in leaf explants (a) embossing toward outside (b) callus initiation of 3rd leaf in MS with 2 mg l^{-1} BAP and 3 mg l^{-1} NAA media and (c) MS with 3 mg l^{-1}BAP and 0.1 mg l^{-1} NAA

Callus initiation was first observed at cut ends of the leaf and most ex-plants were curled and formed into a whitish or greenish yellow friable calli by that time. Seran *et al* (2006) reported, primary calli with compact and yellowish appearance induced somatic embryos. Friable calli initiation was commenced in MS media in the presence of 2 mg l^{-1} BAP and 3 mg l^{-1} NAA when 3rd leaf segment of TRI 2024 was used as an explants after 02nd week from culturing. Leaf calli were observed in 3 mg l^{-1} BAP and 0.1 mg l^{-1} NAA within 04th weeks. However, intensity of callus induction was comparatively low. Gunesekara and Evans (2000) stated that ratio of auxin and cytokinin was found to be critical for inducing callus at high frequency. A significant difference was observed between cultivars and the two media, while maturity stage of leaf did not show any impact on callus initiation at 95% confidence level. Further, cultivar and medium interaction was significant (p=0.0004) with regard to callus initiation.

Callus initiation was high in cultivar TRI 2024 in MS medium with 2 mg l^{-1} BAP and 3 mg l^{-1} NAA of 03rd maturity stage of leaf (Figure 03). High quantity of callus was obtained in MS media with 2 mg l^{-1} BAP and 3 mg l^{-1} NAA. In addition, callus of 03rd leaf responded better than 02nd leaf for both TRI 2024 and TRI 2043 in 2 mg l^{-1}BAP and 3 mg l^{-1} NAA media. This may be due to the less polyphenol content of 03rd leaf (23.1%) than the 02nd leaf (27%). The polyphenol composition of tea undergoes changes with leaf age and activity, where the optimum activity is with 01st leaf (Banerjee, 1992). Further, 2 mg l^{-1} BAP and 3 mg l^{-1} NAA medium was appropriate for callus induction of leaf ex-plants than the 3 mg l^{-1} BAP and 0.1mg l^{-1} NAA medium. Most of the calli were in initial stage and 76.9% of calli were in friable stage. Qualities of calli were difficult to identify in 1st and 2nd categories due to less amount of calli produced. Due to the time limitation of the study, only the callus initiation was observed while subsequent stages were not observed. Seran *et al.,* (2006) have reported that nearly 16 weeks is required to complete the callusing stages. Hence it is suggested to continue observations.

(a)



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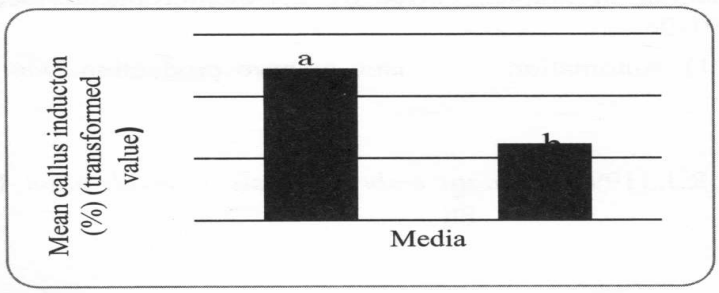

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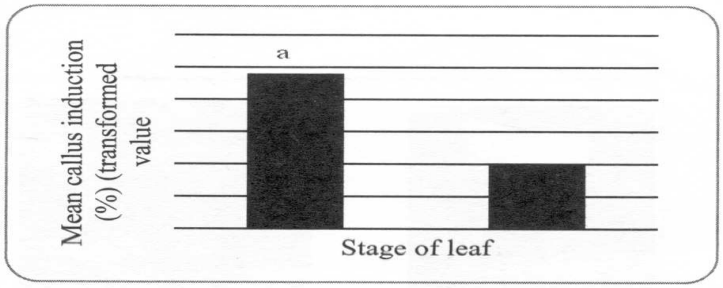

Figure 3: Mean callus induction among different (a) cultivar, (b) medium and (c) maturity stage

5 Conclusions and Recommendations

Somatic embryogenesis of tea varies among different cultivars, maturity of ex-plants and growth regulator composition of the medium. MS with 3 mg l^{-1} BAP and NAA 0.1 mg l^{-1} is the best medium for embryo induction of mature cotyledons in both cultivars. Among the two cultivars tested, TRI 2043 produced large number of embryos in the above medium. MS with 2 mg l^{-1} BAP and 3 mg l^{-1} NAA is suitable for callus induction of leaf explants which is the first step of somatic embryogenesis from leaves. Among the two tested cultivars, TRI 2024 responded well for callus induction in the above medium irrespective of maturity stage of leaves. When MS with 2 mg l^{-1} BAP and 3 mg l^{-1} NAA medium used for callus proliferation, TRI 2043 recorded higher proliferation than TRI 2024. However, the stage of sub culture of calli did not show any impact on callus proliferation. Study suggests testing more cultivars with an extended experimental period using, ex-plant with known maturity to produce consistent results and to optimize the protocol.

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