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Callus induction and organogenesis from leaf explants of Tectonagrandis

[1]M. LW. O. M. Karunaratne, [2]S. E. Peries and [2]W. C. P. Egodawatta*

[1]Department of Plant Sciences, Faculty of Agriculture, Rajarata University of Sri Lanka, Anuradhapura, Sri Lanka
[2]Department of Crop Science, Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka

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ABSTRACT

Organogenesis and embryogenesis of Tectonagrandis was attempted in this study with leaves collected from stumps and mature trees as explants. Tissue contamination and browning were the main problems in in vitro establishment of stumps. Leaf explants from stumps were sterilized using different concentrations of Clorox™, fungicide and adding alcohol. The sterilization based on dipping in 0.05% Bavistin™ as well as adding it in 0.05% concentration to culture medium was more efficient as it produced 98% of vital explants. Keep cultures in dark condition for 3days produced 96% browning free cultures. Different Cytokinins (6-benzyladenine and kinetin) and Auxines (NAA)at a range of various concentrations in Murashige and Skoog (MS) medium have been investigated for organogenesis. Medium supplemented with 0.5 mg/l NAA and 1.5 BAP mg/l produced compact and fibrous callus under light condition after two weeks. Callus initiation was not observed in MS medium supplemented with BAP, Kinetin and NAA. The explants which were obtained from L_2growth stage (second leaf from the tip) produced 96% of yellow colour globular shapes callus. Callus proliferated from 2, 4–D was introduced to no hormone medium, $GA_3$and liquid MS medium and observed different type of callus formation. This study provides callus induction protocol derived from T.grandis leaf explants as a basis for future studies.

Key words: Leaf explants, Organogenesis, *Tectonagrandis*

INTRODUCTION

*Tectonagrandis*L.f. belongs to the family Verbenaceae, which is an economically important, large deciduous, reaching over 30 m in height in favorable conditions. Teak enjoys a worldwide reputation as paragon among the most prized tropical timbers and is predominantly distributed in tropical and sub-tropical regions of Southeast Asia including Sri Lanka. Its extraordinarily wood working qualities and durability make it ideal for variety of purposes such as deckhouses, bridge building, rails, latches, weather doors, and other construction in contact with water such as docks, quays, piers and floodgates. Teak is a slow growing tree with low germination rate (usually less than 50%) that make difficult to propagate naturally and does not meet the demand of timber at present. To overcome this problem *in vitro* propagation has been successfully applied to teak, and became an alternative tool to overcome some problems occurring in sexual regeneration. Currently, mass propagation of selected teak clones is possible through *in vitro* multiple shoot production. Regenerative organs such as pre-existing shoots, meristem shoot-tips, nodal segments or seedling organs have been widely used as explants (Gupta *et al*., 1980; Mascarenhas*et al*., 1987; Apavatjrut*et a*l., 1988; Devi *et al*., 1994). Most of the protocols were developed to multiply shoots by means of shoot tip and axillary bud cultures. Common problems including exudation of phenolic substances into culture medium and riskof contaminations from field-sourced materials are often encountered. Also many papers has shown that the process of repeated cycles of sub-culturing increases the risk of in vitro shoots exhibiting vitrification phenomena, genetic abnormalities and reduced regeneration capacity.

The development of an in vitro regeneration procedure is required not only for the propagation of superior genotypes, but also for the regeneration of genetically improved plants. Adventitious shoot formation from callus tissues has been proposed for the regeneration of genetically engineered tissues of many species (Siemens and Schieder, 1996; Tawfik and Noga, 2001). Leaves are the best type of explants for due to its availability, easiness of extracting, low probability of contaminations and greater regeneration ability. Hence, this study organogenesis and embryogenesis was attempted using leaf explants of *Tectona grandis.* The objective of the study was to induce callus from leaf explant of *T. grandis*, then to proceed production of shoots and roots.

MATERIALS AND METHODS

The experiment was conducted at the tissue culture laboratory of Department of Crop Science, Faculty of Agriculture, and University of Peradeniya in year 2012. The leaves were collected from stump stocks maintained at a plant house.

Experiment 01: **Effect of sterilization method for Leaves collected from Stumps.**
The sever problem associated with cultures of *T. grandis* leaves were the higher rate of contaminations after introduction to a culture medium (Plate 2). Therefore, experiment 01 was carried out to study the effect of sterilization treatments on contamination for in vitro cultured leaf explants (Plate 1). NaOCl was the main disinfection material used in establishing aseptic culture conditions.NaOClwasin concentrations ranged from 5% to 10%. The incubation times of explants in sodium hypochloride showed differences due to its concentrations. There was also a need to use methyl 2-benzimidazolecarbamate fungicide asan extra disinfectant solutions to eradicate the fungal contaminants.

T1- Washing under running tap water [10 min] +5% Clorox containing 2 drops of Tween 20 [10 min] +5% Clorox + washed 3times using autoclaved water
T2- Washing under running tap water [10 min] +10% Clorox containing 2 drops of Tween 20 [10 min] +10% Clorox[10 Min.] + washed 3times using autoclaved water
T3 - Washing under running tap water [10 min] +10% Clorox containing 2 drops of Tween 20 [10 min] +70% ethanol [30sec.]
T4 - Washing under running tap water [10 min] +10% Clorox containing 2 drops of Tween 20 [10 min]+ 10% Clorox [10min] + washed 3times using autoclaved water + Dipped in 0.05% Fungicide
T5 - Washing under running tap water [10 min] +10% Clorox containing 2 drops of Tween 20 [10 min]+ 10% Clorox [10min] + washed 3times using autoclaved water + Dipped in 0.05% Fungicide + Cultured in 0.05% Fungicide added medium
T6 - Washing under running tap water [10 min] +5% Clorox containing 2 drops of Tween 20 [10 min] +5% Clorox + washed 3times using autoclaved water + Dipped in 0.05% Fungicide
T7 - Washing under running tap water [10 min] +5% Clorox containing 2 drops of Tween 20 [10 min]+ 5% Clorox [10min] + washed 3times using autoclaved water + Dipped in 0.05% Fungicide + Cultured in 0.05% Fungicide added medium

After introducing to the culture medium cultures were kept in dark condition for three days to minimize browning.

Experiment 02: **Effect of different combinations of BAP and NAA on regeneration of leaf culture.**
Sterilized leaves of experiment 01 were used as explants. Leaves (L_2) in a size of $1cm^2$ were cultured on MS medium supplemented with plant growth regulators. Explants were cultured in culture tubes, which contained 10ml of MS medium. Four hormone combinations of BAP and NAA were used (Table 01).

Table 01. Growth regulator combination used in organogenesis of leaf culture

TREATMENT	BAP (mg/l)	NAA(mg/l)
T_1	0	0
T_2	0.5	0.1
T_3	1	0.2
T_4	1.5	0.5

Each treatment was replicated thirty times. In each treatment, out of 30 replicates 15 were kept in dark and others were kept in light condition for 16 hrs. After formation of friable white callus and attained to a desirable size, callus was transferred to glass containers contained 20ml of MS medium. When the medium was reducing callus were sub cultured in same medium.

Experiment 03: **Effect of combination of BAP, Kinetin and NAA on regeneration of leaf cultures.**
Teak leaves immerged on stumps at L_2 growth stage were taken as explants. Explants were cultured in flat bottom glass tubes contained 10ml of MS medium consisting hormonal combination as given in Table 02.

Table 02. Growth regulator combination of BAP, Kinetin and NAA on regeneration of leaf cultures

TREATMENT	BAP (mg/l)	KINETIN (mg/l)
T_1	0	0
T_2	1	1
T_3	2	2
T_4	4	4

Leaves of a size of $1cm^2$ (*i.e.*leaf pieces were taken around the mid rib and the rest around main veins) were cultured in each test tube. Four levels of BAP and Kinetin were used, while keeping NAA (0.01mg/l)at a constant. Each treatment was replicated twenty times. For each treatment, out of 20 replicates, 10 were kept in dark and rest were kept in light for 16hrs.

Experiment 04: **Embryogenesis of leaves**

MS media was prepared using 0.5, 1.0, 1.5and 2.0mg/l 2-4D. L_2stage of *T.grandis* leaves were used as explants. Leaves in a size of $1cm^2$ were cultured on 10ml of medium each culture vessels. Each treatment replicated thirty times. For each treatment, out of 30 replicates, 15 were kept in dark andrest were kept in light for 16 hrs. After the emergence of callus cultures were transferred to hormone free 20 ml MS medium in glass vessels.

After a period of two months, callus were transferred to cultures including MS medium containing 0.5mg/l GA_3(*n=25*), MS liquid medium (Hormone free)(*n=25*),Hormone free MS(*n=25*). Callus were sub-cultured immediately after observing any signs of browning.

Experiment 05: **Effect of different stages of leaves on regeneration**

Four different stages of leaves were used to conduct this experiment. These stages were; Bud -L_0, 1^{st} leaf - L_1, 2^{nd} leaf - L_2, 3^{rd} leaf - L_3, and4^{th}leaf - L_4(Plate 3).Four different growth stages (L_1, L_2, L_3, L_4) of leaves were used to investigate the best growth stage for callus induction. After sterilization procedure, leaves in a size of $1cm^2$ were cultured on 10ml of medium contained MS medium supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA. Each treatment was replicated 25 times.

RESULTS AND DISCUSSION

Tissue culture has facilitated large-scale plant multiplication in woody plants aided by somatic embryogenesis and organogenesis or micropropagation (Jain *et al*, 1995, 1999, 2000; Ahuja, 1992). In this study embryogenesis and organogenesis was attempted using leaves as explants. Callus production was severely restricted to immature leaves closer to terminal bud. Those leaves were light green in colour, flexible and easily breakable and also smooth in texture.

Selection of explants is a fundamental step in clonal propagation of any plant, particularly for woody perennial species that displaysdistinctive stages of juvenility and maturity. The type and age of the explant are always important for the success in callus induction. In *T. grandis*, for callus induction and successful growth of callus, leaf explant should be taken from leaves closer to the shoot apex, which are immature and has 100% meristematic action. The severe difficulty of using vegetative propagules in recalcitrant woody plants are associated with phase change and maturation of meristems (Libby,1974). Since maturation is believed to occur in meristems (Schaffalitsky de Muckadell, 1959) clonal propagules from meristems of mature plants are physiologically older than those from juvenile plants and often displays such undesired characteristics as plagiotropic growth, reduce growth rate,*etc* (Bonga, 1980). Therefore, before taking the explant from the *T. grandis* trees, rejuvenation should be done in order to inducejuvenile meristems to a point, where it becomes capable of embryogenesis and organogenesis.

High rate of fungal contaminations was observed in initial cultures, although specimens were collected from a planthouse. Fungal contaminants proliferated particularly most of the proximal ends the leaf .The contamination was suspected to be mostly endogenous in origin, since the contaminant was not directly in contact with media. In *T. grandis* fungal contamination appeared to be dominant. With the use of anti-microbial for pretreatments of explants or including in culture medium, certain reduction in number of contaminated explants was observed. Surface sterilization procedure followed in disinfecting leaf explants were very effective as it resulted were very limited contaminations(Figure 01). Highest number of contamination free cultures were observe in T_5 - Washing under running tap water [10 min] +5% Clorox containing 2 drops of Tween 20 [10 min] +5% Clorox + Dipped in 0.05% Fungicide + Cultured in 0.05% Fungicide added medium and quantitatively it was 96%. However, the survival percentage was only 50% (Figure 01).

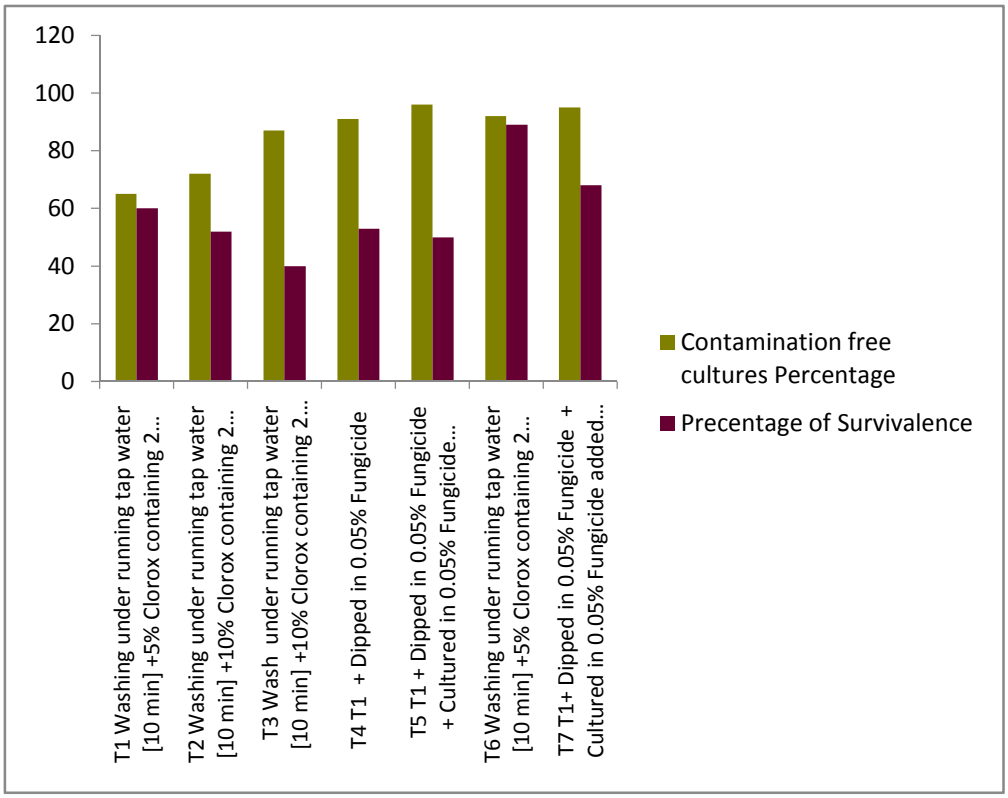

Figure 1. Effect of different sterilization method for leaf explants

Most effective treatment was T_6-Washing under running tap water [10 min] +5% Clorox containing 2 drops of Tween 20 [10 min] +5% Clorox + washed 3times using autoclaved water + Dipped in 0.05% Fungicide, which produced 95% contamination free cultures and resulted 89% survival cultures (Figure 1 and Plate 4). In tree tissue culture, long time exposure to surface sterilization of tissues was the reason of ex-plant browning and subsequent ex-plant death (Plate 5). This was overcame by keeping all cultures in dark for 3days. This was usually due to phenolic compounds produced in tissues damaged by surface sterilization and during dissection process. Nonetheless, after establishment browning was observed in very few cultures (2-3 max).

Plant growth regulators showed a significant impact on percentage of explants forming callus on callus formation media ($p<0.01$). Formation of soft, white and non-embryogenic callus was observed initially on explants in MS medium supplemented with BAP and NAA. After sterilization and 4-5 days from inoculation, cultured leaf pieces became slightly expanded and glossy. This type of callus mostly occurred at the margins of explants (Plate 6).Further, this callus continued to occur randomly at various stages during in vitro regeneration.

The highest percentages of callus formation (93%) were obtained from explants cultured on MS medium containing 1.0 mg/lBAP and 0.2 mg/ l NAA. For *in vitro* cultured leaf explants, medium with 0.5 mg/ l BAP and 0.2mg/l NAA was found to be quite favorable for induction of callus and callus induction was 75% (Figure 2). Interesting and unique aspect was the formation of yellow, globular, compact callus which initiated when the explants were sub-cultured onto hormone free MS medium (Plate 7). After one week of inoculation, cultured explants pieces were enlarged and swelled, however, they remained in green colour. Then the explants were gradually covered by thin layer of white compact callus starting from wounded edge. White colour thin and hard callus layers was observed in cultures at the end of second week after inoculation. Even though,no shoots were produced, callus initiation and growth of callus was fast. Lakshmi Sita and Chatopadhyay (1980) have reported the development of embryo like structures of *T. grandis*. Mascarenhas*et al*. (1993) have obtained globular callus from different explants (apical meristem, *in vitro* developed leaves, stems, shoots tips and axillary buds). This callus turned green when exposed to light.

A cell suspension was initiated by placing friable callus (3mg/100ml) into liquid culture medium and allowed to shake at 100 rpm. Friable callus was produced after four weeks, due to high friability of *T. grandis* callus. These calluses were chopped in laminar flow and placed in the liquid medium. After placing it into liquid culture medium, it also gradually turned into brown colour. Therefore, frequent sub culturing was done by adding ascorbic acid (100mg/l) to the liquid medium to prevent browning. Browning was prevented by removing of phenolic compound

secreted into the medium and which also enhanced the survival rate. If these brown exudates were remained in the medium for sometimes, it would have promoted dying of cells by interfering with the metabolic activities of cells.
It was necessary to subculture frequently in order to remove the toxic phenolic substances secreted to the medium. After four weeks from explant inoculation, first subculture was carried out, cultures in 2,4-D medium were sub cultured in hormone free medium both liquid and solid medium, 0.5 mg/l GA_3. At that time growth of callus changed to nodular like structures. Callus derived from hormone free MS medium were pale yellow in colour and continued to proliferate a large callus mass. Establishing medium that wassupplemented with 2.0mg/l 2,4- D showed highest number of callus induction in both light and dark explants after 3 weeks, which was 93.3% for both conditions. Meantime, 0.5mg/l 2,4-D (T1) and 1.0 mg/l 2,4-D (T2) showed the lowest callus induction percentage (Figure 3). End of 5th week,two different callus types was visible,fibrous callus which was white in colour powdery nature and hard white colour callus(Plate 8- A and B). All most all explants cultured on 2,4- D (2 mg/l) medium gave rise to friable callus. A single auxins such as NAA, IAA, IBA, 2, 4- D and picloram were also able to induce somatic embryogenesis of *Myrtuscommunis*(Canhoto*et al*., 1999) and*Sesamumindicum* (Mary and Jayabalan, 1997).

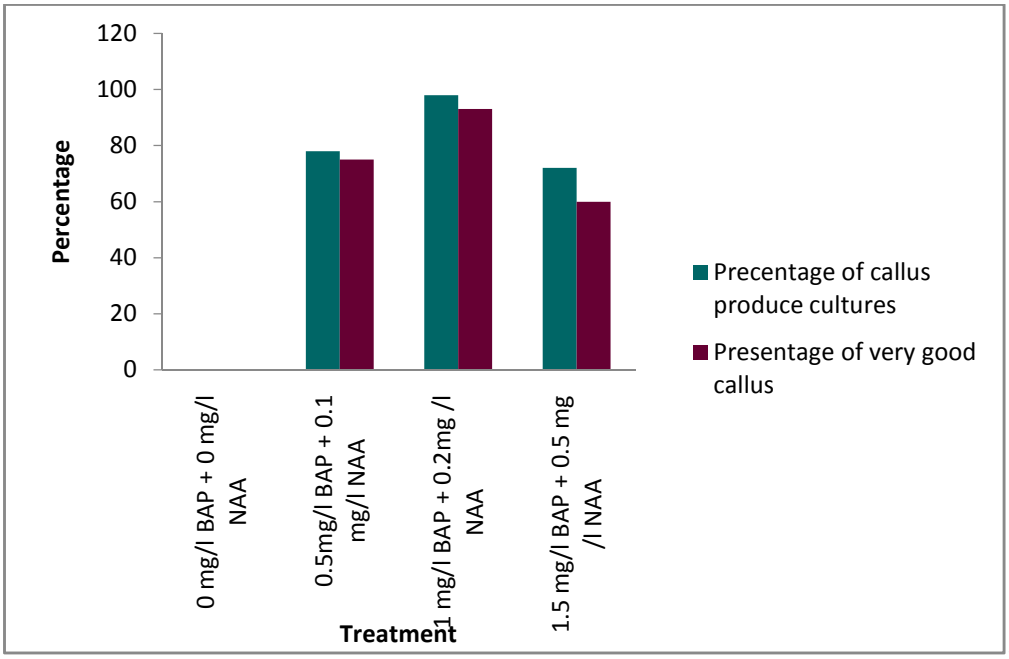

Figure 2. Effects of combinations of BAP and NAA on node establishment

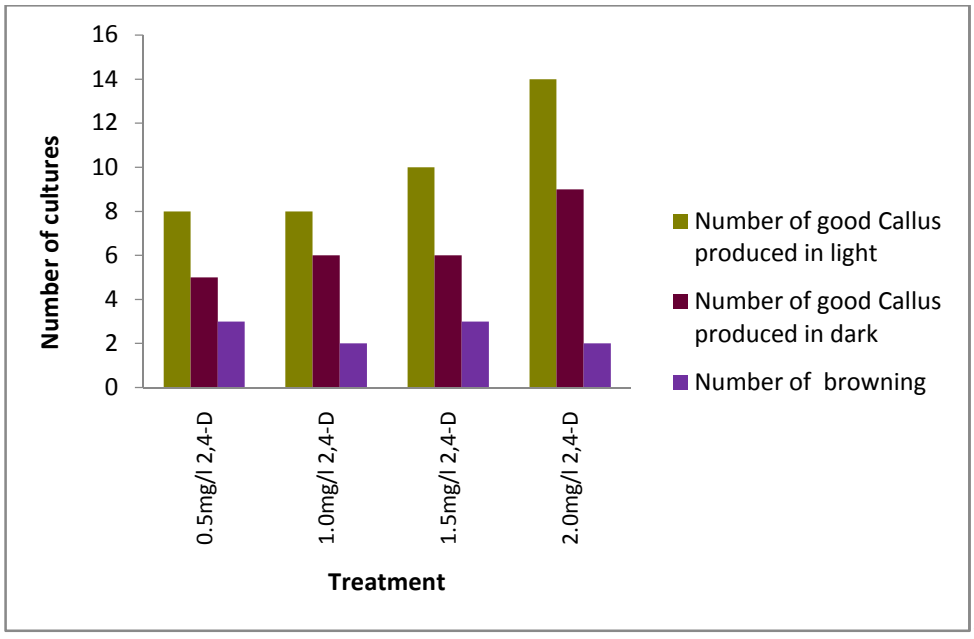

Figure 4. Effect of 2,4-d for *teactona grandis* callus induction

Cultures that were kept in the dark, induction of callus was very poor and mass was smallespecially in medium contained 2,4-D (2 mg/l) After 7th week, no changes occurred in friable callus, however mass was increasing rapidly. These cultures were transferred to two different new media containing 1 mg/l GA_3, 5 mg/l ABA (Absisic acid). Hormones such asGibberellic acid (GA) and Abscisic acid (ABA) are also known to affect growth of the plant and to interact with each other (Bewley& Fountain, 1972). In some studies, BAP and GA_3 were able to regenerate somatic embryos directly. SupplementingBAP and GA_3 individually or in combination into the medium for somatic embryo germinationof *Centellaasiatica*(Paramageethame*t al*., 2004), *Catharanthusroseus* (Junaid*et al*., 2006) and *Proteacynaroides* (Wu *et al.,* 2007) were successful examples. Supplementing GA_3 2 weeks after culturing, loosely arranged callus became light brown and compacted and the shape was changed to round (equal to a mustard seed)(Plate9).In these cultures, rate of callus mass increase was low compared to no hormone medium.

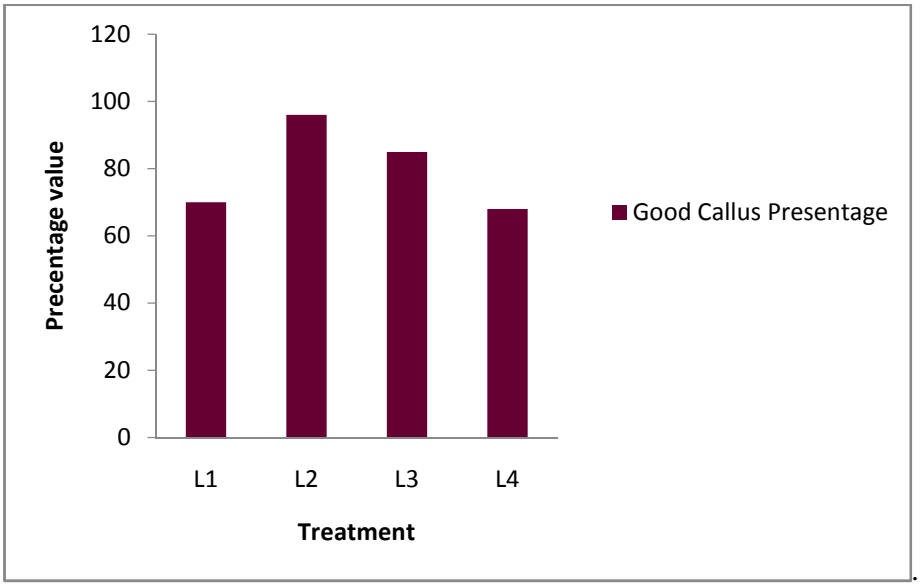

Figure 3. Effect of the best growth stage for *teactona grandis* callus induction

After introducing to ABA containing medium callus changed its' shape and colour with the time to globular and to dark brown (Plate 10). End of the 2nd week, browning was observed in most of cultures and most of them were prone to death. Sub-culturing was carried out with one week intervals,however, it was not effective in controlling browning. This may due to inability to maintain calli mass in white colour at initial stage, although callus were sub-cultured into a new medium. Both GA_3 and ABA containing media, callus mass became green and harder and this was observed after transferring to a new medium. Even though, calli became dark brown in colour after 4 weeks of time, calli again transferred to hormone free medium.One week after transferring, sub-cultured calli produced new callus mass on top of the former callus.

PLATE 1 Leaf ex-Plants

PLATE 2. Contaminated leaf ex-plants

PLATE 3 Different growth stages of Leaves

PLATE 4. Contaminated free culture

PLATE 5 Browning and death of ex-plant

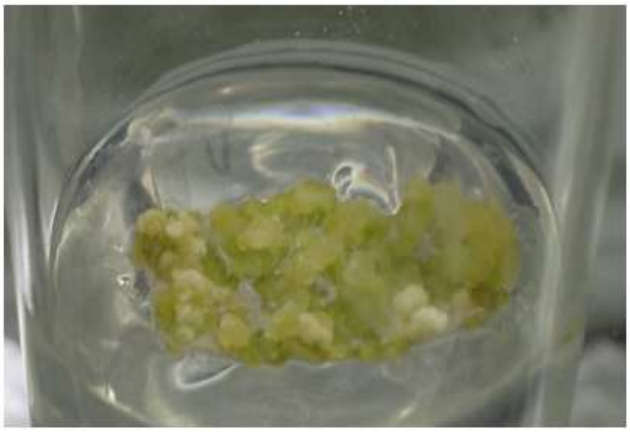

PLATE 6. Non embryonic callus formation

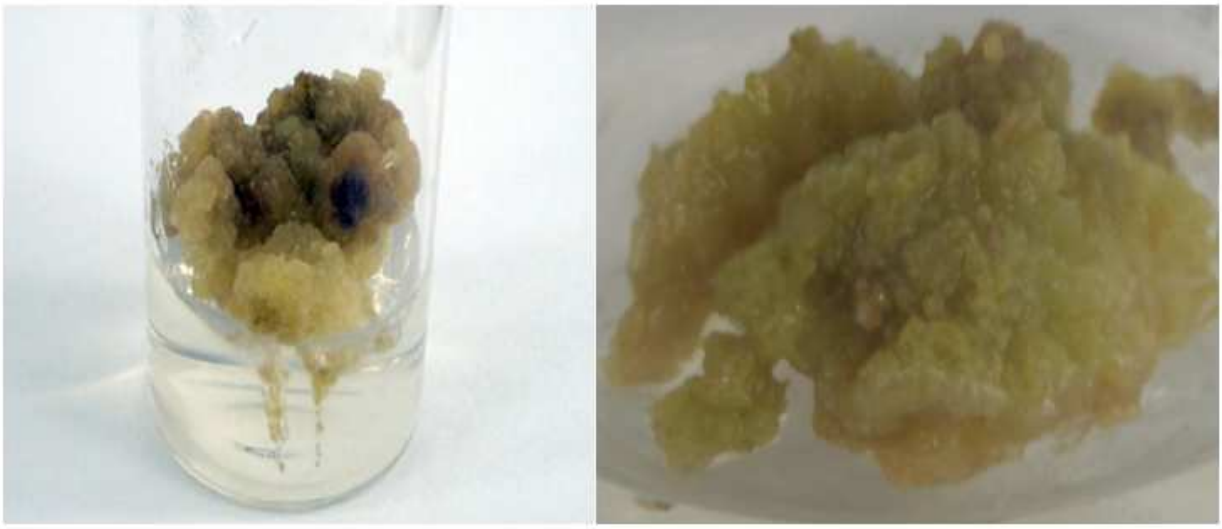

PLATE 7 Callus sub cultured in hormone free culture

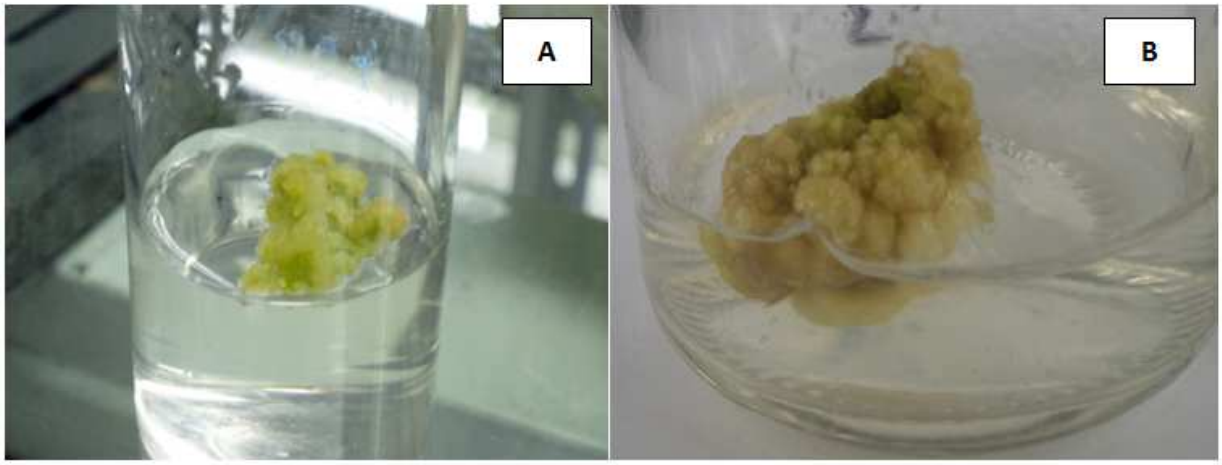

PLATE 8 Two different callus produced in 2,4-D medium

PLATE 9 Callus generated from GA3 ABA medium

PLATE 10. Callus generated from GA3 medium

Development of callus was not observed in any of explants, which were established in BAP, Kinetin and NAA. However, in treatment T_3 explants remained green in colourfor 3 month of period without any change.High frequency of plant regeneration from leaf explants depends on its' growth stage. This was tested using with four different growth stages of the leaf explants. The best leaf was the second leaf from the tip. It produced 95% of good callus, where leaf 3 from the tip produced 85% of good callus (Figure 3).

CONCLUSION

Only few studies have been recorded about callus induction of *Tectonagrandis*from leafexplants and in Sri Lankathis study was the first attempt recorded organogenesis using leaf explants. A simple and efficient protocol for callus induction and plant regeneration from *invitro* cultured leaves. For future studies, the ideal explant is second leaf from the tip of rejuvenated plantlets, and these studies needed to be concentrate on organogenesis and embryogenesis in optimized conditions. Thus, this study will provide a platform for organogenesis of *Tectonagrandis* for commercial production.

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