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Somatic embryogenesis induction of litchi (*Litchi chinensis* Sonn.) from leaves of mature trees

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Abstract

Induction and development somatic embryos of litchi (*Litchi chinensis* Sonn.) from leaflet explants obtained from mature litchi trees were investigated. Somatic embryogenesis was significantly influenced by the cultivar, plant growth regulators, position of leaflet and orientation of leaf explants. Adaxial-face up orientation of leaf explants significantly enhanced embryogenesis in comparison with adaxial-face down orientation. Maximum number of somatic embryos were obtained using median position from basal leaflet with adaxial-face up orientation cultured on Murashige and Skoog (MS) medium supplemented with 1.0 mg/l 2, 4 dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l 6-benzylaminopurine (BA) (37.67). Two types of salts media (MS and Gamborg's B5) as well as different sucrose concentrations were examined for development and maturation of somatic embryos. MS salts medium with 45g/l sucrose was effective on size of mature somatic embryos (8.00 > 0.5 cm SE and 6.33 < 0.5 cm SE) compared with B5 salts medium (4.33 > 0.5 cm SE and 4.66 < 0.5 cm SE) on the same concentration of sucrose. Development of somatic embryos occurred on the media without growth regulators, heart and torpedo stage somatic embryos were obtained. Somatic embryos enlarged more and turned to milky opaque in color and finally reached to mature milky cotyledonary stage. Scanning electron microscopy revealed different morphological development of somatic embryos. In addition, Anatomical observation showed differences in the embryogenic and non-embryogenic cells. Furthermore, anatomical observation for development stages of somatic embryos.

Keywords: Explant position, explant orientation, *Litchi chinensis*, leaves, somatic embryogenesis, 2,4-D.

INTRODUCTION

Litchi (*Litchi chinensis* Sonn.) is an evergreen tree, originated in China and now widely spread in the tropical and subtropical regions of the world [1]. The litchi belongs to the family Sapindaceae which includes the other important tropical and subtropical fruit trees species such as longan (*Dimocarpus longan* Lour.) and rambuttan (*Nephelium lappaceum* L.) [2]. Litchi fruit has strong antioxidant, anti-cancerous, antidiabetic, antimicrobial, anti-inflammatory activities along with hepatic protection properties [3]. Litchi fruit has deliciously flavored, sweet, juicy and pearly white flesh which is a rich source of vitamin C [4].

Litchi chinensis is commercially grown in some countries with tremendous export potential and it plays an important role in their economy [5]. In Egypt, the exportation of Litchi, Persimmon, Annona and Passion fruit valued by 672 tons [6].

The high demand and the high prices paid for litchi has created new interest in the growing of this fruit with an increase in the demand for propagation materials [4]. Litchi can be propagated by seeds. Major problems of litchi seed their short life span and rapid loss of viability under desiccation. Seeds may keep well within fruit up to a month but it starts losing viability even within a day after separation from the fruit, also the seedling obtained from seeds are not advisable because seedling are genetically diverse and most of the trees bear fruit after 5-12 years coupled with irregular bearing and poor fruit quality [7-8] furthermore, vegetative propagation methods are slow and inefficient [9].

Litchi is difficult to propagate using *in vitro* techniques and it is lagged behind due to its recalcitrant nature [9]. Durzan [10] showed that problems in micropropagation of litchi is the polyphenols into the medium by the tissues, this polyphenol produce substances that kill the tissues. Another problem is increasing contamination which is difficult to remove prior to culture. Both of these problems are considerably encountered with hardwood species and the usual process to develop the experimental culture techniques

by using youngster tissues from seeds germinated *in vitro*. This method gives very clean material and juvenile tissues which commonly respond well when culture. With litchi, so far each of experiments using new tissues has not been successful in micropropagation [11].

Litchi is closely related to longan (*Dimocarpus longan* Lour) [12]. Longan organogenesis and shoot proliferation are still poor and this technique cannot be used for mass propagation. Culture of explants that excised from mature trees via organogenesis is very difficult. Usually, adventitious buds could be induced but they die in the subsequent of subculture. Sometimes, adventitious buds proliferated but rooting is still difficult [13]. Somatic embryogenesis resolved this problem by production bipolar embryos with root and shoot [14].

Somatic embryogenesis of litchi using leaves was reported by Puchooa [15], Raharjo and Litz [16, 17] and Ma *et al*. [18]. Several attempted to induce somatic embryogenesis from anther of litchi such as Yu and Chen [19], Deng [20], Xie *et al*. [21], Wang *et al*. [22] and Guo *et al.* [23] and from zygotic embryos of litchi such as Yu and Chen [19], Yu *et al.* [24], Das and Rahman [25] and Das *et al*. [26].

In Egypt, to date there have been no reports on somatic embryogenesis induction of *Litchi chinensis* from leaves of mature trees as a resource of litchi biotechnology. Raharjo and Litz [17] reported that somatic embryogenesis from litchi leaves of mature trees is critical for genetic transformation and mutagenesis to improve elite cultivars for future efforts to transform litchi cultivars with genes that mediate certain horticultural traits. Here, this study was a trial to find the factors affecting the induction of somatic embryogenesis from leaves of mature litchi trees. Moreover, determining the optimal developmental stage and discovering methods for production the high frequency of somatic embryos that could be used in future to improve *Litchi chinensis* cultivars through biotechnology.

MATERIALS AND METHODS

This study was carried out during 2015 to 2017 in Somatic Embryogenesis and Haploid Plants Laboratory, Plant Biotechnology Department, Genetic Engineering and Biotechnology Institute (GEBRI), Sadat City University, Egypt.

Explant preparation.

Leaves of mature litchi trees (about 12-year-old) Brewster and Mauritius cultivars were collected from Nemous farm located in Al-Katta Desert, Giza governorate, Egypt during May (2015 -2017). Leaflets separated from compound leaves and washed in running tap water. In Lab, leaflets carried out inside a laminar airflow cabinet. Leaflets disinfected with 30% commercial Clorox (5% sodium hypochlorite) for 15 min along with 2-3drops of Tween 20. Finally, leaflets rinsed three times with sterile distilled water. Leaflets cut to (Apical-Median-Basal) positions with midrib and these parts used as explants in the experiments.

Experiment (1): Effect of 2,4-D concentrations with cultivars (Brewster and Mauritius) on induction of somatic embryogenesis.

This experiment was established to study the response of tested genotypes under different concentrations of 2,4-D for somatic embryogenesis induction. Explants of Brewster and Mauritius cultivars were used. Ten replicates were performed for each treatment and represented by two explants in each replicate (jar as a replicate). Explants were placed on Murashige and Skoog [27] (MS) Medium containing various concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) at (0.0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) plus 30 g/l sucrose, 200 mg/l L-glutamine, 500 mg/l Casein hydrolisate, 1.0 g/l active charcoal (AC), 100 mg/l of ascorbic acid, 150 mg/l citric acid and 3.0 g/l gelrite .The medium pH was adjusted to 5.7-5.8 and autoclaved at 121°C and 1.2 kg/cm^2 air pressure for 20 min. Then, it was incubated at 25 ± 2°C under total darkness condition. The explants were sub-cultured monthly. Percent of explants that formed somatic embryos and number of somatic embryos per explants were calculated after 90 days.

Experiment (2): Effect of 2,4-D and BA concentrations with cultivars (Brewster and Mauritius) on induction of somatic embryogenesis.

Explants of two cultivars Brewster and Mauritius were used and cultured on MS medium supplemented with (0.0, 1.0, 2.0 and 3.0 mg/l) 2,4-D in combination with (0.0, 0.5, 1.0 and 1.5 mg/l) BA plus 30 g/l sucrose, 200 mg/l L-glutamine, 500 mg/l Casein hydrolisate, 1.0 g/l AC, 150 mg/l citric acid, 100 mg/l of ascorbic acid and 3.0 g/l gelrite. Incubation was done at 25 ± 2°C under total darkness condition. This experiment was designed for somatic embryogenesis induction using 2,4-D combined with cytokinin (BA). Percent of explants that formed somatic embryos and number of somatic embryos per explants were calculated after 90 days.

Experiment (3): Effect of leaflet position, explant position and orientation on somatic embryogenesis induction.

Leaves of Brewster litchi cultivar are compound with six alternate leaflets. This experiment aimed to detect the best leaflet position of leaf and best explant position from leaflets in somatic embryogenesis induction. Preparation of explants as were described above.

A- Leaflet position and explant position.

For test leaflet position and explant position of leaflets. Leaflets from Brewster litchi cultivar were prepared to (Apical, Median, Basal) positions and used as explants. Explants cultured on MS medium supplemented with (1.0 mg/l 2,4-D + 0.5 mg/l BA) plus 30 g/l sucrose, 200 mg/l L-glutamine, 500 mg/l Casein hydrolisate, 1.0 g/l AC, 150 mg/l citric acid, 100 mg/l of ascorbic acid and 3.0g/l gelrite. Incubation was performed at 25 ± 2°C under total darkness condition. Number of somatic embryos per explant was counted after 90 days.

B- ***Explant orientation.***

Median explants from basal leaflets of Brewster litchi cultivar were tested with adaxial side up or down orientation. Incubation conditions as described in last experiment. Number of somatic embryos per explant orientation was counted after 90 days.

Experiment (4): Effect of salts media and sucrose concentrations on the development and maturation of somatic embryos (SE).

Somatic embryos of Brewster litchi cultivar were cultured on two types of salt media (MS or Gamborg's B5) **(**Gamborg *et al*. [28]) free of growth regulators and supplemented with different sucrose concentrations (30, 45 and 60g/l) plus 200 mg/l L-glutamine, 500 mg/l Casein hydrolisate, 1.0 g/l AC and 3.0g/l gelrite to study the effect of two types of salts media with sucrose concentrations on the development and maturation of somatic embryos. Sizes of mature opaque milky cotyledonary somatic embryos (> 0.5 cm SE, < 0.5 cm SE) per explants were counted after 8 weeks from culture.

Scanning Electron Microscopy (SEM).

Embryogenic callus and somatic embryos were fixed in sodium phosphate buffer (pH 7.4, 0.2 M) and 2.5 % (v/v) glutaraldehyde and incubated at 25 °C for 4 h. The samples were rinsed with sodium phosphate buffer and dehydrated in acetone gradient series for 15 min and dried for 72h at 40 °C. After drying, samples were coated with gold-palladium alloy. Tissues were examined under a scanning electron microscopy (Carl Zeiss scanning microscopy).

Histological observation.

For histological studies, somatic embryos fixed in formalin: ethanol: acetic acid (1:16:1). Following dehydration in an ethanol /xylene series the material was infiltrated and embedded in wax. Sections were cut by using a rotary microtome at 20µM thickness, dried onto slides using xylene and rehydrated in a descending ethanol series. Finally, the sections were stained with safranin / light green. The sections were prepared by the method suggested by Sass [29] (Nassar and Sahhar [30]). All

photographs were taken by Nikon Camera of the Carl Zeiss Jena microscope.

Statistical analysis.

All experiments were carried in completely randomized design (CRD) with ten replications (Ten replicates were performed for each treatment and represented by two explants in each replicate (jar as a replicate). The data were analyzed by one- or two-ways analysis of variance and the mean values were compared using the Fisher's least significant difference test (LSD test) at 5% according to the method described by Steel and Torrie [31].

RESULTS AND DISCUSSION

Experiment (1): Effect of 2,4-D concentrations with cultivars (Brewster and Mauritius) on induction of somatic embryogenesis.

Table (1) showed the main effect of 2,4-D concentrations with two cultivars (Brewster and Mauritius) on the number of somatic embryos/explants. The results showed that, no somatic embryos were obtained in the absence of growth regulators. The highest mean number of somatic embryos (2.33) was recorded at 2.0 mg/l 2,4-D followed by (1.33, 1.15 and 1.00) at 1.0, 0.5 and 3.0 mg/l 2,4-D respectively, without significant differences, while the lowest mean number was recorded (0.83) at 4.0 mg/l 2,4-D.

In our initial experiment, brown compact callus formed on the explants of two cultivars (Brewster and Mauritius) after 4 weeks from culture of leaflet explants on the medium containing various concentrations of 2,4-D. Somatic embryos were obtained on callus of Brewster cultivar only after 90 days from culture. Somatic embryos developed and become white in color when cultured on medium free of growth regulator (Fig.1). Similar observation by puchooa [15] on the same plant who described that, somatic embryogenesis of litchi leaves obtained on medium supplemented with 2,4-D (1.5 mg/l) and the development occurred by culture on medium devoid of 2,4-D. The highest explants induction of somatic embryogenesis overall embryogenesis rate was afforded by medium with 2.0 mg/l 2,4-D (50%) or with 1.0 mg/l or with 3.0 mg/l 2,4-D (40%). The lowest explants induction of somatic embryogenesis rate was without 2,4-D (0%) as shown in Fig (2).

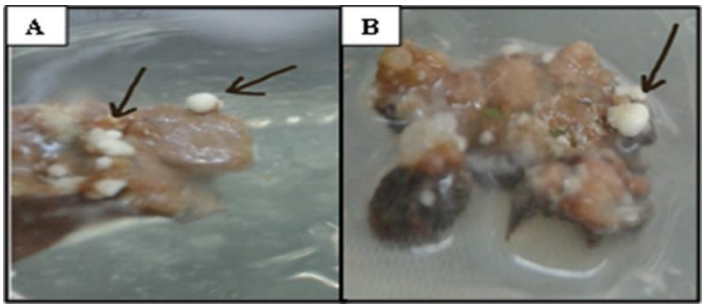

Fig (1): Indirect somatic embryogenesis formation from leaflet explant of Brewster cultivar after 90 days from culturing on MS medium supplemented with 2,4-D. (A): Brown embryogenic callus with increased rate of somatic embryos formation produced by (2.0 mg/l) 2,4-D. (B): Brown embryogenic callus with lower rate of somatic embryos formation produced by increasing of 2,4-D about (2.0 mg/l).

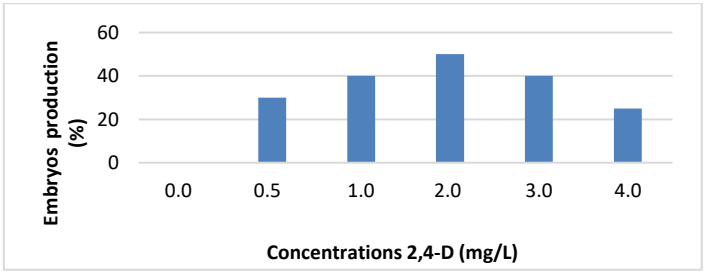

Fig (2): The influence of 2,4-D concentrations on *Litchi chinensis* cv. Brewster somatic embryos production rate.

As for the interaction (2,4-D concentrations and cultivars), a higher mean number of somatic embryos /explants was recorded (4.66) on Brewster cultivar with 2.0 mg/l 2,4-D followed by 2.66, 2.33 and 2.00 without significant differences with 1.0, 0.5 and 3.0 mg/l 2,4-D respectively. The lower mean number of somatic embryos /explants was recorded (1.66) with Brewster cultivar at 4.0 mg/l 2,4-D. Mauritius cultivar failed to produce somatic embryos with all concentrations of 2,4-D (Table1), this result is in agreement with those of Raharjo and Litz [16,17] they reported that the effect of litchi cultivar on induction of somatic embryogenesis was influential factor. Therefore, somatic embryogenesis from litchi leaflets occurred on Brewster cultivar but the Mauritius cultivar failed to induce somatic embryos. Somatic embryogenesis is generally believed to be triggered by an auxin. 2,4-D has been widely used to be effective for somatic embryogenesis for many plants [32]. The effect of 2,4-D and genotypes has been discussed by Fehér [33].

Data in Table (1) showed that the mean number of somatic embryos was increased (4.66) with increasing the concentration of 2,4-D up to 2.0 mg/l then, the mean number of produced embryos decreased (2.00 and 1.66) as the concentration of 2,4-D increased to 3.0 and 4.0 mg/l 2,4-D respectively, similar result was obtained by Aboshama [14] on *Cassia nodosa* somatic embryogenesis using 2,4-D.

Table (1): Effect of 2,4-D concentrations with genotypes on somatic embryogenesis induction.

Concentrations 2,4-D (mg/l)	Mean number of somatic embryos per explant		Mean (B)
	Brewster	Mauritius	
0.0	0.00	0.00	0.00
0.5	2.33	0.00	1.15
1.0	2.66	0.00	1.33
2.0	4.66	0.00	2.33
3.0	2.00	0.00	1.00
4.0	1.66	0.00	0.83
Mean (A)	2.22	0.00	
LSD at 5% A	0.30		
B	0.52		
AxB	0.74		

Experiment (2): Effect of 2,4-D and BA concentrations with cultivars (Brewster and Mauritius) on induction of somatic embryogenesis.

In present experiment 2,4-D combined with cytokinin (BA) were used for induction of somatic embryogenesis. No somatic embryos were obtained in absence of growth regulators (Table 2).

Concentrations of 2,4-D and BA revealed that, the highest mean number of somatic embryos was recorded (3.34) at 1.0 mg/l 2,4-D combined with 0.5 mg/l BA followed by 2.33, 2.01 and 1.17 with significant differences, while the lowest mean number was recorded (1.00) at 2.0 mg/l 2,4-D combined with 1.5 mg/l BA (Table 2).

Responding of the two cultivars for induction of somatic embryogenesis showed that the highest mean number of somatic embryos was scored with Brewster cultivar (1.85) while no response of somatic embryogenesis was induced with Mauritius cultivar (Table 2).

Interaction between 2,4-D combined with BA and the cultivars revealed that, higher mean number of somatic embryos /explant was recorded in Brewster cultivar (6.68) using 1.0 mg/l 2,4-D combined with 0.5 mg/l BA followed by 4.66, 4.02 and 2.34 with significant differences, while the lowest mean number of somatic embryos /explant was recorded with Brewster cultivar (2.00) at 2.0 mg/l 2,4-D combined with 1.0 or 1.5 mg/l BA. Using 2 mg/l 2,4-D alone resulted in lower somatic embryos/explant (4.33) compared to 1.0 mg/l 2,4-D with 0.5 mg/l BA which gave (6.68). Using BA in culture medium increases cell division and differentiation [32]. Similar results were obtained in the Sapindaceae family by Kim *et al*. [34] they concluded that using 2,4-D combined with BA was better than using 2,4-D alone for induction of somatic embryogenesis from leaves of soapberry (*Sapindus mukorossi* Gaertn). Our results revealed that the highest percentage of somatic embryogenesis/ explants was induced by

Table (2): Effect of 2,4-D and BA concentrations with genotypes on number of somatic embryos/explants.

Concentrations (mg/l)		Mean number of somatic embryos/explants		Mean (B)
2,4- D	BA	Brewster	Mauritius	
0.0	0.0	0.00	0.00	0.00
	0.5	0.00	0.00	0.00
	1.0	0.00	0.00	0.00
	1.5	0.00	0.00	0.00
1.0	0.0	2.33	0.00	1.16
	0.5	6.68	0.00	3.34
	1.0	4.66	0.00	2.33
	1.5	4.02	0.00	2.01
2.0	0.0	4.33	0.00	2.16
	0.5	2.34	0.00	1.17
	1.0	2.00	0.00	1.00
	1.5	2.00	0.00	1.00
3.0	0.0	1.33	0.00	0.66
	0.5	0.00	0.00	0.00
	1.0	0.00	0.00	0.00
	1.5	0.00	0.00	0.00
Mean (A)		1.85	0.00	
LSD at 5%	A	0.47		
	B	1.33		
	AxB	1.88		

medium with 1.0 mg/l 2,4-D combined with 0.5 mg/l BA (55%) or combined with 1mg/l BA (50%). On the other hand, somatic embryogenesis/ explants were induced neither by medium containing 3 mg/l 2,4-D combined with all BA concentrations nor with control (Fig 3).

The results in Table (2) showed that the Mauritius cultivar failed to produce somatic embryos in all concentrations of growth regulators. This result is in agreement with those reported by Raharjo and Litz [17] they reported 2,4-D with cytokinin (BA) were effective for induction of somatic embryogenesis from litchi leaflets of Brewster cultivar however the Mauritius cultivar failed to induce somatic embryos. This variation in the embryogenic response may be due to variations in levels of endogenous growth regulators, quantitative and qualitative genetic differences or epigenetic differences including chromatin condensation [35].

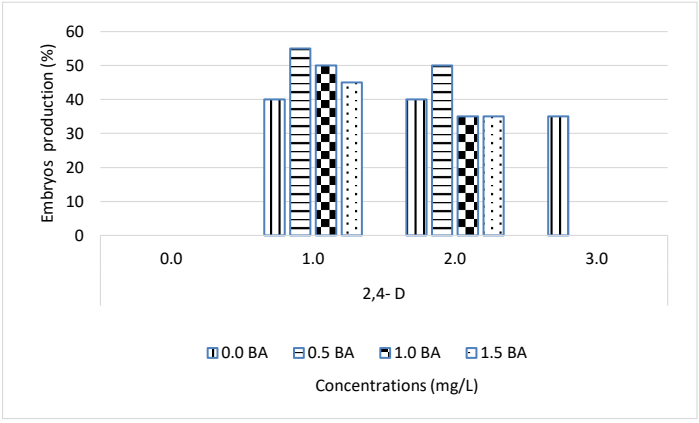

Fig (3): Effect of 2,4-D in combination with BA concentrations on *Litchi chinensis* cv. Brewster somatic embryos production rate

This result indicated that increasing the concentrations of 2,4-D with BA up to 2.0 mg/l 2,4-D plus 1.5 mg/l BA decreased the mean number of somatic embryos to 2.00. No somatic embryos noticed with litchi leaves at 3.0 mg/l 2,4-D in combination with all BA concentrations (0.5,1.0,1.5) (Table 2). This result disagrees with the report presented by Devaraju and Reddy [36] they cultured leaf explants of *Sapindus emarginatus* on various concentrations of 2, 4-D (0.5-1.0 mg/l) combined with BA (0.5-3.0 mg/l) for induction somatic embryogenesis and found that the somatic embryos obtained in all possible combinations.

Furthermore, no somatic embryos were obtained from leaves of litchi using BA alone (Table 2), this result disagree with the report presented by Singh *et al*. [37] they reported that MS medium supplemented with BA alone was able for induction of somatic embryogenesis from leaves of *Sapindus mukorossi* a member of the same family, they referred the reason may be due to presence of high level of endogenous auxin.

Experiment (3): Effect of leaflet position, explant position and orientation on somatic embryogenesis induction.

A- Leaflet position and explant position.

The obtained results from this experiment revealed that significant differences in somatic embryos yield were observed in leaflet position and explant position taken from leaf of Brewster cultivar.

The highest mean number of somatic embryos per explant position was recorded (10.22) at median position followed by apical position and basal position without significant differences (8.88 and 8.33 respectively) (Table 3). This result showed that the explant position was determining factor on the number of somatic embryos, similar result was obtained by Chen and Chang [38] they showed that somatic embryogenesis was significantly affected by explant position from leaf of *Oncidium*.

Table (3): Effect of leaflet position and explant position on somatic embryogenesis induction.

Position \ Leaflets		Mean number of somatic embryos /explants			Means (A)
		Apical leaflet	Median leaflet	Basal leaflet	
Apical position		5.33	8.66	12.67	8.88
Median position		6.66	9.33	14.67	10.22
Basal position		4.66	8.33	12.00	8.33
Means (B)		5.55	8.77	13.11	
LSD at 5%	A	0.58			
	B	0.58			
	AxB	1.02			

Concerning leaflet position and explant position, a higher mean number of somatic embryos /explants was recorded in basal leaflet at median position (14.67) followed by apical position (12.67) and basal position (12.00) in the same leaflet without significant differences. The differences in number of somatic embryos per explant at the same leaflet may be referring to differences in cell differentiation stages. Similar result was reported by Kaouther *et al* [39] they found that the differences between cells of the same leaflet explants lead to different embryogenesis of *Prunus incisa*. In our results, median position derived from basal leaflet, median leaflet and apical leaflet scored 14.67, 9.33 and 6.66 respectively (Table 3). They were significantly higher than apical and basal positions from the same leaflets, this may be due to the median explants contain two cut edges which stimulate the induction of embryogenic callus and subsequently, somatic embryos formation. Similar results were obtained by Puchooa [15] who reported that somatic embryos were formed with 1cm^2 of young tender Tai So litchi leaves as explants. Singh *et al*. [37] found that using 0.36-1.44 cm^2 (square-shaped explant with midrib) was successful for induction somatic embryogenesis from leaves of *Sapindus mukorossi* Gaertn. Furthermore, Anjani and Kumar [4] showed that using cutting from litchi leaves with 1-2 cm for study effect of medium and the explants used on callogenesis of litchi *in vitro*. The lower mean number of somatic embryos /explants was recorded at basal position from apical leaflet (4.66) (Table 3).

B- Explant orientation.

The higher mean number of somatic embryos per explant was recorded with adaxial side up orientation (37.67) and it was significantly higher than adaxial side down orientation on the same concentration of growth regulators that recorded (14.33) (Fig 4). This result was in agreement with the report presented by Raharjo and Litz [17] they reported that induction of somatic embryogenesis from leaflet explants of litchi cv. Brewster occurred with adaxial side up orientation.

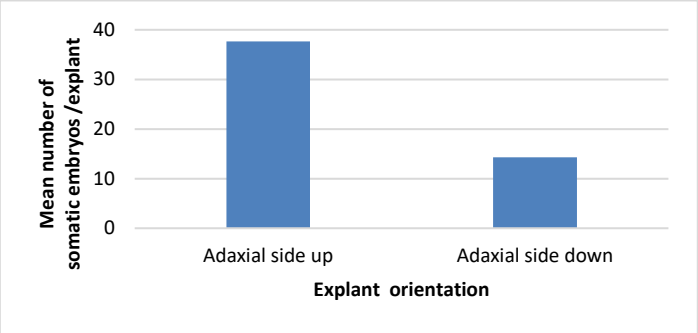

Fig (4): Effect of explant orientation on somatic embryogenesis induction of Brewster litchi cultivar.

Regarding the effect of explant orientation taken from leaflet of Brewster litchi cultivar, embryogenic callus induced and somatic embryos obtained in all surface of explants using median explant from basal leaflets cultured with adaxial side up orientation (Fig 5-A) while culturing with adaxial side down orientation, embryogenic callus induced and somatic embryos obtained on cut ends and midrib region (Fig5-B). Similar result was observed by Ma *et al*. [18] on leaflet explant of litchi and Kim *et al*. [34] on leaf explants of soapberry (*Sapindus mukorossi* Gaertn). This observation may due to the natural facility of nutritional transport and growth factors from the adaxial to the abaxial surface [40]. Adaxial-face-up orientation of leaf explants significantly promoted embryogenesis in comparison with adaxial-face down orientation.

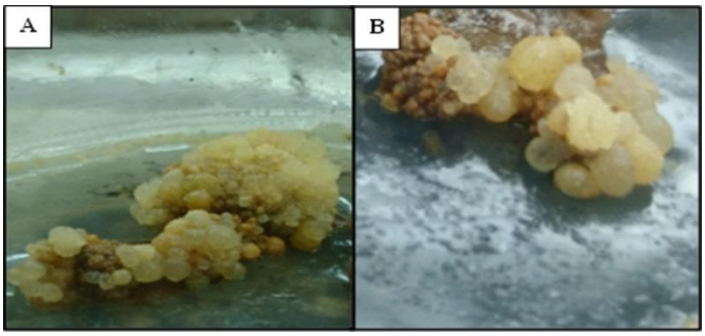

Fig (5): Explant orientation of Brewster cultivar culturing on MS medium and same concentrations of growth regulators (1.0 mg/l 2,4-D + 0.5 mg/l BA). (A): Higher number of somatic embryos obtained on all surface of median explant with adaxial side up orientation. (B): Low number of somatic embryos obtained on cut ends of median explant with adaxial side down orientation.

Experiment (4): Effect of salts media and sucrose concentrations on the development and maturation of somatic embryos (SE).

After 4 weeks of cultured explants of Brewster cultivar on the induction medium, brown compact embryogenic callus induced. Somatic embryos were formed after 90 days from culture. Two types of salts media (MS or B5) with different sucrose concentrations (30, 45 and 60g/l) were tested to investigate the development and maturation of somatic embryos.

Concerning sucrose concentrations, mediated sucrose concentration at 45 g/l gave the highest number of mature milky somatic embryos (5.83) followed by 30 g/l sucrose that recorded (5.25) without significant differences, while the highest sucrose concentration at 60g/l produced the lowest number (3.35) (Table 4). This result showed that the different number of mature opaque milky cotyledonary somatic embryos was obtained with different sucrose concentrations. The results are agreed with the results obtained by Lai *et al*. [13] they showed that culturing of somatic embryos of longan (*Dimocarpus longan* Lour.) on various sucrose concentrations containing medium were the key factors affecting on maturation, number and quality. Carbohydrate during embryo maturation appears to be important for both embryo quality and number, sucrose is most commonly used as carbohydrate [41].

Culturing of somatic embryos on development media, heart and torpedo somatic embryos were observed (Fig6-A) and some of these somatic embryos enlarged further and become opaque milky in color and finally reached to cotyledonary stage after 8 weeks from culture. Most of mature cotyledonary somatic embryos of litchi are closed (Fig6-b, c), this structure of cotyledonary somatic embryos was similar to the somatic embryos that described by Das *et al*. [26] on the same plant.

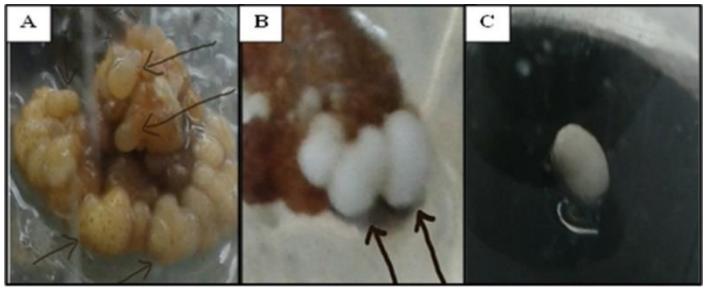

Fig (6): Development and maturation of somatic embryos of Brewster litchi cultivar. (A): Globular and heart somatic embryos of Brewster litchi cultivar after culturing on media free of growth regulators supplemented with different sucrose concentrations (arrows). (B): Mature closed milky cotyledonary somatic embryos of Brewster litchi cultivar after 8 weeks from culturing on development and maturation media. (c): Separation of mature closed milky cotyledonary somatic embryos for germination.

As for the interaction (salts media and sucrose concentrations) highest size of mature milky somatic embryos were recorded on MS salts medium with 45 g/l sucrose (8.00 > 0.5 cm SE and 6.33 < 0.5 cm SE) compared to B5 salts medium (4.33 > 0.5 cm SE and 4.66 < 0.5 cm SE) on the same concentration of sucrose, This result is agreed with the report presented by Raharjo and Litz [16,17] they found that, the development and maturation of somatic embryos from litchi leaflets of Brewster cultivar occurred with high size using MS medium supplemented with 45g/l sucrose. The lowest size of mature milky somatic embryos were recorded on B5 salts medium with 60 g/l sucrose (1.03 > 0.5 cm SE and 5.00 < 0.5 cm SE) compared to MS salts medium (1.70 > 0.5 cm SE and 5.66 < 0.5 cm SE) on the same concentration of sucrose (Table 4). Among all treatments, MS salts medium supplemented with 45 g/l sucrose was most favorable for the development and maturation of somatic embryos.

Table (4): Effect of two types of salts media and sucrose concentrations on mean size of mature milky cotyledonary somatic embryos.

Sucrose concentrations (g/l)	Mean size of mature milky cotyledonary somatic embryos (> 0.5 cm SE, < 0.5 cm SE)				Means (B)
	MS		B5		
	> 0.5 cm SE	< 0.5 cm SE	> 0.5 cm SE	< 0.5 cm SE	
30	6.66	6.33	4.66	3.33	5.25
45	8.00	6.33	4.33	4.66	5.83
60	1.70	5.66	1.03	5.00	3.35
Means (A)	5.45	6.11	3.34	4.33	
LSD 5% A	1.16				
B	1.00				
AXB	2.01				

Mature milky somatic embryos of litchi become hardened after maturation. Similar result was observed by Lai *et al*. [13] they found that after maturation of somatic embryos of longan (*Dimocarpus longan* Lour.), the somatic embryos became white in color and hard. In addition, Aboshama [32] who reported that biochemical changes occurred with *Cajanus cajan* somatic embryos and become hardened after maturation before germinating into plantlets.

Unfortunately, no plantlets were obtained from these somatic embryos of litchi. In this study, the obtained somatic embryos from leaflets of litchi cv. Brewster had cotyledons and root primordia but it failed to germinate and convert into plantlets. This may be due to those somatic embryos

contain inhibitor substances for germination in the tissues. These results are in agreement with the results obtained by Puchooa [15] and Ma *et al*. [18] on somatic embryos of the same plant. Wang *et al*. [42] showed that somatic embryo development of litchi was similar to zygote embryo development. Short life span and rapid loss of zygotic embryos viability have been demonstrated by Menzel [7]; Prasad and Prasad [8], they reported that seeds of litchi may keep well inside fruit up to a month but it losing viability even within a day after separation from the fruit. So that, we believe that somatic embryos of litchi followed the same course of zygotic embryos for losing viability for germination to plantlets. This in fact a frequent problem with hardwood trees and requires further investigations. Further studies are needed for determining the optimal conditions for germination and plantlet recovery from somatic embryos of litchi leaves.

Scanning Electron Microscopy (SEM)

Morphological embryogenic callus and somatic embryos formed on leaf explants of (*Litchi chinensis* Sonn) cv. Brewster were examined by SEM. Moreover, the development of heart stage and root primordia of somatic embryo has been observed (Fig 7).

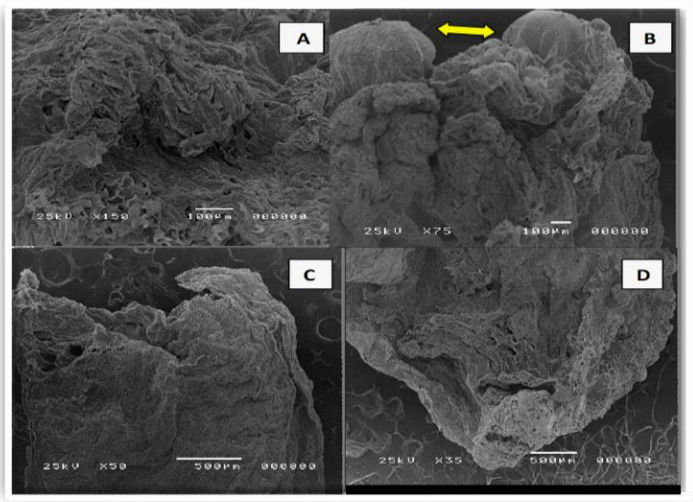

Fig (7): Scanning electron microscopy for development of somatic embryos from leaf explants of (*Litchi chinensis* Sonn) cv. Brewster. (A): Embryogenic callus (x150, bar 100 µm). (B): Globular-stage embryo on the surface of embryogenic tissue (x75, bar 100 µm) (arrow). (C): Development of heart-stage embryo with furrows at the apex (x50, bar 500 µm). (D): Root primordia of somatic embryo with long cylindrical structures and contain of unique notches with constricted end (x35, bar 500 µm).

Histological observation

Anatomical observation using microtome showed differences in the embryogenic cells of callus of Brewster cultivar and non-embryogenic cells of callus of Mauritius cultivar (Fig: 8). Stasolla and Yeung [43] showed that one of the most important events occurred during somatic embryo development is apical meristems observation. Longitudinal sections through *Litchi chinensis* somatic embryos showed the provascular strands connected root and shoot apical meristems. Furthermore, microscopic observation showed globular and heart shape somatic embryo (Fig: 9). The longitudinal section through a root pole of somatic embryo showed the root apical meristem is embedded (Fig: 10).

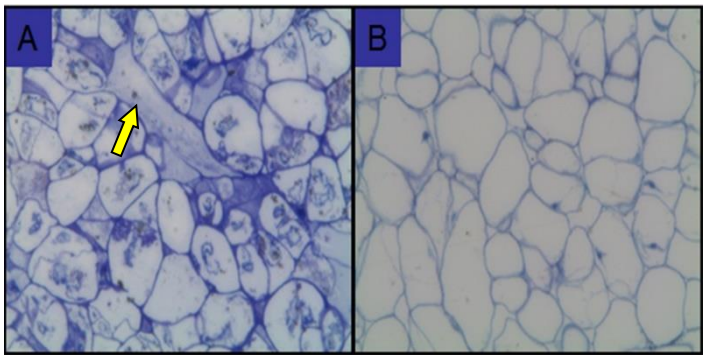

Fig (8): Embryogenic and non-embryogenic cells of two litchi cultivars (Brewster and Mauritius). (A): Embryogenic cells of Brewster cultivar with high cytoplasmic ratio, cell walls are relatively thick and deeply dyed with elongation of embryogenic region (arrow). (B): Non-embryogenic cells of Mauritius cultivar with sparse cytoplasm or almost absent and the cell nucleus were relatively small.

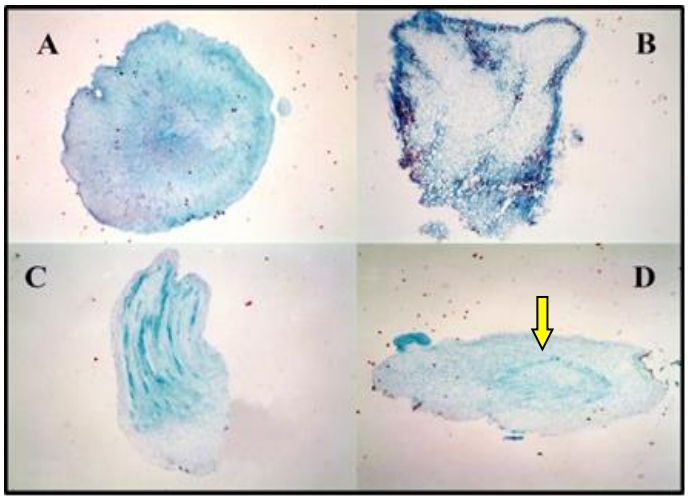

Fig (9): Stages of somatic embryos development of *Litchi chinensis* cv. Brewster; (A): Globular (B): Heart (C): The longitudinal section through cotyledonary somatic embryo showing typical vascular system (D): The longitudinal section through the closed milky cotyledonary somatic embryo showing meristemetic tissues (arrow).

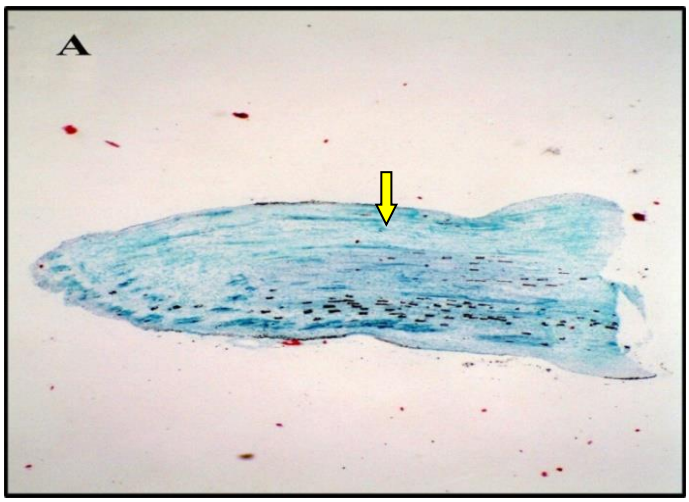

Fig (10): The longitudinal section of somatic embryo of Brewster litchi cultivar showing root meristem (arrow).

CONCLUSION

In conclusion, somatic embryogenesis from one of recalcitrant fruit *in vitro* was achieved from leaflet explants obtained from elite trees. The response of explants to induce somatic embryogenesis of litchi is depending on the cultivar, leaflets position, explant position and orientation. Procedures described her were step towards for induction of

somatic embryogenesis from mature leaves to improvement of litchi trees via biotechnological methods.

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