African Journal of Biotechnology Vol. 3 (11), pp. 576-584, November 2004
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2004 Academic Journals

Full Length Research Paper

In vitro regeneration of lychee (*Litchi chinensis* Sonn.)

D. Puchooa

Faculty of Agriculture, University of Mauritius E-mail:sudeshpeuom@ac.mu.

Accepted 12 August, 2004

***In vitro* plantlet regeneration in the main commercial variety of lychee (*Litchi chinensis* Sonn. cv Tai So) in Mauritius was achieved from callus cultures derived from young, tender leaf explants on Murashige and Skoog (MS) medium. Callogenesis was obtained in all media supplemented with auxin, but was most prominent in media supplemented with 2,4 – D (1.5 mgL^{-1}) with or without benzylamino purine and kinetin. Nodular compact callus obtained in the 2,4 – D and BAP treatment proliferated and differentiated into shoots .When transferred to MS medium supplemented with BAP (2.0 mgL^{-1}) and IAA (3.0 mgL^{-1}). Regenerated shoots produced prominent roots when transferred to MS medium supplemented with IBA (2.0 mgL^{-1}). Regeneration was predominantly through organogenesis. Somatic embryogenesis was observed when callus growing on MS medium supplemented with 2,4–D (1.5 mgL^{-1}) was transferred to medium devoid of 2,4-D.**

Key words: *Litchi chinensis*, micropropagation, somatic embyogenesis.

INTRODUCTION

The lychee (*Litchi chinensis*), a member of the Sapindaceae family, has its origin in China and is now widely spread in the tropical and subtropical regions of the world (Menzel, 1983). It was introduced into Mauritius by Joseph François Charpentier de Cossigny de Palma in 1764. Other introductions were made from India in 1929, from China in 1931 and 1990, from Hawaii in 1972 and from Australia in 1989. The lychee is one of the best fruit trees growing in the subtropics. The high demand and the high prices paid for lychee, especially in the European market, has created new interest in the growing of this fruit with an increase in the demand for propagation material. Traditionally lychee trees have been vegetatively propagated in Mauritius by air-layering. Although this method of propagation has been improved by the use of younger branches, small earth balls and 1, 4-indole-3-butyric acid (IBA), the process is still slow and inefficient. As an alternative to air-layering, micro propagation offers an attractive method for vegetative propagation of lychee. It requires only very small amounts of propagating material and has the potential of providing very large numbers of cloned plants. Fruits that have aborted seed are termed "*chicken tongues*" and are preferred by consumers (Lake, 1988), since these fruits have a high flesh to seed ratio. In crosses involving plants which have tendency to produce these fruits as the female parent, many of the most valuable progeny are lost prior to harvest. Production of plants by culturing embryos prior to abortion would be expected to yield progeny with high proportion showing the chicken tongue character (Anon, 1991).

Lychee is difficult to propagate using *in vitro* techniques but there would be many advantages in clonal propagation since traditional propagation methods are slow and inefficient (Chapman, 1984). One of the problems in the micro propagation of lychee is the secretion of polyphenols into the medium by the tissues. These polyphenols undergo oxidation to produce substances that kill the tissues (Kantharajah et al., 1992). Another problem is that field grown lychee material used as a source of known genetic material, is heavily contaminated with organisms which are difficult to remove prior to culture. Both of these problems are frequently encountered with hardwood species and the usual approach is to develop the experimental culture techniques using young tissues from seeds germinated

in vitro. This technique has the advantage of providing very clean material as well as producing juvenile tissues which usually respond well in culture (Durzan, 1984).

With lychee, so far, even the experiments using juvenile tissues have not been successful in micropropagation (Kantharajah et al., 1992). Fu and Tang (1983) attempted anther cultures of two lychee varieties. While haploid plants have little use, the potential from this technique does exist if diploid plants could be regenerated from haploid callus. The regeneration frequency of the two cultivars used by Fu and Tang (1983) was very low. Kantharajah et al. (1992) attempted developing methods for rescuing and culturing immature embryos and achieving multiplication through induction of adventitious buds from the embryonic shoots. The varieties studied were Bengal, Kwai May Pink and Wai Chee. One of the problems encountered by these workers was the browning of very young tissues.

Although no plantlets were obtained, Kantharajah *et al.* (1992) believe that the induction of multiple shoots from lychee provides one method of clonal propagation and, if successfully applied, could produce up to 15 plants from a single embryo. Das et al. (1999) managed to induce multiple shoot in five genotypes of *Litchi chinensis* Sonn. by direct germination of lychee seeds in MS liquid medium supplemented with benzyl adenine (BA) (20 mgL^{-1}) and supported on filter-paper bridge. Contamination and browning were again the major problems encountered by these workers.

One of the first works on somatic embryogenesis in lychee was by Amin and Razzaque (1995) who managed to induce somatic embryogenesis in the cultures of zygotic embryos of lychee using BA (5 mgL^{-1}) and activated charcoal (1 gL^{-1}). Although about 40% of the *in vitro* formed embryos matured, no plantlets were obtained. In their work on somatic embryos derived from lychee zygotic embryos, Zhou et al. (1996) had some difficulty in germinating the somatic embryos. On the other hand, Yu and Chen (1998) reported the development and maintenance of highly embryogenic suspensions and protoplast isolation for several lychee cultivars. In another work, Yu et al. (2000) managed to successfully culture lychee protoplasts of the cultivar 'Xiafanzhi' from suspensions, only after embedding them in Ca-alginate beads. Although the successful culture of lychee protoplasts could facilitate their use in lychee breeding, the regeneration frequency was low.

Materials and Methods

Source of tissue and preparation of explants: Seedlings of lychee (*Litchi chinensis* Sonn.) 'Tai So' variety were used as the source of tissue for all experiments.The plants were grown in trays in sterile potting media, fed regularly with "Doff Plant Feed™" together with a spraying regime (Table 1) of a pesticide and a systematic fungicide to reduce possible contaminants.

Where possible, the plants were watered without excessive wetting of the aerial portions of the plant. Young leaves from the germinated seedlings were used as explants in all studies.

Table 1. Pesticide treatment of stock plants.

Pesticide (Trade mark)	Application rat (Concentration)
Benomyl	0.5 gL^{-1}
Champion	2.0 mLL^{-1}
Folithion	1.0 gL^{-1}
Lannate	1.0 gL^{-1}
Peropal	1.0 gL^{-1}
Welgro	1.0 gL^{-1}

Preliminary experiment 1 (PE1) - sterilisation

The culture medium devised by Murashige and Skoog (MS, 1962) supplemented with sucrose (20 gL^{-1}) and 2.0 mgL^{-1} 2,4-dichloroxyphenoxy acetic acid (2,4-D) was used in all preliminary studies. The pH of the media was adjusted to 5.8 using sodium hydroxide or hydrochloric acid. Agar (Phytagel) was added to the media at a concentration of 0.25%. A wide range of sterilants, namely, sodium hypochlorite (NaOCl) - commercial bleach "Eau de Javel™" containing 3.5% active ingredient, mercuric chloride, hydrogen peroxide and 95% ethanol were investigated. In all cases the detergent Tween 20 (Polyoxyethylene Sorbitan monolaurate) was added at a concentration of 2 drops per 100 mL of sterilant. The explants were sterilized in various concentrations of these sterilants for different periods of time. The explants were then rinsed three times in sterile distilled water - the first rinse lasting 5 min, the second 15 min and the final rinse lasted for 30 min. All plant tissue visibly damaged by the sterilant was removed. The plant material was cut into suitable sized explants and transferred onto the nutrient medium. The cultures were then transferred to the growth rooms and monitored for contamination. All cultures, unless stated, were incubated in a growth room with a culture environment of 25 ± 2°C with a relative humidity of 40% and a 16 h light / 8 h dark cycle at an intensity of 27 $\mu E\ m^{-2}\ s^{-1}$. From these experiments, a sterilization regime was adopted and used for all future experimentation.

Preliminary experiment 2 (PE2) – browning

The following experiments were carried out to eliminate explant browning *in vitro.*

(i) Addition of polyvinylpyrrolidone (PVP – Mr 10,000) at a range of concentrations between 0.5 and 2.0%.

(ii) Soaking in a mixture of citric and ascorbic acid before inoculation and inclusion of this mixture in the culture medium. The concentration range tested for each was between 50 and 225 mgL^{-1}.

Plant growth regulators

Based on results obtained from the preliminary studies, set of experiments were conducted to find the most appropriate plant

growth regulator supplements to be used for callus initiation apropagation, and plant regeneration.

Explants (1 cm^2) were inoculated onto MS basal salt formulation supplemented with 3% sucrose (w/v), 0.25% Phytagel, 225 mgL^{-1} each of ascorbic and citric acid and a range of combinations of auxins: 2,4-D (0.5 – 2.0 mgL^{-1}), 1-naphthalene acetic acid (NAA) (0.5 – 2.0 mgL^{-1}) and IAA (0.5 – 5.0 mgL^{-1}) and the cytokinins, 6-Benzylamino purine (BAP) (0.5 – 4.0 mgL^{-1}) and kinetin (1.0 – 2.0 mgL^{-1}). The medium pH was adjusted to 5.6 – 5.8 before autoclaving at 121^0C for 15 min and cultures were grown for four weeks at a temperature of 25± 2°C. The experiment was repeated twice with 10 replicates and two explants per replicate.

In the first set of experiments, only the effects of the auxins on callus induction were investigated over four weeks. Based on the results obtained, the effects of combinations of the auxin selected and the two cytokinins on callus induction were investigated over four weeks. This combination was selected for callus propagation. For the regeneration studies, only combinations of BAP and 1,4-indole-3-acetic acid (IAA) were investigated, as according to literature (Gharyal and Maheshwari, 1981), woody trees seem to respond positively towards these growth regulators. The calli (1 g fresh mass) were transferred to MS medium with 3% sucrose (w/v), 0.25% Phytagel, 225 mgL^{-1} of each ascorbic and citric acid and combinations of BAP (0.5 – 4.0 mgL^{-1}) and IAA (0.0 – 5.0 mgL^{-1}). Cultures were grown under white fluorescent light at a photon flux of 27 μEm^{-2} s^{-1}at 16 hour light / 8 h dark period. The effects of 1,4-indole-3-butyric acid (IBA) and NAA in rooting of in vitro raised shoots were also investigated. The concentration range of IBA and NAA tested was 1.0 – 3.0 mgL^{-1}. Control cultures were raised on MS basal medium devoid of any growth regulators. Visual observations of the cultures were made every week and the percentage of cultures showing callusing, shoot bud differentiation / development and/or rooting was recorded.

Somatic Embryogenesis

According to a number of workers (Merkle, 1995; Yu et al., 2000), culturing on medium supplemented with 2,4-D followed by growth of the callus onto medium devoid of 2,4-D, gives rise to somatic embryos and eventually to plantlets. As in this study growing calli, previously grown on medium supplemented with 2,4-D and BAP, and transferred to medium devoid of 2,4-D but supplemented with IAA and BAP gave rise to plantlets, prompted an investigation into the possibility of somatic embryogenesis occurrence. Young lychee explants which were cultured onto MS medium supplemented with 2,4-D (1.5 mgL^{-1}), were transferred onto MS medium devoid of 2,4-D but maintaining the other supplements and grown at the same environmental conditions. After three subcultures (30 day culture interval) histological analyses were done on the calli to check for the presence of somatic embryos.

Specimen preparation for light microscopy

Fixation and dehydration: Sections of the callus, 1 mm thick, were fixed daily in 2% paraformaldehyde / 2.5% glutaraldehyde fixative buffered with 0.1 M sodium cacodylate (pH 7.0) for 16h at 4^0C. The specimens were then washed in the buffer followed by fixation in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer at 4^0C overnight. After washing three times in the buffer, specimens were dehydrated through 10, 20, 30, 50, 70 and 90% ethanol to absolute ethanol.

Embedding: The specimens were transferred overnight through two changes of propylene oxide and on to propylene oxide plus Araldite mixture (Araldite CY 212 : dodeoenyl succinic anhydride: dibutyl phthalate: n-benzyl dimethyl amine - 10:10:1:0.4) in a ratio of 1:1. The propylene oxide was then allowed to evaporate and the Araldite mixture changed three times over a period of two days. The resin specimens were transferred into plastic molds containing the liquid resin and placed into an oven at 60^0C for polymerizing overnight.

Microtomy: The specimen block was rough trimmed to exclude excess plastic matrix and to expose the surface of the specimen. The roughly trimmed block was mounted into a Reichert Ultracut microtome and 1-2 μm sections were cut using glass knives.

Staining: For the staining, the Sass (1967) method was used. Histological observations were made through a light microscope on sections obtained. Photographs were taken using an Olympus BH2 light microscope camera.

Table 2. Percentage of sterile leaf explants after two weeks in culture following treatment with different sterilants.

Sterilant	Time (min)	% Sterile leaf explants
Ethanol (95%)	5	0
	10	2
Hydrogen peroxide (10%)	10	10
	15	40
Mercuric Chloride (0.1%)	10	100*
	15	100*
	30	100*
Sodium Hypochlorite (1%)	5	35
	10	45
	15	45
Sodium Hypochlorite (1.5%)	5	60
	10	65
	15	70
Sodium Hypochlorite (2%)	5	70*
	10	100*
	15	100*

*necrotic explants

RESULTS

Sterilisation (PE1*)* - A prerequisite for successful culture is the establishment of an aseptic technique. Thus, the first experiment in this investigation involved the establishment of suitable sterilisation regimes for the leaf explant. As a large percentage of the contamination was of fungal origin, inclusion of benomyl in the pretreatment was essential for reducing the percentage contamination of the explants. Table 2 summarises the efficiency of the different sterilants.

The more 'violent' sterilization techniques markedly reduced explant vigour with marked shriveling and

necrosis of the explant. As a sterilant, ethanol was ineffective and resulted in partial dehydration of the explant as evidenced in the shrivelling of the plant material. Hydrogen peroxide although not very effective in terms of microbe elimination, did not damage plant tissue as the chemical is easy to remove and is a relatively 'soft' sterilant. While mercuric chloride is probably the most efficient sterilant, it has major drawbacks in that a large amount of tissue damage may occur from over zealous sterilization regimes, as noted. Sodium hypochlorite proved to be the best all round sterilant as it is effective not only in decontamination but is easy to remove resulting in minimal damage to explant tissue. Best results were obtained using 1.5% bleach for 15 min.

This sterilisation regime was therefore selected in subsequent experimentations. It should also be noted that although the 2.0% bleach was effective during decontamination, a high proportion of the explants became necrotic after some time in culture. Although not quantified, the obvious benefit from the inclusion of a detergent in the sterilizing routine was observed. In experiments where Tween 20 was deliberately omitted, an increase in the percentage of contamination was observed. There were clear exudates at the interface between the medium and the explant. However, no microbial presence was detected in them when these exudates were cultured on Luria-Bertani medium.Gram stains were also performed to determine whether microbial interaction was involved. These tests proved negative. The exuded substances may be a long chain polysaccharide as woody species are known to release large amounts of these into the medium after several weeks in culture. This is apparently a result of increased dictyosome numbers in the cultured material (Durzan, 1988).

Table 3. Colour scores used to assess browning.

Rating	Remarks
0	Explant completely brown
1	Explant greenish brown
2	Explant green with necrotic spots
3	Browning along edges, green colour less pronounced
4	Slight browning along cut edges
5	Completely green

Browning (PE2)

In the first set of experiments, the efficiency of PVP (mol wt. 10,000) in eliminating browning using leaf explants was investigated. As culturing in low light has been suggested as a means to reduce and eliminate browning, the effects of two light regimes (complete darkness and 16 h and 8 h light dark to an illumination of 27 $\mu E\ m^{-2}\ s^{-}$) were also investigated. Four different PVP concentrations (0.5, 1.0, 1.5 and 2.0%) were tried under the two light regimes.

Thus, there were eight treatments in all with two controls, one for each light regime. There were eight replicates per treatment. Browning was rated on an arbitrary scale over a period of four weeks as per Table 3.

In the second set of experiments to investigate the effects of antioxidants on browning, citric and ascorbic acids either alone or in combinations were used (0, 25, 150, and 225 mgL^{-1} of each). The cultural conditions were same as in the previous experiment except that the cultures were grown in complete darkness only. There were eight replicates per treatment and browning was rated, as above, over a period of four weeks.

Results showed that PVP, at all concentrations, was unable to control browning of leaf explants. There were no significant differences at 5% level between the explants incubated in the dark and those subjected to an illumination of 27 $\mu E\ m^{-2}\ s^{-1}$ and 16 h light and 8 h dark period. This suggested that PVP was unable to bind to the polyphenols. Effects of PVP of higher molecular weights were not investigated, as they are insoluble.

Using ascorbic and/or citric acid, results showed that when ascorbic acid or citric acid is used singly, they are unable to control browning.However, when used in various combinations, they are very effective. This shows that ascorbic acid and citric acid have a greater penetrative power than PVP. Based on the results, a combination of ascorbic acid (225 mgL^{-1}) and citric acid (225 mgL^{-1}) was added to all culture media in subsequent experimentations. However, the pH of the medium was not allowed to be lower than 5.6.

Phenolics secretion as a result of wounding is more prominent during the few hours following excision. All explant excisions were therefore carried out in a sterile mixture of these two antioxidants (225 mgL^{-1} of each). Browning could be further reduced when following excision the explants were cultured for a limited period of time (1-2 h) in liquid medium supplemented with the two antioxidants. This allowed large amounts of the phenolics to be released into the medium and at the same time allowing a better contact between the explants and the antioxidants so that when they were subsequently cultured onto solidified medium, the inhibitory effects of the phenolics was minimised.

Plant growth regulators

In treatments which responded, callus started from cut ends of the explant and reached a maximum within 20-25 days after inoculation. Among the three auxins tested individually (2,4-D, NAA and IAA) (Table 4) 2,4-D produced profuse, brown, compact and nodular callus (Figure 1). MS medium supplemented with NAA alone

produced yellowish green (Figure 2) / dark brown compact callus. Similarly inclusion of IAA alone in the medium produced either yellowish friable at low concentration to dark brown compact callus at higher concentrations. Maximum callusing response was observed both in 2,4-D (1.5 mgL^{-1}) ± BAP (1.0 mgL^{-1}) and 2,4-D (1.5 mgL^{-1}) + kinetin (1.0 mgL^{-1}) combination (Table 5). Increasing the concentration of any auxin singly became inhibitory or ineffective to callus induction. Results also show that although an auxin alone can initiate callus induction, using a combination of auxin and cytokinin, does enhance callus production.

Callus obtained from young leaf explants, after two subcultures on the combination 2,4-D (1.5 mgL^{-1}) and BAP (1.0 mgL^{-1}) medium, were transferred to MS medium with various concentrations and combinations of BAP and IAA (Table 6). These two growth regulators were chosen because they are known to be effective in the regeneration of woody trees (Gharyal and Maheshwari, 1981). When the cytokinin was used singly in the culture medium, there was low frequency of differentiation.Maximum regeneration capacity was observed when 2 mgL^{-1} BAP and 3 mgL^{-1} IAA was used after 30 days of culture. Shoots were observed after a further two weeks in culture**.**

The effect of IBA and NAA on rooting of in vitro raised shoots is shown in Table 7. These two growth regulators were used because IBA was used by Amin and Razzaque (1995) and NAA was used by Kantharajah et al. (1992), Das et al. (1999) and Yu et al. (2000) in their studies on lychee.

As expected, the auxins stimulated rhizogenesis. The regenerated shoots produced roots when transferred to MS medium supplemented with either IBA or NAA. Highest percentage rooting was obtained with 2.0 mgL^{-1} IBA within three weeks in culture. Although NAA induced rooting, the roots were thin and delicate and the plantlets did not survive the hardening in peat, leca and vermiculite (1:1:1). On the other hand, plantlets in which roots were induced as a result of their treatment with IBA had a survival rate of approximately 40% when hardened.

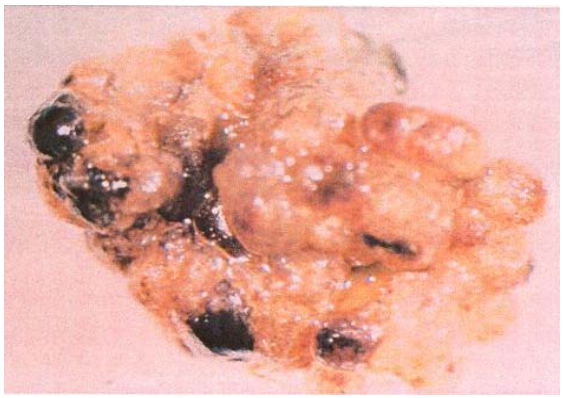

Figure 1. Brown, compact, nodular callus produced by 2,4-D.

Table 4. Effect of auxin on callus induction from young leaf explants of lychee.

Growth regulators (mgL^{-})		Percentage callus induction
2,4-D	0.5	30d
	1.0	55b
	1.5	65a
	2.0	60ab
NAA	0.5	20f
	1.0	35d
	1.5	55a
	2.0	50b
IAA	0.5	20f
	1.0	35e
	1.5	50c
	2.0	60a

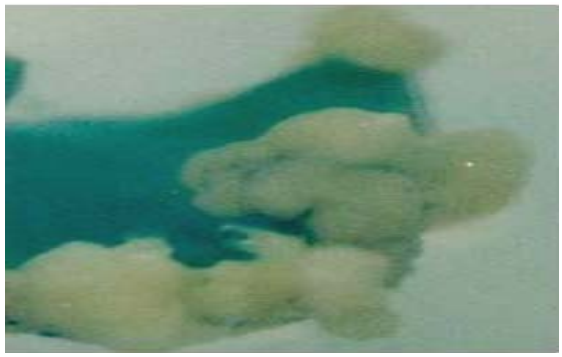

Figure 2. Yellowish green compact callus produced by NAA.

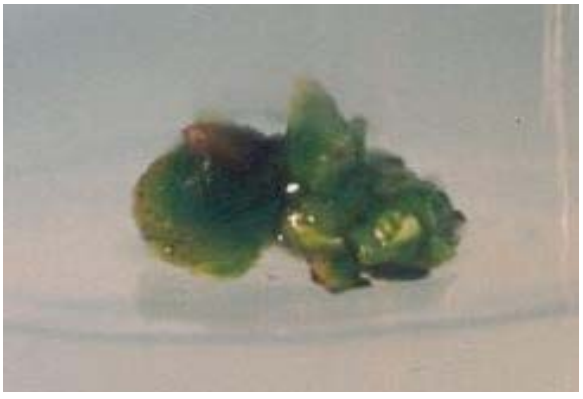

Figure 3. Bud emergence and development on medium supplemented with BAP and IAA.

Somatic Embryogenesis

Observations using light microscope showed that embryogenic callus was formed in the presence of 2,4-D. Callus was composed of large, vacuolated cells with

Table 5. Effect of auxin and cytokinin on callus induction from young leaf explants of lychee

Growth Regulators (mgL^{-1})		% Callus Induction
2,4-D	**BAP**	
0.5	1.0	38e
1.0	1.0	73b
1.5	1.0	83a
2.0	1.0	68bc
0.5	2.0	43c
1.0	2.0	68bc
1.5	2.0	53d
2.0	2.0	48c
2,4-D	**Kinetin**	
0.5	1.0	43c
1.0	1.0	48bc
1.5	1.0	58a
2.0	1.0	43c
0.5	2.0	38cd
1.0	2.0	53b
1.5	2.0	43c
2.0	2.0	38cd

Means followed by the same letters are not significantly different ($p < 0.05$), from each other based on L.S.D.

thick walls and devoid of starch grains. Well-developed meristematic tissues containing small uniform, activelydividing meristem-like cells lacking intercellular spaces were observed (Figure 4). Some of the other observed when callus growing in the presence of 2,4-D was transferred onto medium lacking 2,4-D (Figure 4).

In vitro observations showed that once the callus grown on medium supplemented with 2,4-D was transferred to a medium devoid of 2,4-D, several bud-like structured emerged (Figure 5) but failed to develop into shoots.

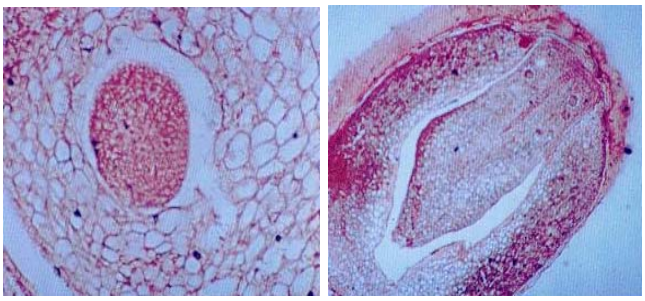

Figure 4 . Stages of development of the somatic embryos. Activity dividing meristem –like cells lacking intracellular spaces.

Table 6. Effect of various combinations of cytokinin (BAP) and auxin (IAA) on regeneration from young leaf derived callus of *Litchi chinensis* Sonn.

Growth Regulators (mgL^{-1})		% Calli forming shoots
IAA	**BAP**	
0.0	0.5	-
0.0	1.0	10h
0.0	2.0	30ef
0.0	4.0	20fg
1.0	0.5	-
1.0	1.0	15g
1.0	2.0	50cd
1.0	4.0	30f
2.0	0.5	-
2.0	1.0	25ef
2.0	2.0	65b
2.0	4.0	40de
3.0	0.5	-
3.0	1.0	35e
3.0	2.0	80a
3.0	4.0	46.6d
4.0	0.5	-
4.0	1.0	25f
4.0	2.0	55c
4.0	4.0	46.6d
5.0	0.5	-
5.0	1.0	20fg
5.0	2.0	46.6d
5.0	4.0	35e

-: No response.
Means followed by the same letters are not significantly different ($p< 0.05$), from each other based on L.S.D.

Table 7. Effect of IBA and NAA on rooting of in vitro raised shoots of lychee.

Growth regulators	Concentration (mgL^{-1})	% Rooting
IBA	1.0	56.6dc
	2.0	76.6a
	3.0	70bc
NAA	1.0	60d
	2.0	66.6c
	3.0	73.3b

Means followed by the same letters are not significantly different at 5% level of significance based on L.S.D.

DISCUSSION

A prerequisite for successful *in vitro* culture is the establishment of an aseptic technique. Sodium hypochlorite (1.5% and treatment time of 15 min) including Tween 20, proved to be the best all round sterilant for the successful sterilisation of young leaf explants of lychee. Browning, which has been reported by several workers to be inhibitory to the culture of lychee *in vitro* (Kantharajah et al., 1992, Das et al., 1999,

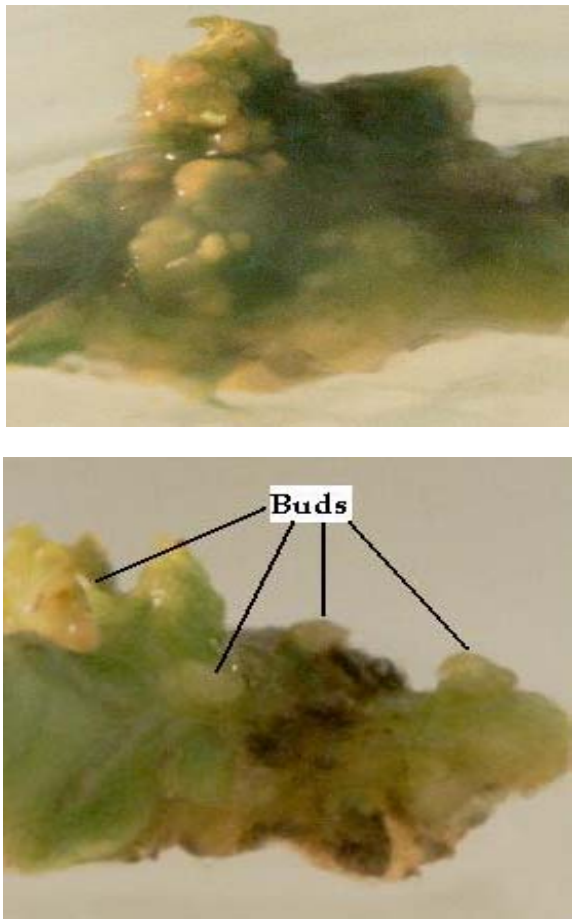

Figure 5. Young globular embryos induced from tissues of primary somatic embryos giving rise to buds (Bar: 1 mm)

Amin and Razzaque 1995),was controlled by the use of a combination of ascorbic acid (225 mgL^{-1}) and citric acid (225 mgL^{-1}) both during manipulation and culture of the explants. Other workers have used either PVP or activated charcoal to control browning.

There is a great deal of variability between plants and the growth regulators necessary for morphogenesis, and the growth regulator(s) requirement of each type of explant should be determined (Hussey, 1975, Hughes, 1981). Although 2,4-D could initiate callus when used alone, using a combination of auxin and cytokinin enhanced callus production. Similarly, for regeneration, when a cytokinin was used singly in the culture medium, there was low frequency of differentiation. Maximum regeneration capacity was observed when 2 mgL^{-1} BAP and 3 mgL^{-1} IAA were used after 30 days of culture. Shoots were observed after a further two weeks in culture. The failure or the low rate of callus producing shoots may be a result of growth regulator carry over or retention that offsets the subsequent experiments, especially the retention of 2,4-D. Das *et al.* (1999) reported 100% germination rate of lychee seeds *in vitro* using 20 mgL^{-1} BAP. Kantharajah et al. (1992) and Amin and Razzaque (1995) in their work on lychee also observed the benefial effect of BAP. In their work on lychee anther culture, Fu and Tang (1983) used 2,4-D and NAA for callus induction and a different cytokinin, (kinetin) but the same auxin (IAA) for regeneration. The results of this study, therefore, show that although auxin (2,4-D) on its own can be use for callus initiation in lychee, a low concentration of 2,4-D in combination with cytokinin (BAP) was more appropriate, and subsequent proliferation and differentiation of shoots from such calli using again BAP but in combination with IAA. Higher concentrations of 2, 4-D were avoided because of its possible role in chromosomal aberration and suppression of morphogenesis (Murashige, 1974).

Often hard to root species only express their rooting capacity, *in vitro*, under suitable conditions via direct or indirect rhizogenesis. Rhizogenesis is a complex phenomenon involving interactions between external factors, endogenous growth regulators levels and nutrition. However, the auxin related factors appear to be of major importance. In general, a high concentration of 2,4-D and NAA seems to favour indirect rhizogenesis, with several cell cycles preceding the recovery of rhizogenetic capacity. Highest percentage rooting was obtained with 2.0 mgL^{-1} IBA within three weeks in culture. Although NAA induced rooting, the roots were thin and delicate and the plantlets did not survive the hardening in peat, leca and vermiculite (1:1:1). On the other hand, plantlets in which roots were induced as a result of their treatment with IBA had a higher survival rate during hardening.

Many reports of somatic embryogenesis in hardwood trees indicate that somatic embryos have been produced, but the cultures fail to demonstrate continued embryo production. Generally these are reports of indirect embryogenesis, where, for example, individual somatic embryos arise from the explanted zygotic embryo cotyledon tissue. Often, only a single population of embryos is produced, some of which may mature and convert to plantlets. This was the case in the work of Das et al. (1999) on lychee. In a case like this, the culture represents a dead end with regard to clonal propagation. On the other hand, the cyclic production of new generations of somatic embryos, also known as repetitive embryogenesis, gives somatic embryogenesis its great potential for mass propagation and gene transfer, since a single culture undergoing repetitive embryogenesis is theoretically capable of generating an unlimited number of somatic embryos. This was the attempt by Yu *et al*. (2000) using lychee protoplasts.

Embryogenic callus was observed in this study on medium supplemented with 2,4-D and these eventually developed when grown on medium devoid of 2,4-D. Embryogenic cultures of some species require continuous exposure to auxin (or other plant growth regulators in some cases) to produce repetitive embryos, while in others, a pulse of auxin, such as 2,4-D, as short as a few days is sufficient to induce repetitive embryo-

genesis (Merkle, 1995). This has been observed in pecan (Wetzstein et al., 1989), black locust (Merkle and Wiecko, 1989) and big leaf magnolia (Merkle and Watson-Pauley, 1993). In a few cases, no exogenous application of plant growth regulator is required to trigger repetitive embryogenesis (Gharyl and Maheshwari, 1981). Once the influence of auxin is removed, the somatic embryos eventually switch to a program of development, maturation and germination, and repetitive embryogenesis stops. This was the case observed in this study. Similar results have also been observed in American chestnut (Merkle et al., 1991) where it was found that for continued production of somatic embryos was dependent on long-term exposure to 2,4-D. However, this is species dependent as prolonged exposure to 2,4-D in some species yields malformed embryos or embryos that fail to complete development (Parrott et al., 1988).

Plantlet regeneration from the somatic embryos was a problem in this study. This is in fact a major problem among hardwood species. Treatments to overcome this vary with species. For example, application of abscisic acid and osmoticum treatments seems to work with coniferous species (Attree et al*.,* 1991; Gupta et al., 1994). One general approach would be to mimic the conditions experienced by zygotic embryos in seeds prior to germination, for example, a cold stratification. Other treatments which have been found to work with hardwood species include direct treatment with benzylaminopurine and addition of ethylene biosynthesis inhibitor silver nitrate to the medium (Mathews and Wetzstein, 1993). This indicates that ethylene accumulation in the culture jar may be inhibitory to the development of plantlets from the somatic embryos. Further investigation is therefore required to test the efficacy of the above, either singly or in combination, on the regeneration of viable lychee plantlets from the somatic embryos.

In conclusion, Lychee plantlets were successfully regenerated *in vitro* using young leaf explants. Sterilisation for 15 min using sodium hypoclorite (1.5%) was sufficient to eliminate contamination. Browning was controlled by using a combination of ascorbic acid (225 mgL^{-1}) and citric acid (225 mgL^{-1}). 2,4-D in combination with cytokinin (BAP) was more efficient in callus initiation while BAP, but in combination with IAA, was more appropriate for the differentiation of shoots from callus. IBA (2.0 mgL^{-1}) promoted rooting of the shoots.

Somatic embryogenesis has been documented in species from most taxonomic groups. It is presumed to be a universal, heritable trait of higher plants, however, details of the genetic control of somatic embryo development remain unclear. In this study, no plantlets were obtained from these somatic embryos unfortunately. This is in fact a frequent problem among hardwood trees and requires further investigations. While somatic embryo genesis has many potential advantages for mass propagation and genetic improvement of lychee, a number of limitations remain to be overcome before embryogenic systems can be applied for operational production of propagules. Detailed study is necessary to unlock the full potential of somatic embryogenesis and make it fully accessible as a tool for breeding and production for hardwood trees.

ACKNOWLEDGEMENT

The author wishes to thank the Faculty of Agriculture, University of Mauritius for supporting this work.

REFERENCES

Amin MN, Razzaque MA (1995). Induction of somatic embryogenesis in the cultures of zygotic embryos of lychee. Bangladesh J. Bot.24: 25 – 29.

Anon (1991). Tissue culture of lychee (*Litchi chinensis* Sonn.). In: CSFRI Information Bulletin 229: 1 – 2.

Attree SM, Moore D, Sawhney VK, Fowke LC (1991). Enhanced maturation and desiccation tolerance of white spruce (*Picea glauca* Voss) somatic embryos: effects of a non-plasmolysing water stress and abscisic acid. Annals of Bot. 68: 519-525

Chapman KR (1984). Sapindaceae: Lychee. *In*: Page P E (Ed), Tropical tree fruits for Australia. Brisbane: Queensland Department of Primary Industries: 179 – 191.

Das DK, Shiva Prakash N, Bhalla-Sarvin N (1999). Multiple shoot induction and plant regeneration in litchi (*Litchi chinensis* Sonn.). Plant Cell Reports 18: 691 – 695.

Durzan DJ (1984). Applications of plant tissue culture to Agriculture and Forestry. An overview. Symposium on Plant Cell and Tissue Culture. University of Tennessee, Knoxville, Sept 9 – 14, pp. 232 – 256.

Durzan DJ (1988). Somatic poly embryogenesis for the multiplication of tree crops. Biotechnol. and Genetic Eng. Rev. 6: 341 – 367.

Fu L , Tang D (1983). Introduction pollen plants of Litchi tree (*Litchi chinensis* Sonn.). Acta Genetica Sinica 10: 369 – 374.

Gharyal PK, Maheshwari C (1981). In vitro differentiation in trees. Naturewissenschaften 68: 379-380.

Gupta PK, Timmis R, Timmis K, Carlson W, Grob J, Welty E (1994). Plantlet regeneration via somatic embryogenesis in Douglas-fir (*Pseudotsuga menziesii*) In: TAPPI 1994 Biol. Sci. Symposium Proceedings, Minneapolis, MN. pp. 35-39.

Hughes KW (1981). In vitro ecology: exogenous factors affecting growth and morphogenesis in plant culture systems. Env. Expt. Bot. 21: 281-288.

Hussey G (1975). Totipotency in tissue explants and callus of some members of the Liliaceae, Iridaceae and Amaryllidaceae. J. Experimental Bot. 26: 256 – 262.

Kantharanjah AS, Mc Conchie CA, Dodd WA (1992). In vitro embryo culture and induction of multiple shoots in lychee. Annals of Bot. 70: 153 – 156.

Lake B (1988). Lychees. Growth in Queensland Industry. Australian Horticul. 863: 80 – 81.

Mathews H, Wetzstein HY (1993). A revised protocol for efficient regeneration of somatic embryos and acclimatization of plantlets in pecan, *Carya illinoensis*. Plant Sci. 91: 101 – 103.

Menzel CM (1983). The control of floral initiation of lychee: A review. Scientia Horticulturae 21: 201 – 215.

Merkle SA (1995). Strategies for dealing with limitations of somatic embryogenesis in hardwood trees. Plant Tissue Culture and Biotechnol. 1(3): 112 – 121.

Merkle SA, Wiecko AT (1989). Regeneration of *Robina pseudoacacia* via somatic embryogenesis. Can. J. Res. 16: 420 –422.

Merkle SA Wieko A, Watson-Pauley BA (1991). Somatic embryogenesis in American Chesnut. Can.J. Res.21: 1678-1701.
Merkle SA, Watson-Pauley BA (1993). Regeneration of bigleaf magnolia by somatic embryogenesis. Hort. Sci. 28: 672-673.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15: 508-509.
Murashige T (1974). Plant propagation through tissue culture. Annual Review of Plant Physiol. 25: 135 – 166.
Parrott WA, Dryden G, Vogt S, Hilderbrand DF, Collins GB, Williams EG (1988). Optimisation of somatic embryogenesis and embryo germination in soybean. *In Vitro* Cell De. Biol. 24: 817 - 820.
Saas JE (Ed) (1967*). In:* Botanical Micro techniques pp 12 – 75, 3rd Ed, Oxford and IBH Publishing, Calcutta, India.
Wetzstein HY, Ault JR, Merkle SA (1989). Further characterization of somatic embryogenesis and plantlet regeneration in pecan (*Carya illinoensis*). Plant Sci. 64: 193 – 201.
Yu CH, Chen Z G (1998). Embryogenic suspension culture and protoplast isolation in litchee. Chinese J.Tropical Crops 19: 16 – 20.
Yu CH, Chen ZG, Lu LX and Lin JW (2000). Somatic embyogenesis and plant regeneration from litchi protoplasts isolated from embryogenic suspensions. Plant Cell, Tissue and Organ Culture 61: 51 – 58.
Zhou LN, Kuang ZS, Ma XJ, Chen JQ, Cao J (1996). The study of factors affecting somatic embryogenesis in young embryo culture of *Litchi chinensis*. J. of Agric. Biotech. 4: 161 – 165.