

Effect of Gelling Agent and Selective Sub-culturing on Hyperhydricity in Anther-Derived Coconut Embryos

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Abstract

Experiments were conducted to compare the liquid medium with the media solidified with agar or phytagel. Selective subculturing and use of the embryo maturation medium supplemented with higher concentration of phytagel (0.5%; w/v) were also tested for reducing the vitrified embryos. Modified Eeuwens Y3 medium was used as the basal medium. By culturing the anthers on the medium solidified with phytagel (0.25%; w/v), direct embryo formation (86.7%) and embryo conversion (21.5%) were significantly increased. Plant regeneration efficiency of anther derived embryos or calluses developed in the liquid culture medium was extremely low (2.4%). Vitrification was further reduced by incorporating 0.5% (w/v) phytagel into the embryo maturation medium. Highest plant regeneration efficiency was obtained by exposing the embryos to 0.5% (w/v) phytagel for 21 days, which reduced vitrification by 42%. Furthermore, selective subculturing of the embryos was effective for reducing vitrification.

Key words: Anther culture, *Cocos nucifera*, Phytagel, histology, doubled haploid

Abbreviations: 2,4-dichlorophenoxyacetic acid (-2,4-D), 6-benzylaminopurine – (BAP), Gibberelic acid – (GA₃), naphthol blue black – (NBB)

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Introduction

Coconut (*Cocos nucifera* L.), an cross pollinating species, is highly heterozygous which is a major constraint in conventional breeding. Availability of homozygous lines would improve the efficiency of generating new cultivars through breeding programs. Pollen embryogenesis allows single-step development of completely homozygous lines from heterozygous parents. A consistent production of embryos or calluses in cultured anthers of coconut has been reported (Perera et al., 2008). The most critical factors that are essential to induce microspore embryogenesis are pollen developmental stage, anther pre-treatment, the properties of the basal culture medium and the genotype of the donor plant. Other factors such as anther density, anther orientation, type and concentration of growth regulators affect the efficiency of the microspore embryogenesis through anther culture. All these factors have already been studied for coconut anther culture (Perera et al., 2008, 2009). Even though some of the embryos or calluses have developed into plantlets, overall plant regeneration efficiency remains very low (less than 10%; Perera et al., 2009).

One of the major drawbacks that limit the success rate of the anther culture technique is the occurrence of vitrification in anther-derived embryos (Perera et al., 2009). Regeneration frequency of the embryos is largely reduced by spontaneous termination of the embryogenic potential in vitrified embryos. Hyperhydricity (Debergh et al., 1992), previously known as vitrification, can result from a number of stress reactions caused by abnormal environmental conditions imposed simultaneously *in vitro* (Chen and Ziv, 2001). These include accumulation of ethylene, CO₂ and other volatile components (Lai et al., 2005), excess cytokinin (Leshem et al., 1988; Ivanova and van Staden, 2008) and ammonium ions (Ivanova and van Staden, 2008) in the culture medium. According to Leshem et al. (1988), hyperhydricity (also referred to as glassiness and vitrification) is an abnormal development of cultured shoot tips into translucent stunted shoots and thickened, turgid and brittle leaves with a glassy appearance.

In the present study the hyperhydric structures were characterised by brittleness, water soaked glassy appearance and tissue breakage. These characteristics could be identified after subculturing the anther-derived embryos into the hormone free medium (embryo maturation medium). This is one of the major drawbacks limiting the success rate of the protocol for doubled haploid (DH) plant production in coconut.

State of the culture medium plays an important role in anther culture technique. Liquid cultures have been most commonly used for anther culture of many crops such as *Triticum aestivum* L. (Zhou and Konzak, 1989) *Linum usitatissimum* L (Kurt and Evans, 1998). The beneficial effects of liquid culture media have been attributed to both physical and chemical advantages such as direct contact with the nutrients and exogenous hormones, quick dispersion of toxic substances released to the medium by dying and dead anthers, and high efficiency of anther plating (Guo et al., 1999). However, the activity of the major proportion of anther-derived embryos or calluses produced in liquid culture media has been terminated due to vitrification (Perera et al., 2009). The total water potential of the medium and the availability of water are major factors in vitrification (Leshem et al., 1988).

The present study was undertaken to test the effect of the solid and liquid androgenesis induction medium on the formation of hyperhydric anther-derived structures. Furthermore, the effect of increased levels of gelling agent for different durations was studied to identify the optimum exposure of the embryos to get minimum hyperhydricity without affecting the regeneration efficiency. Selective subculturing of the embryos (according to the appearance) was also studied to identify the characteristic features of the embryos at the correct transferring stage. A histological study was conducted to identify the hyperhydric structures at cellular level.

Materials and Methods

Rachillae that consisted of male flowers containing the microspores at late uni-nucleate stage (see Perera et al., 2008) were collected from an adult coconut palm (*Cocos nucifera* L.) cv Sri Lanka Tall, grown in a plantation located at Bandirippuwa estate, Coconut Research Institute, Sri Lanka. Middle portions of each rachilla were given heat shock incubation at 38°C for 6 days. Pre-treated anthers were excised from the male flowers and the pooled anthers were surface sterilized by immersion in 2% (v/v) Clorox® solution (prepared using 5.25% commercial Clorox) with two drops of liquid detergent (teepol; approximately 40 µl) for 12 minutes, followed by four rinses with sterilized water under aseptic conditions. Modified Eeuwens Y₃ medium (Karunaratne et al., 1985) was used as the basal medium for androgenesis induction and it was supplemented with 100 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 9% (w/v) sucrose. After adjusting the pH to 5.8 with NaOH, 0.1% (w/v) activated charcoal (BDH acid washed, UK) was added to the media. Media were autoclaved at 121°C for 20 min after adding the solidifying agents (as describe in the following experiments). Media were dispensed into the Petri plates (100 x 10 mm, each containing 25 ml) under aseptic condition. After inoculating the anthers, Petri plates were incubated in the dark at 28°C for eight months.

At monthly intervals mature anther-derived calluses and embryos were sub-cultured onto embryo induction medium, solidified with 0.25% (w/v) phytagel and supplemented with 66 µM 2,4-D, followed by maturation medium (devoid of any growth regulators) and then regeneration medium [containing 5 µM 6-benzylaminopurine (BAP), 0.1 µM 2,4-D and 0.35 µM gibberellic acid (GA₃)], unless otherwise mentioned. The chemicals used in media preparation were supplied by Sigma (USA) unless otherwise mentioned.

All the cultures were maintained in the dark at 28°C until the shoot was elongated after conversion of the embryo. The converted embryos were exposed to light (16 h photoperiod; PAR intensity of 25 µmol m⁻²s⁻¹)

and maintained at 28°C until acclimatisation was achieved.

Effect of liquid and solid media on androgenesis induction

Media gelled with either with 0.65% agar (Park, UK) or 0.25% phytagel (w/v) (Sigma, USA) were compared with the liquid androgenesis induction medium. Solidifying levels were established by preliminary experiments (data not shown) to get similar gelling strength. Thirteen Petri plates, each containing 10 anthers, were used for each treatment (in three repeats). After three months of culture initiation all the calluses and embryos were counted. The androgenic frequency was calculated as the number of calluses or embryos produced per 100 anthers cultured. The performance of these structures in terms of the rates of embryo conversion and vitrification were recorded at each sub-culturing stage in the plant regeneration protocol.

Effect of selective sub culturing of the embryos on plant regeneration

Anther-derived embryos were categorised into two groups according to their external appearance as opaque or translucent. In each group the embryos varied 0.5 – 3 mm length. They were cultured onto the embryo maturation medium and maintained for one month. At the end, the number of hyperhydric embryos, characterised by brittleness and water soaked glassy appearance followed by tissue breakage, was recorded in each category. A total of 41 and 49 embryos were used from each category, translucent and opaque respectively, for the experiments repeated two times.

Effect of phytagel concentration on plant regeneration

A preliminary study was conducted to test the effect of higher concentrations of phytagel in the embryo maturation medium for the occurrence of hyperhydricity. Anther-derived embryos were subcultured onto two embryo maturation media containing either 0.25% (w/v) or 0.5% (w/v) phytagel. After one month, the number of hyperhydric embryos in each medium was recorded. A total of 112 and 80 anther-

derived embryos were used for each 0.25 and 0.5% (w/v) phytagel containing medium respectively in three replicates.

To determine the duration that the embryos were required to be exposed to 0.5% (w/v) phytagel medium, anther-derived embryos were maintained in the embryo maturation medium with 0.5% (w/v) phytagel for 3, 7, 14 and 21 days. Then they were subcultured onto the same medium, supplemented with the normal phytagel level {0.25% (w/v)} for the rest of the period to complete a month. Subculturing on the maturation medium with 0.25% (w/v) phytagel for one month was used as the control treatment. After one month, the hyperhydric embryos for each medium were recorded. A total of 40 -45 embryos were used for each treatment in two replicates.

Histology

Histological studies were conducted to identify differences between the hyperhydric and non-hyperhydric structures. The samples were fixed in FAA solution (50% ethanol: 10% Glacial acetic acid: 10% Formaldehyde, 18:1:1) for 72 hr. Then they were dehydrated in a graded series of 50%, 70%, 95% and 100% ethanol (v/v) for 2 hr in each solution followed by an ethanol – butanol solution (1:1 v/v) and then 100% butanol. Paraffin impregnation was done with 100% butanol / melted paraffin wax (CDH New Delhi, melting point at 59°C) solution (1:1) (v/v) for 3 days followed by 100% Paraffin wax for 3 days at 60°C. Infiltrated samples were sealed in plastic moulds with fresh, melted paraffin wax. Wax embedded samples were sectioned at 5 µm in thickness by using a manual microtome (OSK 9770 Rotary Microtome, Japan). Prior to staining, wax was removed from the slides using sulphur free xylene. De-waxed slides were stained with protein- specific naphthol blue black (NBB). Prepared slides were observed under a light microscope (LEICA CME, Germany) equipped with a camera.

Statistical Analysis

The data were analysed using SAS statistical package (SAS Institute USA, 1999). Chi-square or maximum likelihood analysis of

variance was conducted using the Proc CatMod procedures of PC-SAS according to Compton (1994). Treatment means were compared using orthogonal contrast coefficients.

Results and Discussion

Even though the success rate of androgenesis induction in woody species has achieved limited progress (Germana, 2006; Peixe et al., 2004), a consistent androgenesis could be observed in cultured anthers of coconut. Formation of both embryos and calluses could be observed under similar culture conditions as described by Perera et al. (2008) and the results were also comparable with the anther culture of cucumber (Ashok-Kumar and Murthy, 2004). The embryos and calluses that emerged through the anther lobe were easily dislodged, which suggests the absence of any physical connection with the somatic tissues of the anther wall.

Effect of liquid and semi solid culture media on androgenesis induction and embryo conversion

Pollen embryogenesis could be successfully induced in cultured anthers of coconut in all three media tested. Induction of embryos or calluses could be observed after three months of culture initiation and this continued up to eight months.

Even though the objective of altering the physical state of the culture medium was to reduce the occurrence of vitrification a significant difference among the three tested media was not observed. The vitrification rates were observed as high as 62.2%, 70.5% and 83.1% calli or embryos vitrified in the media solidified with phytagel, agar and liquid media respectively (Table 1).

A significant difference was observed in the aspects of the calluses or embryos produced after sub-culturing anther-derived embryos/calluses in the above three media onto maturation and regeneration media (Table 1). A significantly higher percentage of embryos (86.7%) was produced in the medium solidified with phytagel when compared to the other two media (49.2%; $G^2=17.01$; $p<0.001$ and 53.6%;

$G^2=17.69$; $p<0.001$). When the frequency of direct embryo formation was higher, the conversion rate was also higher (Table 1). The highest embryo conversion frequency of 21.5% was also attributed to the medium solidified with phytigel, being higher than the agar solidified media (9.8%; $G^2=7.49$; $p<0.01$) and liquid media (2.4%; $G^2=13.99$; $p<0.001$). Anthers that remained floating on the liquid medium gave rise to embryos while the calluses produced by the submerged anthers became vitrified and later turned brown.

Vitrification was observed in the different stages of development such as embryos (Fig 1a) calluses (Fig 1b) and converted embryos (Fig 1c, d). Water soaked appearance is the major distinguishable character to differentiate these structures from normal ones (Fig. 1 and 2). When the vitrified callus expands the tissue break is observed within the structure (Fig. 1b). Histological studies clearly indicated the cellular differences in the vitrified structures over the normal ones (Fig. 3). The empty parenchyma cells with large intercellular spaces and tissue break within the structure (Fig. 3 a, c) was observed as the common features in vitrified callus or embryos. The excess water has been shown to be located in the intercellular spaces (Kevers and Gaspar, 1986) reducing cell to cell adhesion which results in breakability of the organs (Gaspar et al., 1995). Lack of vascular bundles further indicated the occurrence of vitrification within the converted embryos (Fig. 3 c) but not within the normal ones (Fig. 3 b). Leshem (1983) indicated that hyperhydric malformation was due to the deficiency in organisation of certain tissues, such as the vascular bundles in carnation.

The physical state of the culture medium is an important factor to be considered in the development of anther culture protocols for many crops. Debergh et al. (1992) indicated that the choice of gelling agent is very important for plant *in vitro* regeneration. In the previous studies liquid cultures have been used for the induction of androgenesis (Perera et al., 2008; 2009). However, due to poor regeneration capacity of the calluses or embryos produced in liquid media, the performance of anthers in both

liquid and solidified media was tested in the present study. The frequency of direct embryo formation was lower in a liquid medium (49.2 %) whereas it could be significantly increased by solidifying the androgenesis induction medium with phytigel (Table 1). The anthers in the liquid media sank to the bottom of the petri plate, and therefore were directly exposed to a higher concentration of 2,4-D that favours callogenesis. However, when the anthers were cultured, with abaxial surface up on solidified medium in direct contact with the medium, callogenesis could be minimised and embryo formation could be increased. Thus the emerging structure developed into an embryo instead of converting into a callus (Perera et al., 2008b). In timothy grass (Guo et al., 1999), wheat (Zhou and Konzak, 1989) and citrus (Chaturvedi and Sharma, 1985), a higher frequency of calluses was obtained in liquid media when compared to solidified media. In the present study, anthers gave rise to embryos only when the anthers remained floating in liquid media (data not shown). A similar observation was made by Chaturvedi and Sharma (1985) in citrus anthers cultured in liquid medium. Several research groups have also observed high yields of microspore calluses in liquid media but their regeneration capacity was lower when compared to agar-solidified media (Zhou and Konzak, 1989). Vitrification was the major drawback observed in coconut anther culture (Perera et al., 2009) and Zhao et al. (2006) also reported that a large portion of regenerated shoots had prominent symptoms of vitrification in wheat. The observations made on the liquid medium of the present study are comparable with the previous studies on coconut anther culture (Perera et al., 2008). Chen and Ziv (2001) indicated that one of the problems encountered in liquid cultures is the phenomenon of hyperhydricity, which results in malformed plants that cannot survive transplanting *ex vitro*.

Plant Regeneration

Effect of selective sub culturing of the embryos

The embryos at different developmental stages, as well as callus, could be observed on the same anther (Figure 2a). The maturity of

Table 1. Effect of liquid and semi solid culture media on the androgenesis induction and aspects of the anther-derived embryos or calluses after subculturing onto maturation medium (modified Eeuwens Y3 medium devoid any growth regulators) and regeneration media

Culture medium	Total structures produced (%) ¹	Percentage of embryos ²	Percentage of converted embryos ³	Percentage of vitrified structures ⁴
Liquid medium (T ₁)	95.4 ±19	49.2 ±10	2.4 ±1	83.1 ±15
Medium solidified with agar (T ₂)	86.2±24	53.6 ±9	9.8 ±2	70.5 ±19
Medium solidified with phytagel (T ₃)	103.9 ±28	86.7 ±23	21.5±5	62.2 ±19
MLAOV	NS	25.88***	18.68***	NS
Contrastes	Chi-square			
T ₁ VS T ₂	NS	NS	3.98*	NS
T ₁ VS T ₃	NS	17.01***	13.99***	NS
T ₂ VS T ₃	NS	17.69***	7.49**	NS

¹ Total number of embryos or calli produced per 100 anthers in the androgenesis induction medium.

² Percentage of the direct embryos calculated from the total structures produced

³ Percentage of the converted embryos in the embryo maturation and regeneration media¹

⁴ Percentage of the vitrified structures from the total structures produced

Mean values of 13 replicates (n=10) and their standard error is stated.

*, **, *** represents the significance at the probability levels of 0.05, 0.01 and 0.001 respectively.

NS refers to non significance.

Figure 1. Aspects of vitrification in anther-derived structures of coconut a. Anther (an) - derived embryo (e) (Bar=388 µm). Note the watery appearance in embryo. **b.** Callus (Bar=1.4 mm). Note the tissue break (Br) when expanding the callus **c.** Partially hyperhydric (V) converted embryo. Non- hyperhydric (NV) area of the embryo is clearly recognized over the water soaked hyperhydric area (Bar=1.1 mm). **d.** Fully hyperhydric converted embryo Note the germination point (Gp) in the haustorium (Bar=1.9 mm)

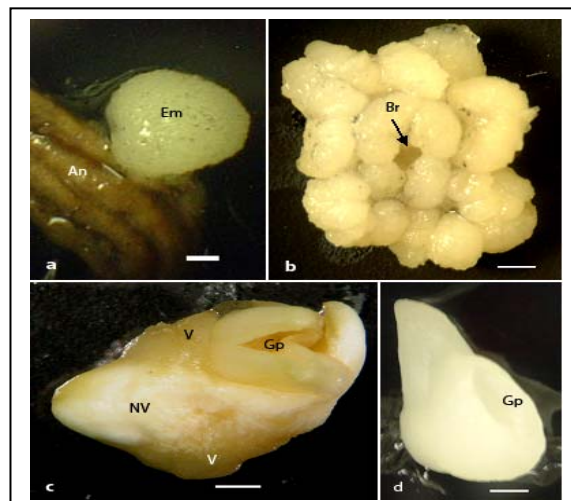


Figure 2. Plant regeneration from anther derived embryos and calluses in coconut

a. An anther (*an*) bearing a callus (*ca*) and direct embryos (*Bar*=2.5 mm). **b.** A converted embryos (*Bar*=2 mm). Note that the shoot (*st*) is emerging through the shoot point (*sp*) of the haustorium (*ha*) and the root (*rt*) development. **c.** Further development of the converted embryo (*Bar*=2 mm). Note that the root system is developed well

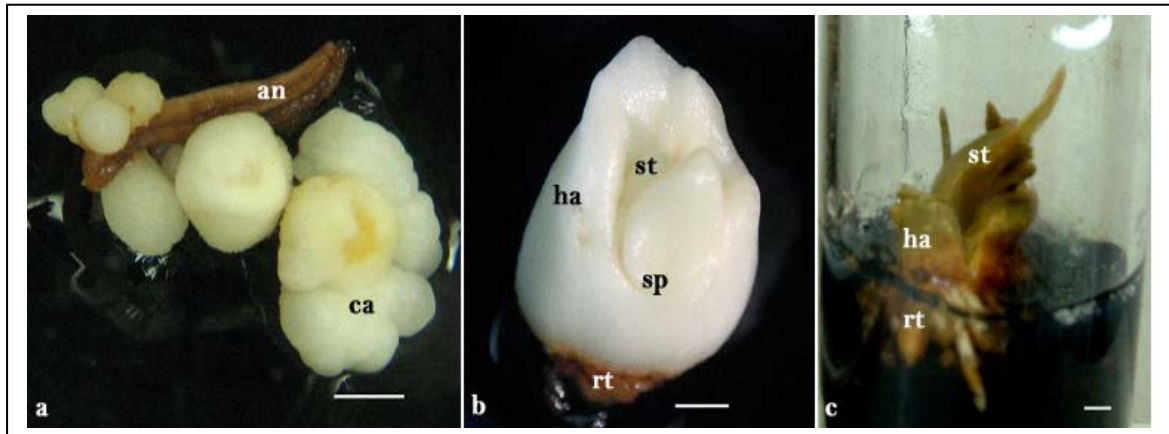
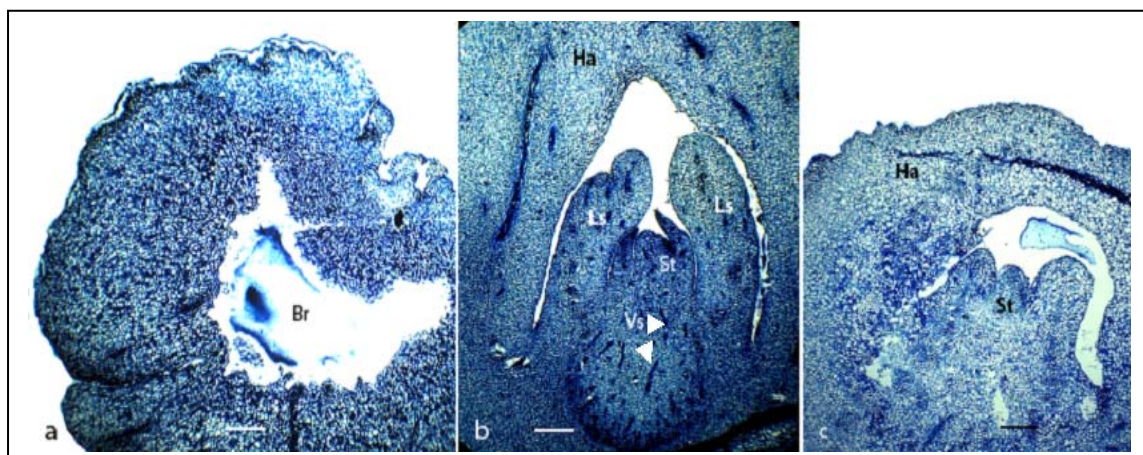


Figure 3. Histological aspects of vitrified anther-derived structures. **a.** A hyperhydric callus (*Bar*=728 μ m). Large intercellular spaces and the tissue break (*Br*) is prominent. **b.** Normal embryo (*Bar*=555 μ m). The developing shoot (*St*) is enclosed by the haustorial tissue (*Ha*) and the leaf sheaths (*Ls*). Vascular bundles (*Vs*) are concentrated around the shoot area (arrow heads). **c.** Hyperhydric shoot containing embryo (*Bar*=672 μ m). Note lack of vascular bundles, tissue breaks and the large intercellular spaces



embryos was determined based on their shape and colour. The mature embryos were white or opaque and triangular or round shape whereas the immature embryos were translucent and round in shape. Further development of these two categories of embryos in the embryo maturation medium indicated a significant reduction of the frequency of vitrification in opaque embryos (14%) over translucent embryos (39%) (Figure 4; $G^2=6.73$; $p<0.01$). This results show the importance of identifying the correct maturity stage of the embryo for subculturing onto the maturation medium in order to get a higher frequency of plant regeneration. Conversion of the appearance of anther-derived embryo from translucent to opaque, demonstrates the maturation of the embryo. The low vitrification in opaque embryos might be attributed to the differentiation of the periphery cells of the embryos. Embryos containing the differentiated cells could have more potential for regeneration; however, selection is tedious and difficult when handling numerous anther-derived embryos.

Effect of a higher concentration of phytigel

Results of the preliminary study clearly revealed that the occurrence of vitrification could be reduced by half (from 35% down to 16%), by subculturing the embryos onto embryo maturation medium supplemented with 0.5% phytigel compared to the control of 0.25% phytigel (Figure 5; $G^2=7.82$; $p<0.01$). Doubling the agar concentration decreased substantially the number of hyperhydric shoots in sunflower (Abdoli et al., 2007). Berrios et al. (1999) mentioned that the medium must be firm enough to support the explants, but if the rigidity is too high it may prevent adequate contact between the medium and the tissue. However, 0.5% level of phytigel provided a sufficient contact with the embryos. Some embryos developed the root and shoot point simultaneously (Figure 2b). Shoots emerged through the shoot point of the mature embryos (Figure 2c) and developed into complete plantlets by continuous subculturing onto the regeneration medium containing 5 μM BAP, 0.1 μM 2,4-D and 0.35 μM GA₃.

The duration of exposure to the medium containing 0.5% phytigel significantly reduced the occurrence of vitrification in the subcultured embryos (Table 2: $G^2=15.21$; $p<0.01$). Exposure to embryo maturation medium with 0.5% phytigel for 7 and 14 days the rate of vitrification could be reduced up to about 40% ($G^2=5.01$ and $G^2=5.08$ respectively; $p<0.05$). The lowest vitrification (24%) was recorded after 21 days ($G^2=13.88$; $p<0.001$) as compared to the control of 66% (Table 2). Formation of single, double or multiple shoots was observed in the converted embryos. Single and double shoots were healthy, whereas multiple shoots became weak after separation from the cluster.

In the present study 371 embryos were produced and 43 embryos converted giving rise to plantlets. The research on coconut anther culture is continuing and the conditions developed are very effective in enhancing the efficiency of microspore embryogenesis.

The phenomenon of vitrification/hyperhydricity is a frequent problem in tissue culture limiting the growth and multiplication *in vitro* and establishment of ex vitro (Debergh et al., 1992). Losses up to 60% of cultured shoots or explants have been reported due to vitrification in carnation micropropagation (Piqueras et al., 2002). The frequency of the production of dihaploid coconut plants via anther culture technique is mainly hampered by the phenomenon of vitrification. Therefore, the identification of the factors responsible for vitrification is an important step in order to achieve a successful use of this technique for a coconut breeding program. Occurrence of vitrification could be reduced substantially by subculturing the embryos onto the embryo maturation medium supplemented with a higher phytigel level (0.5%; w/v). Increasing the phytigel concentration, a water stress condition is created in the medium. The excess water absorption into the embryos could be controlled by suppressing the water availability in the culture medium that could lead to reducing the conversion of embryos into vitrified status. A negative correlation could be observed between the duration of the embryos exposed to the

Figure 4. Effect of type of the embryo on hyperhydricity (Total number of embryos used for each treatment are given in parenthesis)

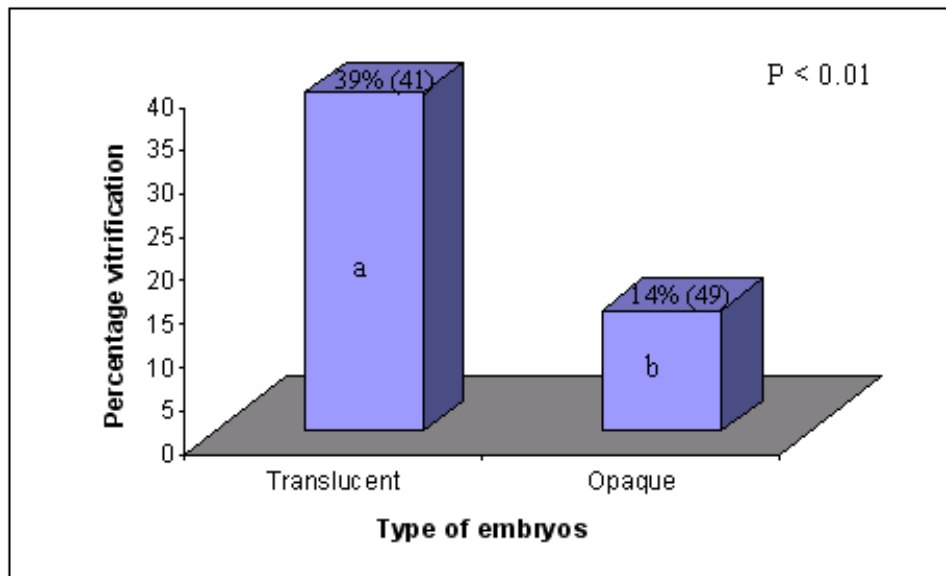


Figure 5. Effect of supplemented concentration of phytigel in to the embryo maturation medium on hyperhydricity (Total number of embryos used for each treatment are given in parenthesis)

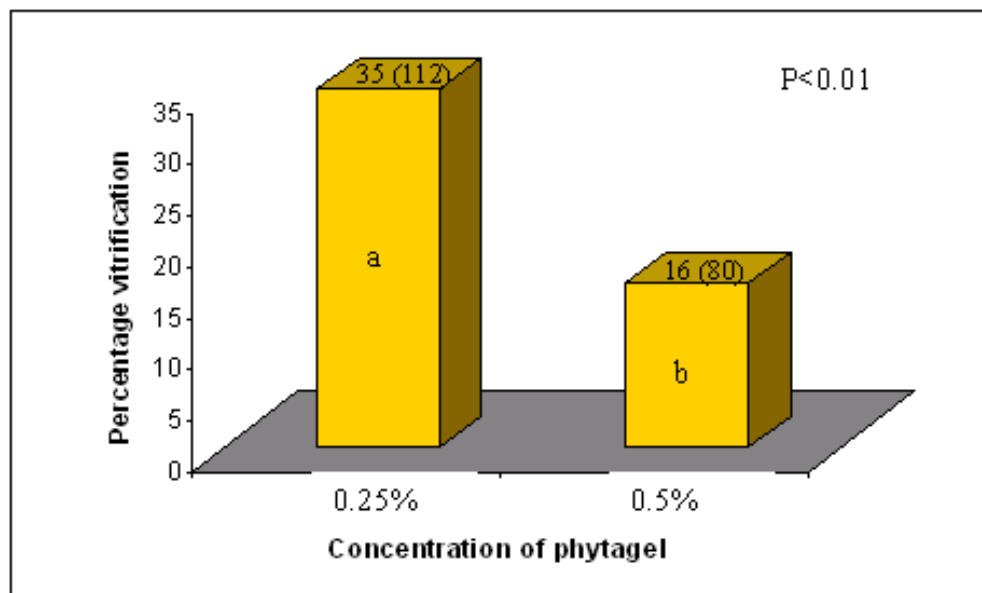


Table 2. Effect of the duration of the embryos exposed to higher phytagel level on vitrification, germination and formation of single/double shoots

Days exposed to higher phytagel level	Total embryos cultured ¹	Percentage vitrification ²	Percentage conversion ³	Formation of single/double shoots
0 (T ₁)	38	66	8	0
3 (T ₂)	41	51	12	40
7 (T ₃)	42	41	17	100
14 (T ₄)	40	40	18	71
21 (T ₅)	46	24	22	70
MLAOV Significant contrasts		15.21** T ₁ vs T ₃ (5.01*) T ₁ vs T ₄ (5.08*) T ₁ vs T ₅ (13.88***)	NS	NS

¹ Total number of embryos used for each treatment in three repetitions (for each replicate 10-15 embryos was used for each treatment)

² Out of the total embryos the percentage of vitrified ones were calculated

³ The percentage of converted embryos resulted in maturation and regeneration medium

higher phytagel levels and the occurrence of vitrification.

In the present study the physical status of the androgenesis induction medium was optimized. The drawback of lower regeneration efficiency in liquid culture medium was successfully overcome by using the medium solidified with phytagel. The frequency of direct embryo formation was significantly increased in phytagel solidified medium (Table 1). The occurrence of embryo vitrification was reduced by selective subculturing and increasing the phytagel concentration in the embryo maturation medium. Regeneration efficiency was increased substantially controlling the vitrification in anther-derived embryos.

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