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EFFICIENT INDUCTION, PROLIFERATION AND REGENERATION OF RUBBER TREE (*HEVEA BRASILIENSIS* MUELL. ARG) VIA SOMATIC EMBRYOGENESIS

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ABSTRACT

An efficient protocol has been established to induce and proliferateembryogenic callus and regenerate plants using anther tissue culture of rubber tree (*Heveabrasiliensis* Muell. Arg), clone *Reyan 7-33-97*. The process of embryo-development from somatic embryos was divided into five stages: embryogenic callus, globular embryos, heart-shaped embryos, torpedo embryos and cotyledon embryos. Efficiency of proliferation and regeneration using embryonic tissues at different physiological stages was determined. A number of conclusions were reached. Friable embryogenic callus was the most suitable tissue for direct long-term proliferation by subsequent subcultures. The rates of proliferation and embryo development did not decrease after two years in tissue culture. Heart-shaped embryos and early embryogenic forms, including embryogenic callus and globular embryos, could proliferate on the media used in subculturing. After repeated proliferation, these tissues could be induced to form mature embryos in 1 - 3 months using MS2 embryogenic tissue induction media. Torpedo embryos and cotyledon embryos could not directly proliferate on MS3 media, but highly embryogenic calli could be induced from them using embryogenic callus induction media (MS4). The highly embryogenic calli could further be induced to form a large number of embryos on MS5 media to achieve an indirect but efficient proliferation. By selecting the same physiological stage of embryonic tissue from the material to be subcultured, the process of somatic embryo development could be controlled to ensure uniform development.

Keywords:*Heveabrasiliensis**, e*mbryogenic tissue, somatic embryo, anther culture.

Abbreviation: MS**:** Murashige and Skoog basal medium; 2,4-D: 2, 4-Dichlorophenoxyacetic acid; KT: Kinetin; 6-BA: 6-Benzyladenine; NAA: Naphthaleneacetic acid; GA3: Gibberellic acid; ABA: Abscisic acid; ZT: Trans-zeatin.

INTRODUCTION

Heveabrasiliensis is the most important commercial source of natural rubber. It is a perennial tree crop with a traditional long breeding cycle of about 30 years. The integration of specific desired characters through conventional breeding is both time-consuming and labor-intensive. In particular, since around 1970, China, Malaysia, India, Indonesia, Sri Lanka and other countries have devoted a lot of manpower, material and financial resources to launch extensive research on the tissue culture of rubber trees and some progress has been made (Nayanakantha *et al.*, 2007 and Venkatachlam *et al.*, 2007). Somatic embryogenesis is one of the powerful tissue culture techniques for mass propagation of elite *Hevea* clones. In recent years, the utilization of Somatic embryogenesis also opens up new avenues for molecular farming through genetic transformation. *Hevea* somatic embryogenesis was first developed in China and Malaysia, using the anther wall as initial mother tissue explants (Venkatachlam *et al.*, 2007). Firstly, the diploid organization was obtained from anther culture (Satchuthananthavale *et al.*, 1972 and Satchuthananthavale, 1973). Then an embryoid body was obtained from anther culture, but it could not be regenerated (Paranjothy *et al.*, 1975 and 1976). China first obtained regenerated rubber tree plants via somatic embryos from anther culture (Wang *et al.*, 1978). The cultivated area of rubber trees derived from somatic embryos was already approximately 70 hectares in 2001(Wang *et al.*, 2001 and Chen *et al.*, 2002). Rubber tree plants developed through somatic embryos have their own roots and are free from the adverse effects of using rootstock and restore its juvenile characteristics. Therefore, the plants are fast growing, have a higher yield of rubber gum and strong resistance characteristics in comparison with the traditional budding seedlings (Wang, *et al.*, 2001 and Chen *et al.*, 2002). The size of plants derived from somatic embryos was 9 % to 20 % larger than the traditional budding seedlings of the same species and production was increased by 20 % to 50 % (Wang *et al.*, 2001 and Chen *et al.*, 2002).

However, the low rate of embryo formation, together with the problems associated with abnormal embryos directly derived from anther or inner integument callus hinder the commercial production of *Hevea* plants

from somatic embryos (Wu *et al.*, 1997 and Carron *et al.*, 1998). The maximum rate of embryo production reported in China is 22.7 % from anther or inner integument callus (Xiao*et al.*, 1994). In fact, somatic embryogenesis needs further improvement for the commercial application of the system. There has been an ever-increasing interest in the development of plantlets through somatic embryogenesis especially by the advent of genetic transformation (Venkatachalam *et al.*, 2007). French researchers from CIRAD reported obtaining friable embryogenic callus from inner integuments of young rubber tree fruit and the embryogenic characteristics were maintained in culture(Montoro *et al.*, 2003 and Blanc*et al.*, 2006). In order to find out the most suitable explant for genetic transformation, an experiment was performed with different explants such as immature anther and ovule, sixty day old callus and embyrogenic callus derived from immature anther and ovule explants and maximum transformation frequency(62%) was obtained with anther derived embryogenic callus (Rekha *et al.*, 2006). On the other hand, the study finds that rubber somatic embryo is divided into nine types and dicotyledonous embryo is the most normal rubber tree somatic embryo state (Tan *et al.*, 2011 and Li *et al.*, 2012). The primary abnormal and normal embryos derived from anther of rubber trees were used for secondary embryogenesis (Hua *et al.*, 2010 and Asokan*et al.*, 2002). The aim of the investigation reported here was to induce and proliferate all the stages of embryogenic tissues to promote *Hevea* somatic embryogenesis using anther as a promising tool for mass propagation and genetic engineering programs on the base of traditional *Heve*a anther culture, which has not been previously reported.

MATERIALS AND METHODS

Plant material and induction of embryogenic tissue: The embryonic tissues were induced from immature anther calli of clone *Reyan 7-33-97* collected from Hainan, China. The calli were initiated from immature anthers in a modified MS (Murashige and Skoog, 1962) medium (MS1) (Figure1a) containing 1.5 mg/L 2.4- D, 1 mg/L KT, 0.5 mg/L NAA (Table 1) (Chen *et al.*, 2002 and Zhao*et al.*, 2007). After 40～60 days on MS1 medium, the calli were transferred onto embryogenic tissue induction medium (MS2) containing 1 mg/L 6-BA, 1 mg/L KT, 0.3 mg/L NAA, 0.5 mg/L GA3, 0.1 mg/L ABA (Table 1). Cultures were maintained in the dark at 26ºC.

Proliferation of embryogenic tissues of different physiological stages: Because the development of *Hevea* anther somatic embryos from different stages were not synchronized, embryogenic tissues at friable callus, globular, heart-shaped, torpedo-shaped and cotyledon-shaped embryo stages were selected and separated and then subcultured once every 30 days for proliferation in embryogenic tissue proliferation medium (MS3) which contains 1.5 mg/L 2.4- D, 1 mg/L KT, 0.5 mg/L NAA (Table 1).

Induction of proliferated tissues into somatic embryos: Healthy proliferated tissues after 3, 6, 9 and12 months of subculturing were transferred back into embryogenic tissue induction medium (MS2) in order to observe their ability to form somatic embryos. The tissues were subcultured once every 30 days in MS2.

Induction of embryogenic calli from torpedo-shaped and cotyledon embryos: Somatic embryos induced from tissues derived from the torpedo-shaped embryo stage changed in white color. Healthy, white dicotyledonous somatic embryos were selected as explants for induction of embryogeniccalli by cutting them into slices 2 -3mm wide, 3 -5mm in length (Figure 1i). Slices were placed onto high embryogenic callus induction medium (MS4) which contains 1 mg/L 2,4-D,1 mg/L KT, 1mg/L NAA,1 mg/L ZT (Table 1, Figure 1j).

Induction of embryo calli into somatic embryos with high efficiency: In order to induce somatic embryos from embryogeniccalli (from Figure 1j to k), embryogenic calli (Figure 1j) derived from somatic embryo slices were placed onto embryogenic tissue induction medium (MS5) which contains 1 mg/L 6-BA, 0.5 mg/L KT, 0.1 mg/L NAA and 0.5 mg/L GA (Table 1). The efficiency of somatic embryo formation was determined with different induction times on MS5 medium. About 10 somatic embryo slices per plate were placed onto MS5 media for different lengths of time (20, 25, 30, 35, 40, 45, 50, and 55 days). After each incubation time the calli were transferred onto MS2 embryogenic tissue induction medium and the presence of embryos was checked after 1-2 months. At least 10 plates per time were used in this experiment.

Statistical analysis: Data, including the efficiency rates of embrygenic callus and embryo presented in tables 2 and 3, were analyzed using SAS 9.0 system for windows (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Proliferation of embryogenic tissues of different physiological stages: Embryogenic tissues were successfully induced from immature anther calli of clone *Reyan 7-33-97* about 40～60 days after being placed on MS1 medium (Figure 1a). The calli were then transferred onto embryogenic tissue induction medium (MS2). After about 3 months of culturing in the dark, deep yellow transparent friable calli were derived from browning anther callus (Figure1b). After further subculturing in

MS2 medium for 2 months, different types of embryos, such as, globular, heart-shaped, torpedo-shaped and cotyledon-shaped embryos appeared (Figure 1c). However, the development of these somatic embryos were not synchronized.

After 3 months of continuous subculturing, friable embryogenic callus, globular embryos, and heart-shaped embryos proliferated and maintained their original forms in MS3 media. The friable embryogenic callus was maintained as deep yellow transparent and friable callus, the globular embryos and heart-shaped embryos kept their original embryo shape after proliferation for 3 months on MS3 media The pointed arrow in Figure 1c indicates proliferating tissues. The embryos induced from torpedo and cotyledon embryo stages did not appear to proliferate in MS3 medium. The non-proliferating embryogenic tissue is shown in Figure 1c by the diamond headed arrow. Further, the wounded tissues of latter embryos do generate dedifferentiation calli on MS3 medium. The calli's ability of embryo formation will be tested further.

The initial proliferation rate of globular embryos and heart-shaped embryos was more than 300 % in the first subculture on MS3 medium. However, the ability of globular and heart-shaped embryos to proliferate was significantly decreased over the next four months of subculturing in this same medium. The tissues appeared to age, turning white and having a watery consistency. But the proliferation ability of friable callus remained almost the same, with the tissue appearing fresh and healthy for at least 9-months of subculturing (Figure 1d). After 9-months of subculturing on MS3 medium, the friable callus also showed some watering phenomena and their color began to change from yellow to white. However, the aging trend disappeared when the calli were transferred to MS2 medium for 1 or 2 months. When the friable callus was returned to MS3 it continued to proliferate and appeared to be in good condition again (Figure 1h).

To summarize, our results showed that during embryogenesis of rubber tree, friable embryogenic callus, globular embryo and heart-shaped embryo stages can be directly proliferated on MS2 media (Figure 1c). However, once the embryogenic tissues were developed into white torpedo embryos, the tissue can no longer be directly proliferated (Figure 1c). The differentiation of an embryoid needs a long time in traditional somatic embryo tissue culture of *Hevea*, normally 2 - 3 months. During this time replacing the media once or twice with fresh induction medium can significantly increase the rate of embryoid induction (Chen *et al.*, 1986). In our research, we found that the most effective period of medium replacement was when the embryogenic callus first appeared. The most direct effect of this measurement is a significant increase in the amount of embryogenic calli. The proliferation rate of these embryogeniccalli is not only significantly higher compared to the original embryogenic callus derived from anther, but also the rate remained as high after 4-years of subculturing. The proliferation test to different physiological stages of embryonic tissue showed that the friable embryogenic callus was the most appropriate long-term proliferating material. At present, we have subcultured for 4 years and proliferation is still good (Figure 1d -h).

Induction of proliferated tissues into somatic embryos: The proliferated tissues (Figure 1b) after 3 months in MS3 which were suitable for direct subculture were placed into embryogenic tissue induction medium (MS2). A large number of torpedo-shaped embryos appeared after 1 to 3 months in MS2 (indicated by the pointed arrow in Figure 1c). The order of different proliferated tissues developing into torpedo embryos was: heart-shaped embryo, globular embryo, friable embryonic callus. This showed that the proliferated tissues were still able to form embryos.

From the perspective of cytology and histology, most of the embryoid bodies remained at the globular or heart-shaped embryo stage during embryo induction and did not develop into a torpedo-shaped embryo (Wang, 2004). Some literature has reported that the abnormal embryogenesis can be controlled by adding ABA, GA3 in the media (Wu *et al.*, 1997; Chen *et al.*, 1986 and Wu *et al.*, 1994). But these methods did not fundamentally solve the problem. In this paper, we reported that, by selecting a certain physiological stage of embryonic tissues to subculture for proliferation, the embryogenic tissues could quickly be developed into a large number of torpedo-shaped embryos via an appropriate subculture. This is an important step to improve the success rate of embryo induction.

The proliferation time of 3 months was the most appropriate length of subculturing for embryogenic tissues of the globular and heart-shaped stages. Because the proliferated tissues tend to age after 4 months of consecutive subcultures, it is not ideal to use them for long-term proliferation. However, the friable embryogenic callus can be used for long-term proliferation, up to several years and their embryo induction rate was not significantly affected, based on our observations (Figure 1d to h).

Embryogenic recovery of friableembryogenic calli for long-term subculture: The friable calli after 3, 6 and 9 months of proliferation on MS3 media were placed on embryogenic tissue induction medium (MS2). The induction time (1-2 months) and induction rates of embryos from the friable calli was about the same for all three time points. However, the calli appeared to age with a watering phenotype and changed from yellow to white after 9 months of consecutive subcultures in MS3. This

aging trend was reversed when the calli were cultured in MS2 for 1 or 2 months, and then returned to proliferate in MS3. These recovered calli formed a large number of embryos in 1 - 2 months. In general throughout the embryo induction process, friable calli retained high proliferation efficiency and through manipulation of the media, older calli were able to regain the ability to proliferate.

Induction of calli derived from somatic embryo slices into embryos: Embryos after the torpedo-shaped stage which have changed to a white color could not directly manifest in embryogenic tissue proliferation medium (MS3), but the wounded tissues can be used to generate dedifferentiated callus in MS4 medium (improved MS3 medium) and differentiated calli have high ability into embryo.

Highly efficient embryogenic calli were directly induced about 1 month after the slices of white somatic embryos were placed onto the MS4 medium. The regeneration rates of the embryogenic callus derived from white somatic embryo slices (represented by the percentage of slices capable of forming embryogenic callus { Figure 2} and by the percentage of embryos formed from embryogenic callus { Figure 3}) were found to be linked to the number of days on the MS4 medium (Figures 2 and 3). The calli (Figure 1j) derived from slices after 20 - 60 days on MS4 medium with high embryo regeneration were selected for inducing embryos (Figure 1 k-n). The embryogenic calli derived from slices placed on MS4 medium for less than 20 days were few and not suitable for embryo induction. The embryogenic calli from slices after 40 days were plentiful but had low embryo induction, therefore, they should not be used for inducing embryos. The optimal time for the highest efficiency of embryo induction was between 20-40 days.

Effect of proliferation for promoting somatic embryo with synchronous development: Literature reports that rubber plants developed from somatic embryogenesis change back to the juvenile stage, like plants derived from zygotic embryos(Chen*et al.*, 2002). However, somatic embryo development was not synchronous and had no obvious time boundary as for zygotic embryos. Cell division of embryonic mother cells occured in the calli even in the cotyledon embryo stage (Wang, 2004).

On the proliferation medium MS3, friable embryogenic callus, globular embryos, and heart-shaped embryos proliferated and retained their original form (Figure 1c to g). The process of proliferation hindered further development of embryogenic tissue and promoted proliferation of the same type of embryogenic tissue. By selecting uniform embryogenic tissues from the same stage to subculture, the development of somatic embryos could be controlled and promoted to be synchronous (Figure 1c to g).

Table 1. Composition of media used for anther callus induction, embryogenic tissue induction, embryogenic tissue proliferation, efficient embryogenic callus induction and embryogenic tissue induction in *Heveabrasiliensis.*

Name of Constituents	Concentration (in milligrams perliter unless otherwise indicated)				
	MS1[a]	MS2[b]	MS3[c]	MS4[d]	MS5[e]
KNO3	1,600	1,600	1,600	1,600	1,600
NH4NO3	1,000	1,000	1,000	1,000	1,000
MgSO4·7H20	555	555	555	555	555
KH2PO4	425	425	425	425	425
CaCl2·2H20	330	330	330	330	330
Minor	MS	MS	MS	MS	MS
FeNaEDTA	37.5	37.5	37.5	37.5	37.5
Myo inositol	100	100	100	100	100
Glutamic acid	150	150	150	150	150
Glycine	2	2	2	2	2
Chlorhydric acid aneurin(VB1)	10	10	10	10	10
Chlorhydric acid compares Duo alcohol(VB6)	1	1	1	1	1
Nicotinic acid(VB5)	5	5	5	5	5
Folacin	0.5	0.5	0.5	0.5	0.5
D-biotin	0.5	0.5	0.5	0.5	0.5
Coconut water	5%	5%	5%	5%	5%
Malt sugar	–	20g/L	–	–	–
Sucrose	70g/L	50g/L	70g/L	70g/L	70g/L
Phytagel	2.2g/L	2.5g/L	2.2g/L	2.2g/L	2.5g/L
2,4-Dichlorophenoxyacetic acid(2,4-D)	1.5	–	1.5	1	–
Kinetin (KT)	1	1	1	1	0.5

6-Benzyladenine (6-BA)	–	1	–	–	1
α-Naphthaleneacetic acid (NAA)	0.5	0.3	0.5	1	0.1
Gibberellic acid (GA3)	–	0.5	–	–	0.5
Abscisic acid(ABA)	–	0.1	–	–	–
Trans-Zeatin (ZT)	–	–	–	1	–
pH	5.8	5.8	5.8	5.8	5.8

[a]**MS1:** anther callus induction medium; [b]**MS2:**embryogenic tissue induction medium; [c]**MS3:**embryogenic tissue proliferation medium; [d]**MS4:** high embryogenic callus induction medium; [e]**MS5:**embryogenic tissue induction medium

Figure 1 Induction, proliferation and regeneration of embryogenic calli and cotyledon somatic embryos from rubber tree anther tissue culture. a)calli induced from anthers. b)friableembrygenic calli from anther callus. c) Proliferation of embryogenic tissues showing different physiological stages including embryogenic callus, globular embryos, heart-shaped embryos, torpedo embryos and cotyledon embryos. A pointed arrow indicates the proliferating embyogenic tissues, and the diamond tipped arrow indicates unproliferating cotyledon embryos. d) friableembryogenic calli after proliferation for 1 year. e) tog) development of friable embryogeniccalli into embryos. The arrow in fig e) indicates embryo formation while fig f) shows multiple embryo formation and fig g) shows the developed embryo. h)friableembryogenic calli after 4-year proliferation. i)the slices of cotyledon somatic embryo from fig g. j) the high frequency of embryogenic calli derived from cotyledon somatic embryo. k)tom) the high efficiency of cotyledon somatic embryos derived from sliced calli of cotyledon somatic embryo. n)rubber plants regenerated from cotyledon somatic embryos.

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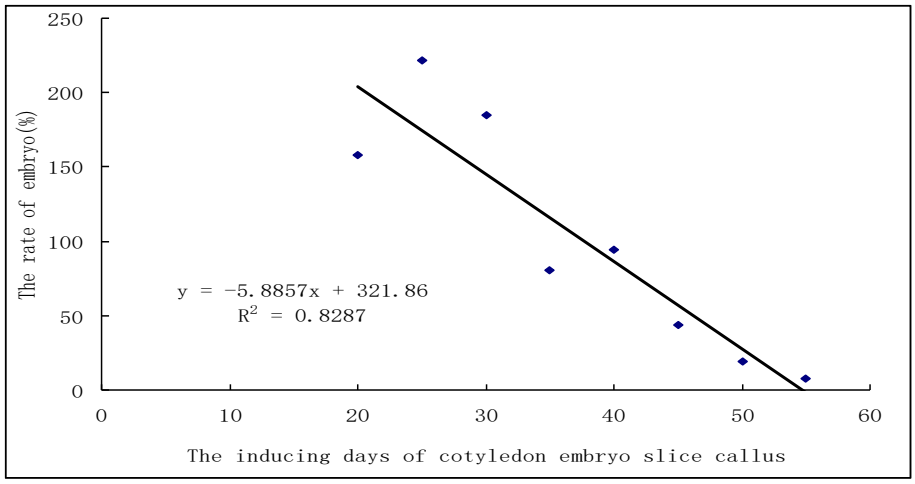

Figure 2. Relationship of induction rate of embryogenic callus with induction time (number of days slices of cotyledon embryo callus were maintained on embryogenic tissue induction medium, MS5)

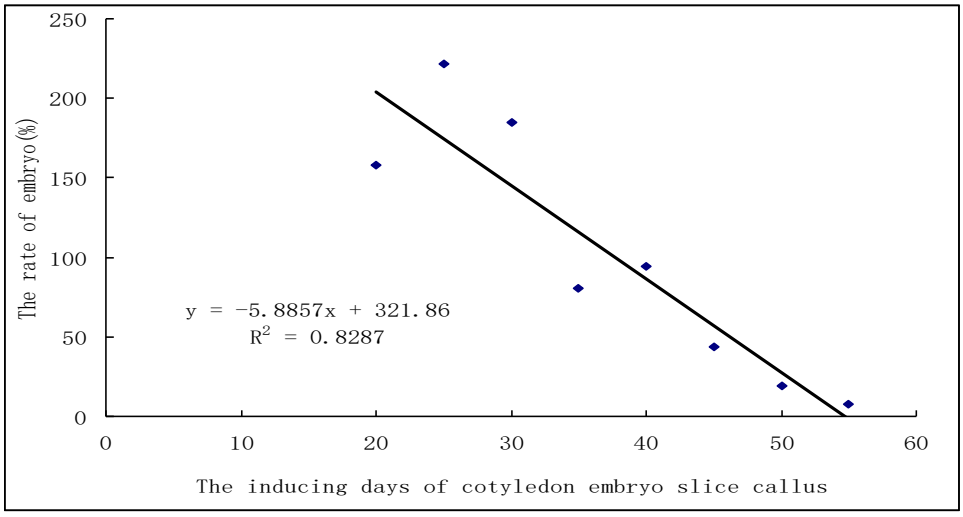

Figure 3. Relationship between induction rate of somatic cotyledon embryos with induction time (number of days slices of cotyledon embryo callus were maintained on embryogenic tissue induction medium, MS5).

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