Global Journal of Biotechnology & Biochemistry 1 (1): 07-15, 2006
ISSN 1990-9241

Optimization of *In vitro* Cyclic Somatic Embryogenesis and Regeneration of the Asian Cultivars of Cassava (*Manihot esculenta* Crantz) for Genetic Manipulation System

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Abstract: A prerequisite for genetic modification of cassava is a reliable transformation system. Establishment of a transformation system for cassava will open up the possibility for genetic manipulation and provide the information needed for handling and introduction of genetically modified cassava clones. Methods for efficient cassava transformation, including plant regeneration *in vitro*, gene delivery, selection of transformed tissues and recovery of transgenic plants, still need to be developed or improved in each cassava cultivar. The two Asian cultivars of cassava; KU 50 (widely grown cultivar with high cyanogens content for non-food purpose) and Hanatee (local cultivar with low cyanogens content for food purpose) were assessed for their ability to produce cyclic somatic embryogenesis from somatic cotyledons. Somatic embryogenesis was induced from different plant materials and various phytohormone concentrations in induction medium and was further developed to the green cotyledons in maturation medium. The developing young green cotyledons could be transferred to organogenesis medium for shoot emerging and plant regeneration. The various kinds of induction media were tested for the most suitable medium which give high cyclic somatic embryogenesis induction frequency in each cassava variety. Cyclic somatic embryogenesis induction frequency were evaluated 60 days after the cotyledons were cultured on each induction medium. The protocol for cyclic somatic embryogenesis induction from different ages of cotyledon had been optimized. The cyclic embryogenesis system can be routinely applied to produce the newly developing young green cotyledons for gene transfer research.

Key words: Asian cassava · cyclic somatic embryogenesis · regeneration

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a tropical crop grown for its starchy thickened roots, mainly in the tropics, for whom it is a staple food. Because of its tolerance to adverse environmental conditions, adaptation to poor soils and its flexible harvesting time, cassava is a vital component in food security in the developing countries. It provides a cheap source of dietary carbohydrate energy to people in the tropics ranking fourth after rice, sugar and maize and six among crops in global production. Cassava has a low protein and high cyanogen content, however, which significantly impact its nutritional value. In addition, cassava yields are often severely reduced by virus diseases and insect pests. It is difficult to address these problems using traditional breeding methods because the allopolyploid cassava plants show high heterozygosity and low natural fertility. Cassava biotechnology offers powerful tools to complement the traditional breeding methods and can extend the genetic pool of useful gene sources beyond the species. Transgene technology also offers the advantage of transferring single or even quantitative traits, without the problems of linkage encountered in traditional breeding. It has potential to alleviate poverty and promote the efficient use of cassava starch in the food industry and for non-food industrial purposes.

Tissue culture methods for cassava have been developed at CIAT for the virus elimination, vegetative propagation, conservation and exchange of germplasm collection [1]. Implementation of molecular and cellular genetic methods in breeding program requires an efficient

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regeneration system from somatic tissues. So far, the availability of regeneration systems has been a limiting factor in cassava genetic manipulation. Since seeds of cassava show high genetic diversity and sexual propagation results in high genetic segregation, immature tissues of *in vitro* vegetatively propagated plants will be chosen as explant. Cassava is a vegetatively propagated plant, therefore plant regeneration from vegetative tissues of well characterized, clonally propagated plants, is important [1, 2].

A prerequisite for genetic engineering of cassava is a reliable transformation system. Begin from year 1996, four groups reported successful production of transgenic cassava plants. The transgenic plants were established through *Agrobacterium*- and Biolistic-mediated transformation methods. The target tissues were either embryogenic suspensions [3, 4] or cotyledons from somatic embryos from which plants were regenerated through organogenesis [5] or through embryogenesis [6, 7]. Since then improved transformation and regeneration of transgenic cassava plants expressing either antibiotic or non-antibiotic selection markers has been reported by several laboratories [8-11]. However, so far there was no report related to genetic transformation of the Asian cultivars of cassava. Since the availability of regeneration systems has been a limiting factor in each cassava cultivar, therefore the two cultivars of widely grown in Asian countries of cassava; KU 50 (high cyanogens content for non-food purpose) and Hanatee (low cyanogens content for food purpose) were assessed for their ability to produce cyclic somatic embryogenesis from somatic cotyledons. The cyclic embryogenesis system can be routinely performed to produce the newly developing young green cotyledons for further optimization of selective condition and gene transfer research. The generation of genetically modified cassava clones should offer a way to increase the use of cassava starch in the food and feed industry and for non-food industrial purposes.

MATERIALS AND METHODS

Plant materials: The axenic culture of the 2 Asian cultivars of cassava, Kasetsart University 50 (KU 50) and Hanatee were prepared. Stems were harvested from Rayong Agronomic Research Center (Department of Agriculture, Thailand) and surface sterilized using 10% v/v Haiter (0.6% sodium hypochlorite solution) for 20 minutes. After washing three times with steriled -reverse osmosis water, shoot apex and lateral buds were excised and cultured on MS medium supplemented with 2% sucrose, 2 μM $CuSO_4$, 0.05 mg l^{-1} BA, 0.05 mg l^{-1} GA, 0.02 mg l^{-1} NAA and solidified with 0.6% bactoagar. The proliferated shoots were repeatedly transferred using node-cutting at 60 days intervals. The node cultures were kept at 25-28°C, 10/14 h photoperiod of 3,000 lux.

Somatic embryo induction: Cutting nodes were cultured on basic medium (MS + 2% sucrose + 2 μM $CuSO_4$ + 0.6% bactoagar; CBM) for 60 days before using to induce somatic embryos. Apical buds, lateral buds and young leaf lobes (immature leaf lobes from shoot tips, 1-6 mm-long) were isolated and used for induction of somatic embryos on induction medium at 25-28°C. Different types of phytohormone in various concentrations were tested for their efficiency in somatic embryo induction medium (CIM = CBM supplemented with picloram, dicamba and 2,4-D at the concentration of 6 and 12 mg l^{-1}) and cultured in dim light at the same condition. In order to maintain the vigorous growth of somatic embryos with minimal callus development, the cultures were transferred onto fresh CIM every second week. After 2-3 cycles, the somatic embryos were harvested and transferred to maturation medium (CMM = CBM supplemented with 0.1 mg l^{-1} BA) to induce embryo maturation and the production of somatic cotyledons.

Cotyledons from germinating primary somatic embryos were used to establish cyclic somatic embryo cultures in the induction medium supplemented with suitable phytohormone concentration (picloram at the concentration of 6 or 12 mg l^{-1}) and cultured in dim light. This routine work has been done to induce more somatic embryos and further maturation.

Cyclic somatic embryogenesis induction from somatic cotyledons: Cyclic somatic embryogenesis induction were induced from different ages of somatic cotyledons in various phytohormone concentrations. Young or mature somatic cotyledons from primary somatic embryos, which were cultured on CMM medium were harvested at different ages (10, 15, 20 and 30 days-old after culture). They were cut into small pieces and transferred to CIM with various concentrations of picloram, 2,4-D or Dicamba to produce cyclic somatic embryogenesis. The cultured were kept at 25-28°C at 10-h photoperiod of 2,000 lux. The cyclic somatic embryogenesis induction frequency were recorded 60 days after the cotyledons were cultured on each induction medium. Moreover the primary somatic embryo induction and cyclic somatic embryogenesis induction frequency were compared.

Organogenesis induction: After 30 days on CIM, the emerging somatic embryos were harvested and transferred to CMM for induction of embryo maturation and production of somatic cotyledons. After another 30 days, the developing young green cotyledon derived from the germinating embryos were harvested, cut into 0.5-cm^2 pieces and transferred to shoot organogenesis induction medium (COM = CBM supplemented with 1 mg l^{-1} BA + 0.5 mg l^{-1} IBA) and cultured in dim light at 25-28°C for induction of shoots or plantlets via organogenesis.

Shoot organogenesis and plant regeneration: After 30 days, shoot primordial developing on the explants were detached from the explants and transferred to shoot elongation medium (CEM = CBM supplemented with 0.4 mg l^{-1} BA). Another 30 days later, the number of elongated shoots was record. Elongated shoots emerging from the shoot primordial were transferred to rooting medium (CRM = CBM supplemented with 0.01 mg l^{-1} NAA) for rooting and further growth.

RESULTS AND DISCUSSION

Since genetic transformation system depend on regeneration via somatic embryogenesis and shoot organogenesis and each cassava cultivar may not respond in those reported condition, it is essential to optimize a protocol for somatic embryogenesis and shoot organogenesis of the Asian widely grown cultivars of cassava. Because plant regeneration via shoot organogenesis from cotyledons of somatic embryos (somatic cotyledons) in cassava is rapid, less genotype-dependent and compatible with both Biolistic and *Agrobacterium*-mediated gene transfer, improvement of the shoot organogenesis frequency will increase the efficiency of cassava genetic transformation. We have optimized the protocol for induction of somatic embryogenesis and shoot organogenesis for cyclic somatic embryogenesis of the cultivar KU50 and Hanatee.

Somatic embryo induction: Different phytohormones with various concentrations; Picloram 6 mg l^{-1} (P6), 12 mg l^{-1} (P12), Dicamba 6 mg l^{-1} (Di6), 12 mg l^{-1} (Di12), 2,4-D 6 mg l^{-1} (D6) and 12 mg l^{-1} (D12) and different plant materials; apical buds (AB), lateral buds (LB) and young leaf lobes (YL), were used to study the effect of somatic embryo induction efficiency in each cultivar of cassava; KU 50 and Hanatee. For cultivar KU 50, somatic embryos could be induced from all plant materials in all phytohormones (Table 1). It should be noted that

Table 1: Effect of phytohormones at various concentrations and different plant materials on somatic embryo induction frequency of cassava cultivar KU 50 after culture for 30 days

Hormone/ concentration (mg l^{-1})	Plant material[a]	Somatic embryos/total explants	Embryo induction frequency (%)
Picloram 6	AB	22/50	44
	LB	21/80	26
	YL	16/40	40
Picloram 12	AB	32/80	40
	LB	3/54	6
	YL	17/40	43
Dicamba 6	AB	4/42	10
	LB	5/46	11
	YL	12/26	46
Dicamba 12	AB	12/47	26
	LB	4/41	10
	YL	17/27	63
2,4-D 6	AB	13/40	33
	LB	4/40	10
	YL	11/26	42
2,4-D 12	AB	5/40	13
	LB	3/40	8
	YL	15/30	50

[a] Plant materials; apical buds (AB), lateral buds (LB) and young leaf lobes (YL)

Table 2: Effect of phytohormones at various concentrations and different plant materials on somatic embryo induction frequency of cassava cultivar Hanatee after culture for 30 days

Hormone/ concentration (mg l^{-1})	Plant material[a]	Somatic embryos/total explants	Embryo induction frequency (%)
Picloram 6	AB	22/60	37
	LB	23/80	29
	YL	19/40	48
Picloram 12	AB	25/50	50
	LB	19/76	25
	YL	19/45	42
Dicamba 6	AB	9/50	18
	LB	5/50	10
	YL	7/40	18
Dicamba 12	AB	11/55	20
	LB	9/75	12
	YL	5/37	14
2,4-D 6	AB	19/52	37
	LB	11/51	22
	YL	8/40	20
2,4-D 12	AB	18/57	32
	LB	7/50	14
	YL	7/35	20

[a]Plant materials; apical buds (AB), lateral buds (LB) and young leaf lobes (YL)

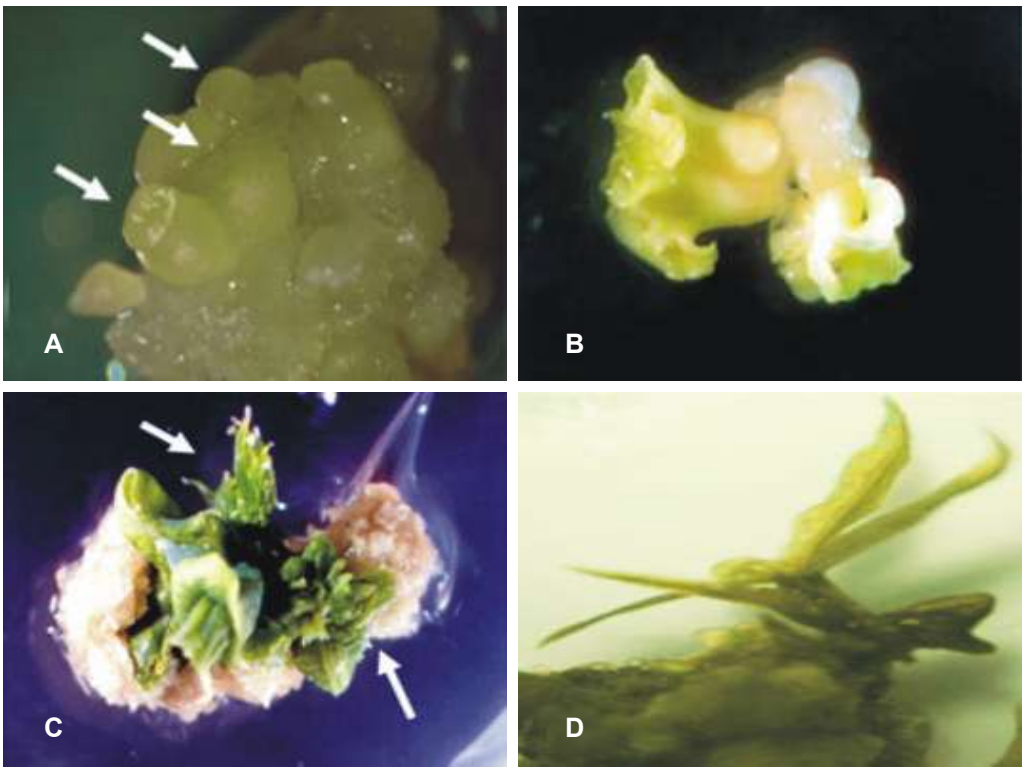

Fig. 1: Somatic embryo induction and plant regeneration of cassava cultivar KU 50
A): Somatic embryos (arrow) induced from lateral bud culture on CIM medium containing Dicamba 6 mg l^{-1}, B): The young green cotyledons development after culture on CMM medium for 30 days, C): Shoots (arrows) emerging from excised cotyledons culture on COM medium for 15 days, D): Further growth of shoot emerging from excised cotyledons culture on COM medium for 30 days.

young leaf lobes had a high trend of somatic embryo induction frequency on all phytohormones at various concentrations. In contrast with lateral buds which gave the lowest frequency of somatic embryo induction in most phytohormones, in which. Picloram 6 mg l^{-1} gave the highest frequency of somatic embryo induction. We found that Picloram gave the relatively high frequency on both apical buds and young leaf lobes as well, while Dicamba and 2,4-D could induce quite high frequency of somatic embryo from young leaf lobes (Table 1 and Fig. 1). As for cultivar Hanatee, somatic embryos could be induced from all plant materials in all phytohormones at various concentrations (Table 2). However, young leaf lobes and apical buds showed a high trend of somatic embryo induction frequency on induction medium with Picloram 6 mg l^{-1} and 12 mg l^{-1} and the frequency was followed by 2,4-D containing medium. Lateral buds had low frequency for somatic embryo induction compared with apical buds and young leaf lobes in all hormones and concentrations especially in the medium containing of Dicamba (Table 2 and Fig. 2).

Regeneration via organogenesis: Adventitious shoots could be induced from cotyledons of somatic embryos after culturing somatic cotyledons on the organogenesis medium. The induction of somatic embryogenesis and adventitious shoots from immature leaves of different cultivar of cassava had also been reported by Guohua and Qiusheng [12]. After a passage on elongation medium, the regenerated shoots were easily rooted in hormone-free medium and could be successfully transplanted to soil. Compared to regeneration via germination of embryos derived from suspensions, shoot organogenesis is faster, requiring less time in tissue culture, which may reduce the risk of somaclonal variation [1].

After somatic embryos of KU 50 and Hanatee were obtained on induction medium, they were subcultured on the same medium every 30 days interval for further growth of somatic embryo. Subsequently they were harvested and transferred to CMM medium. After 15 days, the green cotyledons were found to further develop and were cut to small pieces and transferred to

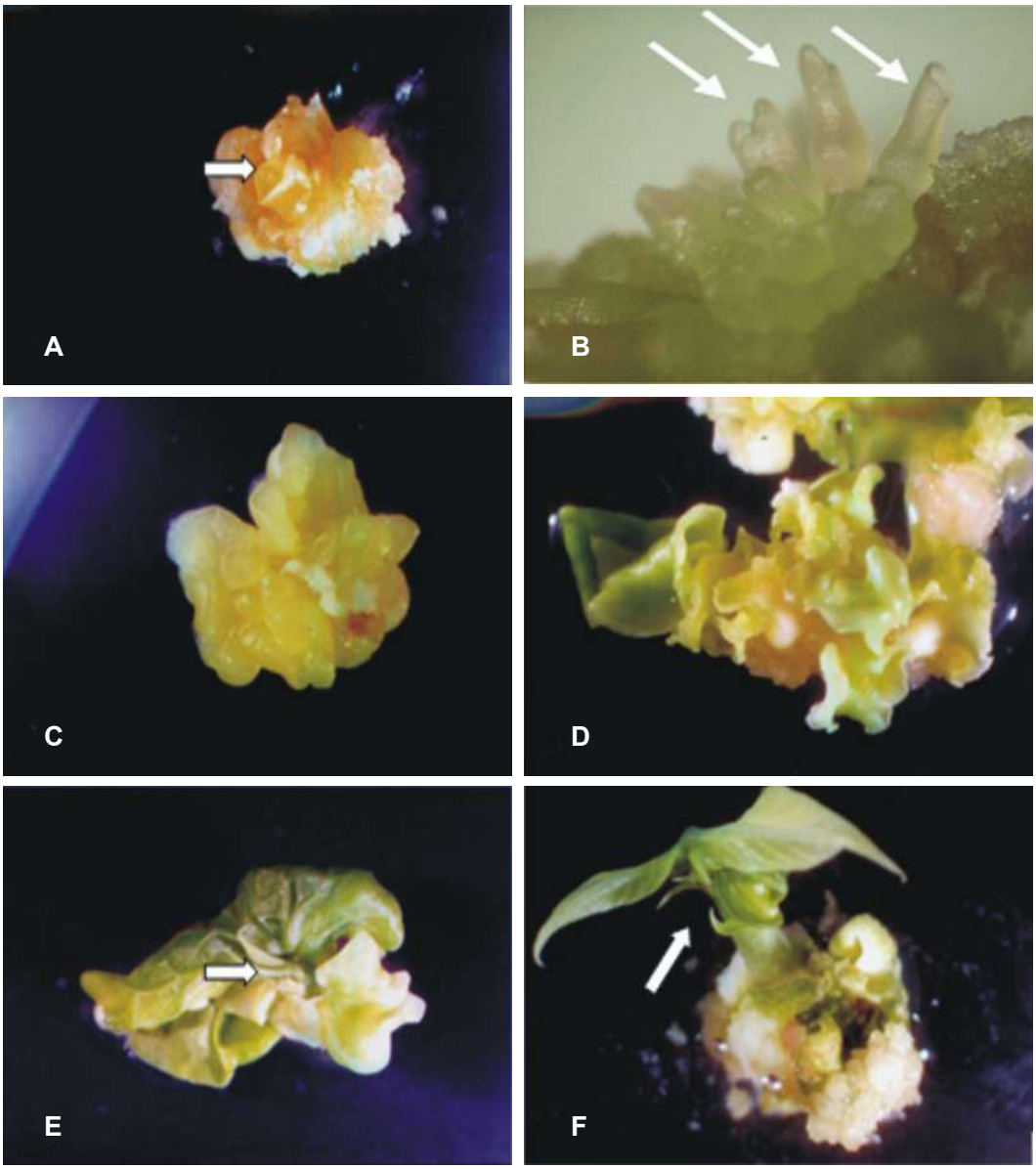

Fig. 2: Somatic embryo induction and plant regeneration of cassava cultivar Hanatee
A): Somatic embryos (arrows) induced from lateral bud culture on CIM medium containing Dicamba 12 mg l^{-1}, B): Somatic embryos (arrows) induced from apical bud culture on CIM medium containing Picloram 12 mg l^{-1}, C): Further growth of somatic embryos (from B) on the same medium after subculture for 30 days, D): The young green cotyledons development after culture the somatic embryo (from C) on CMM medium for 30 days, E and F): Shoots emerging (arrows) from excised cotyledons (from D) culture on COM medium for 20 days.

organogenesis medium. After another 15 days, the shoots emerged from cotyledons on the both cultivars (Fig. 1C and 2F). We have further developed the cyclic somatic embryogenesis induction in both cultivars. Continuous culture of somatic embryos on auxin supplemented medium allows a cyclic system of somatic embryogenesis. Such cultures can be maintained as a continuous explant source for regeneration and transformation studies [13].

Cyclic somatic embryogenesis induction from different ages of cotyledon: Primary somatic embryos can be induced to produce secondary somatic embryos by further sub-culturing on auxin-containing medium. By

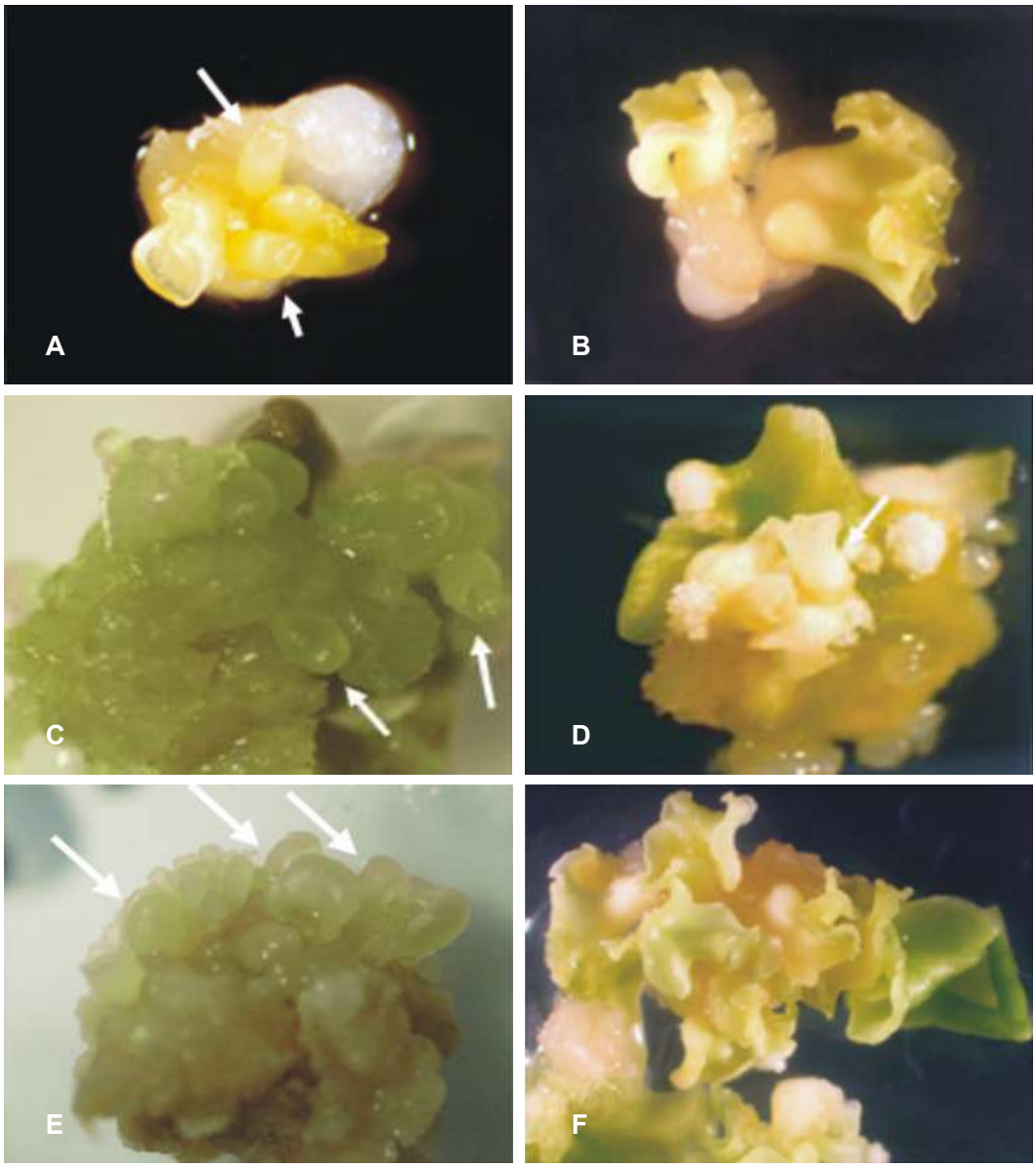

Fig. 3: Primary somatic embryogenesis and cyclic somatic embryogenesis induction of cassava variety KU50 (A-D) and Hanatee (E, F)
A): Primary somatic embryogenesis of KU50 induced from lateral bud on 2,4-D 6 mg l^{-1} induction medium, B): Somatic cotyledons of KU50 obtained from induction of primary somatic embryo on CMM medium, C, D): Cyclic somatic embryogenesis of KU50 induced from somatic cotyledons on 2,4-D 12 mg l^{-1} induction medium, E): Cyclic somatic embryogenesis of Hanatee induced from somatic cotyledons on 2,4-D 12 mg l^{-1} induction medium, F): Mature somatic cotyledons of Hanatee cultured on maturation medium

constant sub-culturing of somatic embryos, a cyclic embryogenesis system can be established in semi-solid medium, where the embryos rarely pass the torpedo stage, until transferred to germination medium. In cassava, both primary and secondary somatic embryos develop from groups of cells, usually located at or near the vascular tissue. However, the multi-cellular origin of cassava somatic embryos makes them poorly suited for genetic engineering. Therefore, in this research we have been using the developing young green cotyledons for gene transfer study. The cyclic embrygenesis system has been routinely performed and the developing young green

Table 3: Cyclic somatic embryogenesis induction frequency from different ages of somatic cotyledons of cassava variety KU 50 on induction medium

Hormone/ concentration (mg l^{-1})	Age of cotyledon (days)	No. of somatic embryo/total explants	Somatic embryo induction (%)
Picloram 6	10	24/28	86
	15	38/42	90
	20	83/94	88
	30	21/28	75
Picloram 12	10	34/41	83
	15	31/42	74
	20	58/83	70
	30	26/31	84

Table 4: Cyclic somatic embryogenesis induction frequency from different ages of somatic cotyledons of cassava variety Hanatee on induction medium

Hormone/ concentration (mg l^{-1})	Age of cotyledon (days)	No. of somatic embryo/total explants	Somatic embryo induction (%)
Picloram 6	10	86/150	57
	15	111/200	56
	20	58/133	44
	30	41/57	72
Picloram 12	10	73/104	70
	15	78/148	53
	20	62/150	41
	30	22/24	92

Table 5: Cyclic somatic embryogenesis induction frequency of cotyledons age 15 and 30 days-old cultured on various kinds of induction medium on cassava variety KU 50

Hormone/ concentration (mg l^{-1})	Age of cotyledon (days)	No. of somatic embryo/total explants	Somatic embryo induction (%)
Picloram 6	15	116/152	76
	30	21/28	75
Picloram 12	15	57/70	81
	30	26/31	84
2,4-D 6	15	66/70	94
	30	30/31	97
2,4-D 12	15	82/84	98
	30	28/29	97
Dicamba 6	15	35/42	83
	30	26/28	93
Dicamba 12	15	39/45	87
	30	26/29	90

cotyledons have been used for further optimization of selective condition and gene transfer study.

As the cyclic embrygenesis system has been routinely performed, the induction frequency of somatic embryogenesis obtained from induction of different developmental stage of cyclic cotyledons is needed to be considered. The two varieties of cassava; KU 50 and Hanatee were assessed for their ability to produce cyclic somatic embryogenesis from cyclic somatic cotyledons. Young or mature cotyledons at different ages (10, 15, 20 and 30 days-old after culture on maturation medium) were collected and cultured on induction media (CIM containing Picloram 6 or 12 mg l^{-1}). Cyclic somatic embryogenesis frequency from cotyledons of KU 50 and Hanatee were evaluated 60 days after the cotyledons were cultured on induction media. Cassava variety KU 50 showed the higher induction frequency of cyclic somatic organogenesis than that of variety Hanatee in both induction media containing Picloram 6 and 12 mg l^{-1}. However, the different ages of cotyledons to be used for initiation of cyclic somatic embryogenesis showed no significant different of induction frequency, as shown in Table 3 for variety KU50 and Table 4 for variety Hanatee.

Cyclic somatic embryogenesis induction from various kinds of induction media: The various kinds of induction media were tested for the most suitable medium which give high cyclic somatic embryogenesis induction frequency in each cassava variety. Two varieties KU 50 and Hanatee were assessed for their ability to produce cyclic somatic embryogenesis from somatic cotyledons. Young and mature cotyledons at the age of 15 and 30 days-old after culture on maturation medium were collected and cultured on various kinds of induction media. Different phytohormones with various concentrations; Picloram 6 mg l^{-1} (P6), 12 mg l^{-1} (P12), Dicamba 6 mg l^{-1} (Di6), 12 mg l^{-1} (Di12), 2,4-D 6 mg l^{-1} (D6) and 12 mg l^{-1} (D12), were added into cassava basal medium.

Cyclic somatic embryogenesis induction frequency from cotyledons of KU 50 and Hanatee were evaluated 60 days after the cotyledons were cultured on each induction medium. Cassava variety KU 50 showed the higher induction frequency of cyclic somatic embryogenesis than that of cassava variety Hanatee in almost all kinds of induction media. It should be noted that the cotyledons of cassava variety KU 50 gave the highest frequency of cyclic somatic embryogenesis induction on both 2,4-D 6 mg l^{-1} (D6) and 12 mg l^{-1} (D12) induction media (Table 5).

Table 6: Cyclic somatic embryogenesis induction frequency of cotyledons age 15 and 30 days-old cultured on various kinds of induction medium on cassava variety Hanatee

Hormone/ concentration (mg l^{-1})	Age of cotyledon (days)	No. of somatic embryo/total explants	Somatic embryo induction (%)
Picloram 6	15	86/150	57
	30	41/57	72
Picloram 12	15	73/104	70
	30	22/24	92
2,4-D 12	15	12/15	80
	30	24/28	86
Dicamba 12	15	15/24	63
	30	19/29	66

Table 7: Primary somatic embryogenesis induced from apical bud (AB), lateral bud (LB) and young leaf lobe (YL) compared with cyclic somatic embryogenesis (SE) induced from somatic cotyledon on different media of cassava variety KU 50

Hormone/ concentration	% Primary somatic embryogenesis			%
(mg l^{-1})	AB	LB	YL	Cyclic SE
Picloram 6	44	26	40	75
Picloram 12	40	6	43	84
2,4-D 6	33	10	42	97
2,4-D 12	13	8	50	97
Dicamba 6	10	11	46	93
Dicamba 12	26	10	63	90

Table 8: Primary somatic embryogenesis induced from apical bud (AB), lateral bud (LB) and young leaf lobe (YL) compared with cyclic somatic embryogenesis (SE) induced from somatic cotyledon on different media of cassava variety Hanatee

	% Primary somatic embryogenesis			%
Media	AB	LB	YL	Cyclic SE
Picloram 6	37	29	48	72
Picloram 12	50	25	42	92
2,4-D 12	32	14	20	86
Dicamba 12	20	12	14	66

The cyclic somatic embryogenesis induction frequency of Hanatee was obtained highly on Picloram 12 mg l^{-1} (P12) and 2,4-D 12 mg l^{-1} (D12) induction media (Table 6).

Comparison of primary somatic embryogenesis induction and cyclic somatic embryogenesis induction on various kinds of induction media: Primary somatic embryogenesis in cassava started with the induction of primary somatic embryos from apical bud (AB), lateral bud (LB) and young leaf lobe (YL). By further sub-culturing on auxin-containing medium, primary somatic embryos can be induced to produce secondary somatic embryos and constantly sub-culturing of somatic embryos can provide the cyclic embryogenesis system. The induction frequency of primary somatic embryogenesis (primary SE) and cyclic somatic embryogenesis (cyclic SE) induction were compared on various kinds of induction media. The apical bud, lateral bud and young leaf lobe of each cassava variety were used to induce for primary SE. The 30 days-old cyclic mature cotyledons of each cassava variety were used to induce for cyclic SE. Different phytohormones with various concentrations; Picloram 6 mg l^{-1} (P6), 12 mg l^{-1} (P12), Dicamba 6 mg l^{-1} (Di6), 12 mg l^{-1} (Di12), 2,4-D 6 mg l^{-1} (D6) and 12 mg l^{-1} (D12), were added into cassava basal medium.

The frequency of somatic embryogenesis induction from cyclic mature cotyledons was higher than that of primary somatic embryogenesis induction in both varieties; KU 50 and Hanatee. The 30 days-old cyclic mature cotyledons of variety KU 50 gave the highest induction frequency of cyclic somatic embryogenesis on both 2,4-D 6 mg l^{-1} (D6) and 12 mg l^{-1} (D12) induction media (Table 7 and Figure 3 A-D). While cyclic somatic embryogenesis induction frequency of Hanatee was obtained highly on Picloram 12 mg l^{-1} (P12) and 2,4-D 12 mg l^{-1} (D12) induction media (Table 8 and Figure 3 E-F).

In conclusion, an efficient and reproducible plant regeneration system has been developed in two Thai cassava cultivars; KU50 and Hanatee. Continuous culture of somatic embryos on auxin supplemented medium allows a cyclic system of somatic embryogenesis. Such cultures can be maintained as a continuous explant source for plant regeneration and transformation studies. Many improved methods have been developed to increase the efficiency of plant regeneration via somatic embryogenesis as the frequency of germination of mature somatic embryos is low. In this study, somatic embryogenesis has been induced from different plant materials: apical buds, lateral buds and young leaf lobes and different phytohormones: Picloram, Dicamba and 2,4D. This system was optimized and the primary somatic embryos were further developed to the green cotyledons in maturation medium and then could be transferred to organogenesis medium for shoot emerging and plant regeneration. The primary somatic cotyledons were used for cyclic somatic embryogenesis induction. The efficient system had been optimized and routinely performed to produce the newly young green cotyledons for gene transfer study.

ACKNOWLEDGEMENTS

We are grateful to Dr. Opas Boonseng, Rayong Field Crop Center, Department of Agriculture, Thailand, for providing us with the identified Asian cassava cultivars. This work was funded by a grant from the National Center for Genetic Engineering and Biotechnology, Thailand (BT-B-06-PG-14-4607).

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