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Full Length Research Paper

Developing an efficient protocol for plant regeneration of manihot esculenta crantz via somatic embryogenesis induction from immature leaf and apical shoots

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In this study, we developed a method for the induction of somatic embryogenesis in two cassava genotypes from Vietnam is described. The explants used were immature leaves and *apical shoots* isolated from *in vitro* 2-3-week plants. Somatic embryogenesis was achieved at high frequencies by the addition in the induction medium of the auxin picloram over a wide range of concentrations. Our results show that the highest rate of somatic embryos formation for both cultivars was obtained by using 12 mg/l picloram supplemented to Murashigh - Skoog (MS) media. For immature leaves: the number of embryos per explant was similar between two cultivars for 4 weeks, the KM94 cultivar gave a higher rate (81,4 ±1,7%) than the KM140 cultivar (70,4 ± 2,9%). For *apical shoots:* the number of embryos per explant was similar between two cultivars for 4 weeks, the KM94 cultivar gave a higher rate (82 ±1.7%) than the KM140 cultivar (59.6 ± 2.9%). Somatic embryos were subsequently transferred onto media (MS supplemented with 0.3 mg/l BAP) for the highest frequency of plantlet regeneration (KM140 - 82,1 ± 3,1% and KM94 - 79,2 ± 2,3%), for immature leaves and (KM140 - 81 ±3.2% and KM94 - 79 ± 2.3%) for *apical shoots.* Shoots with the length of about 1.0-1.5 cm were transferred onto free - hormone MS media for 100% of rooting for 2 weeks. Complete plantlets were cultivated on a mixture of rice husk and sand-soil under ratio 4:6 in a greenhouse. This protocol required 16 to 18 weeks and is entirely appropriate for mass production of various cassava genotypes and further genetic transformation experiments.

Keywords: Cassava (Manihot esculentum Crantz), Callus culture, immature leaf, apical shoots, somatic embryos, regeneration.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most important grain food in Africa countries. In the unsuitable condition such as drain, low nutrients content soil, cassava still provides high content of carbohydrate (Bruijn and Fresco 1989). Beside, cassava used for animal feeding and food industry increased by time. Especially, cassava is the main material for biodiesel producing. Therefore, cassava plays important role for social economy. It's necessary to create new strains of cassava that have high yield and can adapt to extreme conditions (Fukuda 2003).

Gene modified technologies allow scientist to create high-quality strains of cassava. It's may transfer some gene targets into traditional strains that adapted to local conditions. In 2004 Siritunga has shown some experiments about gene transfer in many strains of cassava. This research group has created a new gene modified cassava cultivar that containing a high amount

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of hydroxynitrile lyase (HNL) in leaves and root. The reducing of acetone cyanohydrin and increasing of cyanide evaporation were coupled with increasing of HNL during cutting or grinding cassava's processing. This gene modified cassava is different with cassava cultivar which absent of cyanogen (Siritunga and Sayre 2003). These modified cassavas contain a small amount of cyanogen to discourage herbivores but still safe for human consumption as the food supplement. Guohua has induced the immature leaves by 2,4-Dichlorophenoxyacetic acid (2,4-D) (Ma and Xu 2002) and α-Naphthaleneacetic acid (NAA) to produce embryo (Joseph, Yeoh et al. 2000). Beside, apical shoot and immature leaves also were induced to produce primary soma embryo by picloram (Li, Sautter et al. 1996, Zhang, Phansiri et al. 2001). *In vitro* cell culture that provided cell and tissue as the material for genetic engineering have strong effects on improving quality of genetically modified cassava strains. The starting material (leaves, meristem) were used to induced embryonic somatic through primary pre-embryo. Somatic embryos can transform in many directions such as somatic embryos were directly germinated to seedlings forming, somatic embryos were transformed to the secondary somatic embryos; the somatic embryos were selected, to forming pre-scar tissue, then were culturing to somatic embryos; from pre-scar tissue to transform to protoplast and vice versa. This paper presented the detailed developing process the somatic embryogenesis by induction of immature leaves and apical shoots to regenerate two cassava KM 140 and KM 94 cultivars of Vietnam.

MATERIAL AND METHODS

Two cassava cultivars KM94 and KM140 have supported by Plant Resources Center, the Institute of Agricultural Science Vietnam. The mature stem of 2 strains of cassava KM94 and KM140 were treading in soil. After 4-6 week, the bud meristem shoot was developed into green stem which has 0,1- 0,2 cm in diameter. Then cut this stem into 3- 4 cm in length pieces. Those pieces were cleaned by fresh water in 30 min alternatively with sterilization by 70% alcohol in 30 seconds and 0,1% mercuric chloride in 10 min, then washing the samples with sterilized water for 3 min, repeat the whole process 5 times.

The sterilized samples were grown in Murashigh - Skoog (MS) medium (agarose content) that added 3% of sucrose. For each flask 250 ml, 5 pieces of samples were grown in 50 ml medium/ flask) subculture two months per times. Immature leaves of a new plant (after 12-14 days) and apical shoots of new plant (after 4 weeks were used as samples for tissue culture. The tissue culture were cut into small pieces (2 × 2 mm) then grow in petri dish containing 25 ml of MS medium, added 2% of sucrose, 1 mg/L Copper sulfate (2 mM) and picloram at different concentrations: 1, 3, 6, 9, 12 and 15 mg/L or N^6-benzylaminopurine (BAP) at different concentrations 0; 0.3; 0.6; 0.9; 1.2; 1.5 mg/L to form soma embryos.

Each petri dish grows 10 pieces of samples, 5 dishes for each experiment and repeated 3 times. Firstly, lobed leaves were grown in dark for 1 week then cultured in light condition for next 3 week to produce primary soma embryo. After that, subculturing was repeated two weeks/times in the same medium to produce secondary soma embryo as described before (Li, Sautter et al. 1996, Zhang, Phansiri et al. 2001).

The cotyledons, which were separated from the stage embryos 14-15 days old, were cut into small pieces in 2 × 2 mm. They then were grown in MS medium, added 2% of sucrose, 1 mg/l Copper sulfate (2mM) and indole-3-butyric acid (IBA) in different concentrations 0, 0,3; 0,6; 0,9; 1,2; 1,5 mg/l for 3- 4 week, to evaluate the soma embryos formed seedlings.

To form roots and the plant: The buds which reach 1-1.5 cm length were implanted into to MS medium, added 0.2% sucrose and supplemented with IBA at different concentrations (0; 0,3; 0,6; 0,9; 1,2 mg/L) in 4 weeks. The plant which has the filled roots were transferred into a greenhouse, grown on medium consists of 60% sandy soil and 40% of the smoking husk, in the first 2 weeks avoid direct sunlight.

The mediums were adjusted to pH 5.8, added 7g/l agar, sterilized at 121°C, 1.5 atm in 15 min. The tissues were incubated at 25 ± 2°C, 3000 lux and light supplied for 16h/day.

RESULTS AND DISSCUSSION

Formation of somatic embryo from immature leaves and apical shoots of cassava *in vitro* culture

Normally, the formation of a somatic embryo is induced by auxin (2,4-D, picloram, dicamba) in MS medium (Williams and Maheswaran 1986). 2,4- D is the most common phytohormone used for somatic embryo formation. Beside, picloram was used to induce the formation of a somatic embryo in some kind of plants (Raemakers, Jacobsen et al. 1997, Castillo, Egana et al. 1998, Groll, Mycock et al. 2001, Mendoza and Kaeppler 2002, Kaur and Kothari 2004).

In this research, the somatic embryo was formed in MS medium with added of different concentration of picloram. Immature leaves and apical shoots of two cassava cultivars KM94 and KM140 were used as the medium culture *in vitro* for evaluating the effective of picloram to form a somatic embryo. The samples were grown in medium culture for 2 weeks, the first stage of embryo formation (spherical embryo) were observed in the third week, the second stage (clavate embryo) were observed in fourth week (fig1- leaves and fig2- apical shoots).

Table 1. The influence of picloram's concentration to the formation of somatic embryos from immature leaf of two varieties of cassava KM 94 and KM 140 *in vitro* culture.

Picloram (mg/L)	KM140		KM94	
	Percentage of somatic embryo formation (%)	Number of embryo formed per each explant	Percentage of somatic embryo formation (%)	Number of embryo formed per each explant
1	23.2 ± 1.2	10.3± 2.1	28.6 ± 2.2	15.3 ± 2.6
3	41.3 ± 1.7	18.4 ± 2.0	49.3 ± 2.0	31.3 ± 2.0
6	56.7 ± 1.9	19.2 ± 1.2	70.3 ± 1.2	37.6 ± 1.8
9	61.5 ± 1.7	21.4 ± 1.5	72.5 ± 1.5	39.6 ± 1.2
12	70.4 ± 2.9	27.9 ± 2.5	81.4 ±1.7	42.3 ± 3.0
15	62.8 ± 1.2	24.2 ± 1.8	77. 8 ± 2.6	31.6 ± 2.0

Value somatic embryo/sample was summarized at all shape, such as the round shape, heart shape, rays shape, and cotyledons.
The values were measured after 4 weeks of culture on MS medium supplemented picloram at different concentrations.

Table 2. The influence of picloram's concentration to the formation of somatic embryos from apical shoots of the two varieties of cassava KM 94 and KM 140 *in vitro* culture

Picloram (mg/L)	KM140		KM94	
	Percentage of somatic embryo formation (%)	number of embryo formed per each explant	Percentage of somatic embryo formation (%)	number of embryo formed per each explant
1	16.3 ± 1.2	9.7± 2.6	29.6 ± 2.2	13.3 ± 2.6
3	39.3 ± 1.7	17.3 ± 2.0	51.3 ± 2	29.3 ± 2
6	47.3 ± 1.7	18 ± 1.2	71.3 ± 1.2	36.6 ± 1.8
9	40 ± 1.7	20.7± 1.5	64 ± 1.5	34.6 ± 1.2
12	59.6 ± 2.9	26.7± 2.7	82 ±1.7	29.3 ± 3
15	56.3 ± 1.2	23.3 ± 1.8	76 ± 2.6	31.6 ± 2

Value somatic embryo/sample was summarized at all shape, such as the round shape, heart shape, rays shape, and cotyledons.
The values were measured after 4 weeks of culture on MS medium supplemented picloram at different concentrations.

The table 1 showed that the ability of somatic embryo formation and a number of embryones formed per each explant in all tested picloram's concentration are pretty high. In both cassava strains, the ability of somatic embryo formation and the number of embryones formed per each explant are increased with the increasing of picloram (1- 12mg/L); lowest at picloram's concentration 1 mg/L, and highest at picloram's concentration 12 mg/L.
The ability of somatic embryo formation and a number of embryones formed per each explant for KM 94 cultivar showed higher than KM 140 cultivar at the same concentration of picloram. For immature leaves, at a concentration of 12mg/L. KM 94 cultivar has a percentage of somatic embryo formation and a number of embryones formed per each explant for KM 94 cultivar were 81.4 ± 1.7% and 42.3 ± 3.0, respectively, higher than KM 140 cultivar with two values 70.4 ± 2.9% and 27.9 ± 2.5, respectively. The percentage of somatic embryo formation of KM 94 is higher 11% than KM 140 and the number of embryones formed per each explant was higher 14.4 (Table 1).

For the apical shoots, at a concentration of picloram 12 mg/L, the cultivar KM140 cassava has the embryogenesis rate and a number of embryones formed per each explant of 59.6 ± 2.9% and 26.7 ± 2.7, respectively. However, at a picloram's concentration of 15 mg/L, the somatic embryogenesis and number of embryo formed per each explant began reducing. Meanwhile, cultivar KM94 cassava has the somatic embryogenesis of 22.4% higher than the cultivar KM140 cassava at a picloram's concentration of 12 mg/L. Interestingly, the number of embryones formed per each explant between two cultivar is not so difference, only 2.7 embryos/sample (Table 2).
Our result is consistent with the publication of Zhang (Zhang, Phansiri et al. 2001), and Feitosa (Feitosa, Bastos et al. 2007). These authors indicated picloram's concentration at 12 mg/L exhibited the highest the embryogenesis rate and a number of embryones formed per each explant. Danso suggested that both 2.4 D and picloram are highly effective (90-100%) in somatic embryogenesis process, but the medium culture which

Table 3. The influence of the BAP's concentration to the plantlet regeneration from somatic embryos cultured after 4 weeks from immature leaf

BAP (mg/l)	Plantlet regeneration from somatic embryos (%)	
	KM140	KM94
0.0	45.6 ± 0.7	51.4 ± 2
0.3	82.1 ± 3.1	79.2 ± 2.3
0.6	78.7 ± 2.2	77.3 ± 1.2
0.9	65.2 ± 1.4	71.4± 1.5
1.2	63.4 ± 2.0	61.3 ± 1.45
1.5	54.2 ± 0.7	58.3 ± 2.6

Table 4. The influence of the concentration BAP's to the plantlet regeneration from somatic embryos cultured after 4 weeks from apical shoots

BAP (mg/l)	Plantlet regeneration from somatic embryos (%)	
	KM140	KM94
0	43.6 ± 0.8	50.3 ± 2
0.3	81 ± 3.2	79 ± 2.3
0.6	79 ± 2	77.6 ± 1.2
0.9	67 ± 1.5	71± 1.5
1.2	61 ± 2	60.3 ± 1.45
1.5	55.3 ± 0.8	59.3 ± 2.6

has additional picloram showed a faster somatic embryogenesis process and superior to 2,4 D supplements. This is due to picloram affect cell walls and dilates cells faster than 2,4 D (Danso, Elegba et al. 2010). However, the development of the embryos is induced by NAA faster than 2,4-D, dicamba or picloram (Sofiari, Raemakers et al. 1997).

Growth ability of somatic embryos into small plants

About 3-4 weeks after the formation of somatic embryos and their maturity in medium cultures with different concentrations of picloram, these somatic embryos are moved to cultures with varied BAP concentration (0; 0.3; 0.6; 0.9; 1.2; 1.5 mg/L) in order for growing into complete plants. This is a crucial step for evaluating the efficiency of the plant regeneration, especially in gene transformation because, if the regeneration is poor while a large number of embryos are formed, the gene transferred into tissue but the rate of transgenic plants is also very low.

For immature leaf, Table 3 showed that: at all BAP's concentration, somatic embryos of the both strains KM 140 and KM 94 germinated with the high ratio. Even at BAP's concentration of 0 mg/L, germination still occurred with the ratio of 45.6 ± 0.7 and 51.4 ± 2% for both strains KM 140 and KM 94, respectively. Both strains cassava achieved maximum germination ratio at BAP's concentration of 0,3 mg/L, but the germination ratio was higher for KM 140 cultivar than for KM 94 cultivar (82.1 ± 3.1% to 79.2 ± 2.3%). The germination ratio was decreased in both strains when BAP's concentration increased from 0.6 to 1.5 mg/L.

Regarding of apical shoots: according to table 4, both KM94 and KM140 strains exhibited the germination in each concentration of BAP. KM140 cultivar has the highest ratio of somatic embryo growing into plants (81 ± 3,2%) at BAP's concentration of 0,3 mg/L comparing to medium non-BAP culture (43,6 ± 0,8 %). Similarly, the KM94 cultivar has the highest ratio of the somatic embryo growing into plants (79 ± 2,3%) at BAP's concentration of 0,3 mg/L comparing to medium non-BAP culture (50,3 ± 2%. %). At the same BAP concentration of 0,3 mg/L, there is no difference significantly in the ratio of the somatic embryo growing into plants between KM94 and KM140.

Root formation and plant formation

After 3 weeks in cultivation to form roots, newly germinated cassava buds of KM94 and KM140 reached 1-1,5 cm of length, were moved to MS culture with different IBA concentrations (0; 0.3; 0.6; 0.9; 1.2 mg/L). In each concentration, all buds formed roots (100% ratio). However, in MS culture with not added IBA, roots were formed faster, an amount of 3-5 roots/sample, while medium culture with IBA showed a slower growth (1-2 weeks) and shorter root (Figure 1 & 2).

In *vitro* condition, plants were supplied with adequate nutrition, light conditioning and were grown in sterile medium culture. In order to create a complete plant regeneration process for transformation, the plant

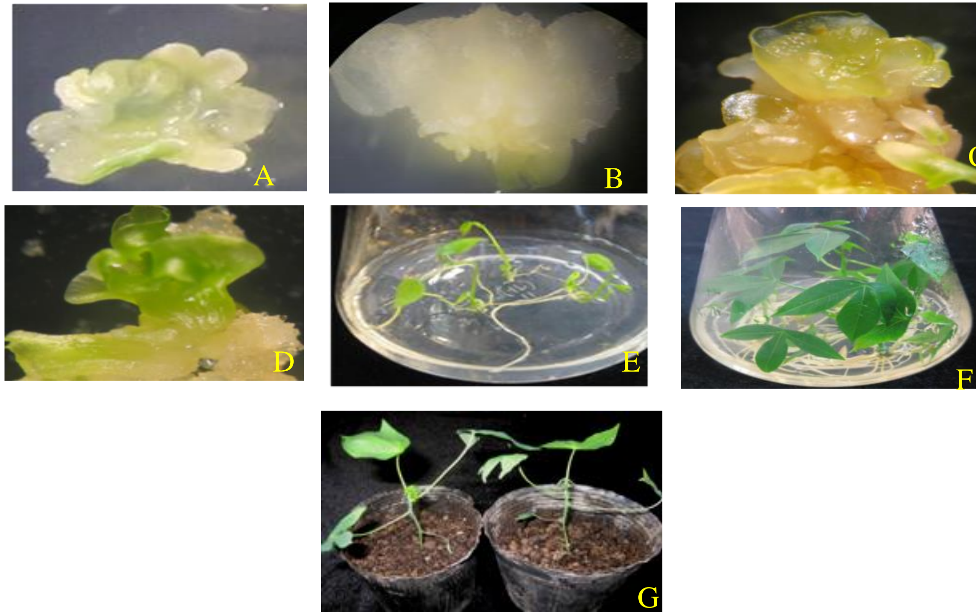

Fig 1. Regenarating of cassava in diferent stages of immature leaves.

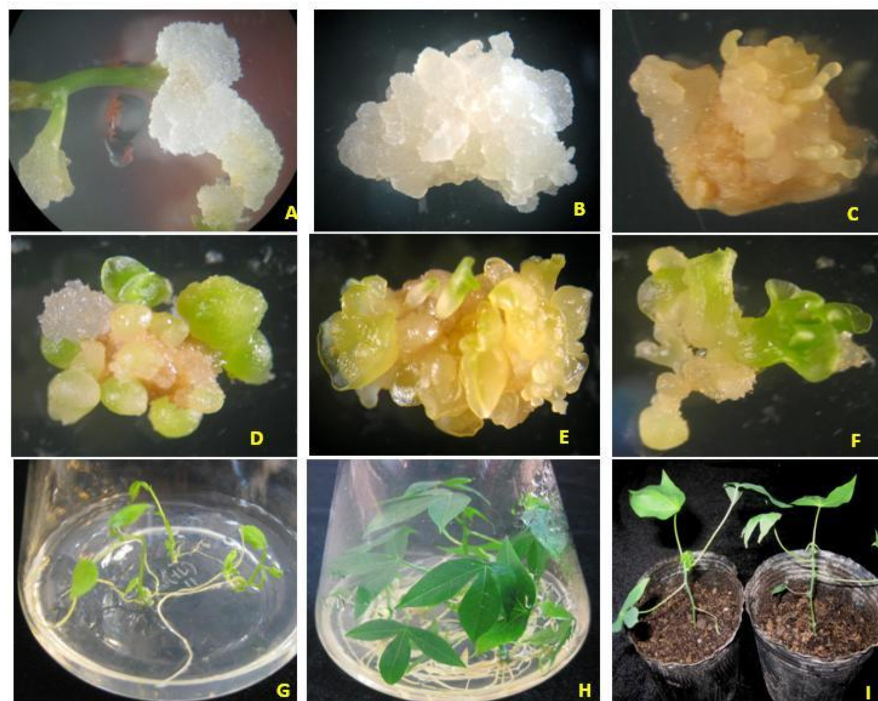

Fig 2. Regenarating of cassava in diferent stages of apical shoot.

regenerating system must be implanted in net house rather than in vitro only. When moving regenerated plants from the lab to the net house, the small plant has to experience slow stages of adapting to the new environment. 4-to-6-centimeter-long regenerated cassavas, which had more than 2 roots, were moved to grown on medium consists of 60% sandy soil and 40% of the smoking husk. In the first 2 weeks, the plants were planted in cultivation room under the temperature of 27oC, the illuminance of 1000-1500 lux and lighting

duration of 12 hours/day. After that, the plants were moved to the net house, supplied with nutrition and water and kept away from direct light in the first week. The generation plant grown in medium consists of 60% sandy soil and 40% reached the rate of survival, growth, and development normal 100%.

A: Callus derived from immature leaves; B: Embryo are forming; C: Soma embryo are germinating; D: Soma embryo are regenerating into new plats; E and F: plant in rooting medium; G: Plant in soil.

A: Callus derived from shoot meristem; B: embryo regenerated from callus; C and D: Somatic embryo; E: Somatic embryo Germinating; F: Somatic embryo regenerate into a new plant; G and H: Plant in rooting medium; I: Plant in soil.

CONCLUSION

A protocol for regeneration of Cassava has been studied. Regeneration of Cassava through somatic embryo from immature leaves and apical shoot has successfully studied in several steps: somatic embryo producing, germination of an embryo, regeneration, rooting and grow in soil.

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