African Journal of Biotechnology Vol. 10(9), pp. 1600-1608, 28 February, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1820
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Effects of cytokinins on secondary somatic embryogenesis of selected clone Rayong 9 of *Manihot esculenta* Crantz for ethanol production

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Accepted 12 January, 2011

In 2005, the Rayong-FCRC released a new high-yielding cassava cultivar designated Rayong 9 (R9) for ethanol production. However, the rate of distribution to farmers has been limited by the traditional vegetative propagation method of this crop which does not always satisfy the needs in planting material. The objective was to improve secondary somatic embryogenesis of the cassava clone Rayong 9 (R9) selected in Thailand by the Rayong Field Crops Research Center (Rayong-FCRC) for its suitability to produce ethanol. Fragments of cotyledon-stage somatic embryos were subcultured onto MS medium supplemented with the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) at 4 mg/l in sight of inducing secondary somatic embryogenesis. Five different cytokinins, 6-benzylaminopurine (BAP), kinetin, zeatin, isopentenyladenine (2-iP) and adenine were added at 1 mg/l to the induction and maturation media to test their efficiency. Onto cytokinin-free media, 70 to 80% of explants produced embryoids, each explant giving 2 to 4 new embryoids within 7 weeks. The conversion rate of the embryoids into plantlets ranged from 11 to 26% depending on the type of cytokinin. With the exception of adenine, the other cytokinins inhibited the intensity of somatic embryogenesis, by 75% in the case of zeatin and 30% in the case of kinetin. Addition of adenine did not significantly improve the number of embryoids per explant. However, at 10, 20 and 40 mg/l adenine tended to improve the process relatively to embryoid sizes and plantlet survival rates in the greenhouse.

Key words: Adenine, biofuel, cassava, cotyledonary-stage, embryoids, icrocuttings, micropropagation.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial shrub of the family Euphorbiaceae grown for its starch-containing tuberized roots which are used for human consumption, animal feed and raw material for the starch industry. Thailand is the world-leading country for cassava industrialization. In 2007, about 1 million hectares were devoted to cassava for which 26 million tons of root tubers were produced (FAOSTAT, 2009). This crop is not only a staple food in this country, but also provides monetary income for about half a million smallholders (Ratanawaraha et al., 2001; Watananonta, 2006). One of the main factors that favored its industrialization was the introduction of high-starch yielding cultivars (Watananonta, 2006; Howeler, 2007).

Recently, the country is exploring a promising new market for its starch, as raw material for production of

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Abbreviation: Rayong-FCRC, Rayong field crops research center; **NR&DC-T,** Nestlé Research and Development Center-Tours.

ethanol used as a biofuel (Nguyen et al., 2008). This industry is a strong growing market, expected to grow at a rate of 30% per year and will require an additional 4 to 6 million tons of fresh root tubers every year (Howeler, 2007). In 2005, the Rayong-FCRC released a new high-yielding cassava cultivar designated Rayong 9 (R9) suitable for ethanol production (Watananonta, 2006).

The distribution rate of new selected cassava clones to the farmers, as the R9 clone, is limited by the traditional vegetative propagation method of this crop which does not always satisfy the needs in planting material (Thro et al., 1999; Escobar et al., 2001). To increase this rate, numerous authors such as Kartha et al. (1974) and Konan et al. (1997) explored the possibility to multiply cassava using *in vitro* microcuttings. Nodal explants onto solid media provide on average 3 to 4 microcuttings by 2 to 3 months. This corresponds to a yearly multiplication rate from 1 to 5,000 (Escobar et al., 2001). With respect to the labor costs, this method is considered to be too expensive for mass propagation purposes (Raemakers et al., 2000).

Since its first description on this species by Stamp and Henshaw (1982), secondary somatic embryogenesis was proposed as an alternative for mass cassava propagation (Szabados et al., 1987; Raemakers et al., 1993; Mathews et al., 1993; Konan et al., 1994; Joseph et al., 1999; Ma and Xu, 2002; Hankoua et al., 2005). However, it has not been really applied for large scale propagation because the frequency of plant regeneration from cassava somatic embryos is usually low.

In plant tissue culture, the type of morphogenetic response is determined by the balance between the auxin and cytokinin hormones in the media. Thus, for numerous plant species, the somatic embryogenesis process is induced in media containing an auxin and cytokinin, generally in a ratio of 4:1 (Van Staden et al., 2008). Fujimura and Komamine (1975) reported the promotive effect of the cytokinin zeatin on somatic embryogenesis in carrot. This cytokinin could be involved in the promotion of cell division. From this, somatic embryogenesis of cassava could be improved by identifying a good hormonal balance (auxin/cytokinin). Surprisingly, in all the cassava works mentioned earlier, the induction media are cytokinin-free. Ma and Xu (2002) reported the complete inhibition of somatic embryogenesis supplementing the cytokinin BAP at 2.0 mg/l to an induction medium containing 2,4-D at 4.0 mg/l.

This study aimed to establish and to improve the secondary somatic embryogenesis for the clone R9 expressing a high production potential of ethanol.

MATERIALS AND METHODS

Plant material and culture conditions

Cuttings of cultivar R9 were obtained in 2007 from the Rayong-FCRC in Thailand and grown in Nestlé Research and Development Center-Tours greenhouse (NR&DC-T, France). The plants were multiplied *in vitro* through nodal segments on standard cassava micropropagation medium comprising basal MS medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/l copper sulphate, 0.05 mg/l naphthalene acetic acid (NAA), 0.02 mg/l 6-benzylaminopurine (BAP), 0.05 mg/l giberellic acid (GA3) and 20 g/l sucrose. The medium was solidified with Gelrite™ at 2.5 g/l. Before autoclaving pH was adjusted to 5.8 with 1.0 N KOH. Microcuttings were maintained at 25°C with a light intensity of 20 µmol m^{-2} s^{-1} (16 h light day period).

Primary and secondary somatic embryogenesis

For primary somatic embryogenesis, young leaf lobes measuring 5 to 10 mm long of the plants growing in greenhouse or microcuttings growing *in vitro* were cultured onto basal MS medium. They were placed in 90 × 10 mm Petri dishes containing 50 ml of induction medium. The latter were supplemented with 0.5 mg/l copper sulphate, 4 mg/l 2,4-D, 20 g/l sucrose and solidified with Gelrite™ at 2.5 g/l.

After 4 weeks, the primary explants with the adjoining tissues were subcultured onto maturation medium consisting of MS salts, Gamborg vitamins (Gamborg et al., 1968), 0.5 mg/l copper sulphate, 2 mg/l glycine, 30 g/l sucrose and 2.5 g/l Gelrite™. The cultures were maintained in the maturation medium mentioned earlier for 3 to 4 weeks at 25°C and with a light intensity of 20 µmol m^{-2} s^{-1}, that is, 16 h light period until the somatic embryos were obtained.

Regarding secondary somatic embryogenesis, cotyledonary-stage embryos collected at the end of the maturation step, were used as source of explants for inducing secondary somatic embryogenesis. They were divided into 3 to 7 small fragments measuring 1 to 3 mm long and transferred onto induction then maturation media. The composition of both media and the culture conditions were identical to the ones described earlier.

Different cytokinins, BAP, kinetin, zeatin, 2-iP and adenine, were tested respectively to compare their effectiveness on cyclic somatic embryogenesis by adding them at 1 mg/l to the induction medium containing 2,4-D at 4 mg/l , then to the maturation medium. A treatment consisted of 10 Petri dishes containing 50 ml of induction medium and inoculated with 5 explants, each Petri dish being a replicate. In total, each treatment comprised 50 explants. After 4 weeks incubation under dark conditions, the cultures were transferred onto maturation media containing the same cytokinins.

The cultures were microscopically examined at the end of the induction and maturation steps to assess the frequency of somatic embryogenesis, defined as the percentage of explants forming at least one embryoid (responsive explants) and its intensity which is defined by the number of embryoids formed per replicate of 5 explants.

Germination and acclimatization

For germination, after 3 weeks of culture on maturation medium, clumps of embryoids were isolated and transferred onto 90 × 10 mm Petri dishes containing 50 ml of germination medium. This medium comprised half-strength MS salts and MS vitamins supplemented with 0.5 mg/l copper sulphate, 0.05 mg/l NAA, 0.02 mg/l BAP, 0.05 mg/l GA3 and 20 g/l sucrose. The medium was solidified with agar at 8 g/l. The parameters evaluated after 5 weeks were the number of shoots and the number of plantlets (structures with shoot and root) scored per replicate. Shoots were rooted by transfer onto standard cassava micropropagation medium.

Concerning acclimatization, plantlets with a well established root

Table 1. Production of primary somatic embryos of R9 cassava clone from greenhouse grown plants and microcuttings.

Origin	Foliar explants	Embryoids
	nbr	nbr
Greenhouse	250	64
Microcutting	40	13

system and a shoot having at least a pair of leaves were transferred to small pots filled with peat. After 1 month, the surviving plants were transferred to larger pots filled with a mixture of peat and soil (50/50 % v/v).

Histological studies

Samples were fixed in 4% glutaraldehyde in phosphate buffer (0.1 M; pH 7.2) for 2 h at 4°C under vacuum and were rinsed in three changes of buffer. They were dehydrated in a graded ethanol series and embedded in technovit 7100 resin (Kulzer Histo-technique kit, Lanonor, Templemars, France) according to Kroes et al. (1998). Specimens were stored at 40°C. Sections were cut at 1.5 µm with a Leica RM2165 microtome, mounted on glass slides, stained with toluidine blue O, mounted after dehydration in a synthetic resin and examined under BH2/RFCA Olympus microscope.

Statistical analysis

Analysis of variance was performed using NCSS software. The treatment means were separate by the Newman and Keuls test at 5% significance level.

RESULTS

Production of primary somatic embryos

At the end of the maturation step, one foliar explant gave rise to 0.2 and 0.3 embryoid, respectively, when it was collected from a greenhouse growing plant or from an *in vitro* growing microcutting (Table 1). As 5 to 10 foliar explants can be prepared from a R9 microcutting, we can estimate that 1 to 3 embryoids can be regenerated within 7 weeks from one microcutting.

Effect of cytokinins on secondary somatic embryogenesis

At the end of the induction step, the frequency of somatic embryogenesis, expressed as a percentage of explants bearing embryoids, ranged from 88 to 100% irrespective of which cytokinin was added (Table 2). However, the number of embryoids per replicate was significantly higher when cytokinins were not added (control) and with adenine.

During the maturation step, the frequency and intensity of somatic embryogenesis decreased for all the treatments, indicating that many of organized embryogenic structures failed to progress beyond the globular stage and/or were overgrown by calli. However, the production of embryoids per replicate remained significantly higher in the presence of adenine. At the end of the maturation, each fragment produced 2.4 embryoids with adenine and 1.7 for the control. The other cytokinins inhibited the intensity of secondary embryogenesis compared with the control with 75% inhibition for zeatin and 30% in the case of the kinetin.

The inhibitory effect of cytokinins was confirmed on the number of plantlets produced at the end of the germination step, with the notable exception of adenine. Shoots were also observed, which could be rescued by rooting them after an additional subculture. In total, 2.3 and 3.1 transplantable plantlets were obtained per replicate, for the control and the adenine treatment, respectively. In both cases, the conversion rate into plantlets of the embryoids counted at the end of the maturation step was 26 %.

Effect of the adenine concentration on secondary somatic embryogenesis

Table 3 shows the results of cultivating small pieces of green somatic embryos in culture media containing 1 to 40 mg/l of adenine. At the end of the induction and maturation steps, only a concentration of 10 mg/l led to a significant increase in the number of embryoids when compared with the control (52.2 instead of 21.6 embryoids per replicate). The embryoids produced on media containing 10 mg/l of adenine were generally bigger than those in the control (Figure 1a, b). Moreover, some typical heart-stage embryoids were frequently observed in these cases contrary to the control or to the lowest adenine concentrations (Figure 1c).

At the end of germination, despite a variation from 0.3 to 1.4 plantlets per replicate, the assessed adenine concentrations did not lead to any significant effect. After rescuing the shoots, the highest number of plantlets per replicate (3.2) was observed where the production of the embryoids occurred in the presence of adenine at 40 mg/l. This treatment was also the best considering the embryoid-to-plantlet conversion rate (13%).

The acclimatization survival rate in the greenhouse varied from 40 to 67%, it tended to be higher for the plantlets issued from the highest adenine concentrations (Table 4) (Figure 2).

Histological study of secondary somatic embryogenesis

After 2 weeks of culture on induction medium containing

Table 2. Frequencies and intensities of R9 secondary somatic embryogenesis and plantlet recoveries as a function of the cytokinin added to the induction and maturation media [a].

Cytokinin	Induction				Maturation				Germination					
	Responding explants		Embryoids structures		Responding explants		Embryoids structures		Foliose		Shoot		Plantlet	
	Average		nbr / replicate		average		nbr / replicate		nbr / replicate		nbr / replicate		nbr / replicate	
Control (0 mg/l)	88	a	18.8	a	70	b	8.7	b	2.6	b	0.7	a	1.6	b
BAP	96	a	11.1	b	25	a	2.9	a	1.9	bc	0.1	a	0.2	a
Kinetine	88	a	9.2	b	55	ab	6.4	ab	3.2	b	0.2	a	0.1	a
Zeatin	90	a	7.8	b	38	ab	2.2	a	2.2	b	0.0	a	0.1	a
2-i P	100	a	12.5	b	32	ab	4.0	ab	3.3	b	0.2	a	0.1	a
Adenine	98	a	20.6	a	68	b	11.9	bc	4.2	ab	1.3	b	1.8	b

[a]Cytokinins were added at 1 mg/l to the induction (with 2,4-D 4 mg/l) then to the maturation media. The evaluation was performed after 4 weeks on induction medium, 3 weeks on maturation medium and 5 weeks on germination media. The data are the average of 10 replicates, each one consisting on 5 fragments of cotyledonary-stage embryos. Data followed by the same letter in superscript are not significantly different at 5% level according Newmann-Keuls tests.

Table 3. Frequencies and intensities of R9 secondary somatic embryogenesis and plantlet recoveries as a function of the adenine concentration [a].

Adenine	Induction				Maturation				Germination					
	Responding explants		Embryoids structures		Responding explants		Embryoids structures		Foliose		Shoot		Plantlet	
mg/l	Average		nbr / replicate		average		nbr / replicate		nbr / replicate		nbr / replicate		nbr / replicate	
0	100	b	30.0	cd	80	b	21.6	a	1.7	a	1.4	ab	0.9	a
1	84	ab	13.1	abcd	42	a	10.6	a	1.7	ab	0.3	a	1.0	a
5	78	ab	10.9	a	60	a	7.4	a	1.0	a	0.4	a	0.3	a
10	100	b	43.5	e	91	b	52.2	b	4.1	d	1.2	ab	1.4	a
20	98	b	23.8	abcd	76	b	16.6	a	3.4	bd	0.8	ab	1.4	a
40	88	b	27.2	bcd	73	b	23.9	a	3.9	d	1.9	b	1.3	a

[a] Adenine was added to the induction (with 2,4-D 4 mg/l) then to the maturation media The evaluation were performed after 4 weeks on induction medium, 3 weeks on maturation media and 5 weeks on germination media. The data are the average of 10 replicates, each one consisting on 5 fragments of cotyledonary-stage embryos. Data followed by the same letter in superscript are not significantly different at 5% level according Newmann-Keuls tests.

adenine at 10 mg/l, the histological sections from cotyledonary explants revealed that, the first cell divisions took place close to the vascular bundles (Figure 3b, c). A week after incubation on inducing medium, large vacuolated cells appeared on the periphery of the explants and numerous isodiametric cells inside (Figure 4a, b, c). These perivascular cells had a high proportion of nucleoplasmic material and were clearly meristematic. They were grouped in meristematic masses inside which intense multiplication zones could be observed at 3 weeks (Figure 4d). Starting from 3 to 4 weeks, differenttiation of adventitious roots, leaves and putative embryoid structures were simultaneously observed (Figure 4e). Typical embryogenic bipolar structures of different stages, having caulinar and root meristems, were frequently identified (Figure 4f, g). Interesting, formation of supernumerary embryoids occurred on already existing differenciated embryoids (Figure 4h). These embryoids showed a well defined separation line between them.

DISCUSSION

The possibility of mass production of cassava plantlets by running secondary embryogenesis was investigated for the clone R9. Our findings were similar to those of

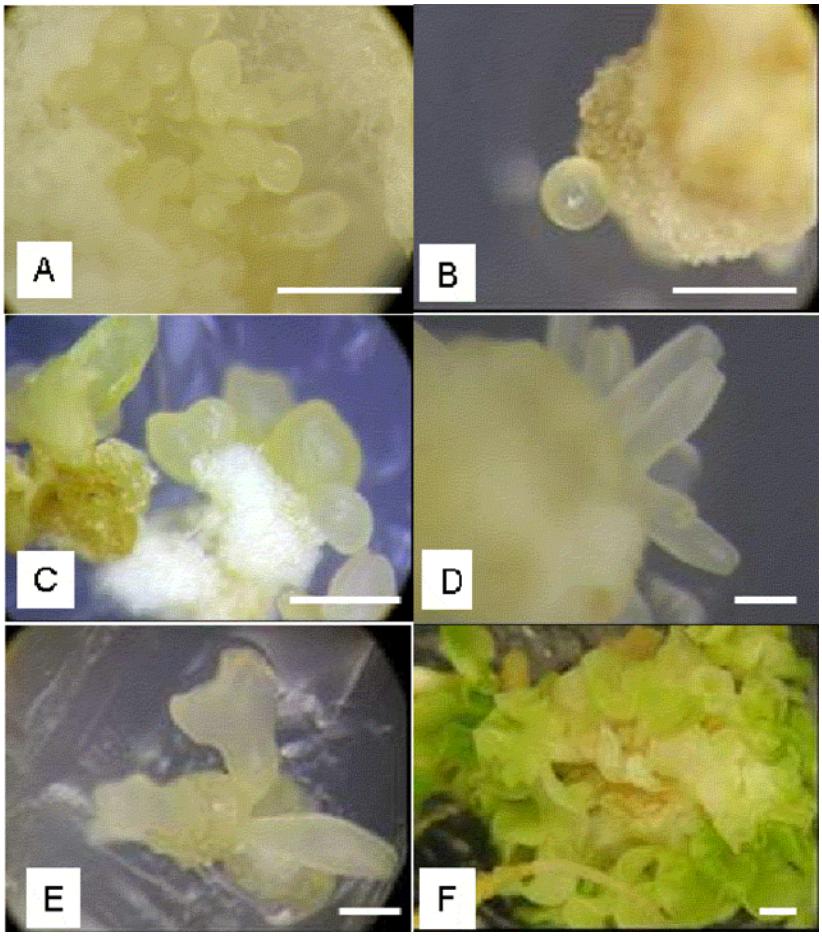

Figure 1. Cyclic somatic embryogenesis of R9 cassava clone: (A) Globular-stage embryos at the end of the induction step without cytokinin; (B) globular-stage embryos with adenine at 10 mg/l ; (C) heart-stage embryos with adenine 10 mg/l; (D) early torpedo-stage embryos with adenine 10 mg/l; (E) late torpedo-stage embryos with adenine 10 mg/l; (F) cotyledonary-stage embryos at the end of the maturation step with adenine 10 mg/l. Scale bar: 1 mm.

Table 4. Acclimatization of cassava R9 plantlets after induction and maturation steps conducted with different adenine concentrations.

Adenine concentration	Plantlets *ex vitro* transferred	Surviving plantlets	Surviving rate
mg/l	nbr	nbr	%
Control (0 mg/l)	22	9	41
1	20	8	40
5	5	2	40
10	12	8	67
20	14	7	50
40	13	7	54

Raemakers et al. (1993) that, each fragment of mature cotyledonary embryos produced significantly more embryoids than foliar explants resulting from micro-cuttings, 2.0 to 4.0 as against 0.3 per explant.

By histological observations, the presence of true embryogenic structures during the induction step could

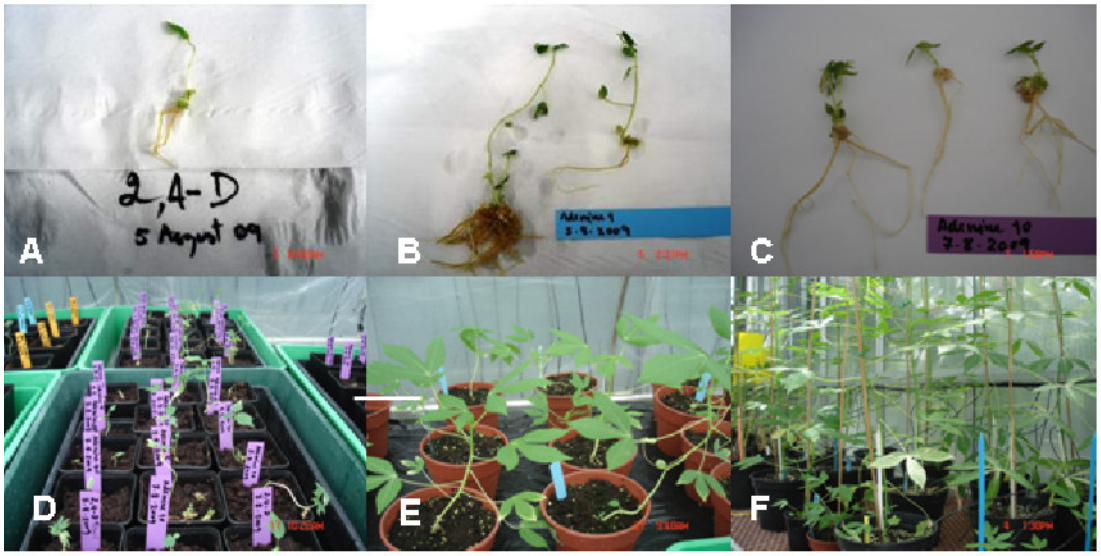

Figure 2. R9 cassava plant recoveries from cyclic somatic embryogenesis process: (A) Plantlets issued from the control (adenine 0 mg/l); (B) plantlets issued from adenine 1 mg/l; (C) plantlets issued from adenine 10 mg/l; (D) acclimatization in greenhouse (2 weeks); (E) plants after 1 month; (F) plants after 3 months.

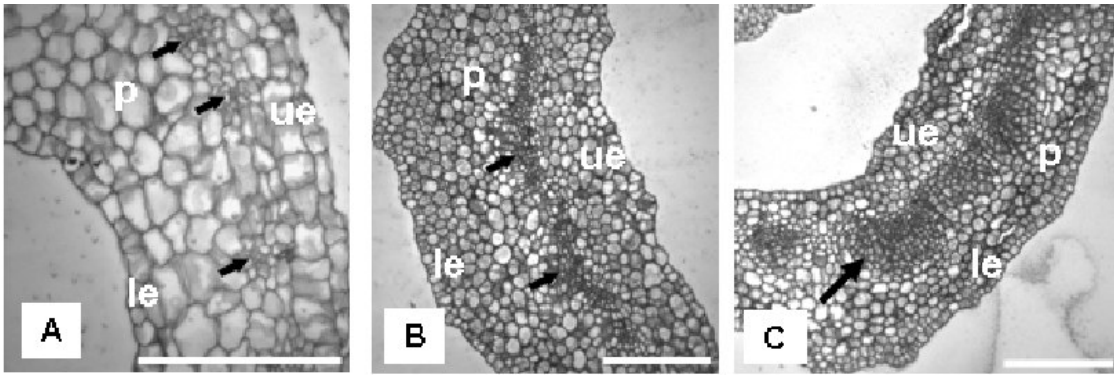

Figure 3. Origin of R9 cassava secondary somatic embryos. (A) Fragments of cotyledons before incubation in the induction medium (control sample): vascular bundle (arrows), ue: upper epidermis, le: lower epidermis, p: parenchyma; (B) first step of cellular multiplication at 2 weeks (arrows); (C) meristematic massifs at 2 weeks (arrow). Scale bar: 100 µm.

be checked. However, this approach confirmed that a cyclic culture consists of a mix of adventitious roots, shoots and embryogenic structures. Other kinds of adventitious structures, leaf-like structures (also called foliose) are also observed. This structural heterogeneity in cassava cultures has been mentioned by several authors (Szabados et al., 1987; Raemakers et al. 1993; Konan et al., 1994).

With the exception of adenine, the application of cytokinins during the induction phase inhibits the development of somatic embryos. The inhibitory effect of exogenous cytokinins on cassava somatic embryogenesis suggests that, this type of hormone is present in the tissues at supra-optimal concentrations. Cassava seems similar to some graminaceous species, as orchardgrass, for which exogenous cytokinins inhibit somatic embryogenesis at very low concentrations (Wenck et al., 1988). In orchadgrass, genotypic differences for somatic embryogenesis are related to endogenous cytokinin levels and anticytokinins positively influence the frequency of embryo formation (Wenck et al., 1988; Somleva et al., 1995). Similarly, high contents in endogenous cytokinins can hamper the embryogenic process in cassava tissues and anticytokinins could eventually be successfully applied to this plant.

The mode of action of adenine is not fully explained

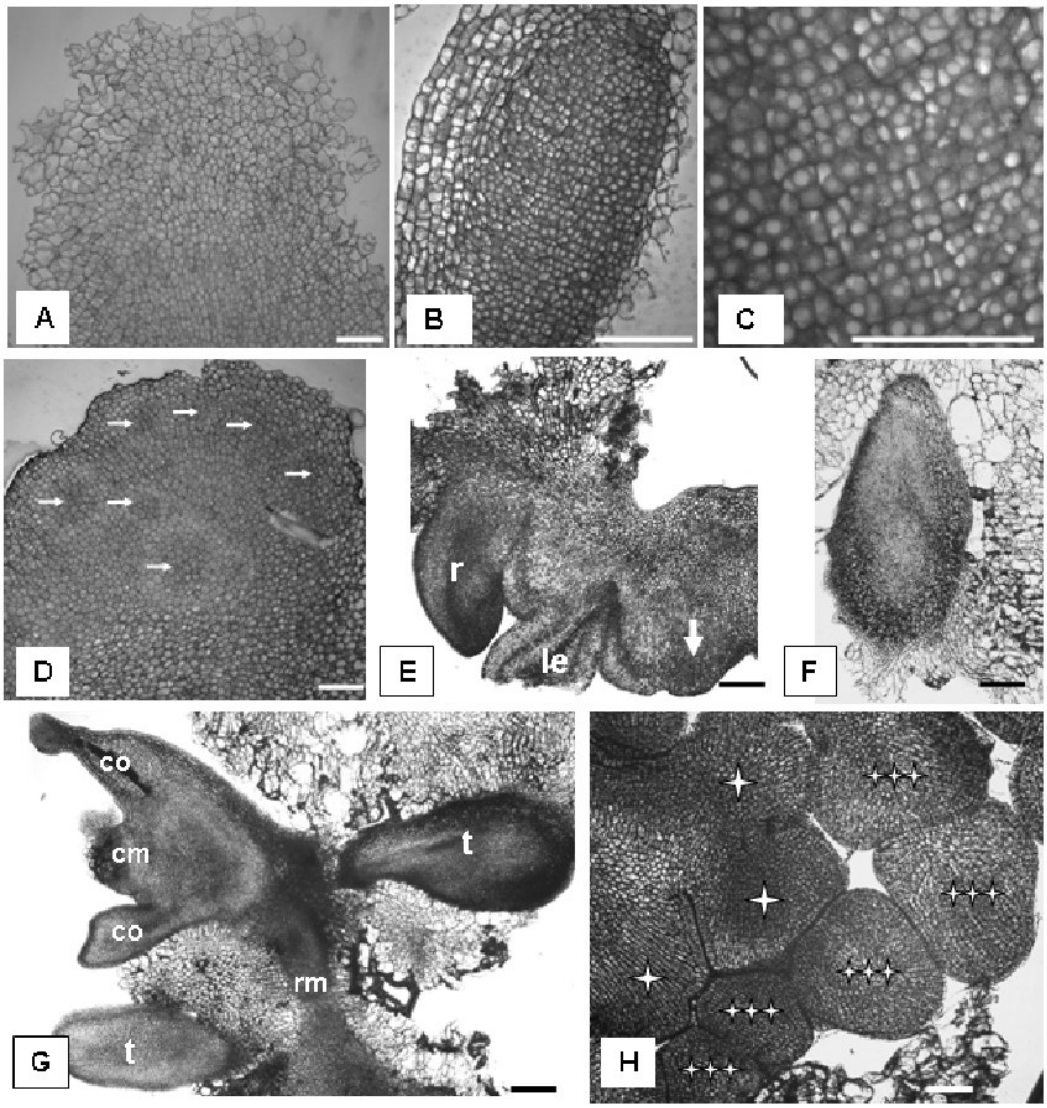

Figure 4. Formation of R9 cassava secondary somatic embryos. (A) Callus cells 1 week after incubation on induction medium; (B) meristematic structures in cotyledons (2 weeks); (C) meristematic cells (2 weeks); (D) intense multiplication zone at 3 weeks (arrow); (E) root and leave differentiation at 4 weeks, r: root differentiation, le: leaves differentiation, cm: caulinar meristem with small and dense cells (arrow); (F) torpedo embryo at 4 weeks; (G) different stages of embryos at 4 weeks, cm: caulinar meristem, rm: root meristem, co: cotyledon, t: torpedo embryos; (H) supernumerary embryo formation (triple star) on somatic embryos (star) differentiated from callus at 5 weeks. Scale bar: 100 µm.

and it is considered as a substrate for the synthesis of natural cytokinins as well as a product of their degradation as reported in a recent review by Van Staden et al. (2008). It is recognized that, the activity of adenine is much less than the true cytokinins and 25 to 100 times the concentrations maybe required producing similar result. On carrot, which is the plant species used as a model for somatic embryogenesis, adenine at 2 mg/l is added to the 2,4-D induction media (Halperin, 1964). In this work, adenine concentrations up to 40 mg/l were applied without any detrimental effect on the induction and maturation of cassava somatic embryos. On the contrary, adenine concentrations higher than 5 mg/l tend to improve the process in terms of embryoid size and plantlet recoveries in the greenhouse. These findings confirm the absence of toxicity of adenine which can probably be used with higher concentrations.

Typically, as one cotyledonary-stage embryo can roughly be cut in 5 fragments (corresponding to one replicate in our experiments), it can be assumed that one R9 embryo produces at least 10 to 20 new embryoids within 7 weeks, from which 2 to 3 plantlets can be rege-

Table 5. Extrapolated yearly multiplication rate of a mass propagation process of the cassava clone R9 based on cyclic somatic embryogenesis.

Duration (weeks)	0	7	35	5	5
Hypothesis:	**Microcutting**	**Primary embryoid**	**Secondary embryoids**	***In vitro* plantets**	**Acclimatized plants**
At each cycle, one embryoid gives:	**nbr**	**nbr**	**nbr**	**nbr**	**nbr**
10 embryoids	1	1	100.000	15.000	7.000
20 embryoids	1	1	3,200,000	480.000	240.000

nerated. These observations are similar to the ones reported on other clones by Raemakers et al. (1993). A yearly multiplication rate of a mass propagation process of the cassava clone R9 based on cyclic somatic embryogenesis can be extrapolated, assuming that: The process starts with one microcutting which will give one embryoid within 7 weeks; each embryoid will give 10 to 20 embryoids at each cycle of 7 weeks; 5 cycles are performed; the embryoid-to-plantlet conversion rate is 15%; the survival in the greenhouse is 50%.

Obviously, the efficiency of the process will depend a lot on the number of embryoids produced at each cycle, from 7,000 to 240,000 plants per year, considering that one embryo will give 10 and 20 embryoids at each cycle respectively (Table 5). However, although high numbers of R9 plantlets can potentially be obtained from secondary embryogenesis, the embryo-to-plantlet conversion rate currently remains too low for a commercial propagation of this clone. Due to that, culture of microcuttings is more efficient, particularly if it can be conducted by temporary immersion in liquid medium as described by Escobar et al. (2001) and Ospina et al. (2007).

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