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Somatic embryogenesis from cultured mature cotyledons of cassava (*Manihot esculenta* Crantz)

Identification of parameters influencing the frequency of embryogenesis

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Abstract

Somatic embryogenesis was obtained from mature cassava cotyledons explants. A two-step medium sequence was developed for efficient embryogenesis. Application of 2,4-D (4 mg l^{-1}) yielded proembryogenic masses which developed into somatic embryos after transfer to a medium containing NAA (0.01 mg l^{-1}), BA (0.1 mg l^{-1}) and GA_3 (0.1 mg l^{-1}). The 2,4-D concentrations used for embryo initiation strongly influenced embryo development. Among the cultivars tested, TMS 30395 was most responsive. Full strength MS basal medium alone or with 4 × MS micro salts was efficient for the formation of somatic embryos. Casein hydrolysate, adenine sulfate, nicotinic acid, glycine, tryptophan, and serine were ineffective for embryo development. High sucrose concentration (6%, w/v) inhibited the induction of somatic embryos, while 6% sucrose was optimal concentration for the development of somatic embryos after an induction treatment using 2% sucrose. Addition of 0.52 mg l^{-1} ABA to the induction media resulted in an increase in somatic embryos production. The ploidy levels of the regenerated plantlets were determined by flow cytometry analysis. Fifty regenerants tested were all tetraploids as the source plants and were morphologically normal. The implications of these results are discussed in relation to genetic transformation using the cotyledons as the explant source.

Abbreviations: ABA – abscisic acid, BA – 6-benzylaminopurine, DAPI – 4′,6-diamidino-2-phenylindole; SR 101 sulforhodamine, GA_3 – gibberellic acid, MCPA – methyl- chlorophenoxyacetic acid, NAA – naphthalen-acetic acid, PCPA – P-chlorophenoxyacetic acid, 2,4-D – 2,4-dichlorophenoxyacetic acid, 2,4,5 T – 2,4,5-trichlorophenoxyacetic acid

Introduction

Research on cassava tissue culture could be useful for vegetative propagation as well as for genetic manipulation studies. Although tissue culture methods are available for cassava (Roca 1984), regeneration from callus culture has been difficult, and is genotype-dependent. Regeneration through somatic embryogenesis has been obtained using cotyledons, embryonic axes of seeds, or young leaflets (Stamp & Henshaw 1982; Stamp 1984; Stamp & Henshaw 1986, 1987; Szabados et al. 1987). Although, an efficient system of secondary embryogenesis in cassava has been reported (Raemakers et al. 1993), primary embryogenesis starting either from young leaflets or zygotic embryo cotyledons is generally inefficient. Variation among varieties as well as the physiological states of the explants has limited routine and high frequency regeneration. This study presents an optimization of somatic embryogenesis from cotyledon explants of several cassava clones of African origin.

Materials and methods

Plant materials and culture conditions

Seeds of African cultivars (TMS 30395, TMS 30555, 85621, 84701, 83375, Tiegba) were obtained from the 'Institut Des Savanes' (IDESSA); Côte d'Ivoire and South American cultivars (Bra 115, Bra 191, Bra 225) were from the 'Centro Internacional de Agricultura Tropical' (CIAT, Colombia). Seeds were surface sterilized for 30 min in 15 % (w/v) calcium hypochlorite, washed in sterile distilled water and soaked overnight in water. Cotyledons were cut transversely, and placed into culture as described by Stamp & Henshaw (1982), using 25 to 30 explants per Petri dish (90 mm, diameter).

Explants were subjected to a two-step culture procedure at 27°C. During the first three weeks of culture (induction or step 1 period), cultures were incubated in the dark for one week before transfer to 16-h photo period under a light intensity of 50-100 µmol photons $m^{-2}s^{-1}$. For each cassava cultivar, experiments were repeated 2 to 6 times over a 2 year period. All data were statistically analysised using the X^2 statistic.

The primary induction medium (step 1 period) contained auxins such as 2,4- D, MCPA, PCPA and 2,4,5-T. Auxins were tested at 2 different concentrations (0.1 and 4 mg l^{-1}) for the induction of somatic embryos, except for 2,4-D which was used at 0.1, 4, 8, 12 and 20 mg l^{-1}. Using MS medium (Murashige & Skoog 1962) containing 4 mg l^{-1} 2,4-D, ABA (at concentrations of 0.26, 0.52 and 1.04 mg l^{-1}), proline, glycine, serine, tryptophan, nicotinic acid, adenine sulfate and casein hydrolysate each at 100 mg l^{-1}, were also assayed together or separately for their effect on somatic embryogenesis during the induction period. Two to 8% sucrose were also evaluated during both step 1 and step 2 periods. Culture media were supplemented with 7% (w/v) Difco-Bacto-Agar and were adjusted to pH 5.7 before autoclaving (20 min at 120°C). During the second-step (2-3 weeks) i.e. for the development of somatic embryos, all the above listed growth regulators were substituted by a phytohormone combination such as 0.01 mg l^{-1} NAA, 0.1 mg l^{-1} BA, and 0.1 mg l^{-1} GA_3.

Cytological studies

Cotyledon tissues were fixed (for 48 h) every two days after culture initiation (0 day) in formalin-acetic acid-ethanol (FAA;1:1: 8, by vol). They were then dehydrated for 15 min in a graded ethanol series (15% to 100%) and embedded in paraffin. Sections (7-10 µm thick) were cut with a rotary microtome and spread on slides with drops of 0.05% gelatine solution. They were then incubated at 37°C for 24 h before removing the paraffin in toluene and decreasing ethanol series (l00° to 15°). After 3 rinses with running water, sections were incubated for 5 min in a solution of 1% periodic acid. (SIGMA, St. Louis, Mo; USA) before staining in Schiff reagent (20 min) and hematoxylin (SIGMA, St. Louis, Mo; USA) for 3 min (Sangwan et al. 1992).

Electron microscopy

For scanning electron microscopy, the samples were fixed in 4% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.6 for 3 hours. After washing (3 times) in 0.1 M cacodylate buffer, the samples were dehydrated in a graded acetone series (15% to 100%) and subjected to critical point drying before metallization with gold and observation with a scanning electron microscope (PHILIPS 505) at 20 kV acceleration.

Flow cytophotometry

For flow cytometry, fresh cotyledons taken from mature seeds of cassava or leaves from in vitro cassava plantlets, were mechanically chopped with petunia leaves (internal reference) using razor blades, following the procedure of Brown et al. (1991). The nuclei were isolated using 0.5 ml of a solution of citric acid (0.1 M) containing 0.5%. The nuclei were isolated using 0.5 ml of a solution of citric acid (0.1M), containing 0.5% Tween 20. The samples were filtered through a 56 µm nylon gauze, and their volume adjusted to 1 ml. Nuclei were incubated for 20-30 min at room temperature and centrifuged (2500 rpm, 5 min). The nuclei were then resuspended for 15 min in 0.2 ml of the citric acid/Tween 20 solution to which was added 1 ml of 0.4 M Na_2HPO_4 solution (pH 9) containing 5 µg l^{-1} of DAPI. This is a slight modification of the original method described by Ulrich & Ulrich 1991. Flow cytometry was performed using a CA II flow cytometer and each histogram is based on analysis of 5. × 10^3 to 10^4 nuclei per experiment. (Brown et al. 1991).

Chromosome counting

Young leaves (0.2-0.5 cm length) from the region of the apical meristem of plants growing in the greenhouse

were removed and fixed in a Carnoy solution for 4 hours. The samples were then stained in acetocarmine at 60-80°C (1 h) and squashed for chromosome countings.

Results

Prior to culture, cotyledons of cassava consisted of uniform epidermal and mesophyll cells, interspersed with vascular tissues (Figure 1 A). After two days of culture on auxin-containing medium (MS with 4 mg l^{-1} 2,4-D), cell divisions were observed. Mitotic activities started first in the vascular regions (Figure 1 B), and after 10 days of culture, meristematic regions were formed (Figure 1 C). Globular structures (Figure 1 D) were evident on the upper surface of the cotyledon explants within 3 weeks of culture. These formations developed into somatic embryos after transfer to second-step medium with reduced phytohormone concentrations. In order to improve the regeneration process, a range of treatments including phytohormonal and nutritional factors were evaluated.

Effects of auxins on the initiation of embryogenesis

Four auxins were tested at concentrations of 0.1 or 4 mg l^{-1} using MS medium. None of the auxins at a concentration of 0.1 mg l^{-1} promoted embryo formation, but the explants expanded in size and occasionally showed root formation. The responses of cotyledon explants of several cultivars following 3-week incubation period on 4 mg l^{-1} auxins are summarized in Table 1. Proembryogenic masses (Figure 1 D) were produced on media containing 4 mg l^{-1} 2,4-D with all the cultivars tested, while PCPA, MCPA and 2,4,5-T showed only slight or no effects. Embryo initials induced on media containing auxins other than 2,4-D never developed into somatic embryos following transfer onto the second-step MS medium. With high concentrations of 2,4-D (> 4 mg l^{-1}), cultivars Tiegba and 85621 produced only callus while other cultivars such as TMS 30395, TMS 30555 and 83375 rarely formed proembryos (data not shown). Moreover, as shown in Table 2 for the cultivar TMS 30395, although the percentage of embryogenic explants increased up to 8 mg l^{-1} of 2,4-D, the density of embryo initials decreased as level of 2,4-D increased from 4 to 20 mg l^{-1}.

Development of somatic embryos

Cotyledon explants with embryo initials were transferred on to the second-step MS medium containing NAA (0.01 mg l^{-1}), BA (0.1 mg l^{-1}) and GA_3 (0.1 mg l^{-1}) for embryo development. Within 2 to 3 weeks of subculture, development of proembryogenic masses into somatic embryos occurred (Figures 1 E, F, G, H and Figures 2 A and B). Phytohormone-free medium during the second-step cultivation did not promote embryo development (0/210 embryogenic explants for all the cultivars tested), whereas combination of NAA (0.01 mg l^{-1}), BA (0.1 mg l^{-1}) and GA_3 (0.1 mg l^{-1}) provided 25.0%, 18.6%, 6.2%, 9.3%, 7.1% and 1.8% of embryo development for the cultivar TMS 30395, TMS 30555, 84701, 83375, 85621 and Tiegba respectively, after an induction treatment using 4 mg l^{-1} 2,4-D. Different stages of embryo development could be observed on embryogenic explants (Figures 1 G and 2 A), indicating an heterogeneous development of embryogenesis in cotyledon culture of cassava. Morphologically abnormal embryos were frequently observed with all the 2,4-D concentrations tested (Table 3) and percentages of somatic embryos with fused cotyledons (Figure 1 H) were higher than those exhibiting one or two cotyledons (Figures 2 A and B). Other embryos had more than two cotyledons (polycotyledonary) and/or partial or total fusion of differentiated embryos, rendering isolation of individual embryos difficult. Foliose and cotyledon-like structures, which developed on some explants, were infrequently (e.g. 5% for cultivar TMS 30395) associated with meristems and gave rise to adventitious buds (Figure 2 C). Bud development was concomitant with embryo development or appeared after subculturing organogenic structures.

Addition of abscisic acid to the induction medium

Addition of abscisic acid to the induction medium supplemented with 4 mg l^{-1} 2,4-D enhanced somatic embryogenesis from cotyledon explants (Table 4). With 2% (w/v) sucrose containing MS medium, optimal embryogenesis occurred at 0.52 mg l^{-1} ABA for the cultivar TMS 30395, resulting in the highest values of both percentage of explants with somatic embryos (35.8%) and the mean number of somatic embryos per explant (9.44 ± 0.78, significant difference). An experiment was conducted for several cassava cultivars to test whether a combination of ABA and high sucrose concentration had a promoting effect on embryo yield.

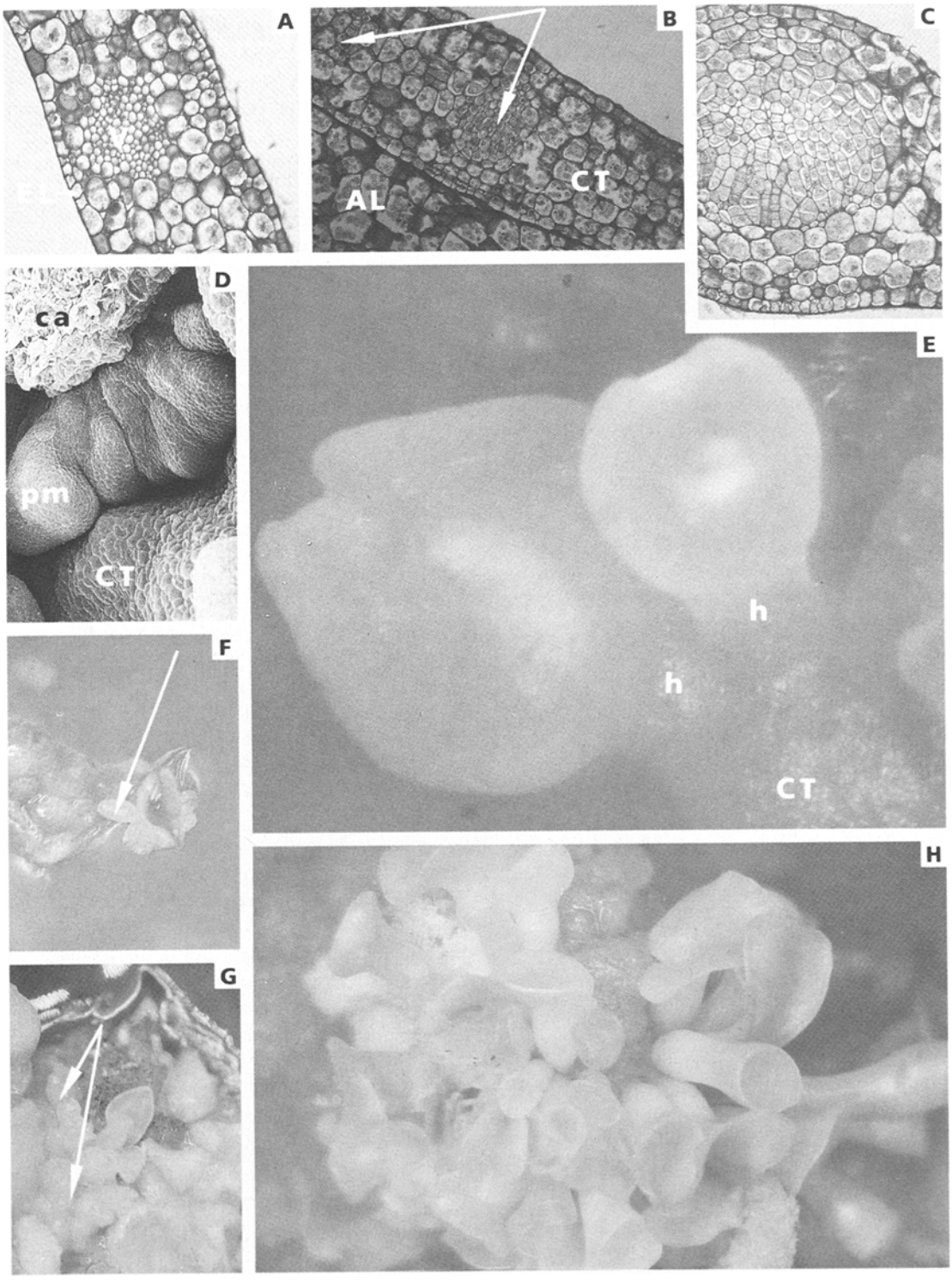

Fig. 1. **(A):** Longitudinal section in a freshly isolated cotyledon of cassava showing vascular region (v). E.L.: Epidermal layer.(× 160.85). **(B):** Mitotic activities in vascular cells (arrows) during the first week of culture. Cell division weakly occurred in the neighbouring cells. CT: Cotyledon tissue, AL: Albumen tissue (× 160.85) **(C):** Intensification of mitotic activities in vascular tissue followed 10 days of culture (× 160.85). **(D):** Globular proembryogenic masses developed on an explant after three weeks of culture on 2,4-D (4 mg l^{-1}) containing medium . ca: callus; pm: proembryogenic mass; CT: cotyledon tissue (× 82.5). **(E):** Evolution and individualization of somatic embryos at heart stage during the second cultivation phase. h; hypocotyl; CT; cotyledonary tissue (× 41.6). **(F):** Arrow, somatic embryo at topedo stage (× 16.6). **(G):** Somatic embryo showing three developed cotyledons, and surrounded (arrows) by several stages of somatic embryos (× 33.2). **(H):** Somatic embryos showing horn-shaped at cotyledonary stage (× 20.8).

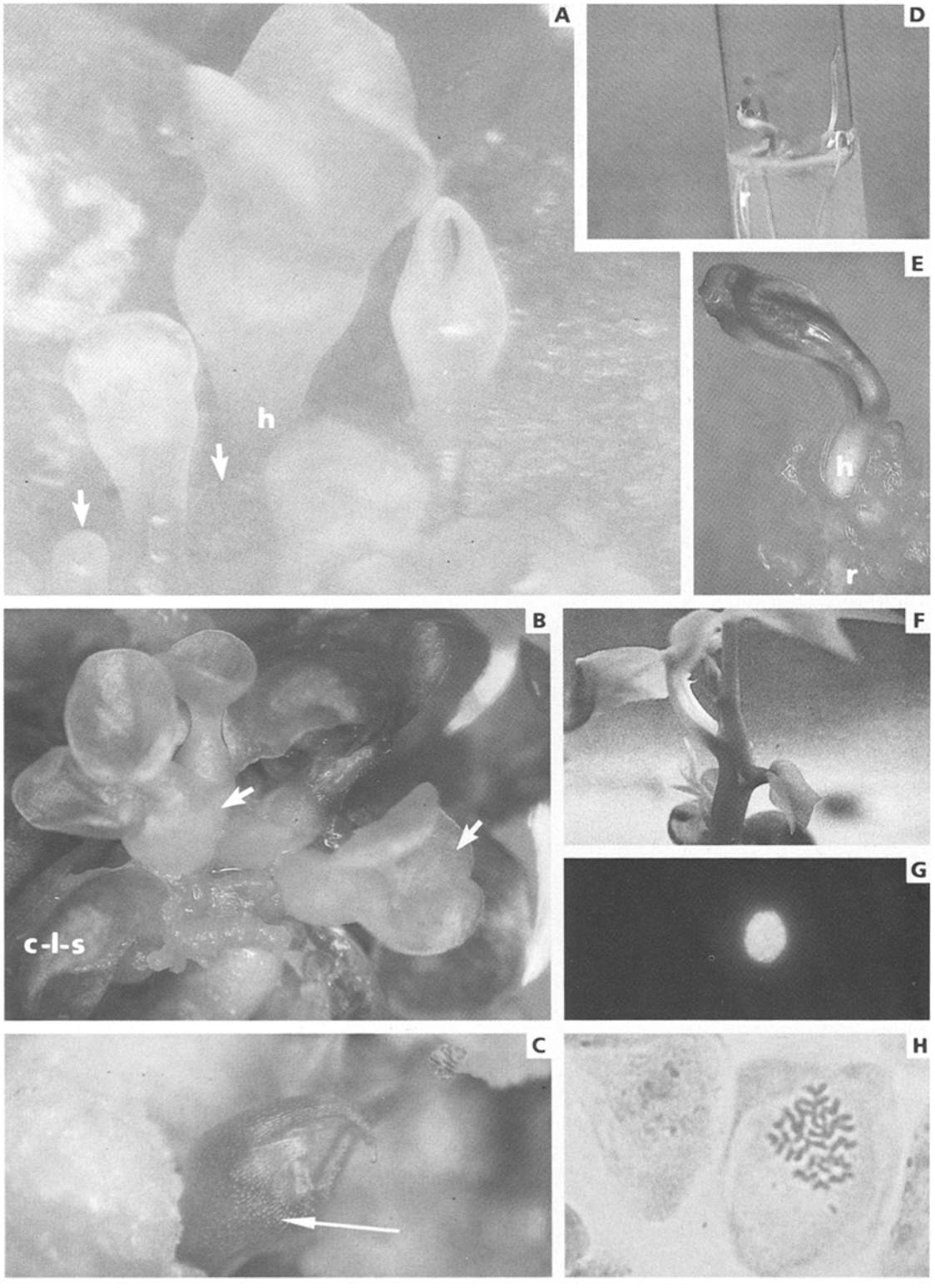

Fig. 2. **(A):** Normal somatic embryos with hypocotyl (h) and two developed cotyledons surrounded by proembryogenic masses (arrows). **(B):** Abnormal somatic embryos showing a fusion in their hypocotyl region and one cotyledon (arrows). c-l-s: cotyledon-like-structures (× 16.6). **(C):** Adventitious bud from a clump of foliose structures. **(D):** Germination of horn-shaped somatic embryos. r: root (× 0.9). **(E):** Germination of a dicotyledonous somatic embryo. h: hypocotyl, r: root (× 4.16). **(F):** Plantlet derived from somatic embryogenesis (x 0.6). **(G):** Nucleus isolated from a cassava leaf; coloration: DAPI; Photography: Rekhert Polyver Microscope (× 187.2). **(H):** Photography of a mitotic cell of a young cassava leaf showing 2n = 36 chromosomes in metaphase (× 1875).

Table 1. Effects of different auxins on the induction of proembryogenic structures from cotyledon explants of cassava.

Number and percentage ( ) of embryogenic explants					
Auxin	Cultivars				
4 mg l^{-1}	TMS 30395	TMS 30555	Tiegba	84701	85621
2,4-D	60/135 ** a	51/64** d	18/64** e	49/80 ag	43/65 dg
	(44.4%)	(79.68%)	(28.12%)	(53.75%)	(66.15%)
MCPA	0/84 b	2/84 bf	0.84 b	N.T.	N.T
	(0.0%)	(2.38%)	(0.0%)		
PCPA	18/92 ce	0.90 b	8/92 f	N.T.	N.T.
	(19.56%)	(0.0%)	(8.69%)		
2,4,5-T	12/94 cf	2/84 bf	0/88 b	N.T.	N.T.
	(12.76%)	(2.38%)	(0.0%)		

Number of proembryogenic explants / Total number of cultured explants.
Results were scored after three weeks of culture using MS medium supplemented with 2% (w.v) sucrose. Numbers followed by the same letters are not statistically different. In each cassava cultivar (across columns), ** indicates significant differences ($p \leq 1\%$) between results obtained with 4 mg l^{-1} 2,4-D and with those obtained on MCPA, PCPA and 2,4,5-T containing media. NS: Not significant difference between cultivars for each auxin treatment (across rows). NT: Not tested.

Table 2. Effects of the 2,4-D concentrations on embryogenesis from cultured cotyledons of cassava TMS 30395.

	*Proembryogenic formations		**Production of developed (mature) somatic embryos	
2,4-D (mg l^{-1})	Number of explants	Explants responding (%)	Number of explants	Explants with embryos (%)
0.1	51	0 (0.0) a	-	-
4.0	135	60 (44.4) b	60	15 (25.0) d e
8.0	112	77 (68.7) c	77	27 (35.0) d
12.0	124	59 (47.5) b	59	8 (13.5) e
20.0	106	46 (43.3) b	46	0 (0.0) f

Explants were cultured on MS medium containing 2% sucrose with 2,4-D as inducted. Across columns, numbers followed by the same letter are not statistically different. *: During primary culture period. **: During second culture period.

The comparative results (Table 4) show that, a combination of 0.52 mg l^{-1} ABA and 3% (w/v) sucrose increased the mean number of somatic embryos per explant for every cassava cultivar tested, especially for the culivars TMS 30555 and BRA 225.

Effects of mineral and organic nutrients on embryogenesis

The results shown in Table 5 indicate that MS basal medium was suitable for embryo development for most of the cultivars tested. Addition of 4 × MS micro salts to basal MS medium increased the frequency of embryogenesis (31.4%) for the cultivar TMS 30395 as compared to the control (13.1%). In most cases, half strength and 2 × MS basal medium reduced embryogenesis. We further optimize the mineral nutrients using

Table 3. Frequency of normal and abnormal somatic embryos at cotyledonary stage developed from cotyledon explants of cassava (TMS 30395).

	Normal somatic embryos	Abnormal somatic embryos		
2,4-D (mgl^{-1}) during the induction period (3 weeks)	Two cotyledons	One cotyledon	Fused cotyledons	Other form
4	9/49	2/49	24/49	14/49
8	13/86	5/86	65/86	3/86
12	2/6	0/6	4/6	0/6
Total of embryos counted (%)	24/141 (17.0%)	7/141 (4.9%)	93/141 (65.9%)**	17/141 (12.0%)

Number counted/Total somatic embryos.

Results were scored at the end of the fifth week after the two-step culture periods on embryogenic explants from a class of experiment. **Indicates a significant difference ($p \leq 1\%$) for somatic embryos with fused cotyledons. Embryogenic cultures were transferred to MS + 2% sucrose + NAA (0.01 $mg\,l^{-1}$), BA (0.1 $mg\,l^{-1}$) and GA_3 (0.1 $mg\,l^{-1}$) in second culture period.

Table 4. Effect of ABA on induction and development of somatic embryos in cultured cotyledon explants of several cassava cultivars. MS medium + 4 $mg\,l^{-1}$ 2,4-D was throughout.

Cultivars	Sucrose concentration (w/v)	ABA ($mg\,l^{-1}$)	Total number of cultured explants	Number and (percentage) of explants with developed somatic embryos	Mean number of somatic embryos per explant
		0.0 (control)	88	24 (26.13%)	2.38 ± 0.65
		0.26	77	20 (25.97)%	3.15 ± 0.98
TMS 30395	2%	0.52	81	29 (35.80%)	9.44 ± 0.78**
		1.04	86	12 (13.95%)	1.25 ± 0.04
BRA 115	2% (control)	0.0	44	15 (34.09%)	5.3 ± 4.1
	3%	0.52	44	14 (31.81%)	6.3 ± 5.1
BRA 191	2% (control)	0.0	75	0 (0.0%)	0.0 ± 0.0
	3%	0.52	74	12 (16.21%)**	6.7 ± 3.1
BRA 225	2% (control)	0.0	46	10 (21.73%)	3.2 ± 1.6
	3%	0.52	41	5 (12.19%)	7.8 ± 3.9**
TMS 30555	2% (control)	0.0	100	0 (0.0%)	0.0 ± 0.0
	3%	0.52	112	6 (5.35%)*	16.2 ± 8.1*
Tiegba	2% (control)	0.0	106	0 (0.0%)	0.0 ± 0.0
	3%	0.52	104	2 (1.92%)	3.0 ± 1.4

Results were scored after 5 weeks of culture. During the primary culture period (3 weeks), cultures were in darkness only during the first week of culture. During 2 weeks, they were cultured on step 2 medium for embryo development in light. For each cultivar, significant differences are * and ** at the 5% and 1% levels respectively as compared to the control.

Table 5. Effects of MS mineral nutrients on development of somatic embryos in the second culture period with several cassava cultivars. The primary induction period was with MS + 4 mg l^{-1} of 2,4-D in media containing 2% sucrose.

Cultivars	Number and percentage () of embryogenic explants							
	Media (MS strength)							MS
	Half-strength	1 × Strength (control)	2 × Strength	MS with 2 × micro salts	MS with 4 × micro salts	MS without macro salts	MS without the micro salts	without macro and micro salts
TMS 30395	4/82*a (4.8%)	19/145 c (13.1%)	0/94*e (0.0%)ns	4/74f (5.4%)ns	22/70*i (31.4%)	0/48** (0.0%)ns	0/48** (0.0%)ns	0/45* (0.0%)
TMS 30355	0/84*b (0.0%)ns	7/113 d (6.2%)ns	0/95*e (0.0%)ns	4/94 fg (4.2%)ns	4/70 j (5.7%)ns	0/50 (0/0%)ns	0/51 (0.0%)ns	0/46 (0.0%)ns
85621	0/90 b (0.0%)ns	1/107 d (0.9%)ns	2/64 e (3.1%)ns	0.68 fg (0.0%)ns	2/60 j (3.3%)ns	N.T.	N.T.	N.T.
83375	0.64*b (0.0%)ns	11/118 c (9.3%)	0.68*e (0.0%)ns	0.86*g (0.0%)ns	2/62 j (3.2%)ns	N.T.	N.T.	N.T.
84701	0/72*b (0.0%)ns	9.126 cd (7.1%)	0/96*e (0.0%)ns	0.84*g (0.0%)ns	2/88 j (2.3%)ns	N.T.	N.T.	N.T.
Tiegba	0.84 b (0.0%)ns	2/106 d (1.8%)ns	0.72 e (0.0%)ns	0/96 fgh (0.0%)ns	2/72 j (2.7%)ns	0/42 (0.0%)ns	0/43 (0.0%)ns	0/48 (0.0%)ns

Number of somatic embryo explants / Total number of cultured explants.

The results were scored at the end of the fifth week of culture after the two-step culture periods. For each cultivar (across rows), significant differences are * and ** for $p \leq 5\%$ and $p \leq 1\%$ respectively, as compared to the control conditions. NS: Not significant differences. For each treatment (across columns), numbers followed by the same letters are not statistically different between cultivars. NT: Not tested. MS macro salts (mg l^{-1}): NH_4NO_3 (1650); KNO_3 (1900); $CaCl_2$, $2H_2O$ (440); $MgSO_4$,$7H_2O$ (370); KH_2PO_4 (170). MS micro salts (mg l^{-1}): KI (0.83); H_3BO_3 (6.2); $MnSO_4$, $4H_2O$ (22.3); $ZnSO_4$, $7H_2O$ (8.6); Na_2MoO_4, $2H_2O$ (0.25); $CuSO_4$, $5H_2O$ (0.025); $CoCl_2$, $6H_2O$ (0.025).

the cultivar TMS 30395. However, variation of both the mineral nitrogen (ammonium and/or nitrate) and KCl concentrations in the culture media did not significantly promote embryogenesis (data not shown). Moreover, organic compounds such us glycine, serine, tryptophan, nicotinic acid, adenine sulfate and casein hydrolysate added at 100 mg l^{-1} together or separately to the MS medium inhibited embryogenesis (Table 6) while 100 mg l^{-1} proline, gave a very low frequency (3.2%) of somatic embryogenesis. The use of sucrose at different levels and in different sequences influenced development of somatic embryos (Fig. 3). Explants cultured with the same concentration of sucrose in both the primary (first step) and secondary (second step) period responded best to 4% sucrose, whereas 6% and 8% sucrose completely inhibited embryo development. In contrast, increasing sucrose levels up to 6% in the second cultivation period significantly enhanced the percentage of explants developing somatic embryos.

Conversion of somatic embryos into plantlets

Isolated somatic embryos or clusters of embryos from cotyledon explants were transferred to phytohormone-free MS medium containing 3% sucrose for germination. Development occurred from all types of somatic embryos. Root growth, followed by formation, enlargement and greening of hypocotyl and cotyledons (Figures 2 D, E) occurred first. However, some isolated embryos ($\leq$ 10 %) showed shoot elongation

Table 6. Effects of organic compounds on embryogenesis from cultured cotyledons of cassava TMS 30395. The primary induction period (step 1) was with MS + 100 mg l^{-1} of each compound + 4 mg l^{-1} of 2,4 D in media containing 2% sucrose.

Organic compounds	Number of cultured explants	Number and (percentage) of proembryogenic formations after 3 weeks of culture (step 1)	Number and (percentage) of responding explants after 2 to 3 weeks of culture in the second step medium (MS + 0.01 mg l^{-1} NAA + 0.1 mg l^{-1} BA + 0.1 mg l^{-1} GA_3).		
			Explants with developed somatic embryos	Explants with roots only	Explants with roots and/or leafy structures
Control	75	29 (38.6)	9 (12.0)	23 (30.6)	10 (13.3)
Glycine	42	9 (21.4) NS	0 (0.0)*	23 (54.7)*	5 (11.9)NS
Adenine sulfate	43	2 (4.6)**	0 (0.0)*	24 (55.8)**	0 (0.0)*
Casein hydrolysate	40	17 (42.5) NS	0 (0.0)*	4 (10.0)*	6 (15.0)NS
Tryptophan	40	18 (45) NS	1 (2.5) NS	2 (5.0)**	12 (30.0)*
Nicotinic acid	40	3 (7.5)**	0 (0.0)*	3 (7.5)**	1 (2.5)NS
Serine	48	0 (0.0)**	0 (0.0)*	15 (31.2)NS	1 (2.0)*
Proline	62	15 (24.1) NS	2 (3.2) NS	24 (38.7)NS	22 (35.4)**
Proline/casein	50	13 (26) NS	0 (0.0)*	9 (18.0)NS	0 (0.0)**
Proline/tryptophan	50	11 (22) NS	0 (0.0)*	11 (22.0)NS	0 (0.0)**
Tryptophan/casein	41	22 (53.6) NS	0 (0.0)*	18 (43.9)NS	5 (12.1)NS
Tryptophan/glycine	45	14 (31.1) NS	0 (0.0)*	15 (33.3)NS	1 (2.2)*
Casein/glycine	51	13 (25.4) NS	0 (0.0)*	6 (11.7)*	1 (1.9)*

Across columns, data were statistically compared to the control conditions (MS medium), and significant differences are * and ** for $p \leq 5\%$ and 1% respectively. NS: Not significant difference.

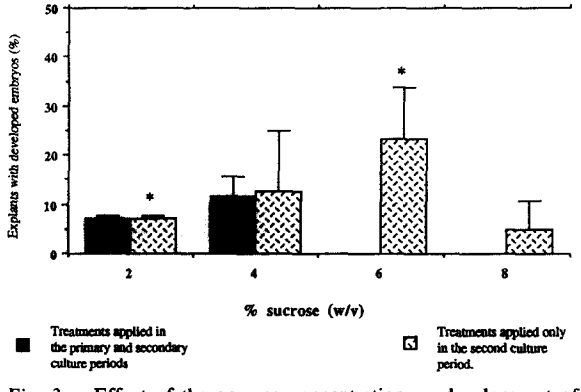

Fig. 3. Effect of the sucrose concentration on development of somatic embryos from cultured cotyledon explants of cassava (Cultivar TMS 30395). Primary induction period (step 1) was with MS medium + 4 mg l^{-1} 2,4-D.

without rooting while most (≥ 90%) of the somatic embryos rooted without shoot elongation. This resulted in a small numbers of embryos converted into plants e.g. rates of conversion for the cultivars TMS 30395, TMS 30555, 84701, 8561 and Tiegba were 11.4%, 12.0%, 10.3%, 4.5% and 0.0% respectively. Moreover, the use of both ABA and high sucrose treatments, did not achieve more conversion of the somatic embryos into plants. Although, some abnormalities (< 5%) were observed during early plant development, these abnormalities gradually dissipated as plants matured.

Ploidy analysis

The regenerated plants (Figure 2 F) were tested for their genetic stability by analyzing the ploidy levels in each of 15 to 20 regenerated plantlets of TMS 30395, TMS 30555, 84701 and 85621. The fluorescence intensities of nuclei (Figure 2 G) isolated after chopping leaves are identical for plantlets obtained from seed germination (control) and plantlets derived from somatic embryogenesis (data not shown). The low CV (coefficient of variation) values (<1.5%) indicate only small changes in the DNA content of leaf nuclei, assuming that there

were homogeneous populations of nuclei in phases G1 and G2. Using the internal reference (petunia leaves containing 2C = 2.85 pg of DNA / nucleus), we estimated the DNA content of the cassava leaf nuclei to be 1.65 pg at stages Go/G 1. This value appears to be similar to those reported by Arumuganathan & Earle (1991). They found nuclear DNA content between 1.43 to 1.72 pg/2C for 17 cassava cultivars. Young leaves of 5 regenerants randomly chosen for each cultivar were also squashed for chromosome counts. No changes in the chromosome numbers, 2n = 36 were observed (Figure 2 H).

Discussion

This study was performed with the aim to optimize nutritional factors to improve somatic embryogenesis from mature zygotic cotyledons of cassava. Unlike the previous observations (Stamp 1987), mitotic activities started in vascular tissues within 4-7 days of incubation and embryogenic tissues and embryo initials were formed after 15-21 days of culture. Our results indicated that the protocol for somatic embryogenesis as described by Stamp & Henshaw (1982) after modification, may offer the possibility of a routine regeneration in several cassava cultivars. However, the frequency of regeneration in cassava, as described frequently in several plant species (Galiba & Yamada 1988; Sellars et al. 1990; Redenbaugh 1990; Goebel-Tourand et al. 1993), depends on the genotype and the culture conditions. For the first factor, our results agreed with previous reports on cultivars originating from South America (Stamp 1984; Szabados et al. 1987). In addition, cassava seeds, as seeds of most of tropical crops have short viability after ripening (Brenda et al. 1986; Gray & Purohit 1991). Thus, viability of seeds is an other important criterion which might influence the frequencies of somatic embryogenesis in cotyledon cultures of cassava.

The type of auxin strongly influenced the embryogenic potential of cotyledon explants. Among the four auxins tested, induction of somatic embryos was mainly achieved with 2,4-D, confirming previous findings (Stamp & Henshaw, 1987). However, we observed that higher concentrations of 2,4-D (> 12 mg l^{-1}) also inhibited embryo development in cassava, as reported in several other plant species (Murashige 1974; Fujimura & Komamine 1975; Gunay & Rao 1978; Finer & Nagasawa 1988), although 25 mg l^{-1} 2,4-D was found to be optimal for soybean (Hartweck et al. 1988).

Abnormal somatic embryos were observed with all the 2,4-D concentrations tested for the cultivar TMS 30395. This indicates that, morphological normalcy of somatic embryos in cassava, presumably does not reflect 2,4-D level in step 1, but is apparently a carry over effect during step 2. Changes in the extent of cell division and premature enlargement in the young embryos beginning at the heart-shaped stage were found to be the origin of variability in cotyledon number and the alterations in cotyledon morphology (Ammirato 1987). In addition, we observed that a large number of globular proembryogenic structures failed to develop into somatic embryos but yielded some other adventitious organs namely 'leafy', cotyledon-like structures, roots and occasionally shoots. This loss of embryogenic competence may be due to the cytokinin BA (0.1 mg l^{-1}) used in the second-step medium. An inhibitory effect of cytokinin on embryo development has been previously reported (Finer & Nagasawa 1988; Kysely & Jacobsen 1990), although studies on the recovery of plants from soybean and grapevine somatic embryos have recommended the use of gibberellic acid or a cytokinin to stimulate or compel the organization of shoot apex (Lazzeri et al. 1985; Ranch et al. 1985; Barwale et al. 1986; Goebel-Tourand et al. 1993). In cassava, it appears that, embryo development during step 2 requires growth regulators, since none of the transferred embryogenic explants on to growth regulator-free MS medium showed somatic embryos. They developed callus, or were unchanged in their development.

Among the various treatments described here, the addition of abscisic acid (0.52 mg l^{-1}) and sucrose (3-4%, w/v) to the MS basal medium supplemented with 2,4-D were the most suitable for embryogenesis in cassava cotyledon explants. ABA and osmoticum (high sucrose level) were recognized to regulate the production of specific proteins during embryogenesis (Obendorf & Wettlaufe 1984; Xu et al. 1990). ABA prevented precocious germination and promoted plant regeneration (Ammirato 1974, 1977,1983; Von Arnold & Hakman 1988; Goebel-Tourand et al. 1993). High sucrose concentration enhanced vigor and embryo conversion in *Medicago sativa* (Anandarajah & McKersie 1990). However, in cassava, such benefit effects of ABA and high sucrose treatments were not observed. Low conversion rates of somatic embryos of grapevine (genotype 41B) were found to be the consequence

of abnormal apical meristem development (Goebel-Tourand et al. 1993).

A current problem in certain higher plants is that cotyledon explants-derived regenerants show a considerable level of somaclonal variations and frequently result in polyploid regenerants (for a review see De Klerk 1990). The analysis of 66 randomly selected regenerants of cassava, revealed that more than 98% were tetraploids as were the source plants. Although, the number of plants, derived from the cotyledons which have been analysed to date is still low, the results of the present investigation indicated that spontaneous variations in ploidy level occur at very low frequency since more than 98% were tetraploids. The relative ease with which cassava can be regenerated, starting from zygotic cotyledons makes the system particularly attractive for further study on *Agrobacterium* mediated genetic transformation, especially when viewed as a system which produce very low level of ploidy variations.

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