

Protocols for callus and somatic embryo initiation for *Hibiscus sabdariffa* L. (Malvaceae): Influence of explant type, sugar, and plant growth regulators

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Abstract

A significant work about callus induction and somatic embryogenesis was realized for *Hibiscus sabdariffa*. Two genotypes (*Hibiscus sabdariffa* var. *sabdariffa* and *Hibiscus sabdariffa* var. *altissima*), 2 sugars (sucrose and glucose) and three concentrations (1 %, 2%, 3%) of each sugar, 3 explant types (root, hypocotyl, cotyledon) were used for tissue culture. Fourteen combinations of plant growth regulators (PGRs) in MS medium and five combinations of PGR in Driver and Kuniyuki (DKW) medium were tested on hypocotyl and cotyledon for callus and somatic embryo formation. The PGR combinations used with MS medium were naphthaleneacetic acid/kinetin (NAA/KIN), 2,4-dichlorophenoxyacetic acid/kinetin (2,4-D/KIN), and naphthaleneacetic acid/6-benzylaminopurine (NAA/BA) and those used with DKW medium were 2,4-dichlorophenoxyacetic acid/thidiazuron (2,4-D/TDZ). Callus formation was initiated on both genotypes with all concentrations of both sugars and PGRs and in all explant types. The best results for callus induction were achieved with 3% sucrose and the hypocotyl and cotyledon explants. Somatic embryos were obtained with DKW medium supplemented with 4 mg/l 2,4-D + 1 mg/l TDZ and 1 mg/l 2,4-D + 0,5 mg/l TDZ.

Keywords: Malvaceae, tTCL, callogenesis, somatic embryos, plant growth regulator, sugar, explant, *Hibiscus sabdariffa* L.

Abbreviations: ANOVA: Analysis of variance; BA: Benzyladenine; Caldiam: callus diameter; DKW: Driver and Kuniyuki medium; Embcal: Embryogenic callus; G: Glucose; KIN: Kinetin; M.S.: Murashige and Skook medium; NAA: Naphtyl-acetic acid; PGRs: Plant growth regulators; S: Sucrose; TDZ: Thidiazuron; tTCLs: Transversal thin cell layers; 2,4-D: 2,4-Dichlorophenoxyacetic acid; % Callog: Percentage of callogenesis.

Introduction

Hibiscus sabdariffa L., popularly known as roselle, is a dicotyledonous autogamous, annual or biannual plant belonging to the Malvaceae family. It is a tetraploid species with $2n = 4x = 72$ (Akpan, 2000) and is widely distributed in the tropics and subtropics of both hemispheres and in many areas of the West Indies and Central America (Morton, 1987). Roselle can be cultiva-

ted in a wide range of soils (deep, fairly fertile sandy loam) and climatic conditions (from sea-level up to 900 m with a rainfall of about 182 cm) and requires only modest labor input. Roselle is grown for nutritional, medicinal, and industrial purposes (Mizukami et al., 1988; Mizukami et al., 1989; Cissé et al., 2009). For example, the calyx is widely used for producing drinks or



Fig 1. A four-month old *Hibiscus sabdariffa* var. *sabdariffa* plant. *H. sabdariffa* is an erect annual or biannual plant with smooth stems, petioles, and pedicels. It has deeply lobed and alternate leaves. The flowers are auxiliary or in terminal racemes. The plant can grow to 180 cm or more.

tea because of its high content of anthocyanins and organic acids (Hong and Wrosted, 1990; Gomez-Leyva et al., 2008) as well as flavor and color additives in the manufacture of jam, liquor, and jellies (Akinhahnsi and Olaleye, 2003). In ethnomedicine, *H. sabdariffa* is traditionally used to deal with several health problems, including hypertension, pyrexia and liver disorders, microorganism growth; it is also used as a diuretic, sedative, or digestive (Faraji and Tarkhani, 1999; Chen et al., 2003; Akinhahnsi and Olaleye, 2003). The fiber obtained from *H. sabdariffa*, which is generally less expensive to produce when compared to fiber from most fiber crops, is a useful substitute for jute and be mixed with jute and spun on jute machinery (Fathima and Balasubramanian, 2006).

Various pathogens adversely affect the growth of roselle plant and its seeds in various parts of the world, including Senegal, the United States of America, and Iran (Boulanger et al., 1984). This crop is susceptible to pathogenic fungal such as *Phoma sabdariffae* for adult stages of development for *H. sabdariffa* and *Fusarium oxysporum* which is the main cause of diseases (foot rot and wilt) in early stages. Since all known roselle cultivars are susceptible to those fungi, there appeared to be little resistance available in roselle genome. Furthermore, several compounds with beneficial health effects are found in *H. sabdariffa*, and tissue cultured *H. sabdariffa* cells could potentially be a source of suitable large scale production of these compounds (Hizukami et al., 1988). In addition, the improvement in somatic embryogenesis methods (Vicent and Martinez, 1998) can allow the use

of somatic embryogenesis in the regeneration of high yielders of fibers and micropropagation of floral varieties for production of natural dye. Modification of *H. sabdariffa* genome using genetic engineering methods would facilitate rapid development of new cultivars with traits that confer resistance to the fungal diseases mentioned above. An efficient *in vitro* plant regeneration method is often considered as a prerequisite before transformation. There are a number of regeneration studies on certain species of *Hibiscus* such as *H. acetosella* through shoot apices (Sakhanokho, 2008), *H. cannabinus* (Reichert et al., 1999) or other species of Malvaceae family such as *Gossypium hirsutum* (Ouma et al., 2004), *Theobroma cacao* (Gultinan et al., 2001; Minyaka et al., 2008). However, there are no reports available on *H. sabdariffa* regeneration via somatic embryogenesis. The *in vitro* regeneration systems currently available for *H. sabdariffa* are based on meristem culture (Gomez-Leyva et al., 2008) or by cuttings (Sié et al., 2008). A direct regeneration protocol targeting seeds were carried out in order to produce some transformed plants (Gassama-Dia et al., 2004). However, the number of transgenic lines was low. Furthermore, such a system is prone to the production of chimeric plants. This is not the case for a transformation system based on somatic embryogenesis because somatic embryos are believed to originate from single cells; therefore, any transformed cell would give rise to a transformed plant. The number of genetically transformed plants through somatic embryogenesis would contribute significantly to genetic improvement of *H. sabdariffa* cultivars in sub-saharian Africa. Moreover, the carrying out of efficient protocol of regeneration of *H. sabdariffa* through somatic embryos would enable the use of somatic embryogenesis in cell selection programs and germplasm cryopreservation. Furthermore, several compounds with beneficial health effects are found in *H. sabdariffa*, and cultured *H. sabdariffa* cells could potentially be a source of suitable large scale production of these compounds (Hizukami et al., 1988). This present study was carried out with the objective to investigate the effect of sugars, genotype, growth regulators, and explant type on *H. sabdariffa* callus and somatic embryo induction.

Material and methods

Plant material

Seeds were collected from *H. sabdariffa* var. *sabdariffa* (Fig.1) and *H. sabdariffa* var. *altissima* plants grown in Korhogo (Northern Côte d'Ivoire) and stored at the University of Abobo-Adjamé in Côte d'Ivoire. They were surface sterilized with 5 % calcium hypochloride (w/v) for 20 min followed by three washes for 5 min each in sterile distilled water.

Table 1. Effect of different sugar concentrations and explants on callogenesis in *Hibiscus sabdariffa* var. *sabdariffa* and *Hibiscus sabdariffa* var. *altissima*.

Genotypes	Sugar (%)	Callogenesis (%)		
		Root	Hypocotyl	Cotyledon
<i>H. sabdariffa</i> var. <i>sabdariffa</i>	S1	28.00 ± 18.00 ab	88.00 ± 10.02 a	100.00 ± 0.00 a
	S2	40.00 ± 2.00 a	84.00 ± 12.02 a	100.00 ± 0.00 a
	S3	40.00 ± 3.50 a	72.00 ± 7.02 a	100.00 ± 0.00a
	G1	8.00 ± 7.21 bc	88.00 ± 7.00 a	100.00 ± 0.00 a
	G2	34.00 ± 16.04 ab	94.00 ± 8.08 a	82.00 ± 2.00 b
	G3	8.00 ± 10.58 bc	74.00 ± 6.03 a	96.00 ± 4.00 a
p ^{significance}		0.009**	0.102 ^{ns}	0.000***
<i>H. sabdariffa</i> var. <i>altissima</i>	S1	19.00 ± 10.44 ab	80.00 ± 9.54 ab	99.00 ± 1.00 a
	S2	0.00 ± 0.00 c	83.00 ± 9.00 ab	99.00 ± 1.00 a
	S3	33.00 ± 16.70 ab	69.00 ± 9.00 c	97.00 ± 2.00 a
	G1	0.00 ± 0.00 c	94.00 ± 1.00 a	99.00 ± 1.00 a
	G2	0.00 ± 0.00 c	82.00 ± 9.84 ab	67.00 ± 30.19 a
	G3	5.00 ± 6.25 bc	76.00 ± 2.00 bc	89.00 ± 6.00 a
p ^{significance}		0.004**	0.036*	0.063 ^{ns}

Means within the same column followed by the same letters are not significantly different according to Duncan's multiple range test at P = 0.05; ns: not significant at 5%; *: significant at 5%; **: significant at 1%; ***: significant at 0.1%; S = sucrose; G = glucose.

Seed germination and experimental protocol

Seeds were germinated on half-strength Murashige and Skoog (1962) salts. The medium contained sucrose (Sigma Chemical Co.) solidified with 5.5 % agar (Sigma Chemical Co.). The pH of the medium was adjusted to 5.8 with 1 M NaOH (Merck) before autoclaving for 20 min at 121 °C. Seeds were germinated in Pyrex test tubes containing each 11 ml of the half-strength MS medium. Seeds were incubated 24 h in darkness and then transferred in a growth chamber at 25 ± 2°C under 14/10 h photoperiod for 7 days. The intensity of light was 2000 lux on average. The 8-day old sterile seedlings were removed from the culture medium and transversal Thin Cell Layers (tTCLs) were obtained by cutting various plant parts (root, hypocotyl and cotyledon) into 0.5 mm sections.

Callus initiation and embryo differentiation

tTCLs were placed in Petri dishes containing callus initiation medium and subsequently embryo differentiation medium. Callus initiation and embryo differentiation were carried out on two basal media, MS and DKW (Driver and Kuniyuki, 1984) supplemented with 3% sucrose and 5% agar.

Influence of sugar and explant types

Two sugars (sucrose, glucose) and 3 concentrations (1 %, 2 %, 3 %) were tested for each genotype. The three explant types used were roots, hypocotyls, and cotyledons. MS basal medium supplemented with 0.2 mg/l NAA and 0.6 mg/l BA was used.

Influence of growth regulators

The medium was supplemented with different combinations of plant growth regulators (PGRs). The first experiment consisted of MS medium supplemented with

various NAA, BA, 2,4-D, and KIN concentrations and used as follows: [NAA (0.1 ; 0.2 ; 0.3 ; 0.4 ; 1 ; 1.5 mg/l) + BA (0.1 ; 0.6 ; 1 ; 3 ; 4 mg/l)], then [NAA (1 ; 2 mg/l) + KIN (0.5 ; 1 ; 2 mg/l)] and finally [2,4-D (0.01 ; 0.1 ; 0.3 mg/l) + KIN (0.05 ; 0.1 ; 0.5 ; 3 mg/l)]. The second experiment consisted of DKW medium supplemented with varying concentrations of 2,4-D (1 ; 2 ; 4 mg/l) and TDZ (0.01 ; 1 mg/l). The experiments were laid out as a completely random design with 24 root, 25 hypocotyl and 15 cotyledon tTCLs per treatment with 3 to 5 repetitions. Petri dishes containing the tTCLs were sealed with Parafilm and incubated for 6 weeks at 25 ± 2 °C under 14/10 h photoperiod for MS medium and total darkness for DKW medium. The pH of the medium was adjusted to 5.8 prior to the addition of the gelling agent. For all experiments, callus initiation percentage, callus diameter, and percentage of embryogenic callus were measured.

Statistical analysis

The results were subjected to analysis of variance (ANOVA) using SPSS 9.01 program. Mean values were separated according to Duncan's multiple test range at P ≤ 0.05. Means are the results of 3 or 5 replicates.

Results and Discussion

Influence of explant type

The responses of various explants (root, hypocotyl and cotyledon) regarding callus induction (% callog) are presented in Table 1. Callus was observed after 6 days on the cut surfaces in all three explant types (Fig. 2a). The ranges for callus production from *H. sabdariffa* var. *sabdariffa* were 8-40, 72-94, and 82-100% for roots, cotyledons, and hypocotyls, respectively while those from *H. sabdariffa* var. *altissima* for the same explants were 0-33, 69-83, and 67-99%, respectively (Table 1).

Table 2. Effect of different NAA/BA combinations and explants on callus and somatic embryo initiation in *Hibiscus sabdariffa* var. *sabdariffa* and *Hibiscus sabdariffa* var. *altissima*.

Genotypes	PGR: NAA/BA (mg/l)	Explant	Callogenesis (%)	Caldiam (mm)	Embal (%)
<i>H. sabdariffa</i> var. <i>altissima</i>	MS ₁ : 0.1/0.1 P ^{significance}	Hypocotyl	41.29 ± 41.95 b	6.00 ± 0.94 b	-
		Cotyledon	97.89 ± 2.88 a 0.039 [*]	7.90 ± 0.42 a 0.007 ^{**}	-
	MS ₂ : 0/0.1 P ^{significance}	Hypocotyl	37.21 ± 17.86 a	3.25 ± 0.29 a	-
		Cotyledon	25.85 ± 10.62 a 0.316 ^{ns}	3.25 ± 0.18 a 1.000 ^{ns}	-
	MS ₃ : 1/3 P ^{significance}	Hypocotyl	96.77 ± 5.59 a	8.60 ± 2.63 a	-
		Cotyledon	98.24 ± 4.29 a 0.633 ^{ns}	11.16 ± 2.06 a 0.103 ^{ns}	-
	MS ₄ : 0.4/4 P ^{significance}	Hypocotyl	91.61 ± 9.84 a	6.90 ± 0.74 a	-
		Cotyledon	100.00 ± 0.00 a 0.203 ^{ns}	7.33 ± 0.76 a 0.458 ^{ns}	-
	MS ₅ : 0.3/3 P ^{significance}	Hypocotyl	84.95 ± 8.12 a	5.67 ± 0.76 a	-
		Cotyledon	86.82 ± 9.19 a 0.804 ^{ns}	5.83 ± 2.52 a 0.802 ^{ns}	-
	S ₆ : 0.2/0.6 P ^{significance}	Hypocotyl	45.83 ± 7.86 a	5.83 ± 2.52 a	-
		Cotyledon	57.57 ± 30.59 a 0.555 ^{ns}	5.83 ± 1.76 a 1.000 ^{ns}	-
	MS ₇ : 1.5/1 P ^{significance}	Hypocotyl	85.05 ± 21.34 a	7.12 ± 0.74 a	-
		Cotyledon	67.80 ± 7.62 a 0.179 ^{ns}	6.25 ± 0.65 a 0.072 ^{ns}	-
<i>H. sabdariffa</i> var. <i>sabdariffa</i>	MS ₁ : 0.1/0.1 P ^{significance}	Hypocotyl	96.77 ± 3.23 a	6.10 ± 0.89 b	-
		Cotyledon	100.00 ± 0.00 a 0.056 ^{ns}	7.90 ± 0.42 a 0.004 ^{**}	-
	MS ₂ : 0/0.1 P ^{significance}	Hypocotyl	38.06 ± 10.05 b	2.80 ± 0.27 b	-
		Cotyledon	81.05 ± 13.21 a 0.000 ^{***}	6.90 ± 0.74 a 0.000 ^{***}	-
	MS ₃ : 1/3 P ^{significance}	Hypocotyl	97.41 ± 2.70 a	7.40 ± 0.55 a	-
		Cotyledon	100.00 ± 0.00 a 0.099 ^{ns}	10.00 ± 1.41 a 0.007 ^{**}	-
	MS ₄ : 0.4/4 P ^{significance}	Hypocotyl	85.16 ± 8.72 b	6.30 ± 0.57 b	-
		Cotyledon	98.50 ± 3.98 a 0.023 [*]	8.42 ± 0.88 a 0.001 ^{***}	-
	MS ₅ : 0.3/3 P ^{significance}	Hypocotyl	91.59 ± 4.47 a	8.13 ± 1.11 a	-
		Cotyledon	97.50 ± 5.00 a 0.129 ^{ns}	7.25 ± 0.65 a 0.130 ^{ns}	-
	MS ₆ : 0.2/0.6 P ^{significance}	Hypocotyl	73.27 ± 13.65 a	7.87 ± 0.48 a	-
		Cotyledon	92.64 ± 8.82 a 0.055 ^{ns}	7.87 ± 0.63 a 1.000 ^{ns}	-
	MS ₇ : 1.5/1 P ^{significance}	Hypocotyl	88.74 ± 10.60 a	6.12 ± 0.85 a	-
		Cotyledon	95.59 ± 2.94 a 0.253 ^{ns}	7.12 ± 0.85 a 0.149 ^{ns}	-

Means within the same column followed by the same letters are not significantly different according to Duncan's multiple range test at P = 0.05; ns: not significant at 5%; * : significant at 5%; ** : significant at 1%; *** : significant at 0.1%.

Overall, more callus was produced with the hypocotyl and cotyledon tTCLs than with roots. These results reflect the existence of a large inter-explant variability in callusing responses. This inter-explant variability has also been reported in other species (Zouine and El-Hadrami, 2004; Dhar and Joshi, 2005; Zouzou et al., 2008). The high callus producing capacity of cotyledon in comparison to hypocotyl and root is probably due to the nutritive reserves and anatomical structure which are similar to leaf. Our results are not in agreement with those published by other authors who showed that hypocotyl was more callogenic compared to root and cotyledon explants (Zhang et al., 2001; Zouzou et al., 2008). Only the hypocotyl and cotyledon explants were retained in this study for the subsequent experiments on callus and somatic embryo initiation in *H. sabdariffa* because they produced significantly more callus than root

explants. However, it is worth mentioning that secondary medicinal metabolites such as ginsenosides have been obtained from root-derived calli in some plant species; the production of such compounds is, however, affected by several factors, such as carbohydrate source and growth regulators (Vanisree et al., 2004). Therefore, a study aimed at maximizing the production of such secondary metabolites should also focus on root-derived calli.

Influence of sugars

Both sugar types and concentration influenced callus induction. Indeed, callus mass was initiated within 6 (glucose) to 11 (sucrose) days directly on the cut surfaces in 3 types of tTCLs on MS basal medium supplemented with 0.2 mg/l NAA, 0.6 mg/l BA (Table 1). For *H. sabd-*

Table 3. Effect of different NAA/KIN combinations and explants on callus and somatic embryo initiation in *Hibiscus sabdariffa* var. *sabdariffa* and *Hibiscus sabdariffa* var. *altissima*.

Genotypes	PGR: NAA/KIN (mg/l)	Explant	Callogenesis (%)	Caldiam (mm)	Embcals (%)
<i>H. sabdariffa</i> var. <i>altissima</i>	MS ¹ : 1/0.5 P ^{significance}	Hypocotyl	96.20 ± 6.94 a	5.80 ± 0.45 a	-
		Cotyledon	93.80 ± 11.28 a	4.90 ± 1.08 a	-
			0.696 ^{ns}	0.087 ^{ns}	
	MS ² : ½ P ^{significance}	Hypocotyl	94.40 ± 4.33 b	5.50 ± 0.35 b	-
		Cotyledon	100.00 ± 0.00 a	8.00 ± 0.61 a	-
			0.045 [*]	0.000 ^{***}	
MS ³ : 2/1 P ^{significance}	Hypocotyl	27.33 ± 13.65 a	3.00 ± 0.50 a	-	
	Cotyledon	3.67 ± 6.35 b	2.89 ± 0.50 a	-	
		0.050 [*]	0.986 ^{ns}		
<i>H. sabdariffa</i> var. <i>sabdariffa</i>	MS ¹ : 1/0.5 P ^{significance}	Hypocotyl	76.20 ± 8.67 a	5.60 ± 0.22 b	-
		Cotyledon	82.00 ± 22.28 a	7.20 ± 0.57 a	-
			0.602 ^{ns}	0.000 ^{***}	
	MS ² : ½ P ^{significance}	Hypocotyl	95.00 ± 6.63 a	5.90 ± 0.65 b	-
		Cotyledon	100.00 ± 0.00 a	7.50 ± 0.71 a	-
			0.130 ^{ns}	0.006 ^{**}	
MS ³ : 2/1 P ^{significance}	Hypocotyl	31.33 ± 25.50 a	3.00 ± 0.50 a	-	
	Cotyledon	2.75 ± 5.50 a	2.00 ± 0.91 a	-	
		0.075 ^{ns}	0.152 ^{ns}		

Means within the same column followed by the same letters are not significantly different according to Duncan's multiple range test at P = 0.05; ns: not significant at 5%; *: significant at 5%; **: significant at 1%; ***: significant at 0.1%.

Table 4. Effect of different 2,4-D/KIN combinations and explants on callus and somatic embryo initiation in *Hibiscus sabdariffa* var. *sabdariffa* and *Hibiscus sabdariffa* var. *altissima*.

Genotypes	PGR: 2,4-D/KIN (mg/l)	Explant	Callogenesis (%)	Caldiam (mm)	Embcals (%)
<i>H. sabdariffa</i> var. <i>altissima</i>	MS ¹ : 0.1/0.5 P ^{significance}	Hypocotyl	66.20 ± 28.50 a	6.50 ± 0.93 a	-
		Cotyledon	29.60 ± 31.44 a	3.90 ± 1.24 b	-
			0.090 ^{ns}	0.006 ^{**}	
	MS ² : 0.01/0.05 P ^{significance}	Hypocotyl	40.50 ± 16.26 a	2.75 ± 0.35 a	-
		Cotyledon	8.75 ± 5.30 b	2.00 ± 0.00 b	-
			0.011 [*]	0.007 ^{**}	
MS ³ : 0.3/3 P ^{significance}	Hypocotyl	73.00 ± 13.44 a	8.25 ± 3.12 a	-	
	Cotyledon	61.00 ± 15.60 a	7.87 ± 1.03 a	-	
		0.288 ^{ns}	0.827 ^{ns}		
MS ⁴ : 0.1/0.1 P ^{significance}	Hypocotyl	30.00 ± 2.83 a	4.00 ± 0.71 a	-	
	Cotyledon	5.00 ± 0.00 b	2.00 ± 0.87 b	-	
		0.000 ^{***}	0.050 [*]		
<i>H. sabdariffa</i> var. <i>sabdariffa</i>	MS ¹ : 0.1/0.5 P ^{significance}	Hypocotyl	69.80 ± 29.65 a	10.80 ± 1.15 a	-
		Cotyledon	38.60 ± 22.69 a	5.70 ± 1.30 b	-
			0.099 ^{ns}	0.000 ^{***}	
	MS ² : 0.01/0.05 P ^{significance}	Hypocotyl	37.00 ± 40.44 a	1.75 ± 1.19 a	-
		Cotyledon	11.25 ± 18.03 a	1.12 ± 1.44 a	-
			0.289 ^{ns}	0.528 ^{ns}	
MS ³ : 0.3/3 P ^{significance}	Hypocotyl	50.00 ± 3.94 b	11.60 ± 2.68 a	-	
	Cotyledon	73.20 ± 4.55 a	8.00 ± 0.35 b	-	
		0.000 ^{***}	0.018 [*]		
MS ⁴ : 0.1/0.1 P ^{significance}	Hypocotyl	78.50 ± 34.59 a	7.00 ± 0.91 a	-	
	Cotyledon	10.75 ± 5.91 b	4.25 ± 1.55 b	-	
		0.008 ^{**}	0.022 [*]		

Means within the same column followed by the same letters are not significantly different according to Duncan's multiple range test at P = 0.05; ns: not significant at 5%; *: significant at 5%; **: significant at 1%; ***: significant at 0.1%.

ariffa var. *sabdariffa*, the percentages of callus induction in sucrose-containing media were 72% (3% sucrose) to 88% (1% sucrose) for hypocotyl explants and 100% callus induction was achieved with all three sucrose concentrations using the cotyledon explants (Table 1). For the same species, the percentages of callus production ranged from 74 to 94% (hypocotyl) and 82 to

100% (cotyledon) for the glucose-containing media. Similar results were observed with *H. sabdariffa* var. *altissima*. In general, the 3% sucrose produced more callus with both genotypes and all three explant types (Table 1). The beneficial effect of sucrose on callogenesis was also reported in many plants (Dhar and Joshi, 2005; Gopi and Vatsala, 2006). The 3% sucrose

Table 5. Effect of different 2,4-D/TDZ combinations and explants on callus and somatic embryo initiation in *Hibiscus sabdariffa* var. *sabdariffa* and *Hibiscus sabdariffa* var. *altissima*.

Genotypes	PGR: 2,4-D/TDZ (mg/l)	Explants	Callogenesis (%)	Cal diam (mm)	Embcal (%)
<i>H. sabdariffa</i> var. <i>altissima</i>	DKW ₁ : 2/1 P ^{significance}	Hypocotyl	100.00 ± 0.00	6.00 ± 2.06 a	-
		Cotyledon	100.00 ± 0.00	8.50 ± 2.55 a	-
			-	0.127 ^{ns}	
	DKW ₂ : 1/0.01 P ^{significance}	Hypocotyl	100.00 ± 0.00	6.62 ± 2.14 a	0.00 ± 0.00 a
		Cotyledon	100.00 ± 0.00	8.12 ± 2.17 a	1.78 ± 3.57 a
			-	0.363 ^{ns}	0.356 ^{ns}
	DKW ₃ : 4/1 P ^{significance}	Hypocotyl	95.00 ± 10.00 a	8.25 ± 2.78 a	11.55 ± 14.06 a
		Cotyledon	100.00 ± 0.00 a	10.37 ± 2.39 a	1.66 ± 3.33 a
			0.391 ^{ns}	0.291 ^{ns}	0.220 ^{ns}
	DKW ₄ : 1/0.5 P ^{significance}	Hypocotyl	100.00 ± 0.00	4.34 ± 3.64 a	10.83 ± 23.42 a
		Cotyledon	100.00 ± 0.00	7.92 ± 2.69 a	12.12 ± 29.69 a
			-	0.082 ^{ns}	0.935 ^{ns}
<i>H. sabdariffa</i> var. <i>sabdariffa</i>	DKW ₁ : 2/1 P ^{significance}	Hypocotyl	100.00 ± 0.00	8.00 ± 0.93 b	-
		Cotyledon	100.00 ± 0.00	13.80 ± 3.96 a	-
			-	0.013 [*]	
	DKW ₂ : 1/0.01 P ^{significance}	Hypocotyl	100.00 ± 0.00	7.90 ± 0.96 a	-
		Cotyledon	100.00 ± 0.00	10.80 ± 3.60 a	-
			-	0.120 ^{ns}	
	DKW ₃ : 4/1 P ^{significance}	Hypocotyl	100.00 ± 0.00	9.92 ± 1.83 b	11.46 ± 28.07 a
		Cotyledon	100.00 ± 0.00	16.58 ± 3.14 a	0.00 ± 0.00 a
			-	0.001 ^{***}	0.363 ^{ns}
	DKW ₄ : 1/0.5 P ^{significance}	Hypocotyl	88.93 ± 9.70 a	7.08 ± 1.02 a	0.87 ± 2.15 a
		Cotyledon	97.92 ± 5.10 a	7.83 ± 2.56 a	2.08 ± 5.10 a
			0.072 ^{ns}	0.520 ^{ns}	0.605 ^{ns}

Means within the same column followed by the same letters are not significantly different according to Duncan's multiple range test at P = 0.05; ns : not significant at 5%; *** : significant at 0.1%.

concentration may have a high osmotic pressure on cytoplasm of cells, thus inducing stress and callogenesis. Similar results were recorded with *Saccharum sp* (Errabii et al., 2006).

Influence of PGRs

Hypocotyl and cotyledon explants were cultured on MS basal medium containing different auxin and cytokinin combinations for callus initiation, callus diameter, and somatic embryo initiation (Tables 2 and 3). The results indicated that all treatments induced callus. However, differences based on PGR regime and explant type were observed. From a total of 15 combinations of PGRs tested, high callus production was obtained in MS medium supplemented with 0.4 mg/l NAA + 4 mg/l BA (MS₄ medium), 1 mg/l NAA + 3 mg/l BA (MS₃ medium) and 1mg/l NAA + 2 mg/l KIN (MS'₂) for *H. sabdariffa* var. *altissima*. The percentage of callus induction obtained with the cotyledon explants was 98.24, 100, and 100 % for MS₃, MS₄ and MS'₂, respectively (Tables 2 and 3). For hypocotyl explants, callus induction was 91.61, 96.77, and 94.40 for MS₄,

MS₃ and MS'₂, respectively for the same genotype. In *H. sabdariffa* var. *sabdariffa*, MS₁ (0.1 mg/l NAA + 0.1 mg/l BA), MS₅ (0.3 mg/l NAA + 3 mg/l BA) and MS'₂ induced high callogenesis (Tables 2 and 3). In this genotype, callus induction obtained with cotyledon explants was 97.50, 97.89, and 100 % in MS₅, MS₁ and MS'₂, respectively, and 91.59, 95.00, and 96.77% in MS₅, MS'₂ and MS₁, respectively for hypocotyl eplants (Tables 2 and 3). The results obtained with the 2,4-D/NAA combinations are in agreement with the response obtained in *Gossypium hirsutum* (Zouzou et al., 2008). In *H. sabdariffa* var. *altissima* (Table 4), callus induction percentages were 66.20% in MS''₁ (0.1 mg/l 2,4-D + 0.5 mg/l KIN) and 73.00 % in MS''₃ (0.3 mg/l 2,4-D + 3 mg/l KIN). For *H. sabdariffa* var. *sabdariffa*, the percentage of callus induction was 69.80% in MS''₁ (0.1 mg/l 2,4-D + 0.5 mg/l KIN) and 50.00% in MS''₃ (0.3 mg/l 2,4-D + 3mg/l KIN). Compared to results obtained with the auxin NAA, the combinations involving 2,4-D seem to be unfavourable to callogenesis induction. Nevertheless, in previous works, it was reported that 2,4-D was an essential growth regulator for the induction of callogenesis in cotton (Trolinder and Goodin, 1988 ; Lee et al., 2004 ; Sun et al., 2006 ; Zouzou et al., 2008) and

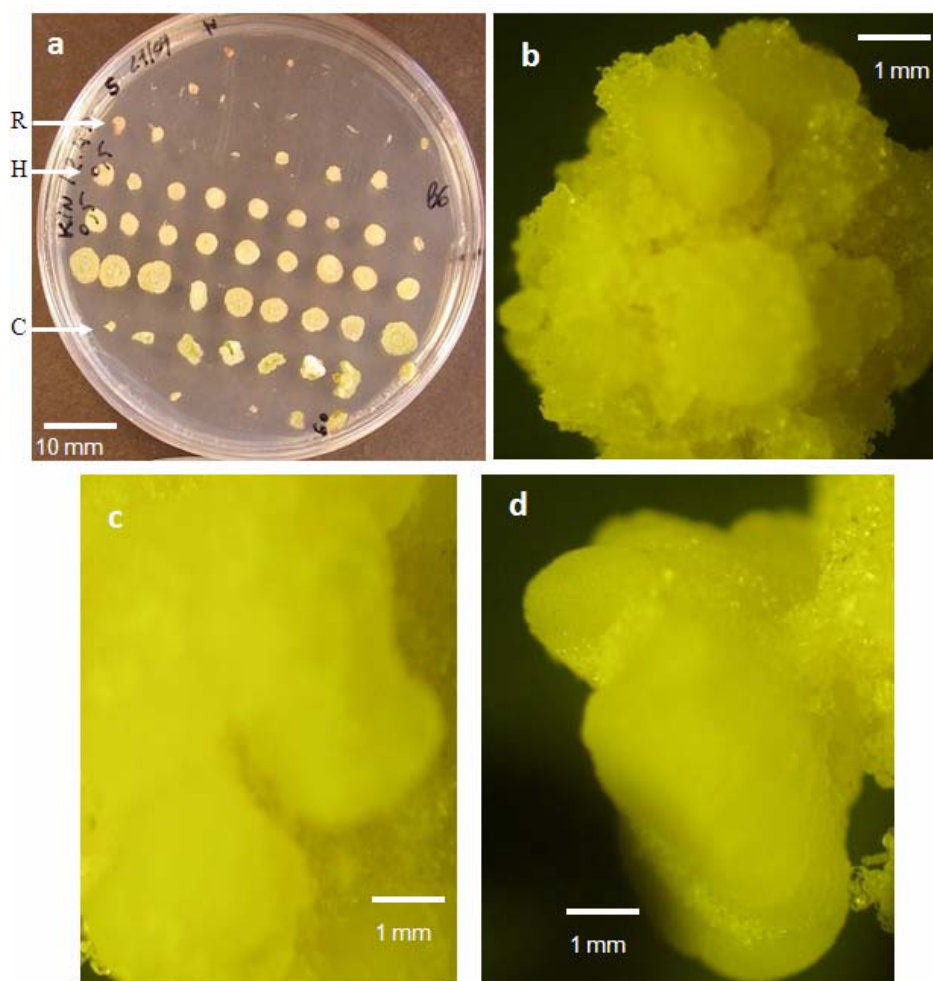


Fig 2. (a-d): Different tissue culture stages from transversal thin cell layers of *Hibiscus sabdariffa*. (a) Calli derived from root (R), hypocotyl (H) and cotyledon (C) tTCLs after 3 weeks; (b) Calli derived from hypocotyl tTCLs after 4 weeks of culture on DKW medium +1 mg/l 2,4-D + 0,5 mg/l TDZ ; (c-d) globular and heart stages of somatic embryo development after 8 weeks on DKW+1 mg/l 2,4-D + 0,5 mg/l TDZ

Gymnema sylvestre (Gopi and Vatsala, 2006). Use of 2,4-D in low concentration or in equilibrium with KIN could reduce excessive root formation. In DKW-based media, different concentrations of 2,4-D and TDZ had an important effect on callus initiation (Table 5). The percentages of callus induction ranged from 88.93 to 100 % for both explant types and genotype in all media. Callus diameter obtained with these media varied from 5.5 mm (MS₂) to 11.16 mm (MS₃) (Tables 2, 3, 4, and 5). Induced calli obtained with MS₃ and MS₄ were well developed and spongy while callus in MS₂ were friable, compact and granular. Calli observed with DKW medium containing 1mg/l 2,4-D/0.5 mg/l TDZ were friable and nodular (Fig. 2b). These morphologic observations are characteristic of embryogenic structure (Kouakou, 2003). Calli from *H. sabdariffa* var. *sabdariffa* explants were white, pale red or reddish white while those from *H. sabdariffa* var. *altissima* explants, were white or whitish green. No somatic embryos were formed on any of the MS-based media (Tables 2, 3, and

4). On the other hand, the different concentrations of 2,4-D and TDZ in DKW basal salt media presented an important effect on somatic embryo initiation (Table 5). Results after 45 days showed that the optimal concentration of PGRs for both explants and genotypes were DKW₃ (4 mg/l 2,4-D + 1 mg/l TDZ) and DKW₄ (1mg/l 2,4-D + 0.5 mg/l KIN). For hypocotyl, the percentage of somatic embryos was 11.55% (DKW₃) and 10.83% (DKW₄) in *H. sabdariffa* var. *altissima* (Fig. 2c-d). Moreover, the percentage of somatic embryos was 11.46 (DKW₃) and 0.87% (DKW₄) in *H. sabdariffa* var. *sabdariffa*. For cotyledon, the optimal medium was DKW₄ in *H. sabdariffa* var. *sabdariffa* (2.08 %) and *H. sabdariffa* var. *altissima* (12.12%). Within the same explant type, a great variability of the induction of the somatic embryos was observed. This result showing a genotype effect in the somatic embryo induction was also highlighted in other Malvaceae species such as *Gossypium hirsutum* L. (Gawel and Robacker, 1990). *H. sabdariffa* is used for a myriad of purposes, but lately an

increasing focus has been placed on the health benefits of its many compounds, including anthocyanins (Mizukami et al., 1988; Mizukami et al., 1989; Maganha et al., 2010). *Hibiscus* anthocyanins are water-soluble and among the most important groups of plant pigments. For example, *Hibiscus* anthocyanins have been reported to significantly reduce oxidative stress induced by *tert*-butylhydroperoxide in rat hepatocytes *in vitro* and *in vivo*, which is an important model in liver injury (Kamei et al., 2003; Maganha et al., 2010). In addition to their antioxidant ability, anthocyanins mediate other physiological functions related to cancer suppression, which has raised interest concerning the pharmaceutical function of these pigments (Meiers et al., 2001; Kamei et al., 1995). *H. sabdariffa* callus is rich in anthocyanin (Mizukami et al., 1988; Mizukami et al., 1989); therefore, the protocols developed here could be used to optimize anthocyanin production in *H. sabdariffa*.

Conclusion

The present investigation reported an efficient and easy-to-handle protocol for embryogenesis through callus for *H. sabdariffa*. The 3% sucrose was the best sugar concentration for callus and somatic embryos initiation. Hypocotyl and cotyledon in MS medium containing 0.1 mg/l 2,4-D + 0.5 mg/l KIN and 0.1 mg/l 2,4-D + 0.1 mg/l KIN were the best explants for callus initiation. However, the DKW media containing 4 mg/l 2,4-D + 1 mg/l TDZ and 1 mg/l 2,4-D + 0.5 mg/l TDZ were the best media to induce the formation of somatic embryos through callus.

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