OPTIMIZATION OF SOMATIC EMBRYOGENESIS CULTURE MEDIUM INDUCTION IN COTTON (*Gossypium hirsutum* L. CV. Y331B-R5) GROWTH IN CÔTE D'IVOIRE

N'GUESSAN[1] Affoué Sylvie Rachelle, AYOLIE[1] Koutoua, KONAN[1] Yao Kouakou Francois

YAPO[1] Sopie Edwige Salomé, KOUADIO[1] Yatty Justin, KOUAKOU[2] Tanoh Hilaire

[1] Université Jean Lorougnon Guédé, UFR Agroforesterie, BP 150 Daloa, Côte d'Ivoire

[2] Université Nangui Abrogoua, UFR Sciences de la Nature, 02 BP 801 Abidjan 02, Côte d'Ivoire

nguessansylvie322@gmail.com

Abstract

The development of an efficient protocol for somatic embryogenesis in cotton is an essential prerequisite for the adoption of genetic techniques transformation for varietal improvement. The present study aims to evaluate the responses to somatic embryogenesis of the Y331B-R5 cotton variety widely grown in Côte d'Ivoire. For this purpose, the hypocotyl of the vitroplants was used as an explant to initiate friable callus, which is used for the induction of somatic embryos. The effect of the medium compound on somatic embryogenesis was evaluated. For this purpose, four carbon sources (maltose; sucrose; fructose and glucose) at 30 g.L^{-1} were tested. Then, the effect of different concentrations (20; 30; 40 and 50 g.L^{-1}) of the best carbon source was evaluated. Then, four amino acids (glutamine; casein hydrolysate; glycine and asparagine) were tested. Finally, phytohormones were added to induce embryos. The results showed that glucose at 30 g.L^{-1} induced the highest level of embryogenic cells (67.34 %). Amino acids tests showed that glutamine (0.5 mg.L^{-1}) was more beneficial (47.31 %). Finally, hormones combination (0.1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} KIN) induced the highest rate (74.25 %). The MIE medium + 30 g.L^{-1} glucose + 0.1 mg L^{-1} 2,4-D + 0.5 mg L^{-1} KIN was the most effective for the induction of somatic embryogenesis in Y331B-R5.

Key words : cotton (*Gossypium hirsutum* L.), carbon source, amino acids, growth regulators, somatic embryogenesis.

1. Introduction

Cotton (*Gossypium hirsutum* L.) occupies an important place in the world economy thanks to its fibre used as a first material in the textile industries (Bachelier, 2016). Four species of cotton are currently cultivated. However, *Gossypium hirsutum* is the most cultivated species and alone covers more than 95 % of world production (Kouakou, 2009). In West Africa, cotton represents a source of income for farmers and contributes to food security in the production areas (ICTSD, 2010).

However, cotton cultivation is appointment abiotic and biotic problems, leading in some cases to a drop in production. These production losses vary from year to year and can reach more than 30 % of production (Miranda *et al*., 2013). Face at this situation, several solutions (genetic improvement by interspecific hybridizations) have been proposed but have failed due to certain genetic barriers (Haouala *et al*., 2010). Indeed, in cotton, genotypic barriers strongly limit *in vitro* regeneration. Thus, most cotton varieties are recalcitrant to *in vitro* regeneration (Sakhanokho *et al*., 2001). However, plant biotechnology through tissue culture is a valuable tool to round these barriers and opens avenus for improvement of this species. Thus, somatic embryos derived from tissue culture represent an ideal material for plant breeding

(Finer and Mc Mullen, 1990). These embryogenic structures lend themselves easily to genetic manipulation and allow the incorporation the interest traits such as resistance to certain diseases (Yapo, 2013). However, the success of somatic embryogenesis depends on several factors that, through their action, can inhibit or promote the induction of embryogenic cells. Thus, in this study, the effect of culture medium composition was evaluated.

This study is a contribution to the establishment of an efficient protocol for somatic embryogenesis and highlights the effect of certains components of the culture medium on the induction of embryogenic cells in cotton.

2. Material and methods

2.1. Plant material

The plant material consisted of cotton seeds (*Gossypium hirsutum* L. cv. Y331B-R5) supplied by the Compagnie Ivoirienne pour le Développement Textile (CIDT), (Korhogo, Côte D'Ivoire).

2.2 Methods

2.2.1. *In vitro* seed disinfection and germination

Cotton seeds, however, were first delinted from the fibres in a glass jar using 30 mL of concentrated sulphuric acid (N'guessan *et al*., 2019). The fibre-free seeds were rinsed thoroughly with tap water and then placed in a beaker in the presence of water. Submerged (viable) seeds were recovered and dried in the open air before disinfection. Cotton seed germination was carried out according to the modified Kouakou, (2003) method. Cotton seeds were disinfected under a laminar flow hood by soaking in 70 % alcohol for 1 minute, then in sodium hypochlorite with 2.4 % active chlorine for 30 minutes. After three rinses with sterile distilled water for five minutes, the seeds were soaked in 150 × 22 (L × Ø in mm) test tubes containing 30 mL of sterile distilled water at a rate of one seed per tube and placed in the dark for 48 hours. Then, using autoclaved tweezers, the seeds with pointed rootlets were removed from the seed coat under the fume hood and cultured on germination medium (Figure 1). This medium consists of ½ MS medium (Murashige and Skoog, 1962) supplemented with 30 $g.L^{-1}$ sucrose (N'guessan *et al*., 2019).

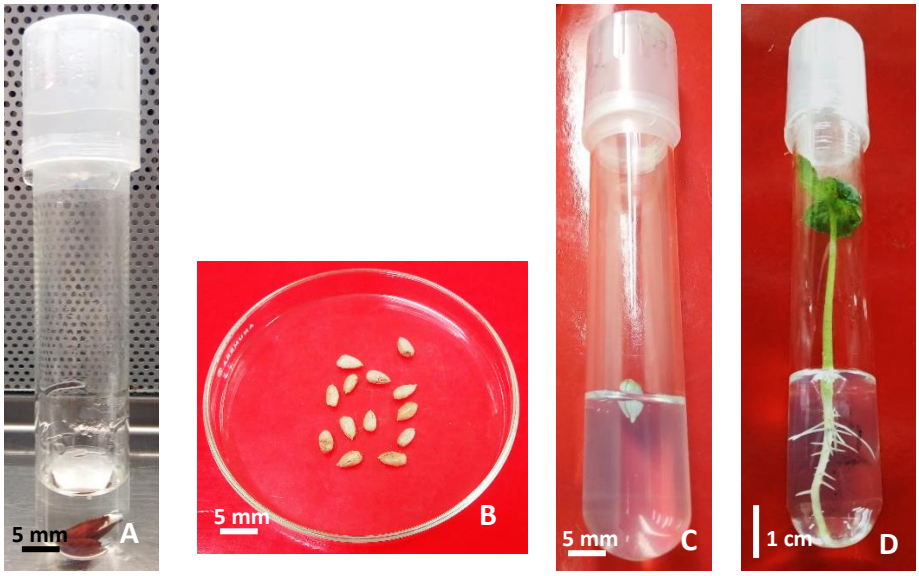

Figure 1 : *In vitro* germination of the cotton plant

A: cotton seed with the radicle pointing after 48 hours in the dark; B: seed with the seed coat removed; C: seed on germination medium, D: 7-day old cotton plant.

2.2.2. Callus induction

After one week on the germination medium, hypocotyl explants (5 mm long) from the resulting vitroplants were used to induce callus on the base medium Murashige and Skoog (MS) (1962) supplemented with Gamborg (Gamborg *et al*., 1968) (MSB5) vitamin B5 supplemented with 30 $mg.L^{-1}$ glucose; 0.1 $mg.L^{-1}$ 2,4-D and 0.5 $mg.L^{-1}$ KIN (N'guessan *et al*., 2019). The pH of the medium was adjusted to 5.8 with 1N NaOH and/or HCl and solidified with 2.2 $g.L^{-1}$ phytagel. After three subcultures of four weeks each on the callogenesis medium, the resulting healthy, friable callus was used as an explant for somatic embryogenesis.

2.2.3. Embryogenic cells induction

2.2.3.1. Medium preparation

The basic somatic embryo induction medium (EIM) is MSB5 free of ammonium nitrate (NH_4NO_3) and with a double concentration of potassium nitrate (KNO_3) EIM: (MSB5 - NH_4NO_3 + 2 KNO_3) (Trolinder and Goodin, 1988). All media were solidified with 2.2 $g.L^{-1}$ phytagel and the pH was adjusted to 5.8 with 1 N NaOH and/or HCl. The prepared media were boiled on a hot plate and dispensed into jars at 30 mL/jar. Sterilisation of the media was carried out in an autoclave for 20 min at 121 °C under a pressure of 1 bar.

2.2.3.1.1. Influence of carbon source on embryogenic cells induction

The medium used for this study was the MIE to which four carbon sources, glucose, fructose, maltose, and sucrose (30 $g.L^{-1}$) were added separately to evaluate their effect on embryo induction. No growth hormones were added to the culture medium. A control medium was prepared with the basal medium, without any carbon source. The carbon source that induced the greatest number of embryos was selected. This carbon source was then tested at different concentrations (20, 30, 40 and 50 $g.L^{-1}$) on the same basal medium (EIM) as before.

2.2.3.1.2. Influence of hormonal regime on embryo induction

The effect of hormonal regimen was evaluated through several combinations of auxins and cytokinins added to the embryo induction medium (EIM). Depending on the combinations made, the different embryogenesis media are designated as follows:

ME1: EIM + carbon source + 2 $mg.L^{-1}$ ANA + 0.5 $mg.L^{-1}$ KIN
ME2: EIM + carbon source + 2 $mg.L^{-1}$ ANA + 0.1 $mg.L^{-1}$ 2,4-D
ME3: EIM + carbon source + 0.1 $mg.L^{-1}$ 2,4-D + 0.5 $mg.L^{-1}$ KIN
MIE4: MIE + carbon source + 0.1 $mg.L^{-1}$ 2,4-D + 0.5 $mg.L^{-1}$ KIN + 0.1 $mg.L^{-1}$ Zeatin
ME5: EIM + carbon source + 0.1 $mg.L^{-1}$ AIA + 0.1 $mg.L^{-1}$ 2,4-D
A control test was carried out without any hormones.

2.2.3.1.3. Influence of amino acids on the induction of embryogenic cells

The effect of amino acids on the induction of embryogenic cells was tested on the same base medium as previously without hormone and with the addition of the best concentration of the carbon source (MIE2). The MIE2 medium was supplemented with glutamine, asparagine, casein hydrolysate, and glycine alone or in combination to induce embryos. The various amino acids were added to the culture medium as follows:

Aa1: MIE2 + 0.5 $mg.L^{-1}$ glutamine
Aa2: MIE2 + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ asparagine
Aa3: MIE2 + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ casein hydrolysate
Aa4: MIE2 + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ asparagine + 2 $mg.L^{-1}$ glycine
Aa5: MIE2 + 0.5 $mg.L^{-1}$ glutamine +0.2 $mg.L^{-1}$ casein hydrolysate + 2 $mg.L^{-1}$ glycine

2.2.3.2. Growing and growth condition

Embryo induction has been carried out according to the method of Kouadio (2018). Approximately 1 g of healthy, friable calluses were weighed into sterile centrifuge tubes under the hood. Callus were cultured in jars containing 30 mL of EIM at a rate of two explants per jar. The jars containing the explants were closed, sealed with parafilm and incubated in the culture chamber. After two subcultures of two weeks each, the jars were tested for evidence of embryogenic or proembryonic structures. All media were incubated in growth roomat under a 12 h photoperiod with cool white fluorescent light at 2000 lux of intensity, and the temperature was maintained at 28 ± 2 °C with 70 % relative humidity

2.2.3.3. Microscopic observations of embryogenic cells

After four weeks of growing, the embryogenic structures were observed on optical microscope DC5.5V/200 mA Lamp: LED (white). For this purpose, callus tweezers from the embryo induction media were taken from each jar and mounted in a drop of distilled water between slide and lamina. Microscopic observations were made at 400 magnification (G x 400). The shape of the embryogenic structures (elongated, round, oval) and their consistency (dense or aerated cytoplasm) were investigated. Eight observations were made for each treatment.

2.2.3.4. Study of embryogenic cells viability

The viability of embryogenic cells was tested using a solution of trypan blue (0.2 %) according to the method of Sumantran (2011). This stain makes it possible to assess the viability of the cells by staining the dead cells blue. Thus, 5 mg of embryogenic callus were weighed and placed in 5 mL of distilled water and gently homogenized by shaking. The resulting suspension was added to 5 mL of the 0.2 % trypan

blue solution and homogenized using a vortex. The resulting homogenate was incubated for 5 minutes. After three successive rinses with distilled water on filter paper, callus tweezers were mounted in a drop of distilled water between slide and slide. Observations were made using the DC5.5V/200 mA microscope, at magnification (Gx400) on a malassez slide.

Each experiment was repeated eight times and viable cells were counted. Cells stained blue are dead cells and those not stained blue are alive.

2.2.3.5. Embryos maturation and germination.

After embryo induction, 500 mg of embryos with nodules were transplanted onto the best embryo induction medium for 4 weeks to induce embryo maturation. At the end of maturation, the embryos are transferred to new medium for another four weeks to initiate embryo germination. The stages of development of the embryos have been investigated using the method of Thiruvengadame *et al* (2006).

2.2.4. Statistical analyses

For each experiment, the rate of embryogenic cell induction, the number of viable and non-viable cells and embryogenic index were determined. To normalize the data, all percentage values were subjected to arcsin (√x) transformation before statistical analysis. The analyses were performed with Statistica version 7.1 software. Analysis of variance (ANOVA) was used to calculate the means. Analyses with one and two classification stantdard were performed on the mean values of the measured parameters. When a significant difference was found between two means, Newman-Keuls test at 5 % threshold was used to classify the means.

3. Results

3.1. Effect of the carbon source on embryogenic cells induction

The results of carbon sources effect reported in Table 1 and showed that the response of explants of embryogenic cell induction was significantly influenced by the carbon source ($p<0.05$). Thus, glucose induced the highest rate of embryos (64.58 %), followed by sucrose (46.7 %), fructose (30.4 %) and maltose (29.94 %). The results of viability test showed that the medium supplemented with glucose induce highest viability rate of induced embryos. Glucose was therefore selected as the best carbon source to assess the effect of glucose concentration on embryogenesis. Thus, analysis of the table 2 shows that 30 $g.L^{-1}$ of glucose gives the highest embryogenic index and embryogenic cell count (0.67 and 67.34 %, respectively). 30 $g.L^{-1}$ glucose was selected for further work.

Table 1. Evaluation of carbon source effect on embryogenic cells induction.

	Paramètres		
carbone source ($g.L^{-1}$)	**Embryogenic cell induction rate**	**Viable cells number**	**No viable cells number**
Indicator	00 ± 00 d	5.9 ± 00 d	94.9 ± 0.21 a
maltose	29.94 ± 0.02 c	37.01 ± 0,04 c	62.99 ± 0.13 b
sucrose	46.74 ± 0.07 b	46.6 ± 0.07 c	53.4 ± 0.08 c
fructose	30.40 ± 0.03 c	60.3 ± 0.12 b	39.7 ± 0.04 c
glucose	64.58 ± 0.15 a	85.9 ± 0.19 a	14.1 ± 0.01 d

In the same column, the means followed by the same letter are not significantly different (Newman-Keuls test at the 5%).

Table 2. Effect of glucose concentration on embryogenic cells induction

	Paramètres	
Glucose concentration ($g.L^{-1}$)	**Embryogenic Index**	**Embryogenic cell induction rate**
Indicator	00 ± 00 d	00 ± 00 d
20	0.39 ± 0.001 c	39.06 ± 0.03 c
30	0.67 ± 0.007 a	67.34 ± 0.17 a
40	0.51 ± 0.003 b	51.47 ± 0.1 b
50	0.47 ± 0.002 c	47.54 ± 0.06 b

In the same column, the means followed by the same letter are not significantly different (Newman-Keuls Test 5%).

3.2. Effect of amino acids on embryogenic cells induction

The analysis of Table 3 shows that amino acids have a significant effect ($p<0.002$) on embryogenic cells induction. Thus, the medium Aa_1 (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine) induced the highest level of embryogenic cells (47.31 %). It is followed by medium Aa_5 (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ casein hydrolysate + 2 $mg.L^{-1}$ glycine) with 37.26 %. The media Aa_3 (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ casein hydrolysate); Aa_4 (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ asparagine + 2 mg. L^{-1} glycine) and Aa_2 (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ asparagine) induced the lowest levels of embryogenic cells (21.47; 25.94 and 27.28 %). The embryogenic index study showed that the Aa_1 medium containing glutamine induce highest embryogenic index (0.47). In contrast, the lowest embryogenic index was recorded with Aa_3 medium (0.21).

Viability tests showed that Aa_1 medium (EIM + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine) induced the highest number of viable cells (81.35 cells) and the lowest number of viable cells was recorded on the control medium (4 cells). The control medium induced the highest number of non-viable cells.

Table 3. Influence of amino acids on the induction of embryogenic cells in the cotton plant

	Paramètres			
Amino Acids	**Embryogenic index**	**Embryogenic cells induction rate**	**Viable cells number**	**No viables cells number**
Indicator	00 ± 00	00 ± 00 d	04 ± 0.00 d	96.01 ± 0.23 a
Aa_1	0.47 ± 0.003 a	47.31 ± 0.07 a	81.35 ± 0.17 a	18.65 ± 0.04 d
Aa_2	0.27 ± 0.001 c	27.28 ± 002 c	31.88 ± 0.03 c	68.12 ± 0.14 b
Aa_3	0.21 ± 0.001 c	21.47 ± 0.02 c	27.86 ± 0.02 c	72.14 ± 0.15 b
Aa_4	0.25 ± 0.001 c	25.91 ± 0.02 c	37.51 ± 004 c	62.49 ± 0.13 b
Aa_5	0.37 ± 0.002 b	37.26 ± 0.03 b	66.25 ± 0.13 b	33.75 ± 0.04 c

In the same column, the means followed by the same letter are not significantly different (Newman-Keuls test at the 5%). Aa_1 (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine); Aa_2 (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ asparagine); Aa_3 (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine + 0.2 mg. L^{-1} casein hydrolysate); Aa_4 (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ asparagine + 2 $mg.L^{-1}$ glycine); Aa_5 (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine + 0.2 $g.L^{-1}$ casein hydrolysate + 2 $mg.L^{-1}$ glycine)

3.3. Effect of hormonal combination on embryogenic cells induction

The results show that the hormonal combination influenced significantly induction rate and embryogenic index (Table 4). Thus, the medium supplemented with 0.5 $mg.L^{-1}$ 2,4D + 0.5 $mg.L^{-1}$ KIN

induced the highest rate of embryos (74.52 %). The medium add 2 mg.L^{-1} ANA + 0.1 mg.L^{-1} KIN induced the lowest rate induction (26.94 %). Similarly, the highest embryogenic index (0.74) was recorded on medium containing 0.5 mg.L^{--1} 2,4-D + 0.5 mg.L^{-1} KIN and the lowest embryogenic index (0.26) was obtained on medium containing 2 mg.L^{-1} ANA + 0.1 mg.L^{-1} KIN. No embryogenic cells were observed on the control medium (Figure 2). The highest number of viable cells (88.79) was induced on medium containing 0.1 mg.L^{-1} 2,4D + 0.5 mg.L^{-1} KIN.

Table 4. Effect of hormonal compound on the induction of embryogenic cells

	Paramètres			
Hormonale compound	**Embryogenic Index**	**Embryogenic cell induction rate**	**Viable cells number**	**No viable cells number**
Indicator	00 ± 00 d	00 ± 00 d	06 ± 0,001 d	94 ± 0.25 a
H_1	0.42 ± 0.006 b	42.57 ± 3.15 b	59.66 ± 0.09 b	40.34 ± 0.05 c
H_2	0.26 ± 0.001c	26.94 ± 2.39 c	36.42 ± 0.04 c	63.58 ± 0.14 b
H_3	0.74 ± 0.08 a	74.52 ± 1.49 a	88.79 ± 0.2 a	11.21 ± 0.01d
H_4	0.43 ± 0.004 b	43.89 ± 2.66 b	64.78 ± 0.13 b	35.22 ± 0.04 c
H_5	0.37 ± 0.001 b	37.10 ± 3.20 b	58.9 ± 0.09 b	41.1 ± 0.08 c

In the same column, the means followed by the same letter are not significantly different (Newman-Keuls test at 5%). H_1: (MIE + 30 g.L^{-1} glucose + 2 mg.L^{-1} ANA+0.1 mg.L^{-1} KIN); (H_2 : (MIE + 30 g.L^{-1} glucose + 4 mg.L^{-1} ANA+1 mg.L^{-1} KIN); H_3 : (MIE + 30 g.L^{-1} glucose + 0.1 mg.L^{-1} 2,4-D+0.5 mg.L^{-1} KIN); H_4 : (MIE + 30 g.L^{-1} glucose + 0.1 mg.L^{-1} 2,4-D+0.5 mg.L^{-1} KIN+0.1 mg.L^{-1} Zéatine) ; H_5 : (MIE + 30 g.L^{-1} glucose + 0.1 mg.L^{-1} AIA+0.1 mg.L^{-1} 2,4-D).

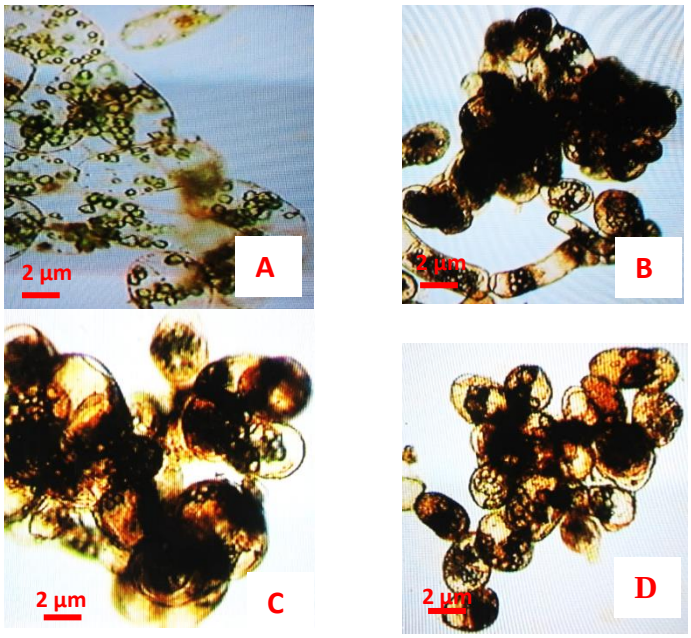

Figure 2 : Cotton embryo induced on different media culture

A: Non-embryonogenic cell control; B: MIE + 30 g.L $^{-1}$ glucose; C: MIE + 30 g.L^{-1} glucose + 0.5 mg.L^{-1} glutamine; D: MIE + 30 g.L^{-1} glucose + 0.1 mg.L^{-1} + 0.5 mg.L^{-1} KIN; GX400

3.4. Embryos maturation and germination

The results of embryo maturation reveal that embryos evolve progressively with the appearance of certain stages of somatic embryogenesis. Culture on the germination medium revealed that these embryos did not germinate, but were alive, as certain stages of somatic embryogenesis were observed (Figure 3).

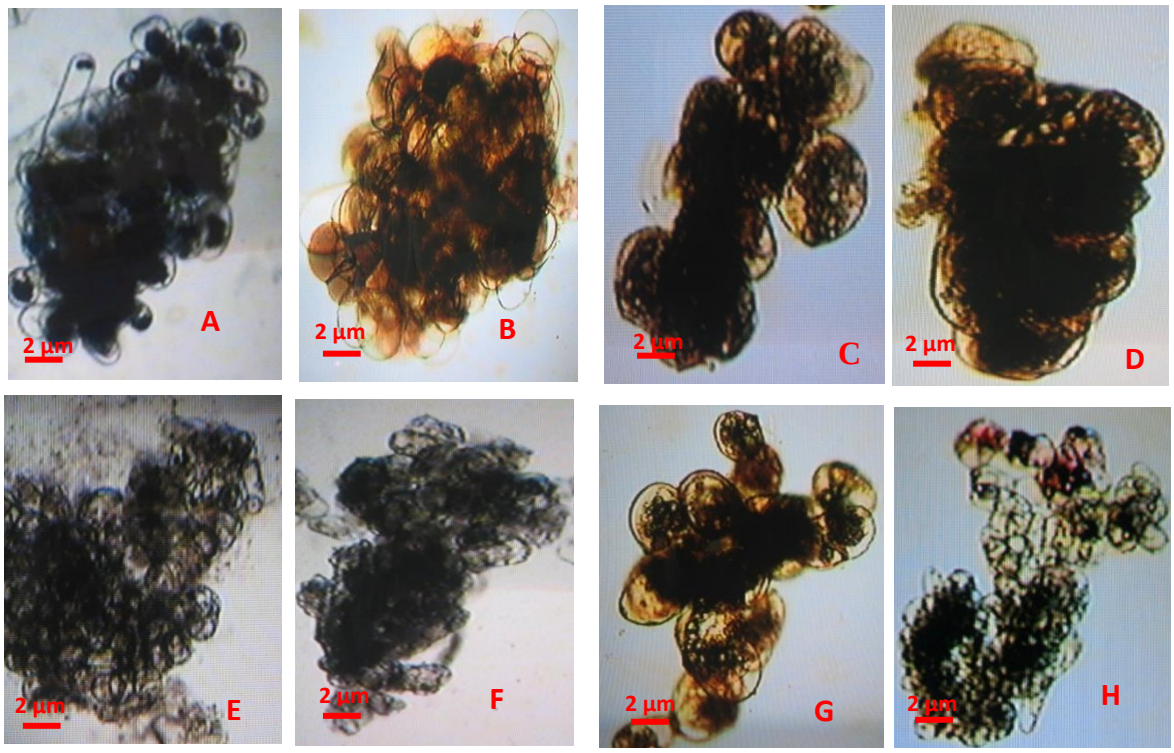

Figure 2: Embryos maturation

A-B: embryo in globular stage; C-D: embryo in cordate stage; E-F: heart stage; G-H: embryo in torpedo/cotyledonary stage; GX400

4. Discussion

Somatic embryogenesis is artificially induced and leads to the formation of an embryo from a somatic cell. The cells nature, culture conditions and other factors play an important role in the acquisition of embryogenic capacity (Najiba *et al*., 2008; Yapo, 2013; Kouadio *et al*., 2017). This study highlights the influence of the composition of the medium on somatic embryogenesis. The results show that the carbon source influences embryogenic cells induction. In *in vitro* culture, sugars are indispensable as they are the only source of energy for the explant. They induce osmotic stress which would be at the basis of the induction of embryogenic structure. Similar observations have been reported by Kouakou, (2009). Similarly, Koné (2010) showed in voandzou that callus regular subcultures in the presence of 30 g.L^{-1} sugar favoured the induction of embryogenic structure. However, cells react differently depending on the source of carbon present in the culture medium. For example, glucose has been shown to have the highest rate of induction of embryogenic cells. Indeed, among carbon sources, glucose is the form most assimilable by plants (Richter, 1993). This would explain the results obtained. Similar results were obtained by Firoozabady and Deboer (2006) and Kouakou (2009) who showed that, glucose used as a carbon source favours the induction of somatic embryos in the cotton. So, in peaches, Declerck *et al*

(1986) showed that glucose and sorbitol gave better results than fructose and sucrose. The action of carbon sources on embryo induction appears to be species-specific. Indeed, in pineapple and olive (*Olea europaea*) Brhadda *et al* (2006); Yapo (2013), and Kouadio (2018) have shown that sucrose promotes the induction and proliferation of embryogenic cells. Glucose appears to be the best source of carbon to induce embryos in cotton.

In terms of glucose concentration, the results showed that a concentration of 30 $g.L^{-1}$ resulted in the highest level of embryogenic cells. The concentration of 30 g.L-1 seems to be the best to induce embryos in a cotton plant. Indeed, high concentrations would lead to an increase in osmotic pressure inducing stress in medium culture. This osmotic pressure leads to a strong absorption of sugars and minerals. It result is an accumulation of sugar in the cells which gives the brown colouring of callus. On the other hand, an increase in osmotic pressure would be harmful to embryos, which are fragile structures. Kumar *et al* (2013) obtained similar results on MS medium supplemented with 30 $g.L^{-1}$ of glucose. In cotton, work has shown that glucose at the concentration of 30 $g.L^{-1}$ is ideal for cell proliferation and embryo induction (Trolinder and Goodin, 1988; Koné, 2010; Kouakou, 2009). In olive trees, Brhadda et al. (2008) showed that sucrose at 30 $g.L^{-1}$ concentration is more favourable for the development of somatic embryos. In addition, authors have shown that the sugar concentration generally used for embryo induction and development in several species is between 20 and 30 $g.L^{-1}$ (Han *et al*., 1989; Rout *et al*., 1991). Moreover, some species (asparagus and chrysanthemum) require higher sugar concentrations of up to 50 $g.L^{-1}$ (Komura *et al*., 1990; May *et al*., 1991). This means that the effect of carbon source concentration is strongly influenced by genotype.

In terms of amino acids, the addition of 0.5 $mg.L^{-1}$ glutamine induced the highest level of embryogenic cells compared to the other media tested. Indeed, once in the culture medium, the amino acids provide a source of nitrogen for the cells to use to stimulate embryogenesis. Likewise, Yapo (2013) uses nitrogen selectively in the form of nitrate for embryo induction. Therefore, the high level of nitrate would have a significant effect on the induction of embryogenic cells. Our results are in agreement with those of Price and Smith (1979), Davidonis and Hamilton (1983) who showed in *Gossypium klotzschianum* that the presence of glutamine in the medium allows a high number of embryos to be obtained. Finer (1988) reported that glutamine promotes embryo induction and proliferation. The presence of casein hydrolysate associated with glycine appears to stimulate the formation of embryogenic cells. These results corroborate those of Rangan, (1984) who observed that casein hydrolysate stimulates embryo induction.

The media Aa_4 (0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ asparagine + 2 $mg.L^{-1}$ glycine); Aa_2 (MIE + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ asparagine) and Aa3 (MIE + 0.5 $mg.L^{-1}$ glutamine + 0.2 $mg.L^{-1}$ casein hydrolysate) have been shown to be unfavourable for embryo induction. This could be explained by the different combinations performed. According to Finer (1988), glutamine stimulates embryo induction. However, when it is combined with asparagine, a decrease in the number of embryogenic cells is observed. Embryo induction medium (EIM) with the addition of 0.5 mg L^{-1} glutamine is more embryogenic in cotton.

The hormonal compound test show that medium supply 0.1 mg L^{-1} 2,4D + 0.5 mg L^{-1} KIN induced the highest level of embryogenic cells. This beneficial effect of this hormonal combination on embryo cells induction is explained by the fact that the absence of NH_4NO_3 in the culture medium causes stress. Thus, in the presence of the 2,4D/KIN couple, which is the reference hormone in the *in vitro* culture of cotton, the induction of embryogenic structures would be favoured. On the other hand, the stress caused by the absence of NH_4NO_3 and the high concentration of KNO_3 increases the number of embryos

(Davidonis and Hamilton (1983); Kumar *et al*., 2013). These results are in agreement with those of Zouzou *et al*. (2008); Kouakou *et al*. (2009); Robinson *et al*. (2011) who showed that in cotton plants the 2,4-D/KIN combination is beneficial for embryos induction. In *in vitro* culture, hormones and more particularly the auxin/cytokinin couple plays an important role in the induction of embryogenesis. Their presence allows the initiation of embryogenesis competence according to Koné (2010). Among synthetic auxins, 2,4D is a hormone capable of inducing somatic embryos when used alone or in combination (Rathore *et al*., 2015; Raju *et al*., 2013). H_1 medium containing 2 mg L^{-1} ANA and 0.5 mg L^{-1} KIN has also been shown to induce embryos. Our results coincide with those of Davidonis and Hamilton (1983) who showed that the ANA/KIN combination was favourable to the induction and proliferation of somatic embryos in certain cotton varieties.

The results of previous tests have clearly shown the ability of callus to induce embryos. Thus, after induction a maturation and germination test was carried out. The results showed that the embryos were able to reach certain stages of embryogenesis but did not germinate. This means that embryogenesis in cotton is strongly linked to the genotype. This genotype effect on *in vitro* regeneration of cotton has already been reported by the work of Trolinder and Goding, (1962); Kouadio, (2004); Kouakou, (2009).

5. Conclusion

The primary objective of this study is to propose a favourable culture medium to somatic embryogenesis in cotton. The results clearly showed that glucose at a concentration of 30 $g.L^{-1}$ is conducive to the induction of somatic embryos, glutamine, induces somatic embryos. Finally, the 2,4-D/KIN combination represents the best hormonal combination for the induction of somatic embryos in cotton plants. At the end of this study, we conclude that glucose (MIE + 30 $g.L^{-1}$ glucose), H_3 (MIE + 30 $g.L^{-1}$ glucose + 0.1 $mg.L^{-1}$ 2,4-D + 0.5 $mg.L^{-1}$ KIN) and (MIE + 30 $g.L^{-1}$ glucose + 0.5 $mg.L^{-1}$ glutamine) are the best media for inducing somatic embryos in cotton.

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