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Effect of genotype, explants, growth regulators and sugars on callus induction in cotton (*Gossypium hirsutum* L.)

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Abstract

Ten cotton (Gossypium hirsutum L.) genotypes were chosen for tissue culture. Callus initiation was genotype dependent, and R405-2000 has the best callogenesis response. Callus was induced from three media, the percentage of callus induction and dry weight of callus varied, but MS was the best callogenesis medium. It appeared that it was much easier to induce callus from hypocotyl than cotyledon or root explants. Induction callus of cotton was varied with hormone regimes. In effect, a proper combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) promoted the callus initiation. Glucose was the best sugar to promote the production of callus. However, the concentrations of glucose were critical to the induction of callus. The optimum glucose concentration for callus induction was 40 g/L. The best medium for the proliferation of callus was MS medium with 0.1 mg/l 2,4-D, 0.5 mg/l KIN and 4% glucose. An efficient protocol for the production of high frequency callus of cotton has been developed.

Keywords: Callogenesis, Explant, *Gossypium hirsutum* L., Nutrient medium, Plant growth regulator, Sugar

Introduction

Much interest is presently focused on tissue culture techniques applied to agricultural plants. To a large extent this interest has been generated by carlson et al. (1972), who obtained a parasexual hybrid plant by plating callus derived from fused protoplasts of two genotypes of tobacco. The methods of producing somatic cell hybrids have opened up exciting possibilities for genetic studies concerning the development of improved agricultural varieties (Davidonis and Hamilton, 1983; Zouzou et al., 2000; Wang et al., 2004; Jin et al, 2006; Burbulis et al., 2007). A basic starting point in such research is to define favorable or near optimal culture conditions for particular species of agronomic plant. Cotton is a cash crop widely cultivated in many countries. Gossypium hirsutum L. is the principal species of cotton currently cultivated, primarily for its fibres that constitute the principal raw material for textile industries. Moreover, cotton seeds have become very interesting sources of proteins for animal feed and human consumption. Genetic improvement of cotton through conventional breeding is limited by several factors including incompatibility barriers and time period for improved variety development (Munro, 1987). Although plant biotechnology seems to be an

attractive way to improve cotton plant, its use requires an effective in vitro culture system using somatic tissues of plant. In vitro culture allows circumventing these difficulties: e.g. callus obtained from explant is an ideal material for genetic transformations (Finer and McMullen, 1990). The first significant work in cotton callogenesis was reported by Beasly (1971), who successfully induced callus from ovule. Three years later, cotyledon achieved the induction of callogenesis from cotton (Davis et al., 1974).

Since then, comprehensive studies are conducted on callogenesis from several cultivars using various explants, growth regulator combinations and carbohydrate source (Davidonis and. Hamilton, 1983; Zouzou et al., 2000; Trolinder and Goodin, 1988; Zhang et al., 2001; Kouadio et al., 2007). Factors involved in the initiation and maintenance of callus Gossypium species have been investigated by number of laboratories (Zouzou et al., 1997; Wu et al, 2004; Ikram, 2005; Rao et al., 2006; Sun et al., 2006; Xie et al., 2007). Zouzou et al. (2000) described favorable conditions for in vitro culture of cotton. Many factors can influence the efficiency of a callogenesis procedure. The main factors determining the tissue culture response in cotton and other recalcitrant crops include genotype (Seabrook and. Douglas, 2001), donor plant (Lu et al., 1984), type of growth regulators (Trolinder and Goodin, 1988; sun et al., 2006), sugar type (Ishii et al., 2004) and culture medium (Popelka and Altpeter, 2001). An in-depth study of such factors would enable the development of genotype-specific culture methods to enhance the tissue culture response of the recalcitrant crops.

The objective of the present study was to investigate the effect of genotype, culture medium, growth regulator, explant type, and carbohydrate source and level on cotton callus induction.

Materials and methods

Plant material

Seeds of cotton (*Gossypium hirsutum* L.), cultivars Deltapine, Guazuncho, ISAGL7, ISA268A, Ka-10, MacNair, M124-10, R405-2000, X449B and W766A were used for the experiments. They were obtained from CNRA (Centre National de Recherche Agronomique, Côte d'Ivoire, West Africa).

Seed germination and cultivation of sterile seedlings

Seeds of cotton were delinted with sulphuric acid. Plump and mature seeds were chosen and surface-sterilized by dipping in 70% (v/v) ethanol (1 min) prior to a 20 min exposure to 2.5% sodium hypochlorite (v/v). After rinsing 3 times with sterile distilled water for 5 min, sterile seeds dipped and kept in sterile water for one day for coats softening. The sterilized seeds without seed coats were then sown in test tubes containing half-strength MS (Murashige and Skoog, 1962) salts with Gamborg vitamins B5 (Gamborg et al., 1968) medium. The medium contained 30 g/L sucrose (Sigma Chemical Co.) solidified with 2.5 g/L gelrite (Sigma Chemical Co.) and 0.75 g/L MgCl2 (Sigma Chemical Co.). The pH of the media was adjusted to 5.8 with 1 M NaOH (Merck) before autoclaving. 10 ml MS media was poured into test tubes (Pyrex) and one seed was germinated in each test tube. Seeds were incubated fro one week in culture room under 16 h photoperiod conditions with the light intensity of approximately 2000 lux, provided by cool white fluorescent lamps at 25 ± 2°C and 70% humidity.

Callus initiation and maintenance

The callus cultures and procedures for maintenance have already been described (Kouadio et al., 2004). Briefly, callogenesis was routinely initiated with hypocotyls of 7-day-old-grown sterile seedlings during 4 weeks on MS medium plus Gamborg vitamins B5 (Gamborg et al., 1968), 30 g/L glucose. The medium was supplemented with 0.1 mg/L 2,4-dichlorophenoxyacetic acid (2.4-D) and 0.5 mg/L kinetin (KIN) at pH of 5.8 and solidified by 2.5 g/L gelrite plus 0.75 g/L MgCl2 (control medium, MSC). This medium has been previously used for cotton tissue culture by Trolinder and Goodin, (1988), Kouadio et al. (2004) and Kouakou et al. (2007).

Effect of genotype

Ten cotton genotypes were cultivated on callogenesis medium MSC to test their callogenesis capacities.

Table 1. Effect of cotton genotype and basal media on callus culture [a]

Genotype	Explant forming callus (%)	Callus dry weight (mg)
R405-2000	95 ± 2,13 a	152.50 ± 4,65 a
ISAGL7	90 ± 1,58 a	101.03 ± 3,41 b
ISA268A	87 ± 2,21 a	70.37 ± 2,34 c
MacNair	71 ± 1,84 b	54.31 ± 1,95 d
X449B	66 ± 1,50 b	45.80 ± 1,43 d
M124-10	51 ± 1,23 c	31.13 ± 1,05 e
Ka-10	48 ± 1,16 c	30.17 ± 0,95 e
W766A	44.33 ± 1,07 c	28.08 ± 0,88 e
Guazuncho	43 ± 0,93 c	26.83 ± 0,85 e
Deltapine	26 ± 0,73 d	14.70 ± 0,75 f
Culture medium		
MC	55 ± 1,60 b	60.11 ± 1,96 b
B5	77 ± 1,87 a	99.67 ± 2, 61 a
MS	96 ± 2,13 c	154.07 ± 3,43 c

[a]Values for each parameter followed by a different letter within each column are significantly different, Newman-Keuls test (P< 0.05). Each value represents the mean of three replicates; ± SD (standard deviation). B5 (Gamborg); MC (McCown); MS (Murashige and Skoog).

Effect of culture medium

The genotype which gave best callogenesis response was tested on three culture media (B5, McCown and MS) frequently used for plant tissue culture (Table 1), under the previously described conditions to optimise the best media and use it in subsequent work.

Effect of growth regulators and explant type

Various concentrations of the four hormone solutions were prepared and added to the best medium culture. 2,4-dichlorophenoxyacetic acid (2,4-D), napthaleneacetic acid (NAA), kinetin (KIN) and zeatin (ZEA) were added at different levels (0.1; 0.5 mg/ L) to the culture medium which was retained by factorial design. Ten combinations of growth regulators were tested. Cotyledon pieces (16~25 mm2 surface area), hypocotyl sections (5-mm length), and root segments (5 mm length) of 7-day-old sterile seedlings were used as explant type.

Effect of sugar type

The hormone regime which showed the best callogenesis potential expression has been retained to study the effect of sugar type on callogenesis. Six sugars (glucose, fructose, galactose, maltose, sucrose and sorbitol) were tested in the study. Six different concentrations of the sugar which could promote callus production (1%, 2%, 3%, 4%, 5% and 6%) were also tested to identify the level of sugar which induce callus the best callus. All explants were incubated in Erlenmeyer flasks, under the same culture conditions of germination. Over 4 weeks period, the ability of the seedlings to develop callus on the above media was observed. Calli were harvested, weighed and freeze-dried for dry weight determination.

Table 2. Comparison between the effect of hormone regime and various explants on cotton callus culture [a]

Hormones	Explants	Explant forming callus (%)	Callus dry weight (mg)
M1: 0.1 mg/L 2,4-D	Cotyledon	67 ± 1,43 a	48.60 ± 0,96 a
	Hypocotyl	90 ± 2,17 b	97.09 ± 1,94 c
	Root	71 ± 1,26 a	55.97 ± 1,13 a
M2: 0.1 mg/L 2,4-D + 0.1 mg/L KIN	Cotyledon	67 ± 1,24 a	60.36± 1,22 a
	Hypocotyl	90 ± 2,10 b	102.29 ± 3,34 b
	Root	71 ± 1,44 a	68.97 ± 1,12 a
M3: 0.1 mg/L 2,4-D + 0.5 mg/L KIN	Cotyledon	78 ± 1,36 a	81.51 ± 1,97 c
	Hypocotyl	94 ± 1,71 b	148.07 ± 4,23 d
	Root	82 ± 1,75 a	94.34 ± 2,51 c
M4: 0.5 mg/L 2,4-D + 0.5 mg/L KIN	Cotyledon	76 ± 1,22 a	60.50 ± 1,08 a
	Hypocotyl	95 ± 1,89 b	94.11 ± 2,12 c
	Root	80 ± 1,33a	70.30 ± 1,63 a
M5: 0.1 mg/L 2,4-D + 0.1 mg/L ZEA	Cotyledon	75 ± 1,21 a	69.95 ± 1,45 a
	Hypocotyl	98 ± 2,19 b	126.41 ± 3,21 e
	Root	81 ± 1,66 a	83.16 ± 1,33 ac
M6: 0.1 mg/L 2,4-D + 0.5 mg/L ZEA	Cotyledon	73 ± 1,36 a	33.45 ± 0,89 f
	Hypocotyl	94 ± 1,84 b	57.61 ± 1,17 g
	Root	78 ± 1,656 a	42.30 ± 1,11gf
M7: 0.1 mg/L NAA + 0.1 mg/L KIN	Cotyledon	77 ± 1,47 a	41.60 ± 0,96 gf
	Hypocotyl	91 ± 1,82 b	63.05 ± 1,17 a
	Root	85 ± 1,77 a	50.18 ± 1,05 g
M8: 0.5 mg/L NAA + 0.5 mg/L KIN	Cotyledon	76 ± 1,56 a	67.29 ± 1,20 a
	Hypocotyl	89 ± 1,49 b	94.12 ± 1,66 c
	Root	77 ± 1,38 a	80.33 ± 1,53 ac
M9: 0.1 mg/L NAA + 0.1 mg/L ZEA	Cotyledon	70 ± 1,31 a	59.15 ± 1,13 g
	Hypocotyl	93 ± 1,88 b	81.29 ± 1,61 ac
	Root	79 ± 1,66 a	68.02 ± 1,52 a
M10: 0.5 mg/L NAA + 0.5 mg/L ZEA	Cotyledon	75 ± 1,45 a	43.61 ± 0,76 gf
	Hypocotyl	90 ± 1,87 b	65.78 ± 1,44 a
	Root	78 ± 1,56 a	53.80 ± 1,19 gf

[a] Values for each parameter followed by a different letter within each column are significantly different, Newman-Keuls test ($P < 0.05$). Each value represents the mean of three replicates; ± SD (standard deviation). 2,4-D (2,4-dichlorophenoxyacetic acid); NAA (napthaleneacetic acid); KIN (kinetin); ZEA (zeatin); M (hormone regime).

Table 3. Effect of carbohydrate sources on cotton callus culture [a]

Sugar type	Explant forming callus (%)	Callus dry weight (mg)
Glucose	96 ± 2,21 a	157.33± 4,11 a
Fructose	73 ± 1,88 b	122.03 ± 3,55 b
Sucrose	54 ± 1,12 c	94.87± 2,67 c
Galactose	27 ± 0,97 d	31.05± 1,17 d
Maltose	23 ± 0,89 d	28.75 ± 1,04 d
Sorbitol	13 ± 0,35 e	14.93 ± 0,66 e

[a]Values for each parameter followed by a different letter within each column are significantly different, Newman-Keuls test (P< 0.05). Each value represents the mean of three replicates; ± SD (standard deviation).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the statistica 7.5 program and significant differences among treatments were compared using Newman-Keuls test. Means are the result of three replicates.

Results and Discussion

Response in tissue culture is highly genotype dependent. Significant genotypic differences in callus initiation response were observed among the ten cotton genotypes investigated. The percentage of callus formed is positively correlated with callus dry weight. Indeed, R405-2000 clearly showed the best callogenesis response and the highest callus dry weight than the others cotton genotypes (Table 1). This positive response of callogenesis was already reported on the Coker (Trolinder and Goodin, 1988; Kouakou, 2003) and Simian (Zhang et al., 2001). Dry weight which represents the mass of cells produced could be considered as good index of cotton callogenesis (Zouzou et al., 2000; Kouadio et al., 2004). Callogenesis positive response of R405-2000 compared to the others cotton genotypes seem to be related to the differential sensitivity of tissue to callogenesis medium. Similarly, several authors mentioned the influence of the genotype in cotton callus initiation (Zouzou et al., 1997; Zouzou et al., 2000; Rao et-al., 2006; Sun et al., 2006; Jin et al., 2006; Kouadio et al., 2007; Kouakou et al., 2007). These genotypic differences with respect to callus initiation were also observed in many other plants (Lee et al., 2004; Wang et al., 2004; Burbulis et al., 2007). Thus, R405-2000 genotype was used to study the effect of four basal media.

One of the most important factors governing the callogenesis is the composition of the culture medium. The basic nutrient requirements of cultured plant cells are very similar to those of whole plants. Several media formulations are commonly used for the majority of all cell and tissue culture work. These media formulations include those described by Murashige and Skoog (1962), Gamborg et al. (1968), McCown (Llyod and McCown, 1980). Murashige and Skoog's medium (MS) and Gamborg's medium (B5) are all highly concentrated in macronutrients, while McCown's medium (MC) formulation contains less of macronutrients. There was significant difference between calli formed among media (see Table 1). MS medium shows the highest percentage and dry weight callus followed by B5 medium and MC medium which had the lowest. This basic formulation is suitable to obtain vigorous callus. This reactivity difference of MS medium seems to be in relation with the calcium and nitrogen concentrations. In effect, calcium and nitrogen level in MS medium was respectively eight times and four times more important than B5 medium (zouzou et al., 2000). The lowest result obtained

Table 4. Effect of glucose level on cotton callus culture [a]

Glucose level (%)	Explant forming callus (%)	Callus dry weight (mg)
1	37 ± 1,56 a	21.98 ± 0,73 a
2	51 ± 1,17 b	63.66 ± 1,66 b
3	95 ± 1,98 c	155.85 ± 3,82 c
4	97± 2,21c	175.40 ± 4,20 d
5	93 ± 2,10 c	133.82 ± 2,86 e
6	79 ± 1,88 d	102.13 ± 2,93 f

[a] Values for each parameter followed by a different letter within each column are significantly different, Newman-Keuls test (P< 0.05). Each value represents the mean of three replicates; ± SD (standard deviation).

from cotton callogenesis with MC medium can be due to the deficiency of phosphorus and nitrogen in the medium. It was reported that this specificity of each medium can influence callus initiation and growth (Zouzou et al., 2000; Kouakou, 2003). Inorganic nitrogen has a determining action on callogenesis (Trolinder and Goodin, 1988; Grimes and Hodges, 2000) and this probably explains the differences of callus dry weight MS and other media. Consequently MS medium was used for the following studies.

A range of hormone regimes were tested for callus initiation via hypocotyl, cotyledon and root segments. The results indicated that all treatments induced callus (Table 2). However, differences based on hormone regimes and nature of the explant were observed. The induction percentage of callus initiation and dry weight of callus formed were increased with 0.1 mg/l 2,4-D + 0.5 mg/l KIN (M3). Although 0.1 mg/l 2,4-D + 0.1 mg/l ZEA (M5) could induce explant to produce callus, the induced callus turned brown. Furthermore, low concentration of 2,4-D and high concentration of KIN stimulated the proliferation of cotton callus, but NAA and ZEA ones were disadvantageous. In previous reports, 2,4-D was an essential hormone for the induction of callogenesis in cotton and other plants (Davidonis and Hamilton, 1983; Zhang, 2000; Lee et al, 2004 ; Wang et al., 2004 ; Sun et al., 2006; Burbulis et al., 2007).The current investigation indicates that apparently only auxin (Trolinder and Goodin, 1987) or cytokinin (Zhang et al., 2001) is necessary to obtain callus. Whether this is true for certain Gossypium species, it should be noted that auxin and cytokinin combination is suitable to obtain more vigorous and friable callus.

In this study, we have found 0.1 mg/l 2,4-D and 0.5 mg/l KIN was proper combination of hormones to cotton callus induction. These results were in accordance with those of several authors which reported the efficiency of this hormone regime cotton callus induction (Trolinder and Goodin, 1988; Zouzou et al., 2000; Kouadio et al., 2007; Kouakou et al., 2007). The callus formed was also different amongst the explants, with hypocotyl callus giving the highest percentage and dry weight hypocotyl explants were most responsive to callus initiation. These results are in agreement with others results published by several authors who showed that hypocotyl is more callogenic compared to root and cotyledon (Peeters and Swennen, 1993; Zhang et al., 2001). Explant type, and probably its anatomical structure seems to play a significant role in cotton callus initiation. Variation in callus forming ability of different explant types has been reported in many others plants (Ishii et al., 2004; Zouine and El hadrami, 2004). Callogenesis specificity of explant type would be explained by their differential reactivity to media components (Zouzou et al., 1997; Kouakou, 2003; Ikram, 2005).

Sugar influence cells proliferation and differentiation according to Swankar et al. (1986); however, sugar don't have the same effects on callogenesis. Indeed, our study shows that the percentage of induction and dry weigh of callus are more significant with glucose followed by fructose and sucrose. Galactose and maltose showed lowest results (Table 3). These results are comparable with those showing that glucose product friable and voluminous callus compared to other sugars (Zouzou et al., 1997). The beneficial effect of glucose on callogenesis has also been mentioned in many plants (Kouadio et al., 2004; Ishii et al., 2004; Sun et al., 2006; Burbulis et al., 2007; Kouakou et al., 2007). Indeed, glucose is the assimilated form of sugars by plant cells and the most important

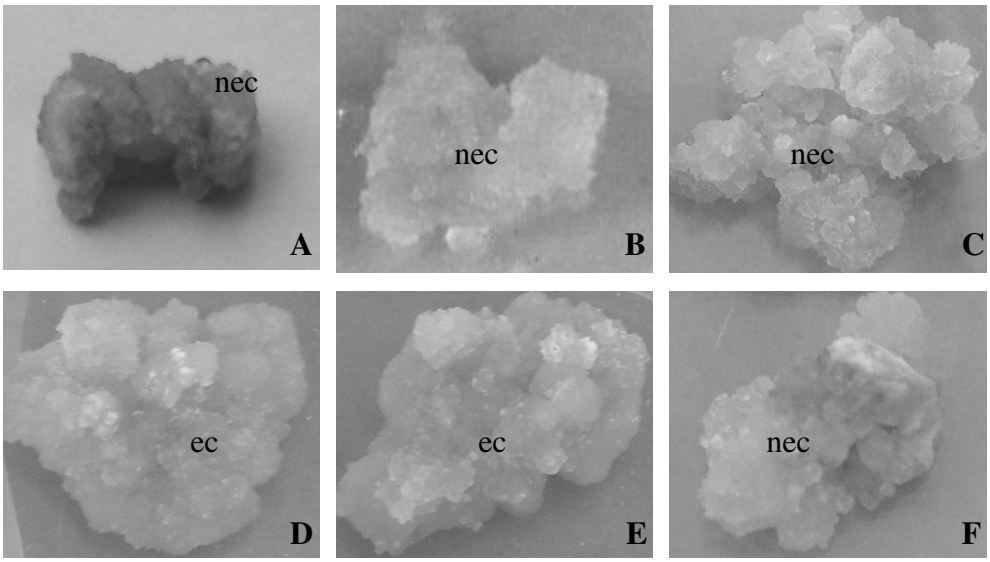

Fig 1. Cotton callus induction under different levels of glucose in MS medium [a] (MS, Murashige and Skoog medium) A, glucose 1 %; B, glucose 2 %; C, glucose 3 %; D, glucose 4 %; E, glucose 5 %; F, glucose 6 %; ec, embryogenic callus; nec, non embryogenic callus.

source of energy production (Richter, 1993). Fructose is also an assimilated sugar by plant cells but its reactivity seems to be less compared to glucose. Sucrose is an important biological reservoir for the two previous cited sugars. Sucrose is an analogous of glucose as regards to the physico-chemical properties but, with a low reactivity. An acid medium (pH 5.8), hydrolyses this sugar and breaks it into glucose and fructose which are assimilable by plant cells. They were probably a competition between these two sugars for their assimilation by the cells that makes sucrose less active and consequently less auspicious to cotton callogenesis. Our results showed that maltose and galactose have no beneficial effect on callogenesis, certainly because these two sugars are nonassimilable forms by plant cells. We found that glucose medium inhibited browning in agreement with works of some researchers (Standstedt, 1975). With glucose less browning and better callus proliferation occurred. Consequently, glucose was sugar which induced the better response to cotton callogenesis. We are currently expanding our studies to include the examination of glucose content on callus initiation. Primary results indicate that there is tremendous variation in callus initiation under different concentrations from glucose (Table 4). The percentage of callus induction was high and nearly identical with 3, 4 and 5% of glucose. However, callus dry weight analysis revealed that 4% of glucose produced highest callus dry weight followed by 3% of glucose and 5% of glucose. This glucose level seems to provide an adequate osmotic pressure that would permit absorption of mineral nutrients presents in medium which according to several authors are essential to cells growth (Buffard-Morel, 1968; Rabéchault et al., 1974). Several types of callus were distinguishable based on the physical appearance under different levels of glucose (Fig.1). Calli on medium containing 1 and 2% of glucose were green and compact, whereas those coming from 3 and 6% of glucose were green yellow, friable and browned. In contrast, calli from medium supplemented with 4 or 5% of glucose were green grayish, friable and no necrosis. These morphologic observations are characteristic of embryogenic structure induction according several authors (Nomura and Koumamime, 1995; Kouakou, 2003; Thiruvengadam et al., 2006).

Conclusion

Conditions for initiation of callus in cotton (Gossypium hirsutum L.) were optimized in this study. Callogenesis showed range of responses depending on medium formulation, growth regulators combinations and concentrations, sugar

type and level. It has resulted from our study that MS medium containing 0.1 mg/l 2,4-D, 0.5 mg/l KIN and glucose at 4 % was optimal to induce cotton callus. The induction of callus from hypocotyl was better than that from cotyledon and root explants. Callogenesis was genotype dependent in cotton.

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