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Full Length Research Paper

Effect of nitrates on embryo induction efficiency in cotton (*Gossypium hirsutum* L.)

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Cotton (*Gossypium hirsutum* L.) cv Coker-312 callus culture was assessed in terms of its usefulness as a system for investigating the effect of nitrates from different chemical compounds of nitrogen on embryo induction percentage in calli as the plant growth and cell differentiation mainly based on nitrogen. Both sources and amount of nitrogen in *in vitro* medium have significant effects mainly on cell growth, embryogenesis and the production of anthocyanin. Anthocyanin production is the best indication of inhibition of cell growth in *in vitro* culture of cotton. Embryo induction rate was high when NH_4NO_3 was eliminated from the medium but in the presence of KNO_3. The dicotyledenary embryos were developed with in 5 weeks, these embryos developed into normal plantlets immediately when they were cultured on a simple MS basal medium supplemented with 3% glucose.

Key words: *Gossypium hirsutum* L, callus induction, somatic embryogenesis, nitrogen sources, plant regeneration.

INTRODUCTION

Efficient *in vitro* techniques for regeneration of large numbers of plantlets from cotton are limited when compared to other major commercial crops. Price and Smith (1979) were the first to report somatic embryogenesis in the cotton, *Gossypium koltzchianum*, although complete plants could not be regenerated. Davidonis and Hamilton (1983) first described plant regeneration from two year old callus of *Gossypium hirsutum* L. cv Coker 310 via somatic embryogenesis. Since then, significant progress has been reported in cotton tissue culture (Zhang and Feng, 1992; Zhang, 1994b). In vitro cultured cotton cells have been induced to undergo somatic embryogenesis in numerous laboratories using varied strategies (Shoemaker et al., 1986; Chen et al., 1987; Trolinder and Goodin, 1987; Zhang and Wang, 1989; Voo et al., 1991; Zhang, 1994a; Zhang et al., 1996; Zhang and Konzak, 1999).

Regenerated plants have been obtained from explants such as hypocotyl, cotyledon, root (Zhang, 1994a; Zhang, 2000), anther (Zhang et al., 1996), and from various cotton species (Zhang, 1994b). Somatic embryogenesis and plant regeneration systems have been established from cotton tissue, protoplasts and ovules (Zhang and Li, 1992; Feng and Zhang, 1994; Zhang, 1995). Regeneration procedures have been used to obtain genetically modified plants after *Agrobacterium*-mediated transformation of hypocotyls (Umbeck et al., 1987; Leelavathi et al., 2004) and cotyledons (Firoozabady et al., 1987) or by transformation of particle bombardment (Finer and McMullen, 1990; Rajasekaran et al., 2000).

Although efficient plantlets regeneration from embryogenic calli through somatic embryogenesis has been improved significantly in recent years, some difficulties still remain. Rajasekaran et al. (1996) obtained regenerative plantlets via somatic embryogenesis from

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; ZT, Zeatin; KT, Kinetin; GA3, Gibberillic acid; MS, Murashige and Skoog's basal medium (1962).

cotton T25, GSA 78 and Acala, while Kumar and Pental (1998), GonzalezBenito et al. (1997) and Zhang et al. (1999) produced plantlets from MCU-5 and CRI 12, respectively. Most of these cultivars, except MCU-5 and CRI 12, are obsolete. Moreover, the frequency of regeneration was low in these varieties.

Nitrogen has a key role in plant growth and development because it has direct effects on rate of cell growth, differentiation and totipotency (Kirbey et al., 1987). Nitrates are good sources of nitrogen supply to plants (Shanjani, 2003; Gould et al., 1991) because it is readily taken up and metabolized by the cells and affects on a number of developmental processes leading to root branching, seed breaking, bud dormancy and apical domonance. The reduction of nitrogen supply, often initiates sexual development (Trewavas, 1983).

In this article, the effects of NH_4NO_3 and KNO_3 on callus proliferation, somatic embryogenesis and plantlets development was studied in cotton (*G. hirsutum* L.) cv cocker-312. Our findings may be helpful in the study of cotton tissue culture and will be of great value in future research studies.

MATERIALS AND METHODS

Mature seeds of cotton (*G. hirsutum* L.) cv Coker-312 were chosen and surface sterilized with 30% commercial bleach [5.25% (v/v) NaOCl] for 30 min and then washed three times in sterile distilled water. These surface sterilized seeds were sown on MS (Murashige and Skoog, 1962) basal medium supplemented with vitamins B_5 (Gamberg et al. 1968) vitamins], 3% glucose and 3.60 g/L phytagel. For germination, culture was placed under dark conditions at 28±2ºC for 72 h. After radical emergence from the seeds they were shifted to photoperiodic conditions (approximately 2,000 lx).

Hypocotyl (3-5 mm) sections were excised from 6-8 days old sterile seedlings that were used for callus induction by culturing on MS medium supplemented different combinations of auxin (2, 4-D) and cytokinin (KT) and 3% glucose for 11 weeks. The embryogenic callus proliferation was observed through application of KNO_3 and NH_4NO_3 (separately and in a combination of both) in callus induction (MS_2) medium.

Embryogenic callus cell lines with high frequency of proliferation were chosen and transferred onto embryo induction medium, such that somatic embryogenesis was induced through the application of hormones (ZT and activated charcoal separately) and also with the changes in microenvironment by various means such as metabolic stress. First they were cultured on MS medium [½ strength (Kumria et al., 2003)], then the KNO_3 and NH_4NO_3 were added (separately and in combination). The calli were cultured as 7 replicates (100±10) per plate for each media.

All cultures were maintained at 28 ± 2℃ under a light intensity of approximately 2000 lx provided by growth rooms with 16 h photoperiod. All media were supplemented with 30 g/L glucose and were solidified with 3.6 g/L phytagel. The pH of all the media was adjusted to 5.7 to 5.8 before autoclaving at 121℃ for 15 min.

After 5 weeks of culture on the embryo induction medium, the anthocyanin contents were measured. Pigments were extracted by preserving samples overnight in a solution containing 1 g/L HCL-methanol at 4℃. the supernatant was saparated by centrifugation at 100 X g for 5 min and its optical density was measured at 530 mm with spectophotometer (Hitachi U-2000, Japan). Anthocyanin contents were calculated with the extinction coefficient ($E^{11\%}_{1cm}$= 566 at 530 mm) of cyanide-3-glucoside in the same solvent. The results were expressed as µg/g fresh weight of calli.

RESULTS AND DISCUSSION

There is need at this time to develop a protocol to attain an efficient callus induction, its proliferation and plant regeneration system for cotton, as there is increasing percentage of transgenic cotton being grown worldwide. Different hormonal combinations of auxin (2,4-D) and cytokinin (KT) at varying concentrations have been previously tested in basal MS medium (Trolinder and Goodin, 1987, Kumria et al 2003, Rajasekaran et al 2000, Leelavathi et al., 2004). According to these reports, 2,4-D was an essential hormone for embryogenesis in cotton and other plants (McKersie and Brown, 1996, GonzalezBenito et al., 1997, Guis et al., 1998, Choi et al., 1999, Zhang, 2000). In this study, we have induced embryogenic callus by using auxin (2,4-D) and cytokinin (KT) combinations to develop maximum calli within a short period. According to ours and others' results (Davidonis and Hamilton, 1983, Trolinder and Goodin, 1986, Chen et al., 1987, Zhang and Li, 1992, Kumar and Pental, 1998), the addition of 2,4-D could promote the induction and proliferation of cotton callus, as it has a negative effect on differentiation and germination of somatic embryos. A bulk of embryogenic callus was obtained within 11 weeks of culture (Table 1) from MS_{2e} and MS_{2f} (Figure 1a).

Seven replicates for each medium were scored for callus induction efficiency (%). The proliferation of the callus is higher with the application of KNO_3. A reduction in the callus peroliferation was observed when NH_4NO_3 was added to the medium. Cell growth was averted and anthocyanin production (red pigmentation) was also noted in the callus induction medium (Figure 1b).

The cultures of many woody species with full strength MS salts have shown inhibitory effect by the medium on organized cell growth, a toxicity that can be reduced by lowering the amount of ammonium or total nitrogen (Bonga and Von Aderkas, 1992). Complete elimination of KNO_3 reduces percentage of callus production (Kirby *et al.,* 1987). Therefore with the addition of KNO_3 in MS_2 medium, callus proliferation was increased but this was inhibited with the addition of NH_4NO_3. Well proliferating embryogenic callus from the callus induction medium (MS_{2e} and MS_{2f}) was cultured on embryo induction medium for somatic embryogenesis (Table 2).

After 4 weeks, the embryogenic callus produced somatic embryos at their different developmental stages on MS_{3a} and MS_{3b} (0.1 mg/L ZT and 2-g/L activated charcoal, respectively) (Kolganova et al. 1992; Zheng and Konzak 1999). Such a capability has been observed also on MS medium with 1.90 g/L KNO_3 (Finer 1988, Gawel and Robacker 1990, Kumar and Pental 1998, Kumria et al., 2003).

Table 1. Callus induction and proliferation in cotton (*Gossypium hirsutum L.*) cv Coker-312 from hypocotyl explant at various hormonal concentrations.

Medium	Hormones (mg/L)		Callus induction and its proliferation, 11 weeks culture					
	2,4-D	KT	No. of explants	No. of calli induced	Callus induction (%)	*Callus (mg)	Callus Wt (g)	Callus growth ratio (%)
MS_{2a}	0.1	0.1	30	28	93.33	99.00	3.10	31.31
MS_{2b}	0.1	0.2	30	28	93.33	99.00	3.10	31.31
MS_{2c}	0.1	0.3	28	27	96.71	92.42	6.18	66.68
MS_{2d}	0.1	0.4	30	16	53.33	96.90	3.65	37.67
MS_{2e}	0.1	0.5	35	35	100.00	98.70	5.80	58.76
MS_{2f}	MS_{2e} + KNO_3 (1.90 g/L)		32	29	90.62	100.30	7.26	72.35
MS_{2g}	MS_{2e} + NH_4NO_3 (1.90 g/L)		27	25	92.59	99.73	2.88	28.91

Calli were cultured as 7 replicates (100±10) per medium, *Callus WT (mg): original weight of calli in milligrams; Callus Wt (g): weight of the callus in grams after 11 weeks; Growth ratio% = (original weight of callus)/(final weight of callus after 11 weeks of culture) x 100.

Table 2. The effect of various embryo induction media on somatic embryo induction in cotton (*Gossypium hirsutum* L.) cv Cocker-312 callus (after 28 day of culture).

Medium	Treatments	Number of embryo types after 4 weeks culture		Embryo maturation (%)	Anthocyanin (µg/g)
		Globular	*Cotyledenary*		
MS_0		100.0	4.62	4.62	104.04±3.49
MS_{3a}	MSo+0.5mg/L ZT	78.	0.82	1.05	/
MS_{3b}	MSo+2.0g/L AC	74.0	3.05	4.12	/
MS_{3c}	MSo+KNO_3 (1.90 g/L)	70.2	19.30	27.49	350.54±4.51
MS_{3d}	1/2 MSo+KNO_3(1.90 g/L)	79.1	8.03	10.15	302.83±7.35
MS_{3e}	1/2 MSo+KNO_3 (0.95 g/L)	88.5	18.77	21.22	252.25±5.34
MS_{3f}	1/2 MSo+1/2KNO_3 + 1/2NH_4NO_3	44.1	3.93	8.92	420.41±3.65
MS_{3g}	1/2 MSo+ NH_4NO_3 (0.95 g/L)	20.1	0.25	1.28	540.93±4.58
MS_{3h}	1/2 MSo+ NH_4NO_3 (1.90 g/L)	34.2	0.04	0.20	620.62±7.48
MS_{3i}	MSo+ NH_4NO_3 (1.90 g/L)	0.0	0.00	0.0	658.70±5.52

Embryo maturation (%)= cotyledenary embryos/globular embryos x 100

Davidonis and Hamilton (1983) reported decrease in nitrogen and sugar triggers embryo maturation. The dilution of the media concentration sustained the cell division and growth of the calli but led to direct differentiation. Furthermore, with the full strength MS medium, high embryo maturation was noted most probably because of higher nutrient requirement for the maturation of vigorously developing somatic embryos.

Both the nitrogen source and the ratio of NO_3^- /NH_4^+ play an important role in cell growth embryo induction and appearance/production of anthocyanin (Kim and Kim, 2002). With the addition of NH_4NO_3, the rate of the cell growth and embryo induction % in embryogenic callus considerably decreased but the anthocyanin production was increased (Shanjani et al., 2003). In this medium, we observed that cells were almost dying, while growth occurs well even in MS_0 medium. But when NH_4NO_3 (1.90 g/L) was reduced to a half concentration (0.95 g/L) then some increases in the cell growth rate and embryo induction was observed. However it resulted in a decrease in anthocyanin production, although higher than in MS_0 medium. When a combination of both KNO_3 and NH_4NO_3 were added to the embryo induction medium, there was a considerable increase in the rate of the cell

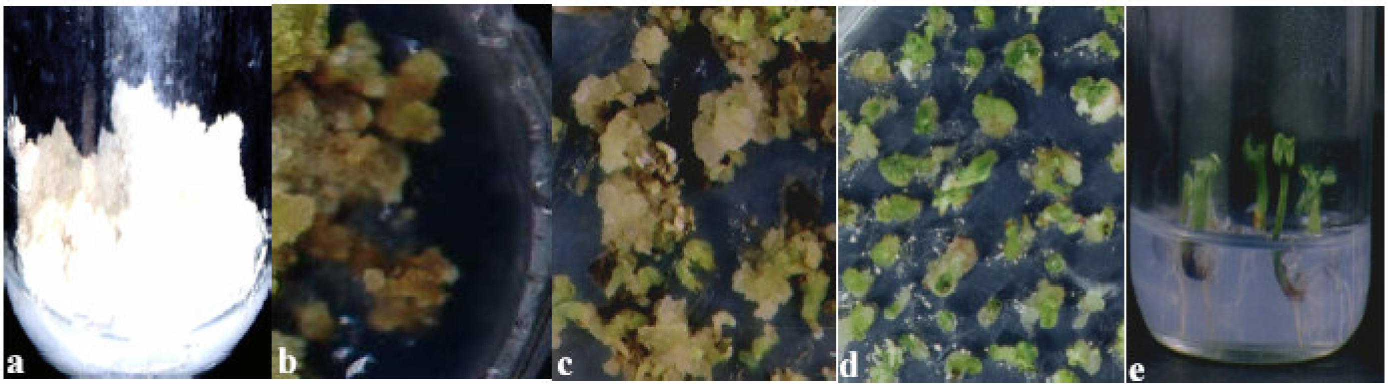

Figure 1. Different stages of development of plantlets from callus throughsomatic embryogenesis in cotton cv Cocker-312. (a) Callus proliferation on MS_{2f}. (b) Callus with anthocyanin production. (c) Embryogenesis in callus. (d) Embryo maturation. (e) Multicotyledonary embryos.

growth and somatic embryogenesis (Figure 1c), but decrease in anthcyanin production (Table 2). There is some indication that in addition to the importance of NO_3^- in the enhancement of the cell growth and somatic embryo induction %age, K^+ may also play a key role in these processes. When KNO_3 (1.90 g/L) was added to the embryo induction medium, both cell growth and embryo induction rate were high, but anthcyanin production was low. Meanwhile KNO_3 may be more helpful in somatic embryo maturation rather then somatic embryo induction (Figure 1d). According to Mishra et al. (2003), the accumulation of small amounts of anthocyanins in callus and embryo cultures was a good indicator of regeneration because the embryogenic callus under stress of the KNO_3 is converted to the somatic embryos with the development of anthocyanin. However, other reports indicate that anthocyanin production may be influenced by different factors such as UV, light, nitrogen source, type of sugar, osmotic stress, temperature, elicitor conditioning and phytohormone conditions (Zhang et al., 1998). According to Kim and Kim (2002) when either NH_3^+ or NO_3^- was lacking, cell growth decreased that leading to anthocyanin development. When NO_3^- was lacking, cell growth increased slightly and anthocyanin contents become relatively low. It was thought that NO_3^- affected cell growth while NH_4^+ affected anthocyanin production. Anthocyanin accumulation begins when there was no multiplication of cells, and when cell multiplication starts, anthocyanin accumulation diminishes.

Anthocyanin catabolism is closely related to cell membrane integrity. With cellular lysis, the anthocyanins stored in anthocyanoplasts or vacuoles are released into the culture medium and are quickly metabolized (Guardiola et al., 1995). Therefore, during embryoid development cell growth inhibition may be caused by the synthesis of anthocyanin. In most cases, the synthesis of secondary products is lost when the cells are differentiating and growing rapidly in culture (Ozeki and Komamine, 1981). In the case of cotton, anthocynin production in the callus is the best indicator of physiochemical stress. If the culture is not under stress, there is no detectable production of anthocyanin with developing embryoids. We observed that within 4 months, a large number of embryos have developed, and most were multicotyledenary (Figure 1e), which were devoid of the ability for the development of shoot. The dicotyledenary embryos have the ability to develop shoot, and these were shifted to rooting medium supplemented with GA_3 (0.05mg/L). The rooted plants with well developed shoot were transferred to soil and then shifted to glass house containment after plant hardening.

Somatic embryogensis is the important step in cotton plant regeneration. With the addition of extra NH_4NO_3 in the embryo induction medium the activity of glutamine synthase enzyme is inhibited, causing a large build up of ammonia in plant cell (Tachibana 1986). This leads to the depletion of crucial amino acids and enzymes including those involved in the photosynthetic process. This inhibition causes a decrease in cell growth, embryogensis and production of anthocynin. The addition of KNO_3 and removal of NH_4NO_3 showed adverse results. Due to the addition of the NH_4NO_3, the developing embryos showed abnormalities in their development and morphology leading to the death of globular embryos, development of multicotylednary embryos and stunted stem/hypocotyl, with and/or without rooting, embryos are bleached at the later stages of the development. If dicotyledenary embryos develops with a small hypocotyl, they produce a bulk of roots, when transferred to the rooting medium.

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