SOMATIC EMBRYOGENESIS OF AN EARLY COTTON CULTIVAR[1]

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ABSTRACT - Somatic embryogenesis in cotton has been previously reported in a limited number of genotypes, predominantly Coker cultivars. Before tissue culture techniques are widely applied to cotton improvement programs, regeneration must be possible for a broad range of genotypes. The aim of the present work was to develop a method to obtain somatic embryoids of an early cotton cultivar (*Gossypium hirsutum* L. race *latifolium* Hutch cv. CNPA Precoce 2) and their subsequent development. Callus induction was attempted with cotyledon and hypocotyl explants. These explants were cultured on MS medium supplemented with five concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and N^6-(2-isopentenyl)-adenine (2iP) either alone or in combination. Based on callus appearance (light brown and granular), four different growth regulator combinations were selected for further callus development. Callus was subcultured on 2.45 µM 2iP and subsequently transferred to 0.45 and 22.50 µM 2,4-D. Somatic embryos of different sizes and shapes subsequently appeared on MS medium supplemented with 2 g L^{-1} glutamine and no growth regulators. Plantlets were developed from those embryoids. Plant regeneration through somatic embryogenesis is achieved for the first time in the early cultivar CNPA Precoce 2.

Index terms: callus, somatic embryos, plant regeneration.

EMBRIOGÊNESE SOMÁTICA NA CULTIVAR PRECOCE DO ALGODÃO

RESUMO - A embriogênese somática no algodão tem sido observada num número limitado de genótipos, predominantemente nas cultivares Coker. Como as técnicas de cultura de tecido são freqüentemente utilizadas nos programas de melhoramento do algodão, a regeneração das plântulas deve ser possível num grande número de genótipos. A finalidade do presente trabalho foi desenvolver um método para obtenção de embriões somáticos na cultivar precoce de algodão (*Gossypium hirsutum* L. raça *latifolium* Hutch cv. CNPA Precoce 2) e seu subseqüente desenvolvimento. Estudou-se a indução de calo nos explantes de cotilédone e de hipocótilo. Estes explantes foram cultivados em meio MS suplementado com cinco concentracões de ácido 2,4-diclorofenoxiacético (2,4-D) e N^6-Δ^2-isopentenil adenina (2iP) isolado ou em combinação. Com base na aparência dos calos (bege e granulado), foram selecionadas quatro diferentes combinações dos reguladores de crescimento para o melhor desenvolvimento dos calos. Os calos foram induzidos em 2,45 µM de 2iP, e, posteriormente transferidos para 0,45 e 22,50 µM 2,4-D. Embriões somáticos de diferentes formas e tamanhos aparecem subseqüentemente em meio MS desprovido de reguladores de crescimento e com 2 g L^{-1} glutamina. Várias plântulas se desenvolveram a partir destes embriões somáticos. A regeneração da planta por meio da embriogênese somática foi conseguida, pela primeira vez, na cultivar CNPA Precoce 2.

Termos para indexação: calos, embriões somáticos, regeneração da planta.

INTRODUCTION

Somatic embryogenesis in cotton (*Gossypium hirsutum* L.) has been previously reported (Davidonis & Hamilton, 1983; Gawel et al., 1986; Trolinder & Goodin, 1987, 1988a, 1988b; Finer, 1988; Voo et al., 1991), but only in a limited number

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of genotypes, predominantly Coker cultivars. Before tissue culture techniques are widely applied to cotton improvement programs, regeneration must be possible for a broad range of genotypes. Embryogenic and non-embryogenic plants have been crossed, showing that embryogenesis is a heritable trait, but further research must be carried out to understand the genetic model (Gawel & Robacker, 1990a).

Early cotton cultivars are of great interest due to their avoidance of plagues such as boll weevil (*Anthonomus grandis* Boh.), which attacks cotton flower buds and capsules, specially in North and South America. The development of tissue culture techniques for those cultivars would be of great importance for cotton breeding programs.

The objective of the present work was to establish a method to obtain somatic embryos of an early cotton cultivar, CNPA Precoce 2, and their subsequent growth into plants.

MATERIAL AND METHODS

Explant source

Cotton (*Gossypium hirsutum* L. race *latifolium* Hutch) seeds of cultivar CNPA Precoce 2 were obtained from the "Centro Nacional de Pesquisa de Algodão-Embrapa", Campina Grande, Brazil. Acid delinted seeds were rinsed in running water 15 min, soaked 15 min in 1% (w/v) NaOCl with one drop of "Tween 20" per 100 mL, and rinsed again three times in sterile distilled water. Disinfested seeds were transferred to test tubes containing 15 mL MS medium (Murashige & Skoog, 1962) solidified with 7 g L^{-1} Difco Bacto-agar; pH was adjusted to 5.7-5.8 before autoclaving. Test tubes were incubated at 25°C with a 16 h light/8 h darkness photoperiod (50 µmol m^{-2} s^{-1}).

Callus induction

Four days after germination, cotyledon (3x3 mm) or hypocotyl (4 mm long) explants were excised and each one cultured in a Petri dish (6 cm diameter). Each dish contained 10 mL semi-solid MS medium with 30 g L^{-1} glucose, and 1.6 g L^{-1} gelrite (Sigma Phytagel™) as the gelling agent, adding 0.75 g L^{-1} $MgCl_2$ to the medium. This gelling agent was used in all subsequent media. Basal medium was supplemented with all possible combinations of 0.00, 0.45, 2.25, 4.50, 22.50 and 45.00 µM 2,4-dichlorophenoxyacetic acid (2,4-D) with 0.00, 0.49, 2.45, 4.90, 24.50 and 49.00 µM N^6-(2-isopentenyl)-adenine (2iP). The pH was adjusted to 5.7-5.8 before autoclaving. Ten replicates were used for each treatment and type of explant. Cultures were incubated at 30°C with a 16 h light/8 h darkness photoperiod (50 µmol m^{-2} s^{-1}). These conditions were mantained in further steps.

Callus proliferation

Four weeks after initiation, callus induction was evaluated. Calli which did not look necrotic or dark brown were transferred (removing from the original explant) to media which consisted of MS salts, B_5 vitamins (Gamborg et al., 1968), 30 g L^{-1} sucrose instead of glucose, and supplemented with the same growth regulator combinations. The selected calli were transferred twice to the same medium at four-week intervals. Calli from four different growth regulator combinations were selected because of their light brown colour and partially friable appearance to attempt embryogenesis. This type of callus has been reported in the literature to be precursor of embryogenic cultures in some cotton cultivars (Gawel et al., 1986; Schoemaker et al., 1986; Finer, 1988). Portions of these calli were cultured onto MS medium with the same salts and vitamins as in the previous step, but with 20 g L^{-1} sucrose and 0.45 µM 2,4-D. Four weeks later calli were transfered to higher 2,4-D concentration (22.5 µM).

Embryogenesis induction

After four more weeks, calli (1 g on each Petri dish) were subcultured onto basal medium (MS salts, B_5 vitamins and 20 g L^{-1} sucrose) alone or supplemented with 2 g L^{-1} glutamine, or with 2 g L^{-1} glutamine + + 1.9 g L^{-1} KNO_3.

Embryoid conversion

Four weeks later embryoids were observed on some cultures and they were transferred to media consisting of half strength MS salts and B_5 vitamins supplemented with: 1) 20 g L^{-1} sucrose + 0.2 mg L^{-1} GA_3; 2) 10 g L^{-1} sucrose + + 0.1 mg L^{-1} GA_3; or 3) 10 g L^{-1} sucrose + 3.8 g L^{-1} KNO_3 (1/2 MS without NH_4NO_3).

RESULTS AND DISCUSSION

Different basal media and growth regulator concentrations have been used for callus induction in *Gossypium hirsutum*. The basal medium used in

this work has been employed in several studies (Trolinder & Goodin, 1987; Finer, 1988; Trolinder & Xhixian, 1989; Voo et al., 1991). The growth regulators were chosen after preliminary tests for the cultivar CNPA Precoce 2. As 2,4-D and, especially, 2iP concentrations increased, callus colour became darker and more necrotic portions were observed.

Only 16 of the 36 tested media were subsequently studied. The rest of the treatments were discarded due to the dark brown colour of calli. Again, only calli with the best appearance (granular, partially friable, light brown) were subsequently subcultured. This type of callus was obtained on 2.45 µM 2iP; 0.45 µM 2,4-D + 0.49 µM 2iP; 0.45 µM 2,4-D + + 2.45 µM 2iP; and 2.25 µM 2,4-D + 0.49 µM 2iP.

After a subculture period (four weeks) on 0.45 µM 2,4-D and then on 22.5 µM 2,4-D, calli were transferred to the three different embryo induction media. Embryos were observed only on calli of hypocotyl origin, which had been induced on 2.45 µM 2iP (Fig. 1A). Glutamine induced somatic embryogenesis in CNPA Precoce 2, as 72 embryoids of different appearance and size were excised from 60% of the callus cultures grown on medium containing this amino acid (Fig. 1B). Glutamine had shown to improve embryoid development (Finer, 1988), although later studies have indicated that the number of recoverable embryoids was reduced (Voo et al., 1991). Moreover, our study showed that the addition of 1.9 g L^{-1} KNO_3 decreased the embryogenic response: only three embryoids were observed on 20% of the calli. However this salt appeared to have the opposite effect on embryo conversion. Of the three media studied, best results were obtained on medium 1/2 MS (without NH_4NO_3) + B_5 vitamins + 10 g L^{-1} sucrose + 3.8 g L^{-1} KNO_3. On that medium, 25% of the embryoids developed into small plantlets (Fig. 1C), while the addition of GA_3 without KNO_3 decreased the number of embryoids to 13%. Similarly, Trolinder & Goodin (1988b) observed higher percentages of embryoid development when medium with no growth regulators and higher KNO_3 concentration was employed. However, in the present study, some of the recovered plantlets showed an abnormal morphology.

FIG. 1. A) Embryogenic callus of *Gossypium hirsutum* cultivar CNPA Precoce 2. Callus was induced on 2.45 µM 2iP, transferred three times to the same medium and subsequently to 0.45 µM 2,4-D, to 22.5 µM 2,4-D and to a medium with 2 g L^{-1} glutamine and without growth regulators; B) Somatic embryoids excised from callus of Fig. 1A (bar= 1 mm); C) Plantlet developed from somatic embryos, eight weeks after culture on 1/2 MS salts + B_5 vitamins + + 10 g L^{-1} sucrose + 3.8 g L^{-1} KNO_3.

Several studies have shown a great interaction between genotype and callus induction medium in the embryogenic response of cultures (Gawel et al., 1986; Trolinder & Xhixian, 1989; Gawel & Robacker, 1990a, 1990b). Protocols for somatic embryogenesis induction tested on several cultivars are being developed in laboratory. Further research is needed especially in the embryoid conversion and plant development stages, as early germination of embryos may be an important limiting factor.

CONCLUSIONS

1. Somatic embryogenesis was obtained for the first time in the early cultivar CNPA Precoce 2.

2. A method for indirect somatic embryogenesis is established for the cotton cultivar CNPA Precoce 2: callus is induced from hypocotyl on medium supplemented with 2.45 µM 2iP, transferred at four weeks intervals to 0.45 µM 2,4-D and afterwards to 22.5 µM 2,4-D; embryoids develop when callus is cultured on medium lacking growth regulators but with 2 g L^{-1} glutamine.

3. Embryoids are germinated on medium 1/2 MS (without NH_4NO_3) + B_5 vitamins + 10 g L^{-1} sucrose + + 3.8 g L^{-1} KNO_3, and plantlets are obtained.

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REFERENCES

DAVIDONIS, G.H.; HAMILTON, R.H. Plant regeneration from callus tissue of *Gossypium hirsutum* L. **Plant Science Letters**, v.32, p.89-93, 1983.

FINER, J.J. Plant regeneration from somatic embryogenic suspension cultures of cotton (*Gossypium hirsutum* L.). **Plant Cell Reports**, v.7, p.399-402, 1988.

GAMBORG, O.; MILLER, R.; OJIMA, K. Nutrient requirements of suspension cultures of soybean root cells. **Experimental Cell Research**, v.50, p.151-158, 1968.

GAWEL, N.J.; RAO, A.P.; ROBACKER, C.D. Somatic embryogenesis from leaf and petiole callus cultures of *Gossypium hirsutum* L. **Plant Cell Reports**, v.5, p.457-459, 1986.

GAWEL, N.J.; ROBACKER, C.D. Genetic control of somatic embryogenesis in cotton petiole callus cultures. **Euphytica**, v.49, p.249-253, 1990a.

GAWEL, N.J.; ROBACKER, C.D. Somatic embryogenesis in two *Gossypium hirsutum* genotypes on semisolid versus liquid proliferation media. **Plant Cell, Tissue and Organ Culture**, v.23, p.201-204, 1990b.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. **Physiologia Plantarum**, v.15, p.473-497, 1962.

SCHOEMAKER, R.C.; COUCHE, L.J.; GALBRAITH, D.W. Characterization of somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). **Plant Cell Reports**, v.3, p.178-181, 1986.

TROLINDER, N.L.; GOODIN, J.R. Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). **Plant Cell Reports**, v.6, p.231-234, 1987.

TROLINDER, N.L.; GOODIN, J.R. Somatic embryogenesis in cotton (*Gossypium*). I. Effects of source of explant and hormone regime. **Plant Cell, Tissue and Organ Culture**, v.12, p.31-42, 1988a.

TROLINDER, N.L.; GOODIN, J.R. Somatic embryogenesis in cotton (*Gossypium*). II. Requirements for embryo development and plant regeneration. **Plant Cell, Tissue and Organ Culture**, v.12, p.43-53, 1988b.

TROLINDER, N.L.; XHIXIAN, C. Genotype specificity of the somatic embryogenesis response in cotton. **Plant Cell Reports**, v.8, p.133-136, 1989.

VOO, K.S.; RUGH, C.L.; KAMALAY, J.C. Indirect somatic embryogenesis and plant recovery from cotton (*Gossypium hirsutum* L.). **In Vitro Cellular Developmental Biology**, v.27, p.117-124, 1991.