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SOMATIC EMBRYO INITIATION AND GERMINATION IN DIPLOID COTTON (*GOSSYPIUM ARBOREUM* L.)

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Summary

The diploid cotton species can constitute a valuable gene pool for the more agronomically desirable cultivated tetraploid cultivars and offer better opportunities to study gene structure and function through gene knockouts. In order to exploit these advantages, a regeneration system is required to achieve these transformation-based goals. Carbohydrate source and concentration were evaluated to improve somatic embryo (SE) production and desiccation treatments to improve the conversion efficiency of SEs to plants in a diploid *Gossypium arboreum* accession, A_2-9 (PI-529712). Improved SE numbers and their subsequent conversion into plantlets was achieved with a Murashige and Skoog (MS)/sucrose-based medium M_2 [0.04 *M* sucrose, 0.3 μ*M* α-naphthaleneacetic acid (NAA)]. On this medium, 219 embryos per g initiated, and close to 11% of these embryos germinated into plantlets. Neither a 5-d desiccation treatment of embryogenic callus previously cultured in liquid medium nor filter paper insertion improved the numbers of SEs induced or their conversion to plantlets. A 3-d desiccation period resulted in improved plant regeneration. When immature *G. arboreum* SEs induced on M_1 (0.2 *M* glucose, 2.6 μ*M* NAA, and 0.2 μ*M* kinetin) medium underwent a 3-d desiccation treatment, 49% of these immature SEs were converted to plantlets after a 4-wk period on M_2 medium. These improved results will help to pave the way for future genetic transformation and associated gene structure and function studies utilizing *G. arboreum*. These results, in particular the 3-d desiccation treatment, can also be incorporated into regeneration protocols to improve the regeneration efficiency of other *Gossypium* species.

Key words: somatic embryogenesis; desiccation; *Gossypium arboreum*; tissue culture.

Introduction

The genus *Gossypium* consists of about 50 species, only four of which (*G. hirsutum* L., *G. barbadense* L., *G. arboreum* L., and *G. herbaceum* L.) are domesticated and produce spinnable fiber. *G. hirsutum* and *G. barbadense* are tetraploid, 'AD genome' species, and the remaining two are diploid, 'A genome' species. *G. hirsutum* cultivars (Upland cotton) provide the bulk of the commercial cotton. The two diploid species are cultivated as rain-fed crops in India, the only country where all four cultivated species are grown (International Cotton Advisory Committee, 1994) and where about 25–30% of cotton produced consists of *G. arboreum* (Choudhary and Laroia, 2001). Despite their poor agronomic traits, the diploid species have other attractive attributes that can be useful for the cotton industry. *G. arboreum* accessions and land races could play a key role in cotton improvement because they have been reported to be resistant to several pests and diseases, ranging from bollworms (Dhawan et al., 1991) to fungal diseases (Wheeler et al., 1999), rust (Blank, 1971), and virus diseases (Dogra, 1998). In addition, diploid cottons offer a better opportunity than the tetraploids to elucidate gene structure and function through gene knockout studies.

Transfer of desirable genes from *G. arboreum* to either *G. barbadense* or *G. hirsutum* could be achieved through interspecific hybridization; however, this approach is time consuming and often results in sterile plants due to the triploid nature of the F_1 hybrids. On the other hand, the recalcitrance of the *Gossypium* species, in particular *G. arboreum*, to tissue culture constitutes a major hindrance to the transfer of desirable characteristics to the cultivated tetraploid species through genetic engineering. In addition, the majority of somatic embryos (SEs) derived from tissue culture typically fail to mature and/or develop into normal plants. This problem is even more evident in cotton, especially diploid cottons, that require lengthy culturing times to generate plants, a process that is often conducive to the production of undesirable somaclones. Therefore, increasing the number of SEs that produce plants is an important consideration in developing a tissue culture protocol for transformation.

Generally, it is necessary to transfer embryogenic cultures to a medium with reduced hormone concentration and nutrient content, or with no hormone, to initiate and/or mature SEs. Other methods used for these purposes include exertion of stressful conditions, such as heat, cold, and temporary starvation. Ventilation of cultures

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has been shown to enhance SE initiation and germination in carrot (Lee et al., 1997). Subjecting the cultured tissues to periods of stress such as desiccation (Parrott et al., 1988; Yehosua et al., 1992; Kazuko and Kazuko, 1994; Liu et al., 1994; Pomeroy et al., 1994; Timbert et al., 1996; Bomal and Tremblay, 1999), cold (Rajasekaran and Mullins, 1979; Ritala et al., 2001), and heat (Zimmerman et al., 1989) appears to improve the conversion of SEs into mature embryos and subsequently to plants. It has been reported that high light conditions led to a reduction in callus proliferation and subsequently to tissue death, whereas low light conditions were more conducive to callus growth and even to callus differentiation to plantlet stage in *G. arboreum* (Smith et al., 1977). In addition, the role of carbohydrate sources on embryogenesis and embryo maturation has been investigated in several plant species as well (Ladyman and Girard, 1992; Teng, 1997; Blanc et al., 1999; Paek and Hahn, 2000).

Recently, we reported somatic embryogenesis and plant regeneration in a *G. arboreum* accession, namely A_2-9 (PI-529712) (Sakhanokho et al., 2001a). However, SE production and maturation, as well as plantlet acclimatization, were sporadic, but our recently published results suggested that somatic embryogenesis could be improved through manipulation of carbon and nitrogen sources (Sakhanokho et al., 2001a). We decided to further investigate the effects of medium type, desiccation, and separation of callus and agar medium by filter paper on SE initiation and germination.

Materials and Methods

Seeds of the *G. arboreum* accession A_2-9 (PI-529712) were obtained from Dr. A. E. Percival, Curator of the National Cotton Germplasm Collection, USDA/ARS, College Station, Texas. Explants used for callus initiation consisted of 5-mm hypocotyl sections, which were cut longitudinally before transfer face-down onto callus initiation medium. The tissue culture method followed our previously published protocol (Sakhanokho et al., 2001b). Two sets of experiments were conducted with regard to initiation and conversion of SEs to plantlets.

Experiment 1. In an attempt to improve both initiation and germination of SEs, potential embryogenic calluses (Sakhanokho et al., 2001b), derived from hypocotyl sections, were transferred into liquid somatic embryo initiation medium (EIML) and shaken at 130 rpm under a 16 h/8 h light/dark cycle at 28°C for 4 wk. EIML medium is similar to $EMMS_2$ described below except, unlike $EMMS_2$, it is devoid of hormones and gelling agent. The cultures were checked on a regular basis to monitor development of somatic embryos.

After the liquid step, embryogenic calluses (0.5–3 g) were transferred onto three different media for SE germination or maturation. These media were $EMMS_2$, S15g.05NAA, and EGM_2. The first two media were described earlier (Sakhanokho et al., 2001b). Briefly, $EMMS_2$ medium consisted of MS (Murashige and Skoog, 1962) salts in which NH_4NO_3 was removed and the amount of KNO_3 was doubled and supplemented with 0.03 m*M* thiamine, 0.6 m*M* *myo*-inositol, 8.1 μ*M* nicotinic acid, 4.9 μ*M* pyridoxine, 6.8 m*M* glutamine, 3.3 m*M* asparagine, and 0.17 *M* glucose. The medium S15g.05NAA consisted of MS salts supplemented with 0.3 μ*M* NAA and sucrose (0.04 *M*) instead of glucose as the carbon source. EGM_2, developed during this study, was made of MS salts, 0.5 μ*M* indole-3-acetic acid (IAA), 0.01 *M* sucrose, 3.7 m*M* $MgCl_2$, and 2 g l^{-1} Gelrite®. The pH was adjusted to 5.8 using 1 *N* NaOH before the addition of the gelling agent Gelrite® and autoclaving at 121°C for 20 min. For the sake of simplicity, $EMMS_2$, S15g.05NAA, and EGM_2 will be referred to as M_1, M_2, and M_3 medium from here on, respectively. In addition to media, two additional treatments were investigated. The treatments consisted of: (1) direct contact of embryogenic callus tissue and media; (2) insertion of Whatman filter paper no. 2 between embryogenic callus tissue and media (F); and (3) 5-d temporary starvation or desiccation of callus tissue (D) before transfer to media. The resulting media treatments were M_1, M_1F, M_1D, M_2, M_2F, M_2D, M_3, M_3F, and M_3D (Table 1). Data on embryo production and maturation were collected after a 4-wk period.

Experiment 2. The purpose of this experiment was to optimize the germination rate of already-initiated immature SEs into plantlets. Following the liquid culture step, embryogenic calluses on M_1 were allowed to develop (after about 30 + d) into immature globular SEs (Fig. 1) lacking well-defined cotyledons, as defined by Firoozabady and DeBoer (1993), and which were then subjected to a 3-d temporary starvation period in empty 12-well cell culture plates before transfer onto fresh M_1, M_1F, M_2, M_2F, M_3, and M_3F media treatments. About 30 immature SEs were transferred onto each plate.

TABLE 1

VARIOUS TREATMENTS USED TO TEST EMBRYO INITIATION AS WELL AS EMBRYO MATURATION IN *GOSSYPIUM ARBOREUM*

		Carbon source		Plant growth regulator		
Treatment	Description	Sucrose (*M*)	Glucose (*M*)	IAA (μ*M*)	NAA (μ*M*)	Kinetin (μ*M*)
M_1	Embryogenic callus from liquid culture directly transferred onto M_1 medium		0.2		2.6	0.2
M_1F	Direct transfer of embryogenic callus onto M_1 plates but with Whatman filter paper between callus and medium		0.2		2.6	0.2
M_1D	Direct transfer of embryogenic callus onto M_1 plates after the callus underwent a 5-d desiccation period		0.2		2.6	0.2
M_2	Embryogenic callus from liquid culture directly transferred onto M_2 medium	0.04			0.3	
M_2F	Direct transfer of embryogenic callus onto M_2 plates but with Whatman filter paper between callus and medium	0.04			0.3	
M_2D	Direct transfer of embryogenic callus onto M_2 plates after the callus underwent a 5-d desiccation period	0.04			0.3	
M_3	Embryogenic callus from liquid culture directly transferred onto M_3 medium	0.01		0.2		
M_3F	Direct transfer of embryogenic callus onto M_3 plates but with Whatman filter paper between the callus and the medium	0.01		0.2		
M_3D	Direct transfer of embryogenic callus onto M_3 plates after the callus underwent a 5-d desiccation period	0.01		0.2		

FIG. 1. *Top left*, Embryogenic callus of *Gossypium arboreum* accession A_2-9 (PI-529712) showing immature SEs with no developed cotyledons. These SEs underwent a 3-d desiccation period before they were transferred onto the various media treatments. *Top right*, Germinated SEs of *G. arboreum*, accession A_2-9 (PI-529712) with developed cotyledons. *Bottom*, Regenerated plants of A_2-9 (PI-529712) undergoing further development in a Magenta jar before transfer to the greenhouse.

Embryo germination frequency was evaluated at the end of a 4-wk period, and only 'mature' SEs with well-defined cotyledons with roots were considered (Fig. 1).

The experimental design for both sets of experiments was a randomized complete design consisting of one cotton accession (PI-529712), three replications, the media treatments, and 20 plates as samples nested within each experimental unit. The SAS software (SAS Institute, Inc., 1999) was used to analyze statistical significance of the treatment means.

Results and Discussion

Embryo initiation and maturation as affected by media treatments. Embryo initiation was higher on sucrose medium (M_2) as the carbohydrate source than on any other treatment (Table 2), a result that supported previous findings (Smith et al., 1977; Shoemaker et al., 1986; Sakhanokho et al., 2001a). To trigger SE initiation, it is often necessary to reduce or eliminate the hormone concentration and the nutrient content of the media supplied to the callus tissues. Exertion of stress (e.g., reduction or elimination of the growth hormones, carbohydrate sources or exposure to heat, cold, or reduced humidity) on embryogenic callus tends to improve the germination and conversion of these SEs into plantlets (e.g., Rajasekaran and Mullins, 1979; Zimmerman et al., 1989; Lee et al., 1997).

In light of the improved results we obtained with sucrose in *G. arboreum* earlier (Sakhanokho et al., 2001a), the embryogenic cultures were grown on reduced sucrose concentration (from 0.04 *M* in M_2 to 0.01 *M* in M_3). In addition, NAA was replaced with a reduced concentration of IAA (0.2 μM) because of its lower auxin activity and improved root development of *G. hirsutum* and *G. barbadense* cultures (Sakhanokho et al., 2001b). Although the number of SEs per g of embryogenic callus on M_3 was lower than that obtained on M_2 treatment, the percentage germination was similar for both treatments (Table 2). On the other hand, neither the

TABLE 2

COMPARATIVE EFFECTS OF NINE MEDIA TREATMENTS ON THE MEAN NUMBER OF SOMATIC EMBRYOS PER GRAM OF EMBRYOGENIC CALLUS AND PERCENTAGE OF GERMINATED EMBRYOS IN *GOSSYPIUM ARBOREUM*

Treatment	Mean no. of embryos per g	Mean percentage of germinated embryos
M_1	169.0 ab	10.1 a
M_1F	120.8 abc	10.5 a
M_1D	2.9 c	9.2 a
M_2	219.1 a	10.6 a
M_2F	163.3 ab	10.6 a
M_2D	64.5 bc	4.8 b
M_3	83.6 bc	10.6 a
M_3F	59.3 bc	0.6 c
M_3D	23.0 c	9.1 a

The embryogenic calluses underwent a 5-d dehydration process before transfer to the various media treatments.

Values represent means of the number of embryos per g of embryogenic callus and percentage of germinated embryos per g obtained for each treatment. Each treatment consisted of 20 plates replicated three times for a total of 60 plates. Means within a *column* with the same *letter* are not significantly different according to Tukey's test ($P = 0.05$).

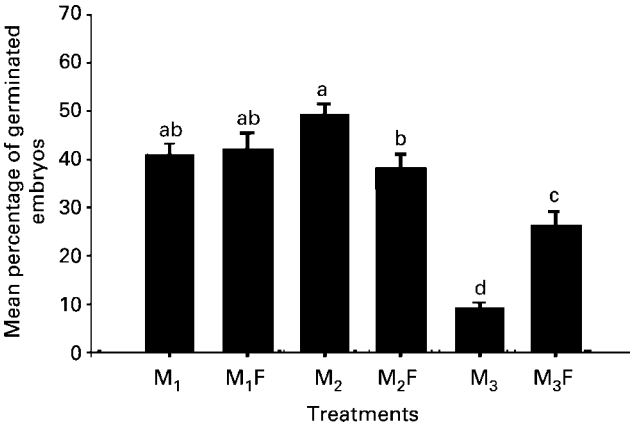

FIG. 2. Mean percentage of *Gossypium arboreum* embryos germinated on various treatments after undergoing a 3-d starvation period and after 4 wk on the various media treatments. Each treatment consisted of 20 plates replicated three times for a total of 60 plates. The means ± standard error were calculated as the average percentages of germinated embryos per g obtained for each treatment. *Bars* with the same *letter* are not significantly different according to Tukey's test ($P = 0.05$).

insertion of the filter paper between the medium and the callus tissue nor the 5-d desiccation period appeared to have a positive effect on SE maturation (Table 2). This is in contrast to the results of Sunilkumar and Rathore (2001) whereby the presence of the filter paper prevented callus formation on the cotton SEs and helped the SEs to root better. In short, this experiment helped to confirm the positive role of sucrose in improved somatic embryogenesis and embryo maturation of *G. arboreum*. On the other hand, stressful conditions exerted on the embryogenic callus through the insertion of filter paper between the tissue and the medium or the 5-d temporary starvation did not improve embryo maturation. The desiccation process in particular produced poor results, suggesting that the 5-d dehydration period was perhaps too long, as some of the calluses showed signs of severe dehydration.

Embryo germination as affected by media treatments. There was no significant difference ($P = 0.05$) in the efficiency of SE conversion for the M_1-based treatments whereas the insertion of the Whatman filter paper no. 2 between the media and the embryos appeared to help improve the germination efficiency for embryos treated with M_3 but not with M_2 (Fig. 2). Although the insertion of the filter paper increased the SE conversion from 9% to 26% in M_3F, it was less effective than in the M_1- and M_2-based treatments, which had conversion rates ranging from 37% to 49%. Similar trends were observed previously in experiment 1, where both SE production and maturation rate were lowest with the M_3 treatments.

Conclusion

The recalcitrance of cotton to tissue culture has been well documented (Smith et al., 1977; Davidonis and Hamilton, 1983; Finer, 1988; Trolinder and Xhixian, 1989; Firoozabady and DeBoer, 1993; Rajasekaran et al., 2000), a problem that is a major impediment to the transformation efforts of this economically important crop. Even though there are a few scattered reports on tissue culture and somatic embryogenesis of *G. arboreum* in the literature, very few of these reports mentioned explicitly somatic embryogenic regeneration of plants in this cotton species. Smith et al. (1977) reported the differentiation of a single plantlet of *G. arboreum* from a cotyledon callus cultured on an MS/sucrose-based medium under low light conditions, but no further detail was provided. Recently, we reported the regeneration of plants from SEs in *G. arboreum* (Sakhanokho et al., 2001a), but some of the problems we encountered were sporadic SE initiation and germination and the difficulty in the *in situ* acclimatization of plantlets, a key step in any plant regeneration scheme. However, this study focused in particular on improving both embryo production and germination efficiency. Both the 5-d desiccation treatment of embryogenic callus previously cultured in liquid medium and the insertion of filter paper between the same type of embryogenic callus tissue and the media failed to improve the numbers of SEs induced or their conversion to plantlets. Also, when immature *G. arboreum* SEs induced on M_1 medium underwent a 3-d desiccation period, 49% of these immature SEs were converted to plantlets after a 4-wk period on M_2 medium (Fig. 2). In light of these results, a protocol is proposed, relying on the liquid culture step previously described (Sakhanokho et al., 2001b). Then M_2 (S15g.05NAA) can be used to induce SEs, which will subsequently be subjected to a 3-d desiccation before transfer onto M_1, M_1F, M_2, or M_2F media.

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