Response to *In Vitro* Regeneration of Immature Zygotic Embryos in Cotton (*Gossypium* spp)

D.N. Vlachostergios, D.G. Roupakias and A.G. Mavromatis
Dept. of Genetics and Plant Breeding, School of Agriculture, Aristotle University of Thessaloniki, Greece.

ABSTRACT

The in vitro culture response of immature zygotic embryos (IZE), from six Gossypium hirsutum and one G. barbadense variety, was studied. Explants were collected from plants grown in the field and the greenhouse. Bolls were collected 12, 14 and 16 days after pollination (DAP) from the field and 10, 12 and 14 DAP from the greenhouse. The young embryos were cultured in vitro in SH and BT supplemented with 2,4-D and kinetin. All genotypes responded well to callogenesis in spite of the environmental conditions where they were grown. Callus formation (%) was higher when the bolls were collected 16 DAP for embryos collected from the field and 14 DAP for embryos collected from the greenhouse. The callus developed was tested for its embryogenic and organogenic potential in several media without positive results. During the callus induction period, it was observed that in some explants the epidermal cells from the upper cotyledon and the hypocotyl were diversified into globular or heart shape embryoids. For their further development, they were transferred to a modified MS medium. Some of the young embryoids formed roots and leaves but they never developed into regular plantlets.

Introduction

Immature zygotic embryos (IZE) have been used for the development of regeneration techniques in a number of species. The use of these explants has allowed the application of tissue culture approaches in species that otherwise were considered extremely recalcitrant to regeneration from somatic cells (Bhaskaran and Smith, 1990). Regeneration from immature zygotic embryos (IZE) could occur either by organogenesis or embryogenesis (Maddock *et al.*, 1983). For both possibilities regeneration could be direct or indirect, having an intermediate callus phase (Bronner *et al.*, 1994).

Cotton (*Gossypium* L. spp.) is considered a typical recalcitrant plant where very few regeneration techniques are commercially applied (Trolinder and Goodin, 1987; Gould *et al.*, 1991). The protocol developed by Trolinder and Goodin (1987) has the main disadvantage of genotype restriction (Trolinder and Chen, 1989). Thus, an alternative regeneration protocol is needed. The main objective of this work was to study the morphogenic potential of immature zygotic embryos in cotton.

Materials and Methods

The genotypes used are given in Table 1. Plants were cultivated under two environments, the glasshouse and the field. Four *G. hirsutum* L. and one *G. barbadense* L. genotype were grown in the glasshouse and six *G. hirsutum* and one *G. barbadense* genotype were grown in the field.

Table 1. *Gossypium* species and genotypes used.

G. hirsutum	*G. barbadense*
Stoneville 506	Menufi
Coker - 315	
Acala SJ-2	
Korina	
Uzbekistan - 2	
C - 6524	

Flowers were self-pollinated and the developing bolls were collected 10, 12 and 14 days after pollination (DAP) from the plants grown in the glasshouse. The bolls from plants grown in the field were harvested 12, 14 and 16 DAP. Young bolls were surface sterilized in 50% ethanol (3min), followed by 30% sodium hypochloride (15min) and one rinse with sterile distilled water. Isolated embryos were transferred to petri dishes and were divided into three blocks according to genotype, age and culture medium. Their length and stage were scored. All cultures were maintained continuously in the dark at 28 ± 2° C for 60 - 70 days.

The induction media used were: SH medium (Stewart and Hsu, 1977) and BT (Beasley and Ting, 1973) supplemented with 2,4-D (1mg/L) and kinetin (KIN) (0.5mg/L). The pH was adjusted at 5.8. In total, 900 IZE from the glasshouse and 1260 from the field were cultured.

Cultures were transferred to fresh medium every 30 days. In order to study the embryogenic callus potential, at the end of the induction period, half of the IZE were subcultured on to MSb + B5 vit medium in liquid and semiliquid form. These IZE were grown under 70-90 μmol $m^{-2}sec^{-1}$ light with a 16h photoperiod and 28 ± 2° C temperature regime. The remaining IZE were

subcultured for organogenesis on MSb + MS vit + NAA (1mg/L) + KIN (0.5mg/L) + adenine (40mg/L) medium. At 60 days they were transferred to a medium containing MSb + MS vit + $CuSO_4$ (0.5mg/L) under the same culture conditions.

The embryoids developed were primarily transferred to M1 medium (MS with ½ MS macroelements and Fe + 1.9g/L KNO_3) under 16h photoperiod and 28 ± 2° C temperature regime. Then, according to their response, they were transferred to one of three media:

- SHb + SHvit general support medium
- SHb + SHvit + IAA (1mg/L) + KIN (1mg/L) shoot formation medium
- SHb + SHvit + $CuSO_4$ (0.5mg/L) + L-glutamine root formation medium

The experiment was designed as a three factor factorial and was analyzed with the statistical program MSTAT.

Results and Discussion

The genotypes responded differently to the different media (Tables 2 and 3). Statistical analysis for the material collected from the glasshouse revealed differences among genotypes, media and stage of development. In general, Coker 315 exhibited the highest response and Menufi the lowest. BT was the best of the two media used and IZE responded better when they were collected 14DAP. Significant differences were also revealed between treatments when the IZE were collected from plants grown in the field. In this case the best general response was observed in Korina and the worst in Stoneville 506. The field material responded well when they were cultured on either medium (SH or BT). The best response, however, was observed in SH at 16 DAP. Finally, genotype x medium x age interaction was also significant. This may indicate that the performance of the genotype is affected by the embryo stage and medium constitution.

A positive correlation was observed between stage of embryo development, embryo length, and callogenesis. Embryos 0.8 - 1.5 mm in length collected 14 DAP from plants grown in the glasshouse had the best callus production. In contrast, embryos collected from plants grown in the field exhibited the highest callogenesis when they were 1.2 - 2.2 mm in length and collected 16 DAP.

Callus produced from IZE exhibited rapid growth and gradually became more friable and green when transferred to a semisolid medium (MSb + B_5 vit). Unfortunately, no somatic embryos resulted. Callus subcultured to liquid media (Trolinder and Goodin, 1987), produced no positive results.

In parallel, IZE were transferred to MS media enriched with MSvit, NAA (1mg/L), KIN (0.5mg/L) and adenine (40mg/L). Root formation was observed only in two genotypes (Acala SJ-2 and Coker-315). In Acala SJ-2 roots were produced on material collected from the glasshouse and the field while in Coker-315 only material collected from the field produced roots (Table 4).

At sixty days the IZE with callus were transferred to MSb enriched with MSvit and $CuSO_4$. It is known that $CuSO_4$ has a root and shoot stimulating capacity (Purnhauser, 1991; Purnhauser and Gyulai, 1993). The presence of $CuSO_4$ in the medium caused a rapid increase in the number and length of existing roots, but it did not induce the formation of new ones. The rapid root growth, however, had a negative effect on shoot formation. Thus, addition of $CuSO_4$ is recommended only after shoot formation.

The most significant observation was the development of heart shaped and globular somatic embryos originating from cotyledonary and hypocotyl epidermal cells. Similar phenomena were reported in sorghum (Dunstan *et al.*, 1979) and sunflower (Bronner *et al.*, 1994). These somatic embryos emerged on IZE 20 - 30 days after they were transferred to the induction media (SH and BT). Embryoids were observed only from Coker-315 and Acala SJ-2 tissues in the material collected from plants grown in the glasshouse (Table 5). In contrast, embryoids were produced from all cultivars grown in the field (Table 6). The highest response was in Acala SJ-2 and Stoneville 506 when the embryos were collected 16 DAP and cultured on BT (Table 6).

When these embryos were transferred to M1 medium, seven of them produced roots. Shoots, however, only developed in three of the rooted embryos (Table 7). Shoot development stopped very soon, and no further growth could be induced.

These results indicate that IZE might have the morphogenic potential needed for the production of a regeneration system in cotton. A critical point, however, is to clarify the composition of the growth medium and other factors affecting somatic embryo development after *in vitro* culture of cotton IZE. For this to be achieved further work is needed.

References

Beasley, C.A., and I.D. Ting. (1973): The effects of plant growth substances on in vitro fiber development from fertilized cotton ovules. Amer. J. Bot. 60:130-139.

Bhaskaran, S., and R. Smith. (1990): Regeneration in cereal tissue culture: A review. Crop Sci. 30:1328-1336.

Bronner, R., G. Jeanin, and G. Hahne. (1994): Early cellular events during organogenesis and somatic embryogenesis induced on immature embryos of sunflower (*Helianthus annuus*). Can. J. Bot. 72:239-248.

Dunstan, D.I., C. Shart, H. Dhaliwal, and E. Thomas. (1979): Further studies on plantlet production from cultured tissues of *Sorghum bicolor*. Protoplasma 101:355-361.

Gould, J., S. Banister, O. Hasegawa, M. Fahima, and R. H. Smith. (1991): Regeneration of *Gossypium hirsutum* and *G. barbadense* from shoot apex tissues for transformation. Plant Cell Rep. 10:12-16.

Maddock, S., V.G. Lancaster, R. Riscott, and J. Franklin. (1983): Plant regeneration from cultured immature embryos and inflorescence of 25 cultivars of wheat (*Triticum aestivum*). J. Exp. Bot. 34:915-926.

Purnhouser, L. (1991): Stimulation of shoot and root regeneration in wheat (*Triticum aestivum*) callus cultures by copper. Cereal Res. Comm. 19:419-423.

Purnhouser, L., and G. Gyulai. (1993): Effect of copper on shoot and root regeneration in wheat, triticale, rape and tobacco tissue cultures. Plant Cell, Tissue and Organ Culture 35:131-139.

Stewart, J.McD., and C.L. Hsu. (1977): In-ovulo culture and seedling development of cotton (*Gossypium hirsutum* L.). Planta 137:113-117.

Trolinder, N.L., and J.R. Goodin. (1987): Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Reports 6:231-234.

Trolinder, N.L., and X.X. Chen. (1989): Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Reports 8:133-136.

Table 2. Callus production (%) after *in vitro* culture on two media and three developmental stages of IZE collected from five cotton cultivars grown in a glasshouse.

Stage	Genotype				
	Menufi	Korina	Coker-315	Acala SJ-2	Stoneville 506
10 DAP	SH: 0	SH: 0	SH: 0	SH: 0	SH: 0
	BT: 0	BT: 24	BT: 6	BT: 71.5	BT: 0
12 DAP	SH: 0	SH: 68	SH: 100	SH: 0	SH: 0
	BT: 91.5	BT: 58	BT: 63	BT: 100	BT: 100
14 DAP	SH: 0	SH: 79	SH: 100	SH: 98	SH: 55
	BT: 100	BT: 100	BT: 100	BT: 100	BT:67.8

Table 3. Callus production (%) after *in vitro* culture in two media and three developmental stages of IZE collected from seven field-grown cotton cultivars.

Stage	Genotype						
	Menufi	Korina	Coker 315	Acala SJ-2	Stone-ville	Uz-2	C-6524
12DAP	SH:0	SH:0	SH:6.1	SH:64	SH:0	SH:98	SH:25
	BT:7.7	BT:6.3	BT:7	BT:0	BT:0	BT:16	BT:0
14DAP	SH:39	SH:100	SH:6	SH:92	SH:96	SH:84	SH:82
	BT:71	BT:100	BT:100	BT:100	BT:93	BT:4	BT:50
16DAP	SH:90	SH:100	SH:100	SH:67	SH:6	SH:100	SH:100
	BT:100	BT:100	BT:100	BT:98	BT:25	BT:100	BT:100

Table 4. Root formation (%) in callus developed from IZE transferred to MSb + MS vit + NAA + KIN + adenine.

Genotype	Plant growth environment	Age (DAP)	Callogenesis medium	Callogenesis %	% Root formation
Acala SJ-2	g	12	BT	52	20
Acala SJ-2	g	14	BT	100	80
Acala SJ-2	f	14	SH	92	20
Coker 315	f	14	BT	100	10

f: field g:glasshouse

Table 5. Somatic embryo production (%) after *in vitro* culture on two media of IZE collected from seven cotton genotypes grown in the glasshouse.

Stage	Genotype				
	Menufi	Korina	Coker-315	Acala SJ-2	Stoneville
12 DAP	SH:0	SH:0	SH:0	SH:0	SH:0
	BT:0	BT:0	BT:3.3	BT:0	BT:0
14DAP	SH:0	SH:0	SH:0	SH:6	SH:0
	BT:0	BT:0	BT:16.5	BT:0	BT:0

Table 6. Somatic embryo production (%) after in vitro culture on two media of IZE collected from seven cotton genotypes grown in the field.

Media	Genotype						
	Menufi	Korina	Coker	Acala SJ-2	Stoneville	Uz-2	C-6524
SH	6.6	3.3	0	26.6	0	0	0
BT	6.6	20	13.3	36.6	33	26.6	6.6

Table 7. Embryogenic response and percentage of embryos responding after in vitro culture in two media of IZE (16 DAP) from five cotton cultivars.

Genotype	Induction medium	Age (DAP)	Embryo response	Embryos responded (%)
Korina	SH	16	R-S	100
Korina	BT	16	R-S	25
Stoneville	BT	16	R-S	18
Stoneville	BT	16	R	18
Acala SJ_2	SH	16	R	12,5
Coker 315	BT	16	R	16,5
Uz-2	BT	16	R	10

R: roots S: shoots