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CALLUS FORMATION AND PLANT REGENERATION FROM TWO COTTON SPECIES (*GOSSYPIUM HIRSUTUM* L., AND *G. BARBADENSE* L.)

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Abstract

In this study carried out during 2000-2001 in Kahramanmaraş, 3,9,15 and 21 days old ovules excised from two different cotton species (*Gossypium hirsutum* L. and *G. barbadense* L.) were cultured in MS (Murashige & Skoog, 1962) medium supplemented with 100 mg/l myoinositol, 30 g/l glucose, 9 g/l agar, 100-120 ml/l coconut water, 0.5 mg/l GA_3, 1 g/l casein hydrolisat, 1 g/l yeast extract and 3 g/l activated charcool. To determine the effects of growth regulators on callus formation, 0.5, 1.5, 2.5, 3.5 mg/l IAA and 0.5, 1.5, 2.5, 3.5 mg/l Kinetin were added to media as 16 combination. For developing of balanced roots and shoots NAA (0.1 mg/l) and 2iP (3 mg/l) were added in the medium. All the obtained calli became brown in 1999, and callus formation was 13.2 % in *G. hirsutum*, 4.4 % in *G. barbadense* in 2000 and callus formation was 85.2 % in *G. hirsutum*, 44.1 % in *G. barbadense* in 2001. In 2000 it was observed that the combinations of 0.5 Kin+0.5 IAA and 0.5 Kin+1.5 IAA induced callus initiation in both 3 and 9 days old ovules in *G. hirsutum*, also in *G. barbadense* the combination of 0.5 Kin+0.5 IAA induced in 9 days old ovules, whereas the combination of 0.5 Kin+1.5 IAA induced in 3 days old ovules. In 2001 the calli formed almost in the all hormone combinations. Furthermore, callus formation were observed from both 3 and 9 days old ovules in *G. hirsutum* species, whereas in *G. barbadense* species callus formation were obtained from only 3 days old ovules. In 2000 and 2001 obtained calli were cultured in MS media supplemented with 0.1 mg/l NAA+3 mg/l 2iP in order to provide developing of balanced roots and shoots. The rootlike structures were observed in some cultures whereas shoot formation were not observed.

Intoduction

Among cotton producing countries, Turkey is in fifth order in terms of cotton seed production with 2 billion ton and in terms of lint production with 755.000 ton (Anon., 1995). The greatest part of income of export from Turkey is only provided from cotton and textile products made from cotton (Anon., 1999). Breeding studies in cotton are very important, because cotton has provided a large scale raw material requirements of textile and food industry in Turkey and at the same time it has provided an additional income from export (Gencer, 1987). The plant breeding methods can be combined with tissue culture methods in order to form genetic variability for desired traits. With the other manner of explaining, the basic objective of researches of plant tissue culture is plant breeding (Evans *et al*., 1981). In breeding, plant tissue culture methods can be used in interspecific hybridization, mutation breeding, hybrid variety breeding, combination breeding, rapid propagation in plant breeding, somatic hybridization and transformation. In addition, plant tissue cultures has also been used in basic researches on many branch of science such as biology, physiology and genetics (Gonulsen, 1987). For example, ovule culture has been used in basic researches such as investigation of fiber

development in cotton and obtaining of fiber *in vitro*. Furthermore, plant tissue cultures can be used for long time conservation of genotypes, obtaining virus free plants, production of secondary compounds, rapid production of plants and shortening of period of dormancy in seeds (Efe & Gencer, 1994). In many plants, particularly problems which occured in hybridization of species which are distance relative can be solved by embryo cultures. If the hybrid embryo can not develop because of incompatibility between embryo and main tissue of endosperm when embryos are cultured in sterile medium they can continue developing. Thus viable and fertile interspecific hybrids can be obtained by means of embryo cultures. Nowadays *in vitro* culture and keeping alive of hybird embryos has been a method used in the whole world. For example in bean, flax, cotton, tomatoes, rice and barley interspecific hybrid plants were obtained by means of this technique (Hatipoğlu, 1995). Similarly, it has also been reported that hybrid embryos were obtained in many studies on embryo culture in cotton. However, from literature review it has been understood that true plant regeneration could not be entirely succeeded in countries in which tissue culture researches in cotton are excessive such as USA, China, India and Russia and that there are some problems in this subject. It has been reported that in cotton breeding huge progress have been made with the studies in fields of gene engineering by using advanced biotechnological methods in the leading countries for science and technology (Stewart, 1991).

Beasley (1971) first reported that calli formed from micropilar region of ovules or from fine tissue pieces cultured from *G. hirsutum* L. Eid *et al*., (1973) cultured fertilized ovules of *G. hirsutum* L., in MS medium which do not contains growth regulators. They reported that normal seedlings were obtained from ovules cultured 5-10 days after anthesis. Stewart & Hsu (1977) cultured cotton ovules for germination in liquid medium at 30°C and in dark. A balanced root and shoot growth from embryos were obtained followed by transfer of ovules to secondary medium after germination in culture conditions of an illumination of 16 hours and at a temperature of 30°C. Morozova & Pontovich (1984) isolated explants of placenta with ovule and without ovule from five *G. hirsutum* varieties and they reported that callus formed in Beasley medium suplemented with GA_3 and IAA. Azizkhodzhaev *et al*., (1987) cultured three days old ovules from interspecific hybridizations and reported that they obtained embryos with leaves like cotyledon and roots. When they subcultured these embryos in the medium suplemented with vitamins but not containing growth regulators, these embryos formed shoots with 3-4 true leaves in length of 2.5-3.5 cm in 30-40 days however roots blackened gradually. Trolinder & Xhixian (1989) investigated 38 species, varieties and races of *Gossypium* in respect to somatic embryogenesis and classified genotypes for reaction to somatic embryogenesis as high, medium, low and non-embryogenic. Idiyatullina & Azizkhodzhaev (1991) cultured ovules isolated from ovaries of two varieties of *G. hirsutum* L., and reported that a number of embryos could reach to mature embryo stage from globular stage due to increasing concentration of IAA and gibberellic acid. Vlachostergios *et al*., (1998) investigated the reactions of immature zigotic embryos of 6 *G. hirsutum* L., and 1 *G. barbadense* L., varieties *in vitro* culture and found that embryos excised from 16 days old bolls taken from field formed more callus. Holmuratov *et al*., (2000) used 20-25 days old embryos from *G. hirsutum* L., as material and found that combining of 2,4-D and BAP stimulated somatic embryogenesis in cultures of calli obtained from immature embryos.

The objective of this study was to determine the effect of some growth regulators, days of ovules and species on callus formation from different day old ovules of *G. hirsutum* L., and *G. barbadense* L.

Materials and Methods

Plant material: In this study carried out during 2000-2001 in Kahramanmaraş, 3, 9, 15 and 21 days old ovules (in 2001 only 3 and 9 days old ovules) of the variety of Ersan-92 (*G. hirsutum* L.) which is one of the local standard varieties in the region of Kahramanmaras of Turkey and Agdas-21 variety which is mutant for earliness (*G. barbadense* L.) and originated in Azerbaijan were used as material.

Preparation of culture media: Explants were cultured in basic MS medium (Murashige & Skoog, 1962) suplemented with myoinositol (100 mg/l), glucose as a carbon source (30 g/l), agar as a gelling agent (9 g/l), coconut water (100-120 ml/l), GA_3 (0.5 mg/l), casein hydrolisat (1g/l) and yeast extract (1 g/l). Furthermore, IAA and Kinetin (0.5, 1.5, 2.5, 3.5 mg/l) were added in culture medium in order to form callus from ovules and for developing of balanced roots and shoots from calli NAA (0.1 mg/l) and 2iP (3 mg/l) were added in the medium. In addition, medium of control group without growth regulators were used.

Sterilization procedure: Culture media were sterilized for 15 minutes at 15 p.s.i., in an autoclave. Equipment were sterilized for 3 hours at 170°C in an autoclave. Bolls were surface sterilized by a 30 second exposure in 70% ethanol followed by a 20 minute exposure in 10% Clorox containing one drop of Tween 20 per 100 ml in sterile bench. Then bolls were rinsed three times in sterile distilled water.

Cutting of bolls, excising and culturing of ovules is shown in Fig. 1. 0.5, 1.5, 2.5, 3.5 mg/l IAA and 0.5, 1.5, 2.5, 3.5 mg/l Kinetin were added to media as 16 combination. There was also the control group without growth regulators. Each combination repeated two times. In each time of excising of ovules (age of ovules) 68 cultures in Petri dishes (2 varieties x 17 hormone doses x 2 replication=68 cultures) were established. Since there were two times of excising of ovules, total culture number were 272 (2 x 68=136). Explants were placed in 10 cm diameter of Petri dishes containing 20 ml of the appropriate medium and cultures were incubated at 25°C, under a 16-hour photoperiod and in luminous intensity of 5000 lux.

Statistical method: In callus cultures obtained from 3 and 9 days old ovules excised from both cotton species, cultures with callus were counted and the rate of callus formation were determined.

Results and Discussion

Results of 2000: From Table 1, it can be seen that only 9 of 136 cultures formed callus in *Gossypium hirsutum* L., which is 13.2% of total cultures. Obtained calli were green and soft. All of calli developed from cultures formed in the 1st and 2nd time of explant taking (from 3 and 9 days old ovules) which is similar to the reports of Vlachostergios *et al*., (1998) and Gill & Bajaj (1987). It would suggest that 3 and 9 days old ovules were

more useful for callus formation. Also callus formation has been observed in 4 of 34 cultures (11.8%) from 3 days old ovules but in 5 of 34 cultures (14.7%) from 9 days old ovules in *G. hirsutum* L. Position of calli obtained from *G. hirsutum* L., for growth regulator combinations and time of explant during 2000 is given in Table 1. Callus formation has been observed from 3 days old ovules in combinations of growth regulators of 0.5 Kin+0.5 IAA, 0.5 Kin+1.5 IAA, 1.5 Kin+0.5 IAA and 1.5 Kin+2.5 IAA, whereas callus formation has been noted from 9 days old ovules in combinations of growth regulators of 0.5 Kin+0.5 IAA, 0.5 Kin+1.5 IAA, 2.5 Kin+1.5 IAA and 2.5 Kin+2.5 IAA. In *G. hirsutum*, it can be said that combinations of 0.5 Kin+0.5 IAA, 0.5 Kin+1.5 IAA stimulated callus formation both in 3 days old and 9 days old ovules. Callus formation has been observed in presence of GA_3 (0.5 mg/l) and different doses of IAA by Morozova & Pontovich (1984).

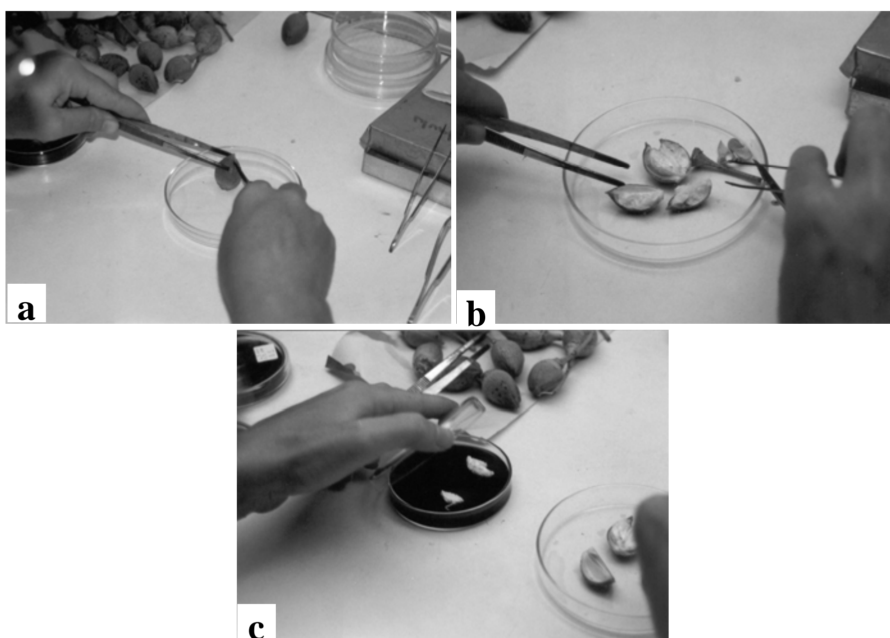

Fig. 1. Cutting of bolls (a) Excising of ovules (b) and Culturing of ovules (c)

Position of calli obtained from *G. barbadense* L., for growth regulator combinations and time of explant taking in 2000 are given in Table 2. Callus formation was observed in 3 of 136 cultures in *G. barbadense* which was 4.4% of total cultures. Obtained calli developed from cultures formed in the 1st and 2nd time of explant taking (from 3 and 9 days old ovules). Findings are similar to the observation of Vlachostergios *et al*., (1998) and Gill & Bajaj (1987). It can be seen that callus formation has been observed in 1 of 34 cultures (2.9 %) from 3 days old ovules but in 2 of 34 cultures (5.9 %) from 9 days old ovules in *G. barbadense* L. Also, it has been noted that callus formation was observed in the combinations of 0.5 Kin+0.5 IAA, 0.5 Kin+1.5 IAA. In *G. barbadense*, it was determined that the combination of 0.5 Kin+0.5 IAA stimulated callus formation in 9 days old ovules, whereas the combination of 0.5 Kin+1.5 IAA stimulated callus formation in 3 days old ovules. Obtained calli were green and soft.

Table 1. Position of calli obtained from *Gossypium hirsutum* L., for growth regulator combinations and time of explant in 2000.

Growth regulator combinations	*Gossypium hirsutum* L.			
	Number of cultures with callus (from 3 days old ovules)	Number of cultures with callus (from 9 days old ovules)	Number of cultures with callus (from 15 days old ovules)	Number of cultures with callus (from 21 days old ovules)
CONTROL	0	0	0	0
0.5 Kin +0.5 IAA	1	2	0	0
0.5 Kin +1.5 IAA	1	1	0	0
0.5 Kin +2.5 IAA	0	0	0	0
0.5 Kin +3.5 IAA	0	0	0	0
1.5 Kin +0.5 IAA	1	0	0	0
1.5 Kin +1.5 IAA	0	0	0	0
1.5 Kin +2.5 IAA	1	0	0	0
1.5 Kin +3.5 IAA	0	0	0	0
2.5 Kin +0.5 IAA	0	0	0	0
2.5 Kin +1.5 IAA	0	1	0	0
2.5 Kin +2.5 IAA	0	1	0	0
2.5 Kin +3.5 IAA	0	0	0	0
3.5 Kin +0.5 IAA	0	0	0	0
3.5 Kin +1.5 IAA	0	0	0	0
3.5 Kin +2.5 IAA	0	0	0	0
3.5 Kin +3.5 IAA	0	0	0	0
Total number of cultures (17 combination x 2 replication)	34	34	34	34
Number of cultures with callus	4	5	0	0
Percentage of cultures with callus (%)	11.8	14.7	0	0
Total number and percentage of cultures with callus (%)	9 (13.2)			

Table 2. Position of calli obtained from *Gossypium barbadense* L., for growth regulator combinations and time of explant in 2000.

Growth regulator combinations	*Gossypium barbadense* L.			
	Number of cultures with callus (from 3 days old ovules)	Number of cultures with callus (from 9 days old ovules)	Number of cultures with callus (from 15 days old ovules)	Number of cultures with callus (from 21 days old ovules)
CONTROL	0	0	0	0
0.5 Kin +0.5 IAA	0	2	0	0
0.5 Kin +1.5 IAA	1	0	0	0
0.5 Kin +2.5 IAA	0	0	0	0
0.5 Kin +3.5 IAA	0	0	0	0
1.5 Kin +0.5 IAA	0	0	0	0
1.5 Kin +1.5 IAA	0	0	0	0
1.5 Kin +2.5 IAA	0	0	0	0
1.5 Kin +3.5 IAA	0	0	0	0
2.5 Kin +0.5 IAA	0	0	0	0
2.5 Kin +1.5 IAA	0	0	0	0
2.5 Kin +2.5 IAA	0	0	0	0
2.5 Kin +3.5 IAA	0	0	0	0
3.5 Kin +0.5 IAA	0	0	0	0
3.5 Kin +1.5 IAA	0	0	0	0
3.5 Kin +2.5 IAA	0	0	0	0
3.5 Kin +3.5 IAA	0	0	0	0
Total number of cultures (17 combination x 2 replication)	34	34	34	34
Number of cultures with callus	1	2	0	0
Percentage of cultures with callus (%)	2.9	5.9	0	0
Total number and percentage of cultures with callus (%)	3 (4.4)			

Number and percentage of calli regarding species and total number and percentage of cultures with callus in 2000 are given in Table 3. When only species are considered, it has called attention that callus formation percentage was 13.2% in *G. hirsutum* species, whereas this rate was 4.4% in *G. barbadense* species. According to this, it can be said that more callus formed in *G. hirsutum* species than *G. barbadense* species which is similar to findings of Trolinder & Xhixian (1989). From the same Table it can be seen that total number of cultures formed callus was 12 (4.4%).

Table 3. Number and percentage of calli regarding species and total number and percentage of cultures with callus in 2000.

	***Gossypium hirsutum* L.**	***Gossypium barbadense* L.**
Total number and percentage of cultures with callus regarding species (%)	9 (13.2)	3 (4.4)
Total number and percentage of cultures with callus (%)	12 (4.4)	
Total number of cultures regarding species	136	136
Total number of cultures	**272**	

Results of 2001: In 2001, it was observed that callus formed in 88 of 136 total cultures (64.7%). Vlachostergios *et al*., (1998) and Gill & Bajaj (1987) also reported that higher callus formation was observed in different media from ovules excised in different days old. Position of calli obtained in 2001 for growth regulator combinations, species and time of explant taking is given in Table 4, from where it can be seen that callus developed in 27 of 34 cultures (79.4%) from 3 days old ovules, but in 31 of 34 cultures (91.1%) from 9 days old ovules in *G. hirsutum* L. Results were similar to the reports of Vlachostergios *et al*., (1998) and Gill & Bajaj (1987). Callus formation was observed in 30 of 34 cultures (88.2 %) from 3 days old ovules, but callus from 9 days old ovules was not observed in the *G. barbadense* L.

Calli were green and soft. When only species are considered, it has been determined that the percentage of callus formation was 85.2% in *G. hirsutum*, but that this value was 44.1% in *G. barbadense*. Trolinder & Xhixian (1989) reported that there were differences between species or varieties for callus formation and embryogenesis. It was determined that callus formation was observed in both 3 and 9 days old ovules in *G. hirsutum* species, whereas in only 3 days old ovules in *G. barbadense* species. Calli developed almost in all combinations of growth regulators.

In both years obtained calli were subcultured in solidified MS medium supplemented with 0.1 mg/l NAA + 3 mg/l 2iP in order to obtain balanced root and shoot formation. While rootlike structures were observed in some cultures, any shoot formation were not observed during three months. Subculture of calli obtained from different growth regulators in MS medium supplemented with 0.1 mg/l NAA + 3 mg/l 2iP is shown in Fig. 3.

Tablo 4. Position of calli obtained in 2001 for growth regulator combinations, species, and time of explant taking.

Growth regulator combinations	*Gossypium hirsutum* L.		*Gossypium barbadense* L.	
	Number of cultures with callus (from 3 days old ovules)	Number of cultures with callus (from 9 days old ovules)	Number of cultures with callus (from 3 days old ovules)	Number of cultures with callus (from 9 days old ovules)
CONTROL	2	2	2	0
0.5 Kin +0.5 IAA	2	2	2	0
0.5 Kin +1.5 IAA	2	2	2	0
0.5 Kin +2.5 IAA	2	2	2	0
0.5 Kin +3.5 IAA	1	2	1	0
1.5 Kin +0.5 IAA	2	2	2	0
1.5 Kin +1.5 IAA	1	2	1	0
1.5 Kin +2.5 IAA	1	2	2	0
1.5 Kin +3.5 IAA	2	2	2	0
2.5 Kin +0.5 IAA	2	0	2	0
2.5 Kin +1.5 IAA	1	2	2	0
2.5 Kin +2.5 IAA	1	2	2	0
2.5 Kin +3.5 IAA	2	2	2	0
3.5 Kin +0.5 IAA	2	2	1	0
3.5 Kin +1.5 IAA	2	1	2	0
3.5 Kin +2.5 IAA	1	2	1	0
3.5 Kin +3.5 IAA	1	2	2	0
Total number of cultures (17 combination x 2 replication)	34	34	34	34
Total number of cultures regarding species	68		68	
Total number of cultures	136			
Number of cultures with callus	27	31	30	0
Percentage of cultures with callus (%)	79.4	91.1	88.2	0
Total number and percentage of cultures with callus regarding species (%)	58 (85.2)		30 (44.1)	
Total Number and percentage of cultures with callus (%)	88 (64.7)			

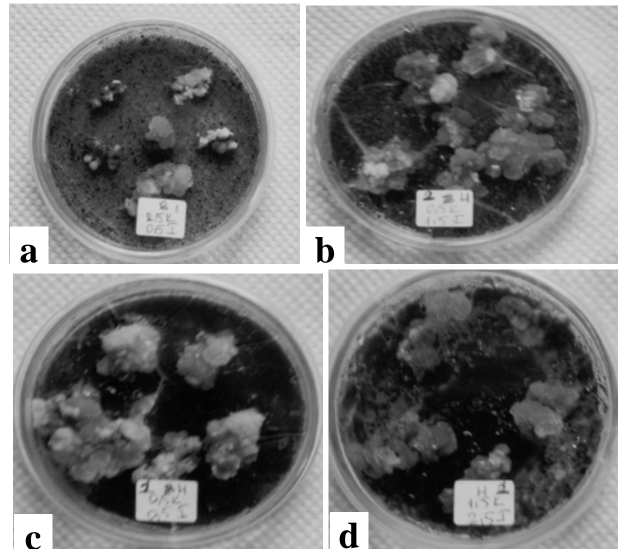

Fig. 2. (a) Calli formed from 3 days old ovules of *G. barbadense* L., in MS medium supplemented with 1.5 mg/l Kin+0.5 mg/l IAA. (b) Calli formed from 9 days old ovules of *G. hirsutum* L. in MS medium supplemented with 0.5 mg/l Kin+1.5 mg/l IAA. (c) Calli formed from 3 days old ovules of *G. hirsutum* L. in MS medium supplemented with 0.5 mg/l Kin+0.5 mg/l IAA. (d) Calli formed from 3 days old ovules of *G. hirsutum* L. in MS medium supplemented with 1.5 mg/l Kin+2.5 mg/l IAA.

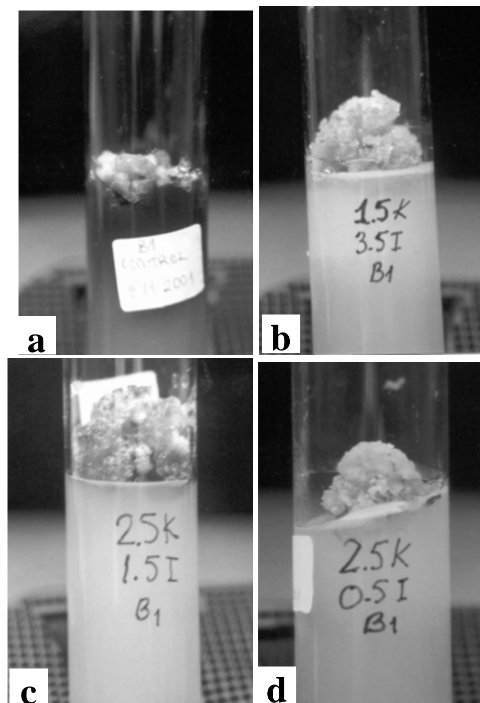

Fig. 3. Subculture of calli obtained from different growth regulators in MS medium supplemented with 0.1 mg/l NAA + 3 mg/l 2iP.

Conclusions

The results of the present study would suggest that 9 days old ovules of *G. hirsutum* were more accustomed for callus formation whereas, in *G. barbadense* 3 days old ovules were more useful for callus formation. Furthermore, *G. hirsutum* formed more callus (85.2%) than *G. barbadense* (44.1%). It has been determined that calli developed almost in all combinations of growth regulators and that obtained callus were green and friable. In both years, calli subcultured in order to form balanced shoot and root developed rootlike structures but did not develop any shoots.

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