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Full Length Research Paper

Comparison of callus induction and somatic embryogenesis of some Iranian cottons (*Gossypium* Spp.) with Coker 312 and histology of somatic embryogenesis

Mahin Ghaemi*[1], Ahmad Majd[2], Fathalah Fallahian[3] and Kamal Ghasemi Bezdi[4]

[1]Department of Biology, PhD Student, Science and Research Branch, Islamic Azad University (IAU), Tehran, Iran.
[2]Department of Biology, Faculty of Science, Tehran North Branch, Islamic Azad University, Tehran, Iran.
[3]Department of Biology, Faculty of Science, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[4]Cotton Research Institute of Iran, Gorgan, Iran.

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Callus induction and somatic embryogenesis from hypocotyl explants of some Iranian cottons spp. (Hashem abad, Kerman, Termez and Sepid) were compared with Coker 312 through induction and formation of embryogenic calli on medium of Murashige and Skoog (MS) with Gamborg vitamins (B_5) supplemented with the following compositions: MSB_1 (0.5 mg/l zeatin), MSB_2 (1 mg/l zeatin), MSB_3 (0.5 mg/l 2,4-dichlorophenoxyacetic acid, 0.1 mg/l kinetin), MSB_4 (1 mg/l 2,4-D, 0.5 mg/l kinetin, 0.5 mg/l zeatin) and MSB_5 (2 mg/l α-naphtalene-3-acetic acid, 1 mg/l kinetin, 0.75 mg/l $MgCl_2$). The optimum medium for the proliferation of embryogenic calli was MS medium containing B_5 vitamins, 1 mg/l 2,4-D, 0.5 mg/l kinetin and 0.5 mg/l zeatin and the optimum medium for the development of somatic embryos was MS medium (NH_4NO_3 was removed and KNO_3 amount doubled) containing B_5 vitamins, 40 g/l sucrose and without hormone. Media MSB_1, MSB_2 and MSB_4 gave the highest percentage (100%) of calli induction in Coker 312 but the lowest induction (46.66%) was observed when Hashem abad explants were cultured in the MSB_3 medium. Embryogenesis percentage of Termez (2.22 to 24.40%), Hashem abad (1.85 to 9.73%) and Sepid (9.06 to 22.28%) genotypes were significantly lower than that of Coker 312 (66.66 to 94.33%). The Kerman genotype did not show embryogenesis. In the histological studies, the different development stages of the embryos (globular, heart, torpedo and cotyledonary) together with callus cells were showed.

Key words: Hypocotyl explants, somatic embryo, *in vitro* regeneration, germination, somatic embryogenesis histology.

INTRODUCTION

Cotton is one of the most important fiber crops in the world. Since cotton is highly susceptible to biotic and abiotic stresses, it requires intensive crop management.Genetic improvement of cotton through conventional breeding is limited by several factors such as lack of useful variation and long time periods that are required. Regeneration through somatic embryogenesis is preferred to organogenesis because of the single-cell origin of the somatic embryos (Merkele et al., 1995), thus, reducing the chimeric transformation events. However, efficient *in vitro* techniques for the regeneration of large numbers of plantlets from cotton are limited when compared with other major commercial

*Corresponding author. E-mail: ghaemi_m86@yahoo.com. Tel/ (Fax):0098-0171-5535704.

Abbreviations: Zt, Zeatin; **Kt,** kinetin; **MS,** Murashige and Skoog medium (1962); **B_5,** Gamborg et al., medium (1968); **GA_3,** giberellic acid; **NAA,** α-naphtalene-3-acetic acid; **2,4-D,** 2,4-dichlorophenoxyacetic acid.

crops. Although plant biotechnology is an attractive means for improving cotton, its use requires an effective regeneration system from somatic tissues of cotton plants.

Price and Smith (1979) reported somatic embryogenesis in cotton (*Gossypium klotzchianum* L.), although, complete plants could not be regenerated. Davidonis and Hamilton (1983) subsequently described plantlet regeneration via somatic embryogenesis from a 2-year-old callus culture of *Gossypium hirsutum* var. Coker 310. This procedure need a long culture period and was difficult to repeat. Since then, several investigators have worked extensively on plant regeneration through somatic embryogenesis in different cotton cultivars (Shoemaker et al., 1986; Chen et al., 1987; Trolinder et al., 1987, 1988, 1989; Finer et al., 1988; Gawel and Robacker, 1990; Zhang et al., 1991, 1996, 2000, 2001; Firoozabady and DeBoer, 1993; Rajasekaran et al., 1996; Sakhanokho et al., 2001; Chaudhary et al., 2003; Kumeria et al., 2003; Leelavathi et al., 2004; Syed Sarfraz et al., 2004, 2005; Ikram-ul-Haq, 2005; WU et al., 2005; Jin et al., 2006; Han et al., 2009).

Compared with many other crops, it is more difficult to obtain somatic embryogenesis and plant regeneration from cotton. Only a limited number of varieties can be induced to produce somatic embryos and regenerative plants and the most responsive lines are Coker varieties, which are no longer under cultivation (Feng et al., 1998). Genotype dependent response restricts the application of cotton biotechnology to cotton breeding and production. Therefore, before plant tissue culture techniques are widely applied to cotton improvement programs, plant regeneration must be possible for a broad range of genotypes. In this study, callus induction, somatic embryogenesis and plant regeneration of Coker 312 were compared with four other cotton genotypes of Iran from different cotton species (*G. hirsutum*, *Gossypium herbaceum* and *Gossypium barbadense*). Meanwhile, the histology of cotton somatic embryogenesis was carried out.

MATERIALS AND METHODS

Plant material

The plant materials investigated in this study were five genotypes of cotton: Hashem abad (*G. herbaceum*), Kerman (*G. herbaceum*), Termez (*G. barbadense*), Sepid (*G. hirsutum*) and Coker 312 (*G. hirsutum*). These cultivars were obtained from the Cotton Research Institute of Iran, Gorgan.

Seed germination

Seeds of cotton were delinted with sulphuric acid (98%). Mature seeds were surface sterilized by 70% ethanol for 1 min and shaken in 30% commercial bleach (5.25% (v/v) NaOCl) by stirring for 10 min. Then, they were washed three times with sterile distilled water. Next they were dipped and kept in sterile distilled water for 8 h to soften the seed coats and allow their complete removal. Subsequently, the sterilized seeds without seed coats were placed in test tubes containing MSB [Murashige and Skoog (MS), 1962) salts with B_5 (Gamborg et al., 1968) vitamins] medium supplemented with 30 g/l sucrose and 7 g/l agar (pH 5.8). Finally, the seeds were germinated at 28 ± 2°C under 16 h photoperiod conditions with the light intensity of approximately 2000 lx.

Induction, selection and proliferation of embryogenic callus

Hypocotyl sections (3 to 5 mm length) of 4 to 7-days-old sterile seedlings were placed in MSB [Murashige and Skoog, 1962) salts with B_5 (Gamborg et al., 1968) vitamins)] medium supplemented with 30 g/l sucrose and various concentrations of the various hormone treatments, MSB_1 (0.5 mg/l Zt, Zhang et al., 2001), MSB_2 (1 mg/l Zt, Zhang et al., 2001), MSB_3 (0.5 mg/l 2,4-D and 0.1 mg/l Kt, Ikram, 2005), MSB_4 (1 mg/l 2,4-D, 0.5 mg/l Kt and 0.5 mg/l Zt, Zhang et al., 2001) and MSB_5 (2 mg/l NAA, 1 mg/l Kt and 0.75 mg/l $MgCl_2$, Sakhanokho et al., 2004) for callus induction. After about 45 days of culture, embryogenic calli were chosen and transferred into MS medium, supplemented with B_5 vitamins, 1 mg/l 2,4-D, 0.5 mg/l Zt and 0.5 mg/l Kt for the proliferation of embryogenic calli, which took 30 days.

Differentiation of somatic embryos and plant regeneration

Embryogenic calli were chosen and transferred on somatic embryo development medium consisted of MS salts in which NH_4NO_3 was removed and the amount of KNO_3 was doubled and supplemented with B_3 vitamins, 40 g/l sucrose and without growth regulators. After about 25 days, the cotyledonary embryos were cultured on MS medium for root/shoot induction, supplemented with B_5 vitamins and 0.1 mg/l GA_3. All cultures were supplemented with 30 g/l sucrose and were solidified with 7 g/l agar. Cultures were transferred on fresh media every 2 weeks. Each culture was incubated at 28 ± 2°C under a light intensity of approximately 2000 lx provided by cool white fluorescent lamps with 16/8 h light/darkness photoperiod. The pH of medium was adjusted to 5.8 before autoclaving at 121°C for 15 min.

Statistical analysis

In each experiment, the percentage of callus and embryo induction was measured by counting per dish. For each treatment, 20 explants were placed in four erlenmeyer flasks with three replications. The ANOVA was performed for each experiment and means were compared using Duncan's multiple range test ($p < 0.05$).

Histological studies

For histological investigations, calli with somatic embryos were fixed in FAA (formalin glacial, acetic acid and ethanol, 10: 5: 85, v/v/v) (Shariatzadeh and Majd, 2001) for 8 h. After fixation, samples in their original place were dehydrated with a graded series of ethanol. Next, they were de-alcoholized with a graded series of toluene. After that, they were embedded in paraffin. The paraffin-embedded samples were then cut 10 µm thick with a rotary microtome. Subsequently, the sections were stained with hematoxylin and eosin. Finally, permanent slides were examined on a light microscope equipped with a camera.

RESULTS AND DISCUSSION

Initiation, selection and proliferation of embryogenic callus

Callus initiation occurred from the cut ends of the explants during 1 to 2 weeks of culture. The results indicated that, all the genotypes on the media (MSB_1, MSB_2, MSB_3, MSB_4 and MSB_5) responded to callus induction within 2 to 4 weeks. The induction percentage of callus for the different media among the various genotypes was also different from the several types of callus, which was distinguishable based on the color and texture (Table 1). Morphology and characteristics of pre-embryogenic calli were previously described (Firoozabady and DeBoer, 1993; Rajasekaran et al., 1996; Sakhanokho et al., 2001). Histological examination showed that, the cells in pre-embryogenic calli had dense cytoplasm, small cells and relatively bigger nuclei, but the cells in non-embryogenic calli were irregular, with the relative size of nuclei been smaller (Data not shown).

The induction percentage of calli ranged from 46.66 to 100% among the different treatments in all the genotypes studied. As for the media, MSB_1 (0.5 mg/l Zt), MSB_2 (1 mg/l Zt) and MSB_4 (1 mg/l 2,4-D, 0.5 mg/l Kt and 0.5 mg/l Zt) gave the highest percentage (100%) of calli induction in Coker 312 but the lowest induction (46.66%) was observed when Hashem abad explants were cultured in the MSB_3 medium (0.5 mg/l 2,4-D and 0.1 mg/l Kt) (Table 1). Significant differences at $p < 0.05$ were found between the percentage of callus induction in some media and genotypes (Table 1). The results indicated that, the type of genotype and medium can affect induction percentage, color and texture of the produced calli (Table1). In some cases, genotypes were different in terms of color and texture of calli in the same medium. Coker 312, Hashem abad and Sepid genotypes in contrast to Termez and Kerman genotypes produced better and bigger calli (Data not shown). Embryogenic calli were generally soft, fragile and granular, but non-embryogenic calli were compact and hard with relatively smooth surface and in some cases they were soft and powdery. In some 250 ml Erlenmeyer flasks, calli resulting from explants were heterogeneous in such a way that some parts of callus had embryogenic cells and other parts had non-embryogenic cells.

A genotype's capability to produce huge amounts of callus is not indicative of its regenerative capacity. Very often, only a small portion of the callus is formed on explant, showing embryogenic potential (Sakhanokho et al., 2001). Excessive root formation was observed in some explants on MSB_5 medium. Trolinder and Goodin (1987) had reported that, root formation decreased the amount of available embryogenic calli.

The limiting step to the successful use of modern techniques in genetic improvement of the major crops has not been transgene insertion itself, but rather the regeneration of viable plants from the transgenic explant material (Murphy, 2003). Thus, first of all, agri-biotechnological research on crops first requires reliable callus induction and then, efficient *in vitro* regeneration system (Abdellatef et al., 2008).

After 30 days of culture, various states of calli differentiation were detected on the media for two types of calli: pre-embryogenic and non-embryogenic (Figure 1). For proliferation, pre-embryogenic calli from the media were selected and were transferred to MS medium supplemented with B_5 vitamins, 1 mg/l 2,4-D, 0.5 mg/l Zt and 0.5 mg/l Kt.

The color and texture of calli in most genotypes changed when transported from the induction medium to the medium of proliferation of calli and development of embryos (Data had shown). The difference in the various cotton genotypes in response to induction and proliferation of calli may be related to the level of endogenous hormones (Tripathy and Reddy, 2002)..

Embryo formation and plant regeneration

Somatic embryogenesis protocols especially for Coker lines and some other genotypes (Zhongmian 12, Simian 3, PD 97019, PD 97021, PD 97100, GA 98033, etc.) were used in cotton. In this study, using the findings of previous studies (Zhang et al., 2001; Sakhanokho et al., 2001, 2004; Ikram, 2005) for induction and proliferation of embryogenic calli and a new method for development of somatic embryos, four recalcitrant genotypes in Iranian cotton was investigated in comparison with Coker 312.

Before calli entered the embryogenic stage, their proliferation had been stopped and as well, as some calli entered the embryogenesis stage, they turned dark brown. Consequently, these calli yielded somatic embryos when transferred to the MS medium (NH_4NO_4 was removed and the amount of KNO_3 was doubled) containing B_5 vitamins, 40 g/l sucrose, 7g/l agar and devoid of growth regulators after 25 days of culture (Figures 1 and 2). Increasing sucrose up to 40 g/l in the MS medium supplemented with B_5 vitamins without hormone can have positive effects on the development and maturity of somatic embryos (Hilarie et al., 2008).

Globular embryos developed into heart-stage and these embryos further developed into late heart-stage, into torpedo-stage and finally, into normal mature cream to greenish cotyledon-stage embryos after 2 weeks (Figures 1 and 2). Among the cotton genotypes, Coker 312, Termez, Sepid and Hashem abad were found to be embryogenic (Table 2). The genotypes Termez, Sepid and Hashem abad produced somatic embryogenesis on MSB_1, MSB_2 and MSB_5 media with frequencies ranging from 1.85 to 23.33%. The calli produced somatic embryogenesis on the MSB_4 medium with frequencies

Table 1. Effect of various media on color, texture and callus induction percentage of five cotton cultivars.

Genotype	Treatment	Texture	Color	Callus induction (% + SD)
	MSB1	Granule	Yellow to light green	70.00 ± 5.00^{b}
	MSB2	Granule	Yellow to light green	65.00 ± 5.00^{b}
Hashem abad	MSB3	Compact	Yellow to brown	46.66 ± 5.77^{c}
	MSB4	Friable	Light yellow to brown	80.00 ± 5.00^{a}
	MSB5	Loose	Cream white	88.33 ± 2.88^{a}
	MSB1	Granule	Cream to green	91.66 ± 2.88^{a}
	MSB2	Friable	light green	91.66 ± 2.88^{a}
Sepid	MSB3	Compact	Cream to white	80.00 ± 5.00^{b}
	MSB4	Loose	White	76.66 ± 2.88^{b}
	MSB5	Granule	Cream to white	83.33 ± 7.63^{ab}
	MSB1	Friable	Yellow to green	81.66 ± 5.77^{a}
	MSB2	Friable	Light yellow	73.33 ± 2.88^{b}
Kerman	MSB3	Compact	Light brown	63.33 ± 2.88^{c}
	MSB4	Loose	Yellow to brown	71.66 ± 2.88^{b}
	MSB5	Compact	Cream white	86.66 ± 2.88^{a}
	MSB1	Friable	Cream green	100 ± 0.00^{a}
	MSB2	Friable	Cream green	98.33 ± 2.88^{a}
Termez	MSB3	Hard	White	75.00 ± 5.00^{c}
	MSB4	Compact	Cream white	88.33± 2.88^{b}
	MSB5	Friable	light green	96.66 ± 2.88 ^{a}
	MSB1	Granule	Yellow to green	100.00 ± 0.00^{a}
	MSB2	Granule	Yellow to green	100.00 ± 0.00^{a}
Coker 312	MSB3	Friable	Light brown	80.00 ± 5.00^{c}
	MSB4	Friable	yellow brown	100.00 ± 0.00^{a}
	MSB5	Friable	Cream green	88.33 ± 2.88 ^{b}

Values represent means ± SD of percentage of callus induction. Means within columns with the same latter were not statistically different at $p < 0.05$ according to the Duncan's multiple rang test (one- way ANOVA). 20 explants/replication and 3 replication /treatment were used.

ranging from 17.36 to 66.66% for Coker 312, Termez and Sepid (Table 2). However, no somatic embryogenesis was observed in the genotype Kerman among all the media.

Coker 312 showed the highest somatic embryogenesis induction (66.66 to 94.33%) on all the media. The genotypes Hashem abad (0 to 9.73%), Termez (2.22 to 24.40%) and Sepid (0 to 22.28%), which showed somatic embryogenesis induction only on the selected media, had much lower percentage than Coker 312 (66.66 to 94.33%). Genotypes Coker 312, Termez and Sepid were tetraploid, but Hashem abad and Kerman genotypes were diploid. The results of the study indicated that, the used tetraploid genotypes were more embryogenic than the diploid genotypes. This result is in agreement with the results of Sakhanokho et al. (1999) (Table 2).

It is believed that different factors influence cotton regeneration such as explant source, medium types, temperature, light intensity and dark conditions (Finer, 1988; Agrawal et al., 1997; Mohuiddin et al., 1997; Gupta et al., 1997; Zhang et al., 2000; Sakhanokho et al., 2001; Mishra et al., 2003; Singh et al., 2003; Ikram-ul-Haq, 2005).

According to previous reports, 2,4-D is necessary for the induction of somatic embryogenesis in cotton and other plants (Davidonis and Hamilton, 1983; Chen et al., 1987; Zhang and Li., 1992; McKersie and Brown, 1996; Guis et al., 1998; Kumar and Pental, 1998; Choi et al., 1999; Zhang et al., 1999, 2000).

Based on the results of this study and the results of Zhang et al. (2001), Zt can improve somatic embryogenesis process in cotton. According to recent advancements regarding somatic embryogenesis and cotton regeneration (Trolinder and Xhixian, 1989; Firoozabady and DeBoer, 1993; Hazra et al., 2000; Sakhanokho et al., 2001; Mishra et al., 2003; Ouma et al., 2004; Jin et al., 2006), as well as the findings of this research which showed that, somatic embryogenesis percentage of Termez, Sepid and Hashem abad were significantly low in comparison to Coker 312, somatic embryogenesis and plant regeneration in species of *Gossypium* genus was closely related to genotype.

Table 2. Comparison of cotton cultivars for the mean percentage of somatic embryogenesis in five media types.

Genotype	Medium	Embryogenic callus with embryos (%)
	MSB1	9.73 ± 4.90[a]
	MSB2	5.34 ± 4.63[ab]
Hashem Abad	MSB3	0.0 ± 0.0[b]
	MSB4	0.0 ± 0.0[b]
	MSB5	1.85 ± 3.20[b]
	MSB1	12.67 ± 2.70[bc]
	MSB2	9.06 ± 3.05[c]
Sepid	MSB3	0.0 ± 0.0[d]
	MSB4	17.36 ± 3.54[ab]
	MSB5	22.28 ± 5.11[a]
	MSB1	0.00 ± 0.00[a]
	MSB2	0.00 ± 0.00[a]
Kerman	MSB3	0.00 ± 0.00[a]
	MSB4	0.00 ± 0.00[a]
	MSB5	0.00 ± 0.00[a]
	MSB1	23.33 ± 2.88[a]
	MSB2	15.26 ± .45[b]
Termez	MSB3	2.22 ± 3.84[c]
	MSB4	24.40 ± 5.84[a]
	MSB5	10.35 ± .30[b]
	MSB1	88.33 ± 2.88[ab]
	MSB2	86.66 ± 2.88[b]
Coker 312	MSB3	79.37 ± 6.35[c]
	MSB4	66.66 ± 2.88[d]
	MSB5	94.33 ± .18[a]

Data are the means of 3 replication, vertical bars represent standard deviation. Means within columns with the same latter were not statistically different at $p < 0.05$ according to the Duncan's multiple range test (one- way ANOVA).

The recalcitrance property in cotton not only slows down the expansion process in transgenic cotton, but also limits its genetic base (Syed Sarfraz et al., 2004). Although, efficiency of regeneration through somatic embryogenesis has been highly developed, there are often many problems with cotton regeneration such as high frequency of abnormal embryos and low proportion of transforming somatic embryos to plantlet (Ikram-ul-Haq, 2005).

After 3 weeks, the regenerated embryos were separated from parental calli and grown on the MS medium supplemented with B_5 vitamins and 0.1 mg/l GA_3 (Figure 1d). Even though integrated root and shoot meristem were seen in the embryos, simultaneous development of root and shoot was infrequently observed. More often, the shoot first emerged from the embryo and subsequently produced roots on the same medium (Figure 1d). The Germination rates of somatic embryos of Hashem abad, Sepid and Termez ranged from 0 to 11.66% among the different treatments, but the germination rates of somatic embryos of Coker 312 was 58.33%.

The mentioned observation supported by previous studies, indicated that individual genotypes within a given species vary greatly in embryogenic capacity (Merkele et al., 1995). Such genotypic differences in embryogenic capacity might reflect current differences in the ability to activate key elements in the embryogenic pathway. In this study, the optimum medium for the proliferation of embryogenic calli was MS medium containing B_5 vitamins, 1 mg/l 2,4-D, 0.5 mg/l Kt and 0.5 mg/l Zt (Zhang et al., 2001) and the optimum medium for the development of somatic embryos was MS medium (KNO_3) containing B_5 vitamins and 40 g/l sucrose without growth regulators.

These results are in agreement with those by Trolinder and Xhixian (1989) and Zhang et al. (2001) who classified cotton varieties on the basis of somatic embryogenesis and plant regeneration into four categories. First, were the varieties with high ability for somatic embryogenesis and

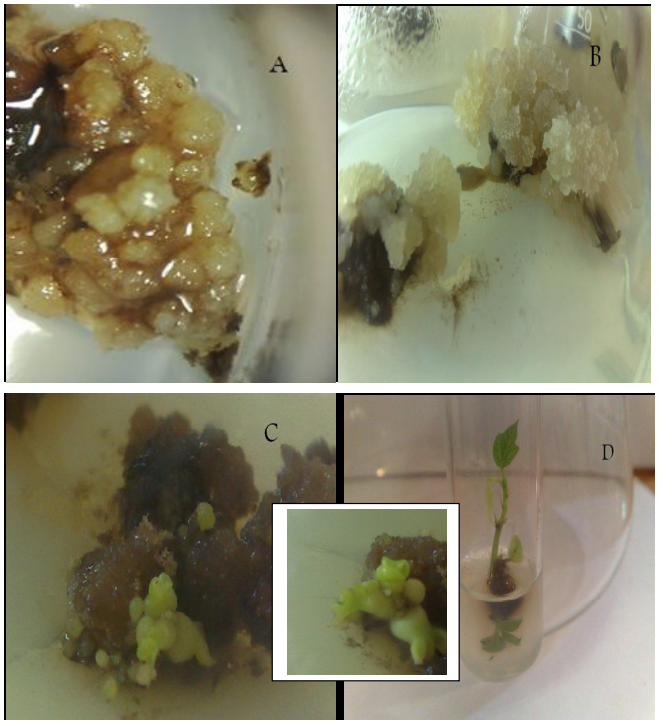

Figure 1. Somatic embryogenesis and plant regeneration of *Gossypium* spp. (A), Pre-embryogenic cultures; (B), non-embryogenic cultures; (C), somatic embryos at various developmental stages; (D) germination of somatic embryos.

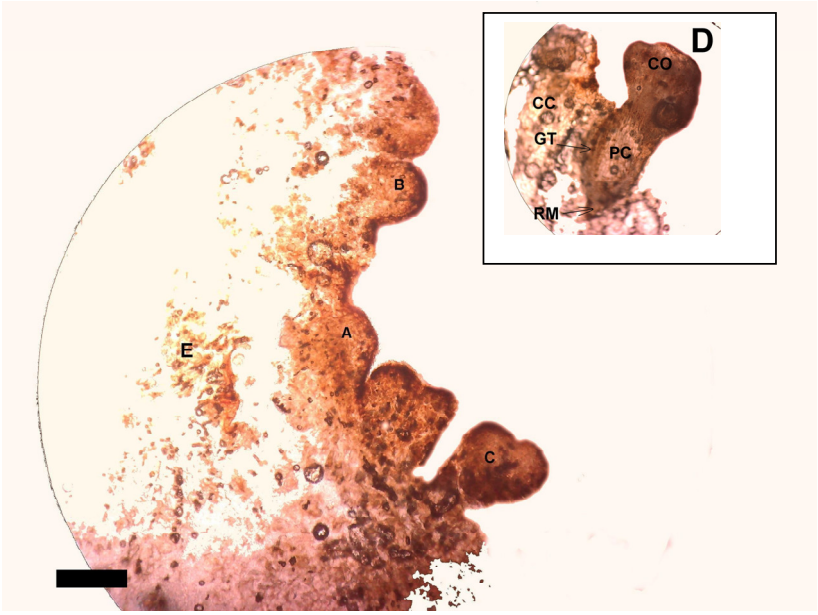

Figure 2. (A to E), Histology of somatic embryogenesis of Coker 312. A and B, longitudinal section of globular embryo development on the surface of the callus; C, longitudinal section of late heart-shaped embryo; D, longitudinal section of somatic embryo (cotyledonary stage) showing distinct cotyledons with root meristem (bar =1 mm); E, non-embryogenic cells; CO, cotyledons; HY, hypocotyl; GT, ground tissue; RM, root meristem; PC, procambium; CC, callus cells.

plant regeneration, such as Coker 201, Coker 312 which have become the model varieties in cotton tissue culture and genetic transformation. The second class was those with moderate ability for somatic embryogenesis and plant regeneration that could produce some embryos and plantlets after many subcultures. Many varieties are included in this class for example, Coker 310, Siokra 1-4, Coker 315, etc. The third class had poor ability, although somatic embryogenesis could be found but showed no regeneration. The fourth class included some genotypes from embryo formation that had not been observed. Termez, Sepid and Hashem abad genotypes that were used in this study belonged to the third class because they had very low somatic embryogenesis and regeneration. Kerman genotype, because did not have somatic embryogenesis, belong to the four class, but Coker 312 which led towards high and normal somatic embryogenesis and plant regeneration belong to the first class.

Histological studies

Histology of somatic embryo-producing location of cotyledons approved that, the induction of the development process was embryogenic and not organogenic in nature. Light microscopic observations of embryogenic accumulation exposed the existence of nodular structures including cytoplasmic cells at the callus surface. The progress of somatic embryos appeared to have evolved through typical globular-, heart-, torpedo-shaped and cotyledonary stage embryo completion. The first and basic sign of embryogenesis was marked by the appearance of globular structures that were joined to the surface of the callus by a separate stalk (Figure 2 a, b). The heart-stage embryo (Figure 2c), which was bilaterally symmetrical also showed a broad suspensor-like stalk. Some of the structures also had vascular texture with unipolar meristems which finally developed into roots. The densely stained meristematic area was often thoroughly surrounded by ground tissue. At this process, completion of clear bipolar embryos with organized shoot and root segment was seen (Figure 2d). The cotyledonary-stage embryos showed the presence of prominent cotyledons (Figure *2*d).

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