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BRIEF COMMUNICATION

Cotton somatic embryo morphology affects its conversion to plant

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Abstract

Somatic embryos differentiated from hypocotyl explant in cotton (*Gossypium hirsutum* L.) exhibited very divergent morphologies. Six different types of somatic embryos based on cotyledon development were observed. The growth hormones (2,4-dichlorophenoxyacetic acid and kinetin) used in induction and maintenance media did not affect embryo rooting and germination. The 95 % conversion of normal embryos (with two cotyledons) was achieved, while an overall conversion was only 38 %. Horn shaped embryos failed to exhibit shoot growth. Poorly developed apical meristems were responsible for lower conversion percentages in some of embryo classes. However, regenerated plants phenotypically resembled to seed grown control plants regardless of somatic embryo morphology.

Additional key words: auxins, cytokinins, *Gossypium hirsutum*, micropropagation.

Embryogenesis is a critical stage in the life cycle of higher plants. Large number of genes must be expressed in a highly coordinated manner to ensure that a single cell develop into an organized, multicellular structure capable of surviving dessication and germinating to produce viable seedling. Even if *in vitro* somatic embryogenesis has great similarities with zygotic embryogenesis, there are also obvious differences.

Davidonis and Hamilton (1983) described first successful regeneration of *Gossypium hirsutum* cv. Coker 310. Although the plants were regenerated, the long incubation period, embryo abnormality and low efficiency of embryo formation were undesirable. Further reports described somatic embryogenesis from leaf and petiole tissues (Gawel *et al*. 1986), development of a suspension culture and optimization of embryo development (Trolinder and Goodin 1987), characterization of somatic embryogenesis (Shoemaker *et al.*, 1986) and transformation and regeneration (Umbeck *et al.* 1987, Firoozabady *et al.* 1987, Wilkins *et al*. 2000). The effects of various factors including source of explant, medium type, combinations of growth regulators, temperature, and irradiance have been investigated (Smith *et al.* 1977, Finer 1988, Zhang and Wang 1989, Sakhanokho *et al.* 2001).

Since the first studies on embryogenesis, abnormalities in somatic embryos and cotyledon development have been observed (Tisserat *et al*. 1979). Only a few studies have looked at the effects of culture environments on embryo morphology and conversion (Wetzstein and Baker 1993). Embryo morphology was influenced by the concentration of auxins in the induction medium. Levi and Sink (1991) noted that higher concentrations of naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D; 1 - 10 mg dm^{-3}) significantly increased the frequency of bipolar embryos; however, addition of 2,4-D resulted in greater abnormalities. On the other hand, lower 2,4-D concentrations (from 50 - 10 μM) resulted in lower yield of somatic embryos but increased the normal somatic embryo production with high rate of conversion in alfalfa (Staurt *et al.* 1985). Some workers reported a relationship between somatic embryo morphology and their conversion to plantlets (Buchheim *et al.* 1989, Wetzstein and Baker 1993).

The somatic embryos differentiated from different explants like hypocotyl, cotyledons, epicotyl, radical portions, and immature zygotic embryos (Hussain *et al.* 2004, 2005, Divya *et al*. 2008, Wang *et al*. 2008) of cotton exhibited very divergent morphologies and the purpose of the present study was to establish a relation-

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Abbreviations: CIM - callus induction medium, 2,4-D - 2,4-dichlorophenoxyacetic acid, GA_3, - gibberellic acid, IAA - indole-3-acetic acid; MM - maintenance medium, NAA - naphthaleneacetic acid.
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ship between somatic embryo morphology and conversion into plants.

Cotton (*Gossypium hirsutum* L.) seeds were delinted and sterilized following the procedure described by Hussain *et al.* (2005) with concentrated H_2SO_4 and 0.1 % $HgCl_2$ followed by 5 washings with autoclaved distilled water. Then the seeds were germinated between two sheets of sterilized filter papers in Petri plates, moistened with 2 - 3 cm^3 of autoclaved distilled water and kept at 30 °C in dark for 72 h.

The established procedure for cotton somatic embryogenesis consists of culturing of hypocotyls (Hussain *et al*. 2005) or immature zygotic embryo explants (Hussain *et al*. 2004) from open pollinated cotton plants for 2 months on callus induction medium (CIM) with 0.1 mg dm^{-3} 2,4-D and 0.5 mg dm^{-3} kinetin, followed by 4-week culture on maintenance medium (MM) with 0.1 mg dm^{-3} 2,4-D and 0.1 mg dm^{-3} kinetin. Both CIM and MM consist of Murashige and Skoog major and minor salts with B5 vitamins (Gamborg *et al*. 1968), 3 % glucose, 0.075 % $MgCl_2$, 0.01 % myoinositol. The pH of the medium was adjusted to 5.8 before autoclaving and all media were solidified with 1.4 g dm^{-3} *Phytagel*. After 8 weeks on CIM and 4 weeks on MM, somatic embryos were classified into six developmental classes according to presence of single or fused cotyledons, axis configuration and distinctness of apex. Embryos were then used for conversion studies.

CIM medium was proved to be the best for induction and growth of somatic embryos from many tissue types in cotton as compared to other combinations of growth regulators (Hussain *et al*. 2004). Therefore, this combination was subsequently used as standard for induction of embryogenic calli, while MM was used for growth and maintenance of somatic embryos.

Somatic embryo germination was carried out as described by Hussain *et al.* (2004) on sterile surgical cotton, saturated with Stewart and Hsu (1977) medium supplemented with 0.1 mg dm^{-3} indole-3-acetic acid (IAA) and 0.1 mg dm^{-3} gibberellic acid (GA_3) under a 16-h photoperiod (irradiance of 130 µmol m^{-2} s^{-1}) and temperature of 27 ± 2 °C. Germination was evaluated after 6 weeks and embryos which at least exhibited root growth (Fig. 1*C*) were transferred to 1:1:1 mix of sand, soil and peat moss in plastic pots and covered with plastic bags for maintenance of high humidity. Plants were gradually acclimated, hardened and transferred to greenhouse.

The callus consisted of a yellow to green, non hard, friable tissues that later separated into distinct structures attached to callus at one side or show a suspensor like structure similarly as showed Vooková and Kormuťák (2006). During culture, the somatic embryos passed through morphological stages usually similar to those in zygotic embryogenesis (Fig. 1; Hussain *et al*. 2004). Griga (2002) had also reported the same finding in *Pisum sativum*. Somatic embryos at this stage were pale yellow; occasionally the apical area of the immature somatic embryo was slightly green (Fig. 1*A*). The green colour associated with globular stage gradually became creamy at torpedo stage (Fig. 1*B*) followed by development of cotyledons, greening of the cotyledons and hypocotyls and formation of the apical areas. Further, somatic embryos exhibited root/shoot polarity (Fig. 1*C*,*D*).

Cotton somatic embryos derived from hypocotyl varied widely in morphology. Somatic embryo morphologies were classified into 6 types: vestigial cotyledon, mono-, di-, poly-, trumpet/fused cotyledon and tubular/horn shaped embryos (Fig. 1 *E-J*). All embryo types were found in the MM media. Our findings are in partial agreement with Buchheim *et al*. (1989) who reported 9 different morphologies in soybean. Kumria *et al*. (2003) have also described a few of such morphologies, such as fused cotyledons, lack of a well defined shoot tip and multiple cotyledons during maturation stage of cotton. A low frequency of somatic embryo maturation in cotton has been reported by several authors (Gawel *et al*. 1986, Shoemaker *et al*. 1986, Gawel and Robacker 1990, Kumar and Pental 1998). The concentration of auxin in the induction media has been reported to influence the morphology of somatic embryos. CIM media did not support different somatic embryo morphologies until and unless, embryogenic callus was transferred to MM medium. These results are in contrast to Ranch *et al.* (1986), who found that higher concentrations of 2,4-D result in an increased production of normal embryos in soybean.

All embryo morphologies were found in MM media which show that auxin concentration in MM media did not affect embryo morphologies. Somatic embryos of all classes can be divided into responsive (showing either root or shoot growth) and non responsive (showing neither root nor shoot growth). Responsive somatic embryos can be further subdivided into embryos showing either root or shoot growth and embryos showing both root and shoot growth. Our results showed that between 5 and 50 % of the embryos in all classes failed to germinate (non responsive) after 6 weeks on germination medium (Table 1). Dicotyledonous embryos showed the highest plantlet percentage (95 %) with both root and shoot polarity, with average root length of 4.5 cm (Fig. 1*D*). In contrast to this, trumpet/fused cotyledonous embryos

Table 1. Percentage of responsive (somatic embryos showing shoot only/ root only/both root and shoot) and non responsive somatic embryos in each embryo category (E, F, G, H, I, J - see Fig. 1)

Embryo category	Responsive shoot	root	shoot and root	root length [cm]	Non responsive
E	-	20	35	3.0	45
F	10	15	45	2.5	30
G	-	-	95	4.5	05
H	15	25	30	4.0	30
I	-	25	25	2.0	50
J	-	55	-	3.0	45

showed the lowest conversion percentage (25 %) with average root length 2.0 cm. Tubular/horn shaped embryos produced the highest percentage of roots (55 %) and completely failed to show any shoot development (Fig. 1*J*). These plantlets did not show shoot development also in soil. We achieved an overall conversion rate of about 38 % while bipolar, normal embryos (dicotyledonous) gave 95 % conversion percentage. This finding is in agreement with that of Lazzeri *et al*. (1987). They obtained a 73 % germination rate for normal embryos compared to 24 % for total population and finally concluded that the ability of somatic embryos to germinate was closely related to embryo normality. On the other hand, Griga (1998) reported that conversion rate was induced by application of 10 μM thidiazuran (TDZ), which induced shoot bud regeneration on embryos without a differentiated shoot apex, allowing to germinate up to 70 % of all harvested somatic embryos with various morphology in pea.

Several reports described the improvement in regeneration efficiency *via* somatic embryogenesis in cotton, but genotype dependent response (Trolinder and Xhixian 1989), prolonged culture period (Jin *et al*. 2006), high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets, and a lack of shoot elongation are the problems still associated with cotton regeneration (Kumria *et al*. 2003). Almost all embryo classes produced root/shoot or both, with the exception of horn shaped embryos (Fig. 1*J*) where embryos produced only roots. This type of horn shaped embryo development has been demonstrated in other embryogenic systems with similar difficulty in plant conversion (Ranch *et al*. 1985, Buchheim *et al*. 1989). It is probably due to poorly developed apical meristems or distorted hypocotyls (Wetzstein and Baker 1993). Likewise, poorly developed apical meristems in sweet potato somatic embryos have been reported but all embryos could produce roots (Chee and Cantliffe 1988)

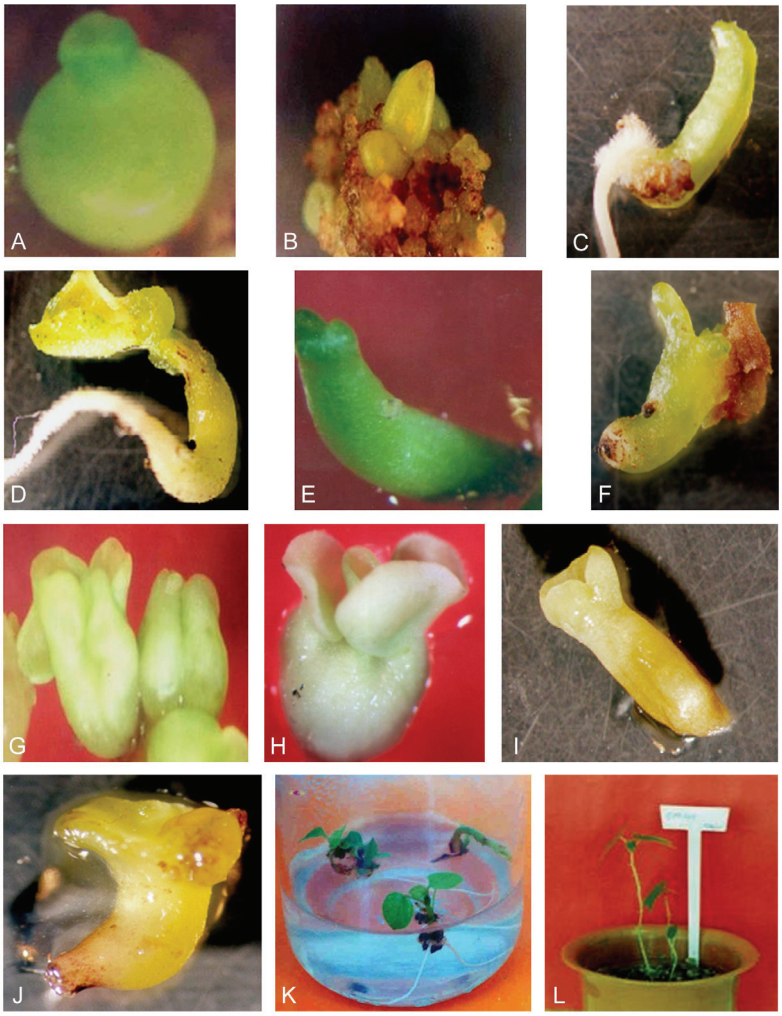

Fig. 1. Different stages during somatic embryogenesis in cotton including different abnormalities: *A* - globular stage, *B* - torpedo stage, *C* - somatic embryo with root, *D* - somatic embryo with root and shoot, *E* - vestigal cotyledon and long hypocotyl, *F* - monocotyledon embryo, *G* - dicotyledons, *H* - polycotyledons, *I* - trumpet/fused cotyledons, *J* - tubular and horn-shaped embryo, *K* - germination of somatic embryos; *L* - plants transferred to pot.

as in this case. Similarly, culture conditions can have marked effect on the development and form of somatic embryos.

After germination, healthy plantlets of 3 - 4 cm height were transferred to a mixture of 1:1:1 sand, silt and peat moss, covered with plastic bags for maintenance of high humidity. These were gradually acclimated, hardened off and transferred to greenhouse after 3 - 4 weeks (Fig. 1*L*).

This study reports the high conversion rate of abnormal somatic embryos into plantlets without any pre-selection, treatment or medium manipulation. Plantlets when transferred to greenhouse phenotypically resembled the seed grown control plants regardless of origin of embryo class. We have evaluated the morphology and conversion performance of different morphological types of somatic embryos in cotton. Pre-selection of highly convertible embryo forms is a possible option. However, this strategy is labor extensive and requires experience; otherwise it may necessitate the culling and loss of numerous somatic embryos. Low conversion rates can be improved in some systems by treatment of somatic embryos with conversion enhancing treatments or media manipulations. For example, Kumria *et al*. (2003) employed nutrient manipulation and dehydration stress which triggered the differentiation process of cotton embryogenic calli and resulted in reduced culture period and conversion of large competent cells into embryos. Therefore, culture manipulations with the goal of high frequency production of quality embryos may be a fruitful approach for the eventual application of currently unusable somatic embryogenenic systems in cotton.

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