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PJBS

ISSN 1028-8880

# Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 8 (1): 141-145, 2005
ISSN 1028-8880


# Recurrent Somatic Embryogenesis and Twin Embryo Production in Cotton

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**Abstract**: Current approaches of cotton improvement include the use of genetic engineering, but progress in this area is limited because of notoriously recalcitrant nature of most elite cotton cultivars in tissue culture. A well-established regeneration system is desired for the improvement of cotton through genetic engineering. As it is reported that somatic embryos have been obtained from the regenerable lines of Coker 312 and Coker 315 but the problem with these varieties was the loss of embryogenic nature of callus with the passage of time. A procedure of recurrent somatic embryogenesis and twin embryo production in *Gossypium hirsutum* L Cv. Coker 312JS is being reported for the first time from the older somatic embryos. Calli were subcultured regularly. Embryogenic capacity was remained stable for more than two years. Similarly, twin embryo production was seen in second cycle of this system. This will help to propagate embryos for the development seed technology and gene transfer system.

**Key words:** Twin embryos, *Gossypium hirsutum*, recurrent somatic embryogenesis, cotton improvement

## INTRODUCTION

Cotton (*Gossypium hirsutum* L.) is an agricultural and technological term used to describe the cultivated species of the genus *Gossypium*, which is placed in the Malvaceae family. As one of the major world crops, cotton has a long history of improvement through breeding, with sustained long-term yield[1]. Most of this progress has been achieved through conventional breeding, but current approaches have been directed at improving both agronomic efficiency and product quality using molecular means. The two major goals of current genetic engineering in cotton are conferring insect resistance and tolerance to more environmentally acceptable herbicides[2]. Efforts to transform cotton have been hampered by the recalcitrant nature of many elite cultivars in terms of regeneration. Therefore, developing a reliable and efficient regeneration system will constitute a major step towards improving this economically important crop through genetic engineering.

Somatic embryogenesis resulting in regeneration of whole plants is an important step in any plant transformation scheme. Successful stable transformation requires that a single cell gives rise to a plant. The ideal transformation scheme is that done via somatic embryogenesis because from callus, each transformed cell has the potential to produce a plant. The first report of regeneration of *G. hirsutum* was by Davidonis and Hamilton[3], who used polyploidy cotyledonary tissue. Somatic embryos developed spontaneously and plants were obtained, but the experimental conditions leading to these results were not well defined. Since then, progress has been made and somatic embryogenesis and regeneration of plants in cotton have been reported by several workers[4-8]. However, regeneration of plants in Gossypium species is highly genotype dependent[5]. Most of successful regeneration studies have utilized the variety, "Coker 312" and related lines. Regeneration from cotton germplasm, in general remains a sought after goal that will pave the way for more efficient transformation in *Gossypium* species. There have been various types of explants used to evaluate regeneration potential, including hypocotyls[4,6,9,10] cotyledons[11-13] and stems[12].

The effects of various factors believed to impact regeneration in cotton have been investigated. These factors include source of explant, medium types, amounts, types and combinations of hormones or growth regulators, temperature, light intensity and dark conditions[8,11,13-15].

Application of plant biotechnology is gaining momentum in developed as well as developing countries to improve the agricultural crops. For an agricultural country like Pakistan, it offers a tremendous scope for its agricultural crops by the application of transgenic plant

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technology. In order to develop a successful programme of practical genetic engineering, it is important to develop a system for the recovery of large numbers of whole plants from the explant tissues.

## MATERIALS AND METHODS

**Seed sterilization:** For delinting of seeds, concentrated commercial $H_2SO_4$ was used @ of 100 mL/1 kg of seeds. After adding acid, the seeds were continuously stirred with the help of spatula for 10-15 min until shiny surface of seeds appeared. Then some water was added and the stirring was continued for a few seconds. Seeds were washed 5 times with tap water to remove the acid completely. At that point, the seeds, which floated at the surface of water, were removed.

Delinted cotton seeds were sterilized using autoclaved magenta boxes. After adding water, few drops of Tween-20 were added and giving vigorous shaking washed the seeds. Three washings with autoclaved water followed this. Surface sterilization was done by using 0.1% $HgCl_2$. The seeds were treated with $HgCl_2$ for 20 min followed by 5 washings with autoclaved distilled water. The seeds were soaked in autoclaved distilled water for one hour. All the sterilization work was performed in a laminar airflow cabinet.

**Callus initiation:** Delinted, sterilized cotton seeds were germinated on sterile blotting papers in petri dishes under 30 μE m-2 s-1 light and 28±2°C. Three days after the emergence of radical, the hypocotyl was sectioned into 4 mm lengths. Similarly, epicotyle and radical portions were also isolated. Cotyledonary leaves were cut into 3-4 mm size. The pH of the media was adjusted to 5.8 prior to autoclaving for 20 min at 121°C and 15 lbs psi pressure. All growth regulators were added to media prior to autoclaving. Explants were placed on the media in petri plates and incubated under a 16:8 hours photoperiod at 60-90 $\mu E\ m^2\ s^{-1}$ and 28±2°C. Calli were visually evaluated one month after initiation and then again after two months of culture. Fast growing callus was dispersed in sterile water and examined under stereomicroscope for the characterization of different stages of embryogenesis, comparable with those observed *in vivo*. The basis of selection of a genotype for further studies was the level of embryogenicity, simplicity, convenience and the time required to achieve it. Similarly, in all subsequent experiments, for the development of a model system, hypocotyl was selected as a best explant for callus initiation in preliminary screening. Callus subculturing was done after every 2 weeks on the same media for further proliferation.

## RESULTS AND DISCUSSION

**Callus initiation:** Many Callus Initiation (CIM) media with different growth hormone combinations (Table 1) were used. One medium[16,17] was selected and used for callus induction the explants used in this study. However, both hypocotyle and cotyledon gave excellent results but hypocotyle were better callus producers. These results are in agreement with those obtained by Smith *et al.*[11]. Furthermore, callus initiation and production was maximized by increasing contact between the cut surface of explant and media after longitudinally dissected explants (hypocotyle) placed on the media. Hypocotyle produced more and friable callus than other explants used. Radicle was found to be the slowest callus producing explant but the callus produced was of fine quality.

Modified MS medium with 01+0.5 mg $L^{-1}$ 2,4-D and kinetin respectively seemed to be the most favourable for the induction and growth of somatic embryos from all embryogenic tissues than other combinations of growth hormones used in this study. MS medium with 0.5 mg $L^{-1}$ kinetin resulted in a higher %age of embryogenic calli, but tissues became brown at an earlier time and callus friability was less. Similarly, embryogenic tissue also lost embryogenecity during subculturing on media containing 0.5 mg $L^{-1}$ kinetin. Media containing 0.1 mg $L^{-1}$ kinetin restricted further growth and development of somatic embryos. So the growth regulator regimes subsequently used as standard for induction of embryogenic calli was 0.1+0.5 mg $L^{-1}$ 2, 4-D and kinetin respectively while media used for the growth and maintenance of somatic embryos was 0.1+0.1 mg $L^{-1}$ 2,4-D and kinetin, respectively.

**Recurrent somatic embryogenesis and twin embryo production:** Recurrent somatic embryogenesis is the production of new somatic embryos from the old germinating somatic embryos. When germinated somatic embryos were transferred on media for plantlet formation (Table 1), two things happened, one is the growth of somatic embryo while other is the production of new somatic embryo directly or with very short period of callusing from the old somatic embryo (Fig. 1). These cultures have been maintained for two years during which this recurrent somatic embryogenesis remained stable producing a lot of somatic embryos continuously as studied by Lupotto[18,19]. Similarly twin embryos were also found among the recurrent somatic embryos (Fig. 2). These twin embryos germinate well but gave rise to abnormal plantlets. Twin embryo production was a rare phenomenon. We encountered a few twin embryos, which result in normal plants when separated and transferred to soil during this study.

A B

Fig:. 1: Recurrent somatic embryogenesis, A and B: Recurrent somatic embryos at different stages. Recurrent somatic embryogenesis a beneficial phenomena cutting lengthy regeneration process short. During this, new somatic embryos produced continuously with (very short) or without an intervening callus phase

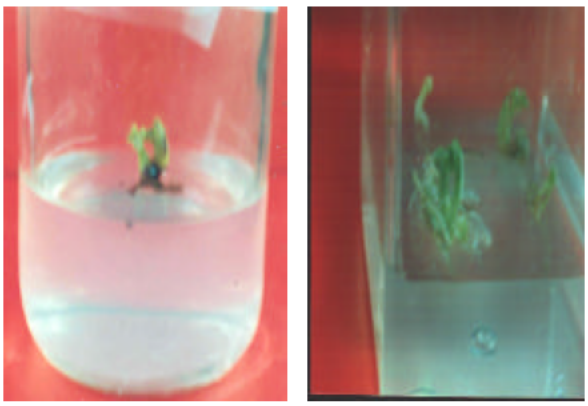

Fig. 2: Twin embryo production in cotton, twin embryos joined together at the base are produced during recurrent somatic embryogenesis. A few produced normal plants when separated and transferred to media

Recurrent somatic embryogenesis was originally described by dos Santos *et al*.[20] and by Lupotto [18,19]in alfalfa. Actually this recurrent somatic embryogenesis is a kind of direct embryogenesis and this sort of embryogenesis appears to be associated with greater genetic and cytological uniformity[21] and it takes less time to complete cycle of embryogenesis than indirect embryogenesis.

Upon transfer of somatic embryos to SEM-I, SEM-II, SEM-III and SEM-IV medium, new somatic embryos repeatedly formed in great number directly on older, regenerating somatic embryos with (very short) or

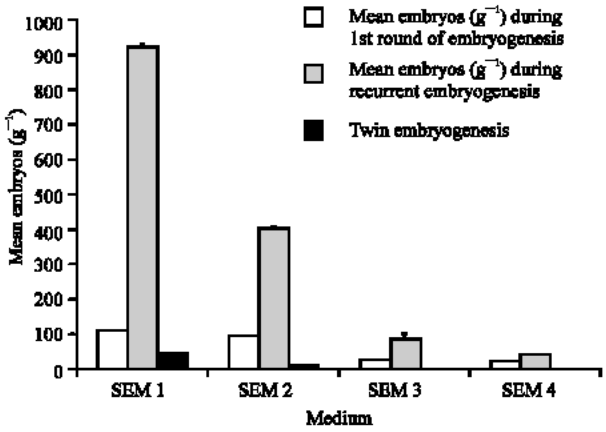


Fig. 3: Values represent means±SD of the number of embryos per gram of embryogenic callus obtained for each treatment. Each treatment consist of 5 plates replicated thrice for a total of 15 plates.

Table 1: Growth hormone concentration used in different medium

| Medium | NAA | 2,4-D | Kinetin |
|---|---|---|---|
| SEM-I | - | 0.5 | 0.1 |
| SEM-II | - | 0.1 | 0.5 |
| SEM-III | 0.1 | - | 0.5 |
| SEM-IV | 0.5 | - | 0.5 |

without an intervening callus phase in a cycle lasting about 30-40 days (Fig. 3), thus reducing time period which is about 100-130 days in 1st cycle of embryogenesis. The embryogenic capacity of these cultures remained stable for two years during which time, these were monitored regularly. New embryogenic culture could very conveniently be started repeatedly from these old cultures. Recurrent somatic embryogenesis has been shown to be a useful system for the transformation of plants[22]. Furthermore, it could be integrated into artificial seed production technology[23-26] as a system capable of providing an unlimited number of somatic embryos for research efforts towards cell engineering, gene transfer and cellular developmental biology[21].

Although cotton somatic embryogenesis has been reported by many workers, recurrent somatic embryogenesis and twin embryo production has not reported yet.

The recalcitrance of commercial cotton varieties to tissue culture has been a major stumbling block for transgenic cotton development. In addition, the fact that current generation of transgenic cotton is based only on the Coker lines could lead to a genetic bottleneck problem. The tissue culture protocol used in these studies has generated large amounts of embryogenic calli and copious somatic embryos. Although the regenerated plants were low, the protocol has the potential to be used for the regeneration of local commercial cultivars. A minimal

amount of genetic change is associated with the protocol as the somatic embryos and regenerated plants were mostly visually genetically uniform.

This system is simple, manipulatable and has shown the potential that large number of somatic embryos can be produced in a short period for studies of gene manipulation, somaclonal variation and developmental studies of embryos.

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