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Somatic Embryo Germination and Plant Development from Immature Zygotic Embryos in Cotton

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Abstract: Successful cotton plant transformation depends on regeneration of plants from transformed cells. Recalcitrance of cotton to tissue culture has not only slowed the development of transgenic cotton but also narrowed its genetic base. Keeping this in view, an efficient *in vitro* plant regeneration system characterized by bulk, rapid and continuous production of somatic embryos using immature zygotic embryos, taken at various stages of development from ovules as explant has been developed in cotton. One of the drawbacks of using this is the requirement of large number of high quality immature zygotic embryos. To address this problem, we have developed a procedure that generates highly homogeneous populations of embryogenic calli by selectively propagating a small number of regeneration proficient calli derived from immature zygotic embryos. Stages showing the early cotyledon development cultured on modified Murashige and Skoog media proliferated intensely. Rapid callogenesis was observed from these immature zygotic embryos. To induce germination and plantlet growth, embryoids were placed on sterile processed cotton, saturated with Stewart and Hsu media. Upon development of roots and leaves, plantlets of 3-4 cms were potted in 1:1:1 mixture of sand, silt and peat moss under high humidity and further hardened under green house conditions. Using this system, we have been able to regenerate approximately 70% of healthy plantlets.

Key words: Callogenesis, somatic embryogenesis, immature zygotic embryos, cotton

INTRODUCTION

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. A prerequisite to transformation of cotton is the development of methods to regenerate efficiently, healthy fertile plants from tissue culture. Tissue culture methods in cotton have lagged behind those for the other major crops, with the exception of soybean (*Glycine max*),[1]. Somatic embryogenesis was observed for the first time, with callus suspension culture of *Daucus carota*. *In vitro* development of somatic embryo had great similarities with zygotic embryogenesis. Despite of the many similarities between zygotic and somatic embryogenesis, there are also obvious differences. Ammirato[2] provided a general discussion of somatic embryogenesis as a method of regeneration.

A number of successful procedures describing regeneration of cotton plants from tissue culture have been reported in recent years. Davidonis and Hamilton[3] described first successful regeneration of *G. hirsutum* L. Cv. 'Coker 310'. Although plants were regenerated, yet the long incubation period, embryo abnormality and low efficiency of embryo formation were undesirable. Numerous reports on plant regeneration in cotton have followed first report, which include induction of embryogenesis from leaf and petiole tissues[4], development of a liquid suspension, optimization of initiation of embryogenesis, embryo development[5], characterization of somatic embryogenesis[1] and transformation and regeneration[6,7]. In all the reports mentioned above, no clear cut characterization of various stages of somatic embryogenesis was given so that one could easily recognize different stages in development of cotton somatic embryos, but these studies include all the stages of somatic embryogenesis occurred during the developmental process.

MATERIALS AND METHODS

Seeds of *G. hirsutum* L. Cv. C-312JS were grown in crop season 2002 under green house conditions. Flowers were tagged on first day of anthesis. Bolls were collected

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from plants after 7-14 days post anthesis, sterilized by dipping in 70% ethanol for 5-10 min with continuous slow shaking, followed by treatment with solution of 0.1+0.1% $HgCl_2$ and SDS for 5 min followed by 3 rinses with sterile distilled water. Zygotic embryos excised under dissecting microscope at various stages of development, gently taken with fine forceps and plated on modified MS media as stated by Trolinder and Goodin[5] for callus induction.

Five immature zygotic embryos were cultured in 30 mL of media plated in 90 mm petri plates. In this experiment, there were three replications and each replication consisted of 4 petri plates (20 immature embryos per replication). This experiment was repeated twice. One experimental set was placed in dark at 28±2°C for one month to induce callogenesis while other experiment was incubated in light under same conditions. The media used for callus induction was that of Murashige and Skoog[8] supplemented with 100 mg L^{-1} myo-inositol, B5 vitamins[18], 0.75 g L^{-1} $MgCl_2$ and 30 g L^{-1} glucose. The medium was gelled with 1.6 g L^{-1} gerite. pH of the media was adjusted to 5.7-5.8 prior to autoclaving. Four growth regulator regims either singly or in combination were evaluated: 0.05, 0.1 mg L^{-1} of 2,4-D and 0.1, 0.5 mg L^{-1} kinetin as described by Trolinder and Goodin[5]. All growth regulators were added to the medium prior to autoclaving.

Callus initiation and maintenance: Calli were evaluated first after 4 weeks and then after 8 weeks of culture and visually scored for appearance and growth of callus. Similarly, calli were observed weekly for embryogenecity. Embryogenic callus was subcultured onto same media for increase and maintenance. During this, callus growth pattern, different stages of callus development, embryo and cotyledonary development was studied for proper characterization of somatic embryogenesis.

Embryo germination and Plant regeneration: Somatic embryo germination was carried out on different supportive materials like sand; sawdust, combination of both, vermiculite and processed cotton saturated with Stewart and Hsu medium with 0.1 mg L^{-1} IAA and 0.1 mg L^{-1} GA_3. Upon development of roots and leaves, the plantlets were transferred to magenta boxes containing half strength hormone free MS media with 1.5% sucrose. After three weeks, healthy plantlets of 3-4 cm height were transferred into 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 green house after 3-4 weeks.

RESULTS AND DISCUSSION

Callus initiation and maintenance: Immature zygotic embryos taken at various stages of development from bolls of *G. hirsutum* L. were classified into five stages: the globular and torpedo stage, followed by heart, cotyledonary and immature stages (Fig. 1). All the stages were cultured on MS basal medium supplemented with 2, 4-D and kinetin separately and in combination. The cotyledonary and immature embryo stages were found to be the most suitable for the initiation of an embryogenic callus. In fact, these stages led to cellular proliferation of 83% and 97%, respectively (Data not shown). When the embryos were taken at earlier stages (globular, torpedo or heart), the frequencies decreased to 0-31%. Similarly, initiation of callus was apparently not affected whether the embryos were intact or sectioned. Plates incubated in light did not show any callusing, first appeared yellow, turned brown and died. First three stages incubated in darkness did not show any callusing and died (turned brown) within 15 days of culture, while cotyledonary and immature stages, first show some growth, elongate, swell up, burst and start callus formation, which was a slow process.

Somatic embryo initiation and development: Somatic embryogenesis is a process by which cells develop into differentiated mature embryos through characteristic stages. Later on, all the calluses were shifted to light after 4 weeks. Again after 4-5 weeks of culture in light, somatic embryos arose as fused masses, differentiated into distinct structures attached to callus at one side (Fig. 2A). All the stages of somatic embryo development were found but slightly larger than those of zygotic embryos. Subculture of embryogenic masses continued to produce additional embryos that were in turn subcultured to get more somatic embryos. Initially, callus production was slow but it was increased greatly with the passage of time. After three months of culture, almost all the callus showed embryo like structures with yellow to greenish nodular structures, which developed, in most cases, into somatic embryos. Some of the embryos developed as far as the cotyledonary stages at which the separation and greening of the cotyledons was observed. At the same time, various malformations were also observed on many somatic embryos.

Somatic embryos rarely have the precise form of zygotic embryos. They are often bipolar (Fig. 2B) with cotyledons of unequal size, with multiple cotyledons or fascinated structures that may be abnormal cotyledons[9-12].

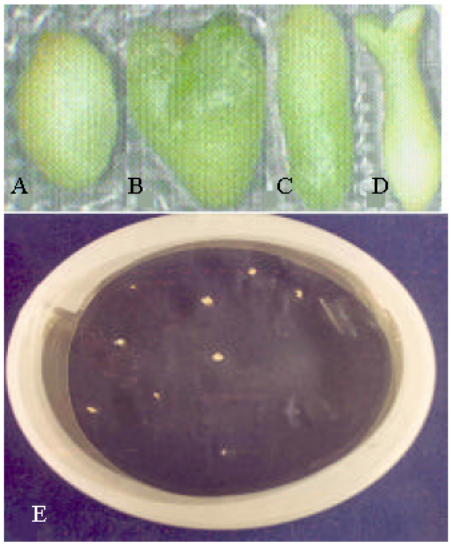

Fig. 1: Different stages of immature zygotic embryos of cotton coloured line diagram showing different stages of immature zygotic embryos isolated from cotton bolls present in the plate. Only late cotyledonary stage is well prominent with expanded cotyledons. Different stages are A: Globular stage B: Heart stage C: Torpedo stage D: Early cotyledonary stage E: Late cotyledonary stage

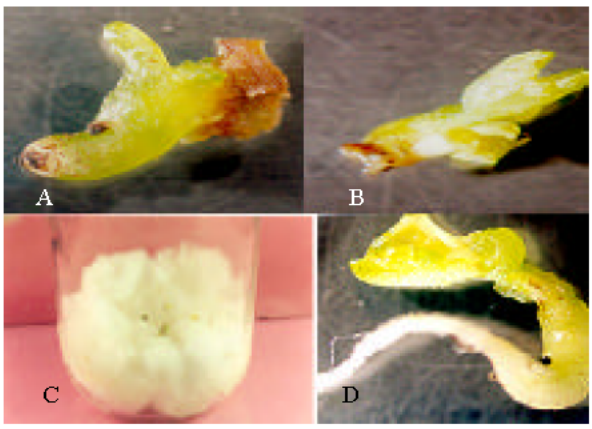

Fig. 2: Different stages of somatic embryo maturation and germination A: Somatic embryo showing root and shoot polarity B: Somatic embryo separated from callus C: Somatic embryos are being germinated on processed cotton D: Germinated somatic embryo with developed root and shoot

Modified MS medium with 0.1+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

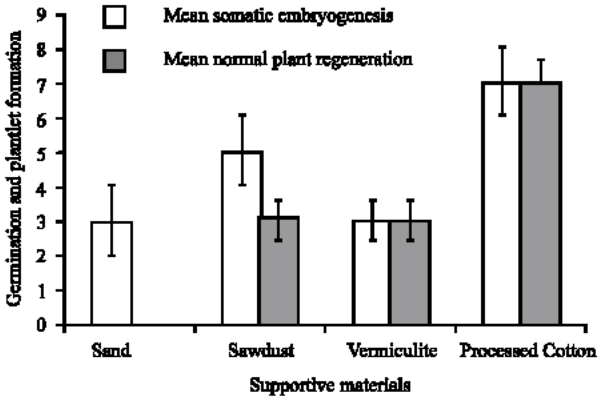

Fig. 3: Different inert supportive materials were used for germination and plantlet formation from somatic embryos

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. Somatic embryos isolated from callus and cultured on semi solidified MS basal medium without growth regulators had further cycle of indirect embryogenesis. Similar observations have been reported for immature zygotic embryos in *Picea gluaca*[13], *Pinus elliottii*[14] and *Pinus patula*[15].

Morphology of somatic embryo: Zygotic and somatic embryos (embryoids) from entirely different origins have a strikingly similar sequence of embryos formation: a globular stage, a heart stage, a torpedo stage. The growth mass derived from immature zygotic embryos consisted of a yellow to green, non-hard, friable tissues that later separated by differential growth into distinct structures attached to callus at one side or by a suspensor like structure. Somatic embryos exhibited root shoot polarity. Subculture of embryogenic masses continued to produce additional embryos.

Embryo germination and development: Previous reports had shown that embryos developed into plants better when placed on vermiculite saturated with medium rather

than on semi solid gelrite/agar media. Additionally, embryo loss due to small size and precocious germination was a problem in somatic embryo growth. Somatic embryos separated from callus and cultured on half strength MS media with out growth hormone had no further development. Similarly, the embryos kept *in situ* (I-e, on callus) show necrosis and general browning of the tissue after longer periods of subculture. We therefore, tried to devise a situation whereby transferred somatic embryos could continue development, germinate properly and form healthy plants. SH[16], BT[17] and MS[8] media have been reported suitable in the normal maturation of somatic embryos by different workers. No worker has given due attention to supportive materials. All had used either solidified medium or vermiculite saturated with media. In this study, we have used different inert materials like sand, sawdust, processed cotton separately and in combination and were successful in regenerating 70% of healthy plantlets (Fig. 3). Upon development of roots and leaves, the plantlets were transferred to magenta boxes containing half strength hormone free MS media with 1.5% sucrose. After three weeks, healthy plantlets of 3-4 cms height were transferred into 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 green house after 3-4 weeks (Fig. 2C and 2D).

Somatic embryogenesis was rapidly induced from immature zygotic embryos of cotton. The young cotyledonary and early immature stages were found to be most appropriate for the induction of undifferentiated tissues (callus). The procedure described in this report for the germination and plant development has an edge over all other procedures reported so far in literature.

This system is simple, manipulatable and has shown production of large number of somatic embryos in a short period, valuable for studies of gene manipulation, plant transformation, somaclonal variation and developmental studies of embryos.

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