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RESEARCH PAPER

An Efficient Culture System for Synchronization Control of Somatic Embryogenesis in Cotton (*Gossypium hirsutum* L.)

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Abstract: Low efficiency of somatic embryogenesis and asynchronous embryo development results in a lot of difficulties to physiological, biochemical, and molecular biological studies of the embryogenesis processes in cotton (*Gossypium hirsutum* L.). A simple and efficient method was developed to improve somatic embryogenesis frequency and synchronous development of mass somatic embryos from cultured cells of the cotton cultivar Coker 201. The embryonic calli obtained after several rounds of subculture were scattered in a liquid medium by shaking for 2 d and then resuspended in the same liquid medium after discarding the larger callus aggregates over a 30 mesh-size-sieve. The suspensions cultured for 14 d were filtered through a 50 mesh-size-sieve and the aggregates over the sieve were incubated for 21 d onto the surface of a Whatman filter paper that was placed on the solid medium containing 2.46 μmol L^{-1} indole-3-butyric acid (IBA) and 0.70 μmol L^{-1} kinetin. The amount of somatic embryos obtained by this system was 15.5-fold and 3-fold higher than that of suspension culture and solid culture without filter papers, respectively. About 70.2% for globular, 52.3% for torpedo-shaped, and 73.0% for cotyledonary embryos were obtained during the culture. The method combining suspension culture and solid culture (with filter paper) proved to be efficient for synchrony of somatic embryogenesis and mass embryo development.

Keywords: cotton; somatic embryogenesis; synchronization control; culture system

Somatic embryogenesis is very similar to zygotic embryogenesis at both morphological and molecular levels and is an important embodiment for totipotency of single somatic cells in higher plants. Since Steward et al. [1] and Reinert [2] first reported independent somatic embryo production from carrot (*Daucus carom* L.) callus cells, somatic embryogenesis has been recognized as a useful system for plant propagation in vitro. It is also a nice model for studying the mechanisms of zygotic embryogenesis and separating specific proteins and genes associated with plant embryo development. However, somatic embryogenesis usually occurs at a low frequency. When somatic embryos are induced from embryogenic callus cells, embryos at different stages of development always coexist in a given culture. This brings a lot of difficulties to the physiological, biochemical, and molecular biological investigations of somatic embryogenesis. To obtain embryos at the same developmental stage, the following methods have been used to select purified embryos at a particular stage from different developmental stages: glass bead filtration with subsequent centrifugation [3], differential sedimentation and filtration through filters with pores of various sizes [4], fractional selection with an equipment of embryonic cell clumps selector [5], filtering with screen disks of various sizes [6], and cold treatment to heterogeneous suspension cultures, followed by sieving through a stainless steel filter [7]. These methods, however, have failed to select somatic embryos or embryogenic cell clusters prior to the globular stage, and separate embryos that have similar diameter, but are at different developmental stages. Among these methods, cold treatment will change the metabolism of embryo development. In recent years, another attempt has been made by making the initial cell population homogeneous in suspension cultures.

Nadel et al. [8] and Torres et al. [9] proposed a synchronized culture method of somatic embryos by abscisic acid (ABA) preconditioning. Hutchinson and Saxena [10] showed that synchronization of embryo development and proliferation of somatic embryos were promoted by acetylsalicylic acid (ASA) combined with thidiazuron (TDZ) treatment. Kumar and Tuli [11] presented a method for developmental synchrony of somatic embryos by a single cycle of myoinositol starvation. However, these methods did not disclose the real information about the mechanisms of somatic embryogenesis at first hand, because

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the effects of these treatments on metabolism during somatic embryogenesis and embryo development were not known. Furthermore, Osuga et al. [12] and Tonon et al. [13] developed a synchronized system for somatic embryogenesis in carrot and *Fraxinus angustifolia*. The initial embryogenic cells or cell clusters that differentiated synchronous embryos at a high frequency were obtained from suspension cultures by sieving and density gradient centrifugation in Ficoll solutions. Unfortunately, this synchronous system of somatic embryogenesis is not only labor intensive, but also has a high risk of contamination because of repetitious filtering and centrifugation.

Cotton (*Gossypium hirsutum* L.) is a species of increasing interest for quality fiber production and is widely grown in many countries. Somatic embryogenesis is an important tool in breeding cotton species because of its great potential in germplasm enhancement [14]. To take the full advantage of somatic embryogenesis, a high-frequency, synchronous embryogenic system is needed. It is essential for investigating the physiological, biochemical, and molecular aspects of embryogenesis, because limited information is available in cotton. Since Davidonis and Hamilton [15] reported plant regeneration via somatic embryogenesis in *G. hirsutum* cv. Coker 310, a number of protocols have been exploited for somatic embryogenesis and plant regeneration from various explants of cotton [16–34]. Although the proficiency in somatic embryogenesis and regeneration of cotton has clearly improved, some technical problems remain. For example, the efficiency of somatic embryogenesis from culture and synchronization in embryo development is low. Of late, a high frequent synchronous embryogenic system in embryogenic callus cultures of *G. hirsutum* cv. Coker 312 has been reported by Kumar and Tuli [11]. Because the development of synchrony relied on a single cycle of myoinositol depletion in liquid culture, the metabolism involved in induction and development of heterogeneous embryoids obtained from this system may be easily changed.

On the basis of embryogenesis inducing culture medium described in previous studies [34, 35], a new method for synchronized and high-frequency somatic embryogenesis in embryogenic callus cultures is reported in the present study. The embryogenic calli with cell division and differentiation can be kept the most identical in this method. In addition, the method avoids negative physiological effects on embryogenic calli or embryoids, such as, cold stress, myoinositol starvation, ABA, and acetylsalicylic acid (ASA) + thidiazuron (TDZ) treatment. The results from this study will be useful in the physiological, biochemical, and molecular investigations of embryo development in cotton.

1 Materials and methods

1.1 Plant materials

Mature seeds of upland cotton (*Gossypium hirsutum* L.) cultivar Coker 201 were ingathered from the farm of Huazhong Agricultural University through self-mating every year, and kept by this laboratory.

1.2 Induction and proliferation of embryogenic callus

Mature seeds were delinted with concentrated sulphuric acid and decoated. They were then surface-sterilized with 0.1% (*W*/*V*) aqueous mercuric chloride ($HgCl_2$) solution for 10 min followed by 4 washes with sterilized distilled water. The surface-sterilized seeds were inoculated onto the MS0 medium for seedling germination and maintained in darkness at 28 ± 1°C. Seven days later, hypocotyls were excised from aseptic seedlings and cut into 5–7 mm segments to be used as explants for somatic embryogenesis. Hypocotyl sections were inoculated on a solid medium MS1 to induce callus. After 35 d, the calli obtained were inoculated on medium MS2 and subcultured every 28 d for the induction of embryogenic calli. Vigorously growing, loose, granular, and light yellow embryogenic calli were transferred onto medium MS3 for proliferation and somatic embryogenesis as described previously by Jin et al. [34]. The embryogenic calli were subcultured every 28 d. The media used in cell culture are shown in Table 1. All the media were autoclaved at 121°C for 15 min. Cultures were maintained in a room at 28 ± 2°C under a 14 h / 10 h light / dark photoperiod (135 μmol m^{-2} s^{-1} provided by cool, white fluorescent lamps). Embryogenic calli with high proliferation activity obtained after 5 or 6 rounds of subculture on fresh MS3 medium were used for induction of developmental synchrony of somatic embryos.

1.3 Improvement of embryogenesis inducing culture media

Six media based on MS3 medium were designed: M1, containing 0.1% KCl; M2, containing 1.5% additional glucose; M3, containing 0.02% $CaCl_2$; M4, containing 0.1% KCl and 1.5% additional glucose; M5, containing 0.1% KCl and 0.02% $CaCl_2$; and M6, containing 0.02% $CaCl_2$ and 1.5% additional glucose. Embryogenic calli obtained after 5 rounds of subculture on MS3 medium were transferred onto these media.

Table 1 Media used in cell culture in cotton

Medium	Basic component	pH
MS0	1/2MS [36] + 1.5% (*W*/*V*) glucose + 0.25% (*W*/*V*) phytagel	6.0
MS1	MS basal salts +B5 vitamins [37]+ 0.45 μmol L^{-1} 2,4-D + 0.46 μmol L^{-1} KT + 0.1% (*W*/*V*) $MgCl_2 \cdot 6H_2O$ + 3% (*W*/*V*) glucose +0.25% (*W*/*V*) phytagel	5.8
MS2	MS basal salts (double KNO_3) +B5 vitamins [37]+ 0.23 μmol L^{-1} 2,4-D + 0.46 μmol L^{-1} KT + 0.1% (*W*/*V*) $MgCl_2 \cdot 6H_2O$ + 3% (*W*/*V*) glucose +0.25% (*W*/*V*) phytagel	5.8
MS3	MS basal salts (double KNO_3 but removal of NH_4NO_3) + B5 vitamins + 2.46 μmol L^{-1} IBA + 0.70 μmol L^{-1} KT +0.1% (*W*/*V*) Glu +0.1% (*W*/*V*) Asn + 3% (*W*/*V*) glucose + 0.25% (*W*/*V*) phytagel	5.9