

Somatic Embryogenesis and Plant Regeneration via Solid-liquid Alternating Culture in Elite Genotypes of Upland Cotton

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Research

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Abstract

Background: Cotton is one of the most genotype-dependent plants for regeneration, in order to expand cotton regeneration genotypes and establish efficient regeneration system platform, Jiwu 2031 (glandless cotton), ND 58 and CAU 102, were selected for studying the highly efficient somatic embryos formation and regeneration via solid-liquid alternating culture system.

Results: In present research, the MSB medium (MS salts adding B5 vitamins) containing 0.571 μM indole-3-acetic acid (IAA), 0.465 μM kinetin (KT) and 0.904 μM 2, 4-dichlorophenoxyacetic acid (2, 4-D) was effective for callus initiation of fourteen Upland cotton with 'Corker 201' as the control. 'Jiwu 2031', 'ND 58' and 'CAU 102' could form somatic embryos and regenerate fertile plants in a solid MSB medium containing 10.31 mM NH_4NO_3 , and supplemented with 0.027 mM glycine, 2.460 μM indole-3-acetic acid (IBA), 0.930 μM KT, 3.784 mM asparagine and 6.843 mM glutamine. Under the solid-liquid alternating culture system, the non-embryonic callus was induced to form embryonic callus and the identical status somatic embryos were obtained in 42 days, and the period for plants regeneration was shortened to 90~120 days, with the higher proportion of normal plant regeneration.

Conclusions: The solid-liquid alternating culture method could increase the rate of embryogenesis and shorten the period of plants regeneration of Upland cotton. This work provides the evidence that the glandless cotton is beneficial for somatic embryogenesis (SE) and plant regeneration.

Background

Upland cotton (*Gossypium hirsutum* L.) is one of the most important economic crops for valuable fiber and seed oil production (Wang et al. 2018). Steady improvements of agronomic traits in cotton have been achieved insuccessive breeding programs, and genetic improvement is time consuming and limited by insufficient genetic variability (Sakhanokho et al. 2001). The effective regeneration system and transgenic technology is a useful tool to introduce foreign genes into cotton as well as further study for genomics. Somatic embryogenesis (SE) -based transformation was recognized as the prominent method for the single-cell origin of somatic embryo (Merkle et al. 1995), which avoids the disadvantages of multi-copy and highly complex transgenic loci of the particle bombardment process (Stanford et al. 1987; Stanford, 2000), and chimeric plant of the shoot tip method, as well as other protocols (Yang and Zhang, 2010).

The first SE in cotton (*Gossypium klotzchianum*) was conducted in 1979, but failed to regenerate a complete plant (Price and Smith, 1979). The regeneration in *G. hirsutum* was first obtained via spontaneous SE from Coker 310 (Davidonis and Hamilton, 1983). Since then, the SE and regeneration of cotton from cotyledon and other tissues have been intensively studied (Trolinder and Goodin, 1987; Trolinder and Chen, 1989; Firoozabady and Deboer, 1993; Sakhanokho et al. 2001; Kumria et al. 2003; Mishra et al. 2003; Wang et al. 2008; Xu et al. 2015).

Progresses have been achieved in *Gossypium* regeneration and a few cultivars of Upland cotton were reported to be able to regenerate efficiently *in vitro*, which were 'Coker 312,' 'Coker201', 'Ekangmian No. 9', 'Simian No. 3', 'YZ-1', *etc* (Trolinder and Goodin, 1987; Jin et al. 2006; Zhang et al. 2000; Zhang et al. 2009). An elite cotton Jin668, with an extremely high regeneration ability, was developed from its maternal inbred Y668 cultivar using a Successive Regeneration Acclimation (SRA) strategy (Li et al. 2019). Different genotypes or explants showed variable results in the process of cotton SE and plant regeneration (Trolinder and Chen, 1989; Seabrook and Douglass, 2001; Ikeuchi et al. 2016). Other factors, such as the components of carbon and nitrogen source, hormones (Wu et al. 2004; Liu et al. 2011) and somatic embryo morphology (Hussain et al. 2009), were also important for the efficiency of SE and regeneration in cotton (Buchheim et al. 1989; Wetzstein and Baker, 1993; Zhang et al. 2009; Juturu et al. 2015). Endogenous or exogenous compound also played important roles on cotton SE, such as thidiazuron (Nanda and Basra, 2013), polyamine (Cheng et al. 2015), hydrogen peroxide (H₂O₂) (Cheng et al. 2015) and reactive oxygen species (ROS) (Zhou et al. 2016). Meanwhile, many genes also acted as regulators with ectopic expression on SE, such as *WUS* (*WUSCHEL*) (Bouchabké-Coussa et al. 2013; Jha et al. 2020), *SERKs* (Pandey and Chaudhary, 2014a; 2014b), *AGL15s* (Yang et al. 2014), *GhPAO* (Cheng et al. 2017). Some useful medium or protocols have been developed for efficient regeneration in cotton (Divya et al. 2008; Stella and Demetrios, 2009; Sun et al. 2009). Researches now focus on SE and plant regeneration of current commercial cotton cultivars in order to conduct cotton genetic improvement without backcross with Coker transgenic lines (Han et al. 2009; Khan et al. 2006; Kumar and Pental 1998; Zhang et al. 2009). Screening for elite genotypes of Upland cotton, especially for glandless genotypes with efficient regeneration capacity and overcoming genotype dependence, which could considerably reduce the time and costs, will be of great importance in cotton genetic improvement programs. Nowadays, with the development of sequencing and emerging technology, the omics data on SE have excellent enhance the theoretical basis on cotton regeneration, including transcriptome (Yang et al. 2012; Xu et al. 2013; Jin et al. 2014; Cheng et al. 2016), proteome (Ge et al. 2015; Zhou et al. 2016), small RNA and degradome sequencing (Yang et al. 2013).

Suspension culture was used in tissue culture for producing embryos and regeneration plants from 'Coker 312' (Trolinder and Goodin, 1987) and 'Coker 310' (Finer, 1988), and used as a platform for genetic transformation (Ke et al. 2012). A system with suspension culture for synchronization control of SE in 'Coker 312' was developed (Sun et al. 2005; Yang and Zhang, 2010), but the efficiencies of the system relied on inositol starvation stress, were short of identity and easily changed the metabolic conditions in the process of SE and development (Trolinder and Goodin, 1987; Kumar and Tuli, 2004).

In this study, fourteen Upland cotton varieties with desired agronomic traits were used to compare the regeneration capacity, including the status of callus and embryonic callus induction as well as proliferation rate. With the cultivar 'Coker 201' was used as control, an efficacious protocol for cotton SE and plant regeneration has been developed to regenerate plantlets from three commercial Upland cotton varieties in several months. An improved protocol based on solid-liquid alternating culture was used to improve SE frequency and synchronous development of mass somatic embryos.

Results

Medium for induction of callus

Upland cotton cultivar 'Coker 201' was used to select the efficiency of callus induction medium. The explants began to swell at both ends of hypocotyls after cultured on MSB medium (Table 1) 5~7 days, the hypocotyls elongated and intumescenced. A mass of callus produced 30~35 days. Induction capacity varied in medium (CIM01- CIM12) with different combinations and concentration of hormones. The colors of the callus diverged from fawn to green or yellow green, etc., and the textures were compact, loose, friable and hardy. The induction frequency of callus was higher in the medium supplemented with high concentration of 2, 4-D (0.904 μM) and higher ratio of 2, 4-D/KT concentration (Table 1), indicated that 2, 4-D was an important hormone for the induction of callus.

IBA or IAA was employed to meliorate the callus state reported before (Wang et al. 2006). Callus could be improved to friable and loose texture in light yellow or green yellow color when the IAA (0.571 μM) or IBA (2.460 μM) were added to the medium containing 2, 4-D and KT, but higher frequency of callus induction was obtained using IAA than that of using IBA (Table 1). The ratio of auxin: cytokinin should not be too high in order to reduce the formation of rhizoid.

Callus with loose and friable texture in yellow and light yellow color were easier to differentiate, this result was coincident with previous report (Han et al. 2009). The CIM06, CIM medium supplied with 2, 4-D (0.904 μM), IAA (0.571 μM) and KT (0.465 μM) (Table 1), generated high frequency callus with desirable color and texture, was confirmed to be the most suitable one for callus induction.

Callus induction among different genotypes of Upland cotton

Explants from fifteen Upland cotton cultivars were used to induce callus in the selected medium CIM06, containing MS + 2, 4-D (0.904 μM) + IAA (0.571 μM) + KT (0.465 μM). No obvious difference was observed among these varieties in terms of the time of the callus initiation, color and texture of infant callus in the process of callus induction. The induction rate of loose callus varied from 40.22% to 100.00% (data not shown). The induction rate of 13 cultivars was as higher than 82%, which confirmed further that the medium CIM06 was suitable for callus induction, except induction rate of C1013 and Yumian No.1 were 40.22% and 48.84%, respectively.

Proliferation rates of callus of different genotypes

Loose callus was subcultured every 30 days on the medium supplemented with 5.16 mM NH_4NO_3 , 1.504 M KNO_3 , 1.48 μM IBA and 0.465 μM KT for proliferation and development. The wet weights of developing callus were recorded each 30 days to calculate the proliferation rate (g/d) of callus.

The fifteen genotypes could be classified as four types based on the proliferation rates of callus (Fig. 1). Category I: Callus proliferated very slowly in dark gray color and soft and wet texture, such as Yumian No. 1, C1006 and C1013. Category II: Callus proliferated in general rate, which was in light yellow color, soft

and wet texture. More than half of the genotypes, such as CAU102, were classified in the group. Category III: Callus of Luwu 401, Jiwu 2031, ND 58 and C1013 proliferated fast, and the callus is light yellow in friable and dry texture. Category IV: Callus of C1028 proliferated in a "crazy" mode with firm and hard texture in green color.

Embryogenic callus induction and somatic embryo formation

Callus of different genotypes were subcultured on medium with MSB1 (1.480 μM IBA, 0.465 μM KT), 6.843 mM glutamine and 3.784 mM asparagine to induce embryogenic callus. After one month subcultured on this medium, various callus differentiations were found among the 15 genotypes. The color, texture, number of callus proliferation and embryogenesis callus varied (Table 2), which can be classified into three types (Fig. 2A-C). **Category I:** 4 varieties, Coker 201, ND 58, Jiwu 2031 and CAU 102 could differentiate into typical embryogenic callus in light yellow or gray color with friable or alveolate structure; **category II:** 9 varieties formed callus in brown or green color with the compact surface, but were hard to differentiate into embryogenic callus on this medium; **category III:** callus from C1028 proliferated in a "crazy" mode in dark green color with loose texture, and could not differentiate into embryogenic callus either. Embryogenic callus from the cultivar which had the ability of redifferentiation could produce somatic embryos after two to three times of subculture on this medium.

Development of somatic embryos and plant regeneration

Embryos formed from embryogenesis were transferred onto medium supplemented with seven combinations and concentrations of hormones plus 10.31 mM NH_4NO_3 , 6.843 mM glutamine, and 3.784 mM asparagine for embryo development. Somatic embryos successfully developed into plants after subculturing on this medium for four to five weeks, but the growth and development of the embryos were obviously different (Fig. 3). The germination rate of the embryos with normal root on the medium without hormones was 41%, indicating it was the best among the seven media used in embryo germination. 0.985 μM IBA and 0.465 μM KT (MSB4) could facilitate embryo germination and maturation. Medium containing high concentrations of hormones (MSB2) could increase the rate of abnormal plantlets. The ratio of somatic embryo dedifferentiation was 22.3%, 34.5% and 28.9% when the embryos germinated on medium supplied with MSB4, MSB5 and MSB6, respectively, which indicated that 2, 4-D could result in somatic embryo dedifferentiation and should not be used during embryo germination. The treatment MSB6 with reduced IBA concentration with 2, 4-D (0.492 μM IBA + 0.465 μM KT + 0.452 μM 2, 4-D) showed reduction in somatic embryo dedifferentiation (28.9%) compared to that of MSB5 (34.5%), which indicated that the relatively lower concentrate of IBA would more proper for embryo development and germination.

Somatic embryos also developed from globular embryo, heart shaped embryo, torpedo shaped embryo, cotyledonary shaped embryo to plantlets (Fig. 2D) as previous report (Sun et al. 2009), and the development process was similar to that of zygotic embryo. After the regenerated plantlets grew four to six leaves and normal roots in the flask, all of the rooted plantlets were successfully transplanted to soil

pots in green house. All plants recovered, flowered and bore seed after two to three months in green house, the regenerated plantlets had normal fertility (Fig. 2E-K). And these results also indicated that SE in Upland cotton would be more useful with low frequency of chimeras and higher proportion of regenerates.

The solid-liquid alternating culture increased inducing efficiency of embryogenetic callus and somatic embryos

The explants of 'Coker 201', 'Jiwu 2031', 'CAU 102' and 'ND 58' began to swell at both ends of hypocotyls after cultured on **CIM** (MS medium supplemented with 0.571 μM IAA, 0.465 μM KT and 0.452 μM 2, 4-D), and a mass of callus produced within 25~30 days (Fig. 4A, the abbreviation for Fig. 4A (a), 4B (a) and 4C (a), similar showed below). After subculture of the initial callus on the same medium for 14 to 28 days, the pre-embryogenetic callus was highly variable in color and texture. Typical colors of non-embryogenetic callus were green, light yellow, white, gray, and brown. The types of callus texture include loose, friable and compact.

For embryogenetic callus initiation and maintenance, pre-embryogenetic callus with loose or friable texture and in gray or light yellow (Fig. 4(b)) were chosen to suspension in MS liquid medium and shake for five minutes to disperse the callus. After removing the large particles with 30-mesh stainless steel sieves, the pre-embryogenetic callus was re-suspended in **EIML** (Fig. 6) and cultured for 14 days until the callus with identical color and texture formed. **EIML** contained halved NH_4NO_3 , doubled KNO_3 , and supplied with 2.46 μM IBA+0.698 μM KT, not similar to the hormone-free medium used in establishing and maintaining embryogenic suspension cultures of cotton cultivar 'Coker 312' (Trolinder and Goodin, 1987). The callus in identical/similar light yellow and loose texture were chosen for further suspension culture in 50 mL fresh **EIML** (Fig. 4(c)). After 14~28 days' inoculation, the embryonic callus or embryogenetic tissues were found in suspension cultures. In this study, the induction frequency of embryogenesis callus by suspension culture was 72.14%, 52.86% and 61.62% in 'Coker 201', 'Jiwu 2031' and 'ND 58', respectively (Table 3), while that in solid culture for Coker 201 was 63.5% (Wu et al. 2004). The induction time to form embryonic callus was shorted from 60~90 days (Wu et al. 2004) to 35~42 days.

For somatic embryo development/maturation, once the embryogenesis callus with similar/identical status of friable texture and yellow color were formed in suspension culture system, embryogenesis callus were filtered with 50-mesh stainless steel sieves, and subcultured onto **EMMS** (with halved NH_4NO_3 , doubled KNO_3 , 2.460 μM IBA, 0.698 μM KT, 6.843 mM glutamine and 3.784 mM asparaginate) (Fig. 4(d)). Within 28 days, large amounts embryos were obtained (Fig. 4(e)). At the initial stage of embryos, the percentage of global-embryos counts for 67.5% which indicated that the highly **synchronous** of embryos development. The embryos in identical status (global embryos) were selected to subculture on the solid medium **EGM** for germination (Fig. 4(f)). Following placement of the mature embryos on **EGM**, hypocotyl and root elongation was first observed in 3~7 days (Fig. 4(g)). The percentages of embryos germination were 47.71%, 39.83% and 46.67% in 'Coker 201', 'Jiwu 2031' and 'ND 58',

respectively (Table 3). Within 14 days, the majority of the embryos had undergone obvious elongation, and then the cotyledons developed, and plantlets regenerated with at least 2 euphylla in an additional month (Fig. 4(g)).

Discussion

If the agronomic traits of the cultivars used as transgenic receptors are not desirable, and it will take years for breeders to improve the yield and fiber qualities of the transformants through backcross. In this study, three elite cotton cultivars (CAU 102, Jiwu 2031 and ND 58) and Coker 201 (as a control) had been identified that have high efficiency of regeneration. Jiwu 2031, ND 58 and CAU 102 were commercial varieties with desired agronomic traits. Jiwu 2031 was a glandless variety that could be used for the production of cotton seed oil and protein. The genotype of Jiwu 2031 showed excellent quality and yield with the pre-frost yield up to 85%~90%, and had good characteristics of early maturity without premature senescence in field comparison experiment. During the later period of adult stage, the Jiwu 2031 displayed strong resistance to stress and blight, and the damage of cotton aphid and leaf spot were light.

The successful regeneration of these elite genotypes provides better choices of cotton cultivars for transformation and great potential of expediting process of transgenic breeding. It seems that only 3 months for the control Coker 201 to regenerate, four to five months for other 3 cultivars using the protocol developed here took longer time (Fig. 5). But considering the time consumed in many backcrossing generations two or three months is really a short time.

All explants from various cotton genotypes could have the regeneration capability via SE according to plant cellular totipotency, but the abilities of SE and plant regeneration in cotton rely greatly on cotton genotypes (Trolinder and Chen, 1989; Seabrook and Douglas, 2001; Yang and Zhang, 2010; Rosspopoff et al. 2017). There are more than 50 species in *Gossypium* genus, and many species have multiple genotypes (Grover et al. 2017). However, only a few genotypes of *G. hirsutum* could be regenerated via SE, which restricted the utilization of biotechnology in cotton transgenesis (Zhang et al. 2009). Most of the genotypes which have the regeneration capability, such as 'Coker 201', 'Coker 312', Ekangmian No. 9', 'Simian No. 3', 'YZ-1', 'Zhong No. 3', etc, were belong to pedigree of Coker, Deltapine and Uganda (Juturu et al. 2015). Here, totally thirty-four genotypes of Upland cotton were used to compared the callus induction ability in our primary research, and fifteen varieties with typical callus were used to compare the ability of SE and plant regeneration, and significant differences were observed among the fifteen cotton genotypes investigated from callus induction to embryo development.

The combinations and concentrations of hormones were important factors for induction of callus. Different hormones combination has been well employed, such as 'Brassinolide (BR) + 2, 4-D', '2, 4-D + KT' and 'IBA + KT' (Firoozabady and DeBoer, 1993; Aasim et al. 2009; Liu et al. 2011; Duan et al. 2012). Zeng et al (2007) identified two phases of chromatin decondensation associated with endogenous auxin: cytokinin dynamic activity, which may underlie dedifferentiation and redifferentiation during SE process.

The results of this research indicated that 2, 4-D was useful for callus induction, which was consistent with the results of previous reports (Zhang, 1991; Wang et al. 2006).

High concentration of exogenous hormones might result in undesirable effects for callus development, such as the formation of rhizoid, due to the disequilibrium between endogenous and exogenous hormones. In this study, we used seven hormone combinations to optimize the medium with appropriate asparagine and glutamine amounts for embryo germination (Fig. 3). Medium supplemented with 2, 4-D had significantly negative effect on embryo generation. Several studies have demonstrated that when decreased or completely removed 2, 4-D, the proliferation of non-embryogenic callus was inhibited, and the proliferation of embryogenic callus was activated and advanced to the initiation of somatic embryos (Voo et al. 1991; Sun et al. 2009). In this study, embryos were transferred onto the medium without hormones could relatively promote embryos to regenerate normal plantlets, with a little bias with the results of Rashid *et al.* (2009). However, higher rate of abnormal plantlets was still observed during embryo development on all kinds of media, indicating further investigation should be performed to avoid this barrier. Meanwhile, the effect of amino acids on embryo development should also be studied (Witjaksono and Litz, 1999; Wu et al. 2004).

The rate of callus proliferation, which was considered as a useful index in cotton callogenesis, has significant difference among genotypes. That normally proliferated callus could advance to embryogenic callus phase and eventually regenerate plantlets, suggesting that the callus proliferated slowly or massively should not be chosen for cotton regeneration. Obvious genotype-dependent differed in embryogenic callus induction, Jiwu 2031 had the highest differentiation efficiency.

The low frequency and **time-consuming** of embryogenetic callus and somatic embryos induction are the main restrict factor during the process of tissue culture of Upland cotton. The liquid suspension medium can high-efficiency promote callus to produced SE and maturation compared to semi solid medium (Juturu et al. 2015). In this protocol with solid-liquid alternating culture, the time to induce and form embryonic callus was reduced from 60~90 days to around 42 days, and the percentage of embryogenesis callus induction was reached as high as 72.14% in 'Coker201'. Within 14 days, the embryo could be formed from the embryonic callus with identical color and texture, and the percentages of embryos germination on solid medium were over 39.00% in all three genotypes. Besides, the ratio of regeneration seedlings in normal development were as high as 17.53%, 18.04% and 18.29% in 'Coker 201', 'Jiwu2031' and 'ND58', respectively, which reduced the high rate of abnormal seedlings during embryo development. And the time for plants regeneration was only cost 90~120 days (Fig. 6), much shorter than 120~150 days cost in the previous method (Sakhanokho et al. 2001; Wu et al. 2004).

Conclusions

This solid-liquid alternating culture protocol provided an efficient method for inducing of embryonic callus and embryos, not only for 'Coker' lines, but also for other excellent genotypes, such as 'Jiwu 2031' and 'ND 58'. And we also found there was a high **synchronism** for embryos development, which provided

an ideal platform for physiological, biochemical, and molecular biological studies of embryogenesis processes in Upland cotton. This method raised the frequency of normal seedlings and shorted the time of plant regeneration in cotton tissue culture, might have the great potential to be used as a platform for genetic transformation.

Methods

Plant materials

Fourteen Upland cotton cultivars with desired agronomic trait (data not shown) were used in this study. A cotton cultivar 'Coker 201', known has high efficiency of embryogenic callus induction and plant regeneration (Sun et al. 2005, Yang and Zhang, 2010), was used as a control in the study. All of the materials used in this research, including the cultivars 'Jiwu 2031', 'ND 58', 'CAU 102', and 'Coker 201' were conserved in Laboratory of Cotton Genetics, Genomics and Breeding, China Agricultural University in Beijing, China.

Seed sterilization and production of sterile seedlings

Matured seeds were delinted with sulfuric acid (98%, v/v), immersed in 70% alcohol for 30 s and shaken in 0.1% HgCl₂ with for 10 min, then rinsed with sterile distilled water for five times, and soaked in sterile water at 25~30°C for 48 hours. Seeds coats were removed before the seeds placing on half strength macronutrient MSB medium comprising water, 0.65% (w/v) agar (Amersico) and 2% (w/v) sucrose in dark at 28±2°C for 5~7 days to germinate (Sakhanokho et al. 2001).

Initiation and proliferation of callus

Hypocotyls from sterile seedlings of 'Coker 201' were cut into 5-8 mm segments in length, and served as the explants to be placed on MSB medium supplemented with twelve combinations of 2,4-dichlorophenoxyacetic acid (2,4-D), IAA (3-indoleacetic acid), indole-3-butyric acid (IBA) and kinetin (KT) (CIM01 -CIM12, Table 1) in order to induce the callus. The medium was selected based on 1) the frequency, and 2) recorded status of callus induced, including the emerging time, texture, color, and proliferation rate of callus (g/d) (Fig. 1). The callus proliferated rates were calculated by net weight of proliferated callus/days of subculture. The soft or friable texture callus in grey and yellow were chosen to subculture on MSB medium contained 10.31 mM NH₄NO₃, 1.504 M KNO₃, and supplemented with 1.48 μM IBA and 0.465 μM KT.

Induction of embryogenic callus and somatic embryos

After subcultured once or twice, the prolific and loose non-embryogenic callus were placed to induce embryogenic callus on above MSB medium but added 6.843 mM glutamine and 3.784 mM asparagine. The embryogenic calli with high proliferation rate were transferred onto same medium to induce somatic embryos (Fig. 2). Somatic embryos were transferred to seven kinds of MSB media contained 10.31 mM

NH_4NO_3 , 1.504 M KNO_3 , and supplemented with 2, 4-D, IAA, IBA and KT at different hormone combinations (see details in Fig. 3), along with 6.843 mM glutamine, 3.784 mM asparagine for embryo growth. The status of embryo development and the ratio of different types of embryo were recorded.

Plant regeneration, plantlets recovery and transplantation

The regenerated normal plantlets were transferred onto the same media for root induction. Apart from the half strength macronutrient MSB medium, all above media were supplied with 3% glucose and 0.27% phytigel, and adjusted the pH to 5.95 before being sterilized by autoclaving at 115°C for 20 min. All of the culture conditions were maintained at $28\pm 2^\circ\text{C}$ under a 16 h photoperiod with a light intensity of 1600LX. Plantlets with strong roots were challenged by unsealing the erlenmeyer flask for 2~3 days, and then were transplanted to soil pot and grown in the greenhouse. The fertility of the regeneration plantlets was surveyed when flowering and the mature seeds were harvested.

Solid-liquid alternating culture

Hypocotyls from sterile seedlings of 'Coker 201', 'Jiwu2031' and 'ND58' were cut into 5~8 mm segments in length and served as the explants to be placed on callus induction medium (**CIM**, MS salts supplemented with 0.571 μM IAA, 0.465 μM KT and 0.452 μM 2, 4-D, pH=5.85) (Fig. 6). Explants were transferred to Petri dishes containing CIM under light intensity of 1600LX and a 16 h/8 h light/dark cycle at $28\pm 2^\circ\text{C}$ for the callus induction and proliferation.

After 28 days, pre-embryogenic callus with soft or friable texture were transferred to suspend in MS liquid medium and shake (120 rpm) for 5 minutes. The large particles were removed with 30-mesh stainless steel sieves, and then the pre-embryogenic callus were suspended, and cultured in 250 mL Erlenmeyer flasks containing 100 mL somatic embryo initiation liquid medium (**EIML**, MS salts with halved NH_4NO_3 , doubled KNO_3 , 0.5 $\text{mg}\cdot\text{L}^{-1}$ IBA, 0.15 $\text{mg}\cdot\text{L}^{-1}$ KT, pH=5.85). These flasks were agitated on a gyratory shaker at 120 rpm and administrated regular under the same environmental condition used for callus induction (at 16 h/8 h light/dark cycle at $28\pm 2^\circ\text{C}$ with 135 $\mu\text{mol m}^{-2} \text{s}^{-1}$ cool white fluorescent light).

After cultured for 14 days, further subculturing involved the replacement of 50 mL of the cell suspension with fresh **EIML** medium at 7-d interval, and suspensions were filtered through 50-mesh stainless steel sieves to separate the bigger callus and small cell clumps. The separated callus was re-suspended in fresh **EIML** medium and cultured for 2-4 intervals (7 days for one interval). Samples from the suspension callus were observed under a microscope to decide subculturing period when the right embryonic callus obtained.

The cultured cells were screened with 50-mesh stainless steel sieves to remove the small cell clumps and then embryonic callus with similar status of texture and color were harvested. The embryonic callus with identical loose and friable status were cultured for embryos initiation on solid somatic embryo development/maturation medium (**EMMS**, pH=5.85), which consisted of the same composition as **EIML** (MS salts with halved NH_4NO_3 , doubled KNO_3 , 2.460 μM IBA, 0.698 μM KT), added 6.843 mM glutamine

and 3.784 mM asparaginate. Mature embryos, which were obtained within 28 days on the solid medium, were placed on embryo germination medium (**EGM**, pH=5.85), which was a modification of the medium used by Davidonis and Hamilton (1983), and contained modified MS salts (no NH_4NO_3 and $2\times \text{KNO}_3$), 6.843 mM glutamine, 3.784 mM asparaginate, 3% glucose, and 0.27% phytagel.

Statistical analysis

The experimental design was completely randomized with three replications per treatment, each treatment consisting of 24 explants or about 3 g of calluses, or 60 somatic embryos for germination. The induction rate of callus was calculated by weight of callus proliferated each day, and induction rates of the loose callus were calculated by the number of hypocotyls with loose calluses/the total number of hypocotyls per treatments. Induction frequency of embryogenic callus was calculated by the number of hypocotyls with embryogenic callus/the total number of hypocotyls in each treatment. The data were analyzed using STATISTICA 5.0 and the means were checked by Duncan's multiple range test at $P=0.05$ or $P=0.01$.

Abbreviations

CIM: callus induction medium; EIML: somatic embryo initiation liquid medium; EMMS: solid somatic embryo development/maturation medium; EGM: embryo germination medium.

Declarations

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Availability of data and material

Please contact author for data requests. All data generated or analyzed in this study included in published article and additional files.

Authors' contributions

ZL performed the experiments, analyzed the data and drafted the manuscript. WW attended discussion and revised the manuscript. JH designed the experiment, provided experimental platform and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1 Callus induction and differentiation of 'Coker201' on MSB medium supplemented with various hormone combinations

Combinations	Hormone combinations μM				Induction rate* (g/d)	Color	Texture	Rhizoid	
	2,4-D	IAA	IBA	KT					
CIM01	0.452	0	0	0.465	0.15* *	d	Green	Compact	No
CIM02	0.904	0	0	0.465	0.42	bc	Gray	Loose	No
CIM03	0.452	0	0	0.233	0.13	d	Green	Hardy	No
CIM04	0.904	0	0	0.233	0.49	b	Yellow green	Loose	No
CIM05	0.452	0.571	0	0.465	0.35	c	Yellow green	Compact	No
CIM06	0.904	0.571	0	0.465	0.61	a	Light yellow	Friable	No
CIM07	0.452	0.571	0	0.233	0.17	d	Brown	Compact	Yes
CIM08	0.904	0.571	0	0.233	0.53	ab	Light yellow	Loose	No
CIM09	0.452	0	2.46	0.465	0.32	c	Light yellow	Loose	No
CIM10	0.904	0	2.46	0.465	0.48	b	Yellow green	Loose	No
CIM11	0.452	0	2.46	0.233	0.21	d	Yellow green	Compact	No
CIM12	0.904	0	2.46	0.233	0.39	c	Yellow green	Loose	Yes

*induction rate was calculated by weight of callus proliferated each day, and detected significance at $P = 0.05$. **Values were the mean of ten replicates, each replicate contains 8 explants.

Table 2 The status of embryogenic callus of different cotton genotypes

Genotypes	Callus color	Callus texture	Number of proliferated calli*	Inducing frequency of embryogenic calli (%)**	Number of the visible matured embryos***
Coker 201	Light yellow	Friable	++ ^a	78.47±4.47	19.5
C1006	Light green	Compact	+++	N.A.	N.A.
Xu244	Light green	Compact	++	N.A.	N.A.
C1011	Dark green	Loose	+++	N.A.	N.A.
C1013	Dark green	Compact	++	N.A.	N.A.
CAU 102	Light yellow	Friable	+	37.08±5.43	9.8
C1028	Dark green	Loose	++++	N.A.	N.A.
C1034	Dark green	Compact	+	N.A.	N.A.
Yumian No.1	Light green	Loose	++++	N.A.	N.A.
Zhongmiansuo 35	Dark green	Compact	++	N.A.	N.A.
Liaomian 17	Dark green	Compact	++	N.A.	N.A.
Jinmian 36	Light green	Compact	++	N.A.	N.A.
Luwu 401	Brown	Friable	+	N.A.	N.A.
Jiwu 2031	Light yellow	Friable	+++	89.04±1.41	21.9
ND 58	Light yellow	Friable	+++	67.32±5.14	15.6

*more "+" means more number of callus; Values were the means of three replicates.

** Induction frequency of embryogenic calli was the average of three replicates, which calculated by the number of hypocotyls with embryogenic callus/the total number of hypocotyls in per treatments.

***Number of visible matured embryos was calculated by the number of observed mature embryos /the number of explants with embryogenic callus.

N.A.: no data available for this study because no test was successfully performed for the genotype.

Table 3 Statistic results of solid-liquid alternating culture

Genotypes	Coker 201	CAU 091	CAU 101
Number of hypocotyl incubation	88.67±1.53*	92.00±4.00	91.33 ±3.51
Number of callus lumps for suspension culture	64.00±5.57	48.67±4.73	56.33 ±6.66
Number of embryos subcultured on germination medium	221.67±28.99	194.67±20.74	196.00±13.11
Number of germinated embryos	105.00±12.53	77.33±10.01	93.00±4.58
Number of regeneration plants	18.33±2.52	14.00±2.65	17.00±3.61
Embryogenic callus induction frequency (%)	72.14±5.43	52.86±3.70	61.62±6.24
Frequency of embryos germination (%)	47.71±6.69	39.83±4.63	46.67±5.63
Ratio of normal seedlings regeneration (%)	17.53±2.22	18.04±1.44	18.29±3.94

Embryogenic Callus induction frequency (%)=Number of callus lumps for suspension culture × 100 / Number of hypocotyl incubation; Frequency of embryos germination (%)=Number of germinated embryos × 100 / Number of embryos subcultured on germination medium; Ratio of normal seedlings regeneration (%)=Number of regeneration plants × 100 / Number of germinated embryos.

*Values in the table are the means ± SD of five replicates (n=5), and ten treats for each replicate.

Figures

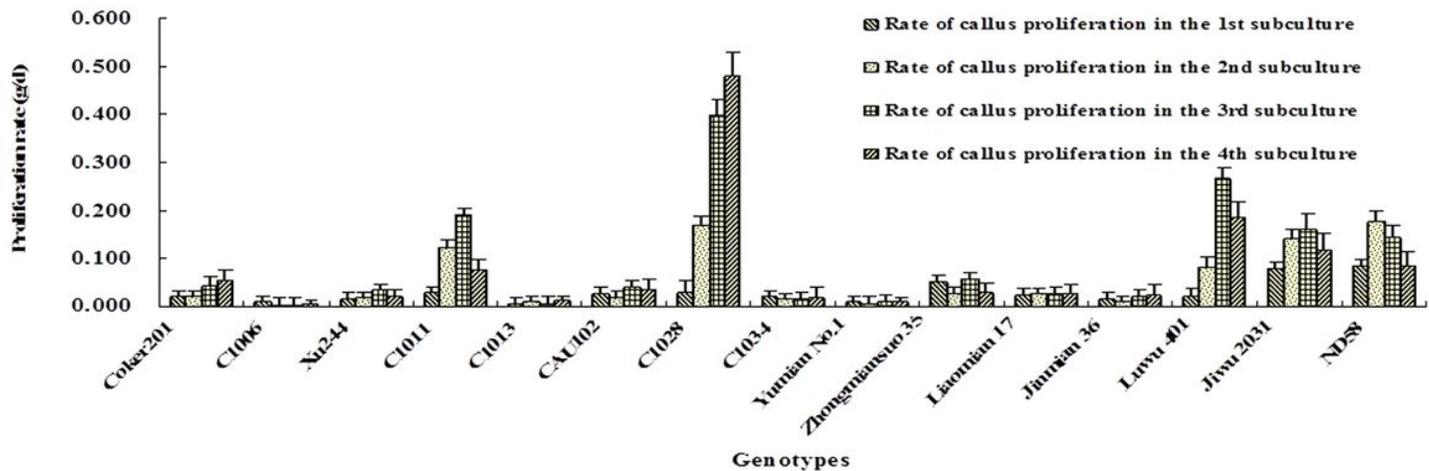


Figure 1

The callus proliferated rates of 15 different genotypes. The callus was subcultured for four times. Values were the means of three replicates, each from the data of callus in 10 bottles. X axis is the different genotypes, and Y axis is the proliferated rates of callus.

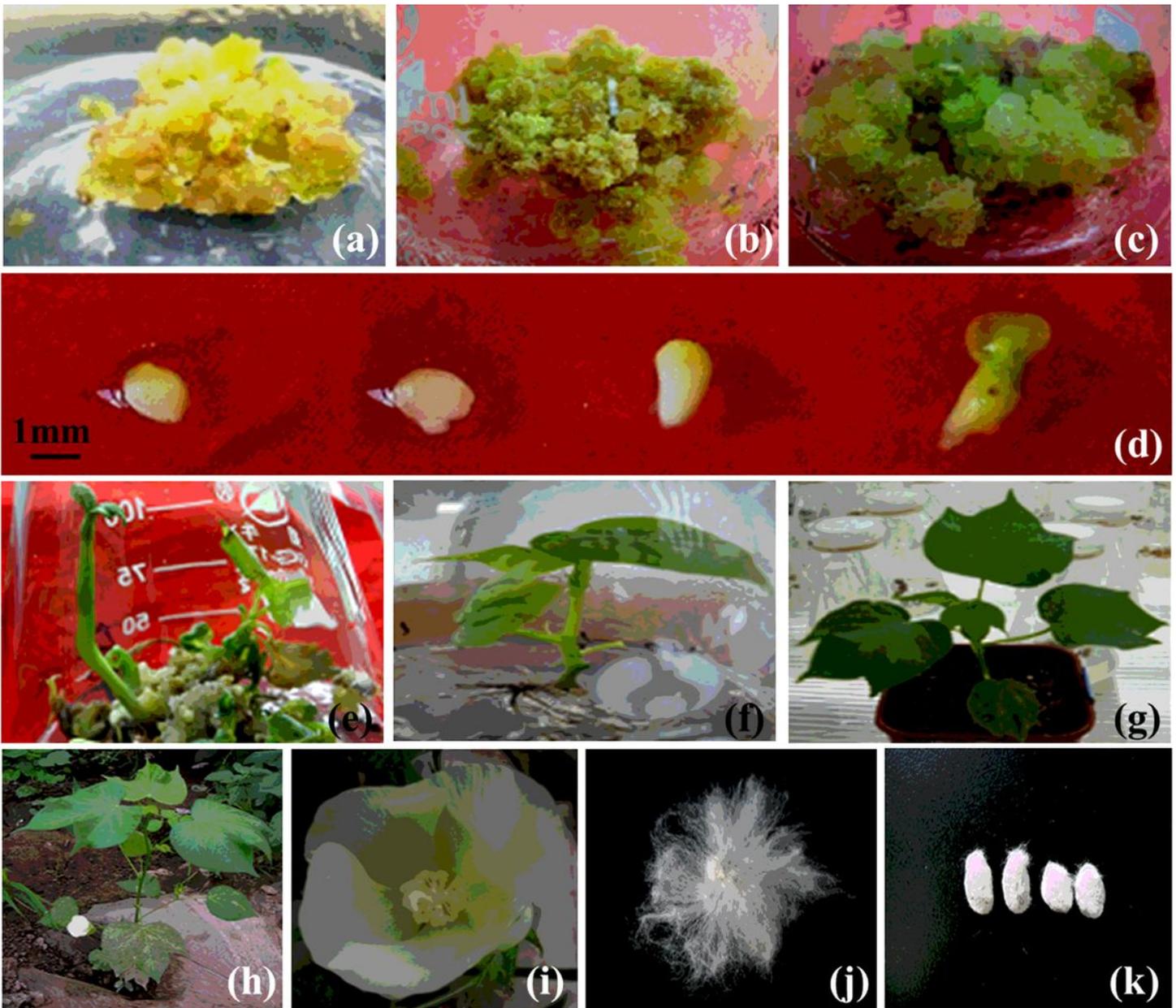


Figure 2

The process of embryogenic callus induction, somatic embryogenesis and plant regeneration. (a)~(c) Embryogenic callus induction. (a) Category I, light yellow or yellow embryogenic callus (Jiwu 2031). (b) Category II, brown or green callus with the compact surface (C1011). (c) Category III, dark green and loose texture callus proliferated massively (C1028). (d) The process of embryo development (Jiwu 2031), left to right: globular embryo, heart embryo, torpedo embryo, and cotyledonary embryo. (e)~(h) Plant regeneration (Jiwu 2031). (i)~(k) Fertile regenerated plants (Jiwu 2031).

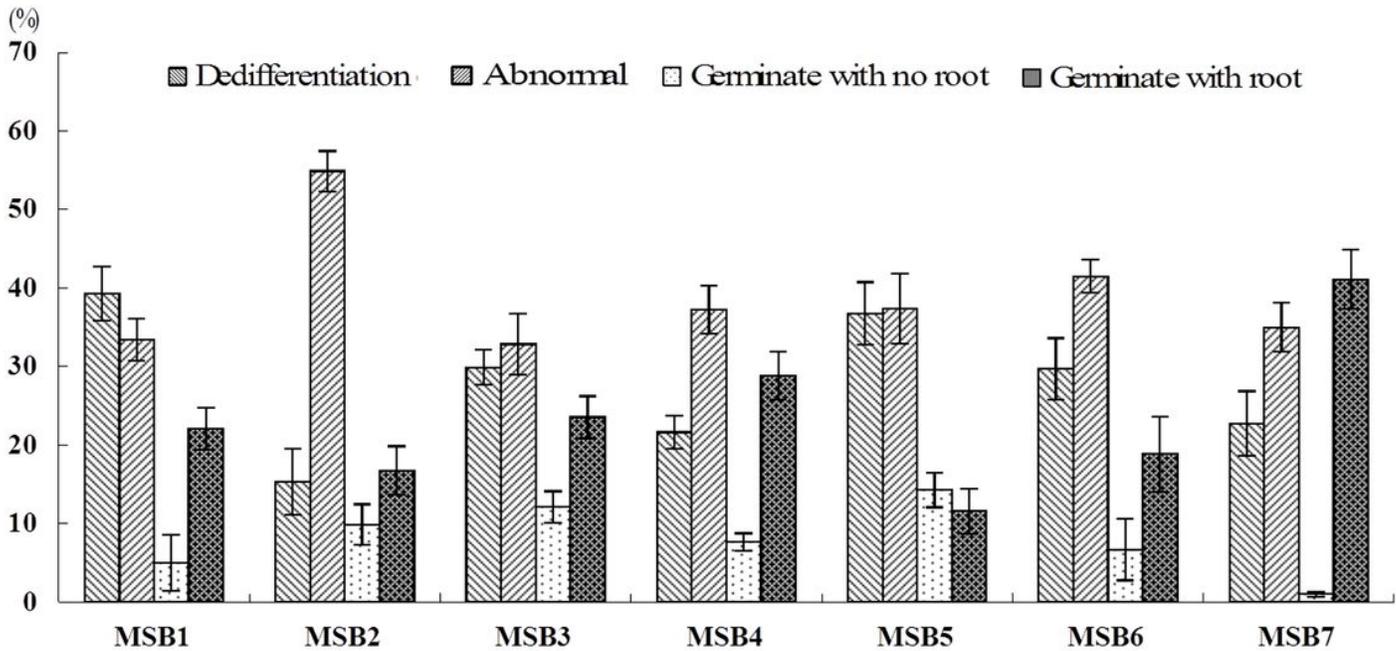


Figure 3

The callus differentiation ratio of developing somatic embryos under various of hormone combinations. The statuses of embryo development were classified into four types: dedifferentiated, abnormal, germination without root and germinated with root. Values are the means of three replicates. X axis is the different MSB mediums contained various hormone combination, and Y axis is the ratio of different statuses of developed embryo under different treatments. MSB1: 1.48 μM IBA + 0.465 μM KT; MSB2: 2.95 μM IBA + 0.929 μM KT; MSB3: 2.46 μM IBA + 0.465 μM KT; MSB4: 0.984 μM IBA + 0.465 μM KT; MSB5: 0.984 μM IBA + 0.465 μM KT + 0.452 μM 2, 4-D; MSB6: 0.492 μM IBA + 0.465 μM KT + 0.452 μM 2, 4-D; MSB7: No hormones.

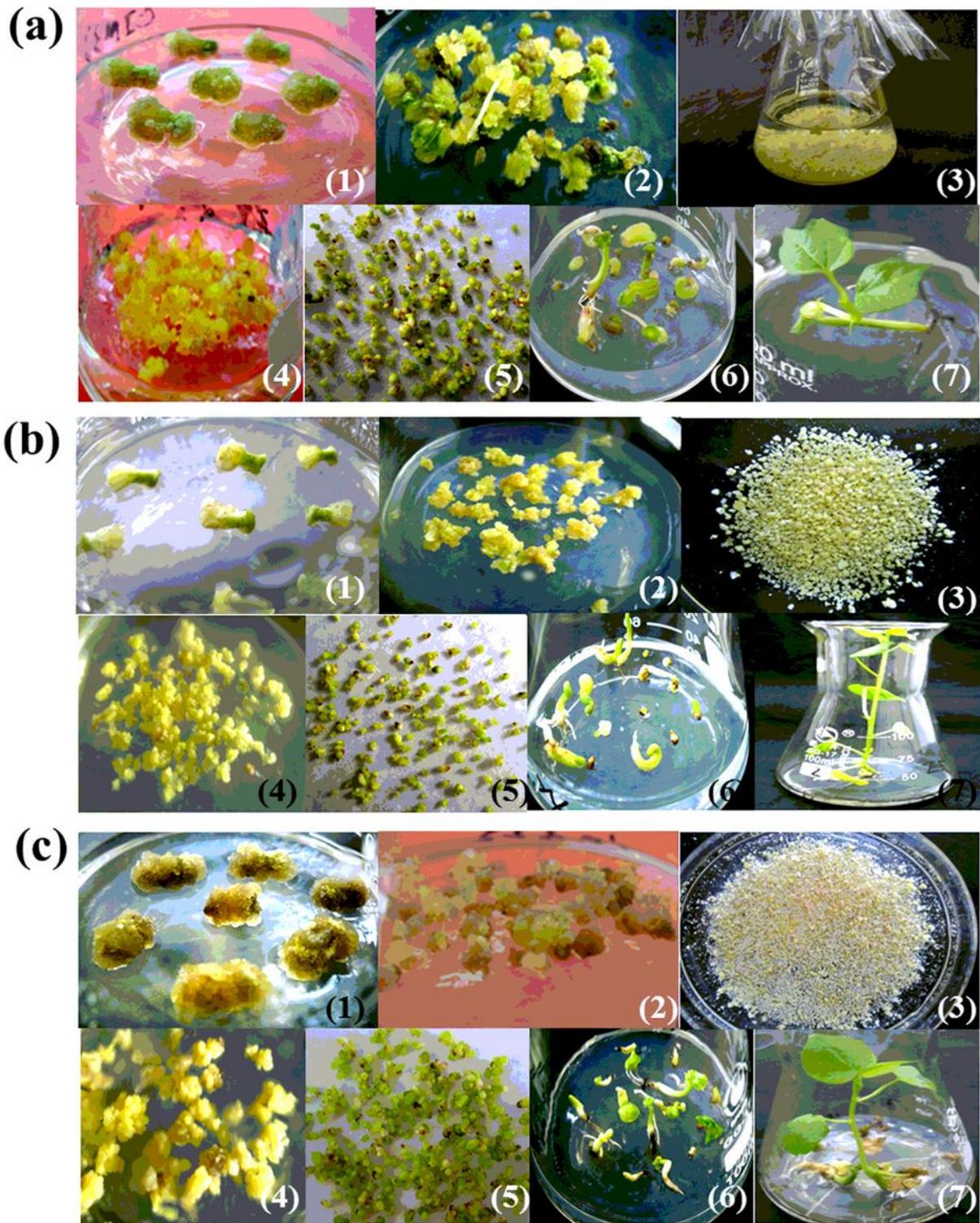


Figure 4

Somatic embryogenesis and plant regeneration via solid-liquid alternating culture in Upland cotton. (a) 'Coker 201'. (b) 'Jiwu 2031'. (c) 'ND58'. (1) Callus induction. (2) Pre-embryogenetic callus proliferation. (3) Suspension culture. (4) Embryogenetic callus. (5) Mature embryos. (6) Embryos germination. (7) Regenerated plantlets.

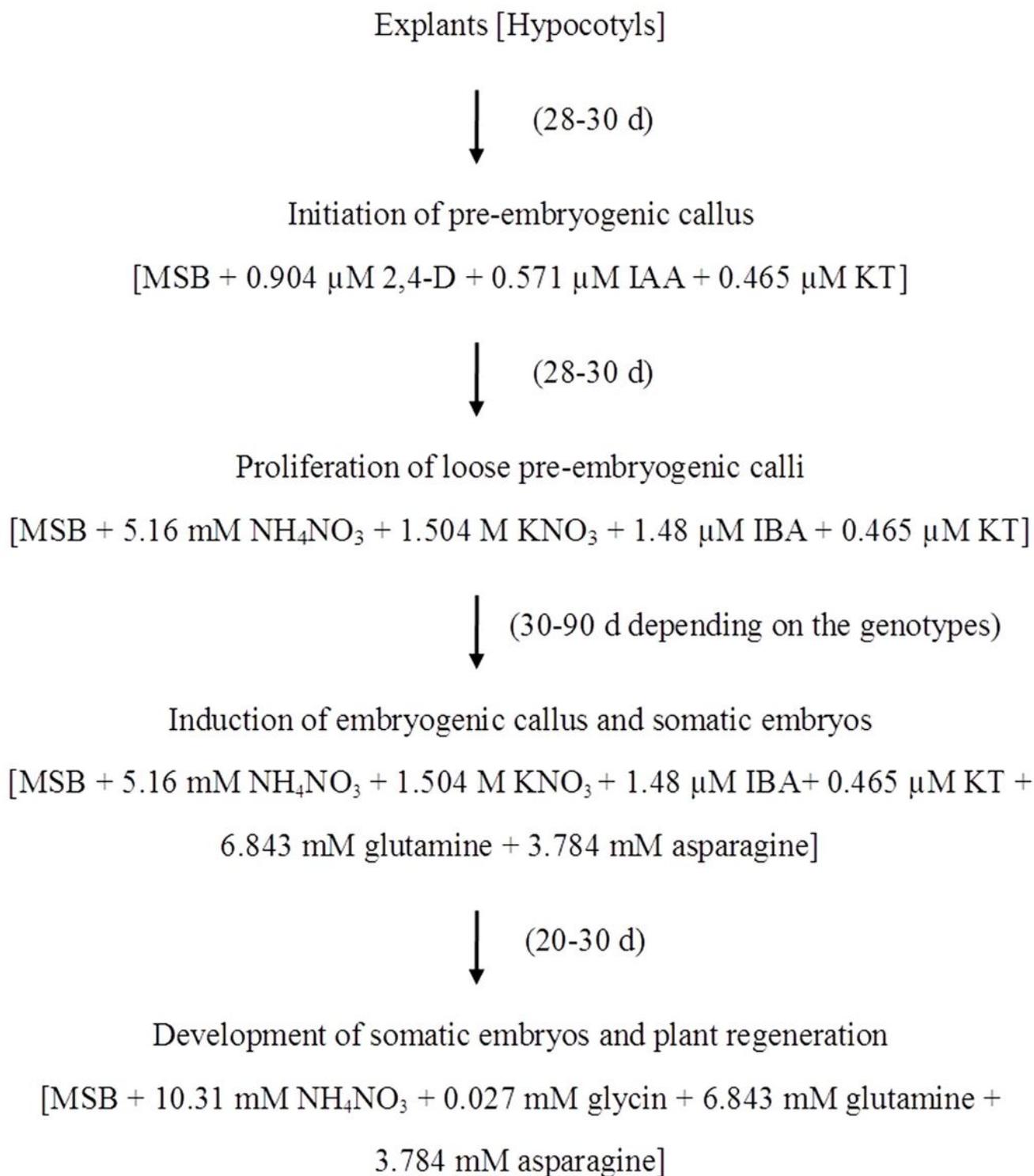


Figure 5

Schematic protocol via solid culture for somatic embryogenesis and plant regeneration of CAU102, Jiwu 2031 and ND 58.

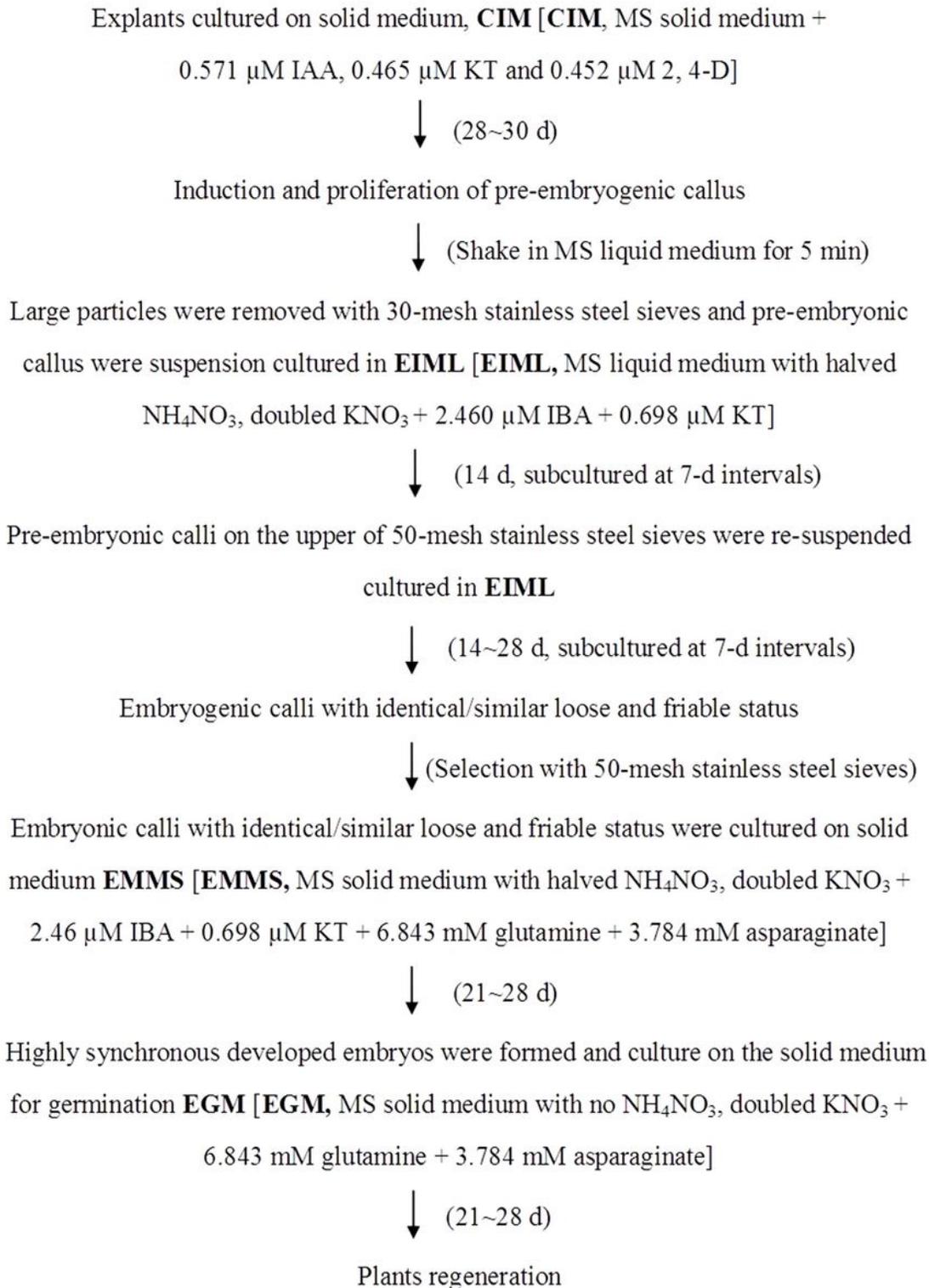


Figure 6

Schematic protocol of solid-liquid alternating culture and plant regeneration in Upland cotton.