# Plant regeneration via somatic embryogenesis in diploid cultivated cotton (*Gossypium arboreum* L.)

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# Abstract

The cultivated diploid cotton species *G. arboreum* offers a better opportunity to elucidate gene structure and function compared to the allotetraploid cotton species through genetic transformation, the reliable and efficient method for high frequency somatic embryogenesis and plant regeneration in *G. arboreum* is urgent need to be established. Callus was induced from hypocotyl, root and cotyledon of *G. arboreum* seedlings on MSB (MS salts and B5 vitamins) medium with 0.09 μM 2,4-D and 2.32 μM KT. The embryogenic callus was induced on $MS_5$ medium from the suspended cultures of several cycles of alternate liquid-solid culture, which was critical step for somatic embryogenesis. The liquid medium of $MS_4$ was supplemented with 0.1g/L NaCl, 0.1g/L KCl and 0.1g/L $CuSO_4$. The solid medium of $MS_5$ for embryogenic callus effective induction was supplemented with 37.59 mM $KNO_3$ + 62.47 μM $NH_4NO_3$ and 2.46 μM IBA + 0.93 μM KT or 0.045 μM 2,4-D + 2.46 μM IBA + 0.465 μM KT. During callus growing on different media, callus was effectively selected for subculture or treatment according to cell morphology to induce embryogenic callus and somatic embryos. Somatic embryo maturation and germination were better on $MS_5$ medium with maltose or glucose + maltose than the single glucose. The regenerated plantlets with well-developed roots were directly transferred to soil or grafted onto the germinated cotton plantlets. The feasible process of plant regeneration via somatic embryogenesis in diploid cultivated species was established and needed to be improved and optimized for the gene functional analysis and gene editing in the diploid cotton species.


# Key Message

Regenerated plants of *G. arboreum*, the cultivated diploid cotton, were obtained via somatic embryogenesis by adjusting the culture mode and medium compositions.

# Main Text

Cultivated cotton is one of the world's most important commercial crops as a source of fiber. The *Gossypium* genus consists of over 50 species, only four of them (*G. hirsutum* L., *G. barbadense* L., *G. arboreum* L. and *G. herbaceum* L.) are widely cultivated and provide spinnable fiber, two of these are tetraploid species with AD genome, and two are diploid species with A genome (Wendel et al. 1992; 2009). All tetraploid cotton species came from interspecific hybridization between the A-genome species, the possible ancestors of/or *G. arboreum* ($A_2$) or *G. herbaceum* ($A_1$) and the D-genome species *G. raimondii* ($D_5$) (Wendel et al. 2009; Li et al. 2014; Du et al. 2018; Huang et al. 2020). *G. arboreum* accessions play a key role in cotton improvement and has been reported to be resistant to several pests (bollworms, thrips and spider mite) and diseases (Dhawan et al. 1991; Stanton et al. 1992; Wheeler at al. 1999; Sakhanokho et al. 2004; Kantartzi et al. 2009; Kulkarni et al. 2009; Miyazaki et al. 2012; Ullah et al. 2014), and provides the reference genome for other cotton species (Li et al. 2014).

Because of the complex allotetraploid nature, the large genome size of 2.5 Gb in *G. hirsutum*, much more complex genetic structure and larger genome compared to the diploid species, most genes have multiple

copies distributed in $A_t$ and $D_t$ subgenomes, makes genetic, genomic and functional analyses extremely complicated and challenging (Paterson et al. 2012; Li et al. 2015; Zhang et al. 2015; Wang et al. 2019). For example, *G. hirsutum* has multiple copies and homologous residings on each $A_t$- and $D_t$-subgenome, perhaps each homologous pair of genes has four alleles that will produce unexpected or more complex mutation profiles as well as the offtarget effects in CRISPR/Cas9-edited plants (Li et al. 2018; Li et al. 2019; Qin et al. 2019; Wang et al. 2018). Off-target mutations in CRISPR/Cas9-edited cells can further add complexity to the mutation analysis, and this is emerging as the major concern for this promising technology. Multiple copies and function overlapped of genes in allotetraploid cotton also significantly reduced the efficiency of RNA interference and even gene knockout, resulted in silencing of endogenous genes when cotton genes over expressed. Diploid cultivated cotton species offer a better opportunity than the allotetraploid cottons to elucidate gene structure and function through gene knockout and gene editing for cotton functional genomics. So the diploid cotton species with reproducible, and highly efficient plant regeneration scheme is a prerequisite for the coming functional genomic era.

One of the main problems facing cotton genetic engineering and functional gene analysis is the scarcity of cultivars or varieties that capable of readily producing regenerated plants via somatic embryogenesis. The recalcitrance of *Gossypium* species, especially diploid cotton *G. arboreum*, constitutes a major hindrance to the transfer of desirable characteristics into the cultivated cotton species through genetic engineering (Sakhanokho 2001). For *G. arboreum*, somatic embryo could initiate and germinate, somatic embryo production and maturation as well as plantlet acclimation were sporadic, regenerated plant still could not obtained through somatic embryogenesis (Sakhanokho et al. 2001; 2004a; Rajasekaran et al. 2004). So the improvement of tissue culture methods to induce efficient transformation in diploid cotton species is very desirable for cotton functional genomics and genetic improvement (Sunilkumar and Rathore 2001; Satyavathi et al. 2002 Huang et al. 2020).

For diploid cotton species, many factors influencing embryogenic callus induction and somatic embryogenesis were studied (Smith et al. 1977; Finer and Smith 1984; Sakhanokho et al. 2004a; Sun et al. 2003, 2006). The regenerated plants were obtained from *G. davidsonii*, *G. klotzschianum*, *G. raimondii* and *G. stocksii* through somatic embryogenesis (Sun et al. 2003, 2006; Wang et al. 2007). On the base of plant regeneration via somatic embryogenesis in wild diploid cotton species, we have investigated the effects of PGRs, suspension culture and the alternate suspension-solid culture on embryogenic callus formation, somatic embryo initiation and conversion to plantlet in *G. arboreum*, and further investigated the morphology of callus at the different stages for callus selection and subculture. A reliable protocol of plant regeneration through somatic embryogenesis described here could be used for development of diploid genetic transformation mediated by *agrobacterium tumefaciens*.

## Materials And Methods

## Seed sterilization, germination and callus induction

Mature seeds of the *G. arboreum* accession ZB-1 (this accession with purple petal base) were surface-sterilized in 0.1% (w/v) $HgCl_2$ for 5–8 min, followed by washing four times with sterile distilled water. The surface-sterilized seeds were cultured on $MS_0$ medium (Table 1, listed the media used in this study) for seedling germination, firstly maintained in darkness at 28 ± 1℃ for 3 days and then grown under LED lights (14 h photoperiod, light intensity 108 μmol $m^{-2}$ $s^{-1}$). Seven days later, hypocotyl sections, cotyledon sections and roots (about 5 to 10 mm) were inoculated on $MS_1$ medium to induce callus; pH was adjusted to 5.8 prior to autoclaving at 121℃ for 15 min. Cultures were maintained at 28℃ (14 h photoperiod) under LED lights.

Table 1
The characteristics of the initial callus and new formed callus from the suspension culture

| | Day of culture | Net fresh weight (g) | Grow rate of fresh callus (g/d) | Net dry weight (g) | Grow rate of dry callus (g/d) | Ratio of dry weight to fresh weigh |
|---|---|---|---|---|---|---|
| Initiated callus | 14 | 4.66 ± 0.339 | 0.33 | 0.29 ± 0.052 | 0.021 | 0.061 |
| | 28 | 3.97 ± 0.279 | 0.14 | 0.18 ± 0.020 | 0.007 | 0.046 |
| Sc1 | 14 | 2.57 ± 0.216 | 0.18 | 0.18 ± 0.015 | 0.013 | 0.070 |
| | 28 | 2.19 ± 0.107 | 0.08 | 0.14 ± 0.021 | 0.005 | 0.063 |
| Sc2 | 14 | 2.04 ± 0.116 | 0.15 | 0.19 ± 0.012 | 0.013 | 0.090 |
| | 28 | 2.19 ± 0.089 | 0.08 | 0.2 ± 0.015 | 0.007 | 0.091 |
| Sc3 | 14 | 1.59 ± 0.148 | 0.11 | 0.16 ± 0.014 | 0.011 | 0.099 |
| | 28 | 1.93 ± 0.212 | 0.07 | 0.19 ± 0.023 | 0.006 | 0.097 |

Sc1: new callus formed from one cycle of the suspension cultures of initial callus (C1 callus) in liquid $MS_4$ medium; Sc2: new callus formed from the two cycles of suspension cultures of SC2; Sc3: new callus formed from the suspended cultures of SC2 callus.

After about 4 weeks, the peripheral cultures covered the explants were transferred to $MS_2$ medium for 2 to 3 subcultures. Partial calli were directly subcultured on $MS_3$ medium. The other partial calli were transferred to liquid $MS_4$ medium for suspension culture about 2 weeks, then the suspended cultures

were transferred onto solid $MS_3$ medium. The new formed calli on $MS_3$ medium were continued to subculture on the $MS_3$ medium or suspension culture in liquid $MS_4$ medium. The alternate solid-liquid culture was used for initial callus or proliferated callus to induce embryogenic callus, and the newly formed callus on $MS_3$ medium was labeled as SC1 for one cycle of suspension culture and solid medium culture and SC2 for two cycles of alternative liquid and solid cultures. The media used in this study were listed in Table S1.

# Embryogenic callus induction and maintenance

The calli were alternate solid-liquid cultured in $MS_3$ solid medium and liquid $MS_4$ medium for several times (liquid medium exchanges every 7 days) according to cell morphology. The suspension culture system was described as the previous report (Sun et al. 2003; 2006). Suspension cultures of different cycles of alternate solid-liquid culture were collected and transferred onto $MS_5$ solid medium to induce embryogenic callus under different combinations of PGRs (Table S2). Embryogenic calli were cultured on $MS_5$ medium with different PGR combinations to explore the appropriate PGRs to maintain embryogenic state and convert into plantlets. The capacity of somatic embryogenesis was observed and scored by the number of embryos per gram fresh weight of embryogenic callus (EC) (No. /g FW) as our previous reports (Sun et al. 2003; 2006).

# Somatic embryogenesis

Friable, gray-green embryogenic cultures were then taken for further proliferate on $MS_5$ medium with 3% (w/v) glucose with different PGR treatments as Table 2, as well as for embryo maturation and germination. $MS_5$ with no PGR (G6) was served as a control. The frequencies of somatic embryo formation were counted as number of embryos per gram of fresh weight of cultures (No./g FW) after 2 subcultures (6–8 weeks).

# Somatic embryo maturation and germination

The somatic embryos with normal morphology were cultured on $MS_5$ medium with different sugar sources for maturation and germination. The $MS_5$ medium containing 3% (w/v) glucose was used as control. The frequencies of somatic embryo maturation and germination were recorded for each of the plates after 4 weeks of culture. Immature somatic embryos were characterized by lack of well-defined cotyledons. Normal somatic embryos were those with a pair of cotyledons and normal morphology (i.e., green and 3 to 12 mm in size). Embryo germination refers to the development of the apical area of the somatic embryo resulting in production of true leaves, and production of plantlets, when germinated embryos produced roots as well (Firoozabady and DeBoer 1993). The germinated embryos were planted on $MS_5$ medium for further germination with a pH of 5.95-6.0. Plantlets with poor root systems were subcultured on $MS_5$ medium again or grafted on the seed-germinated plants.

# Transfer to soil

Regenerated plants with well-developed shoots and roots were transferred to the pots covered by clear plastic bottle for one week, then the plants were hardened and transferred to the field nursery. The regenerated plantlets or shoot tips were also used as scions to graft on rootstock of natural seedlings of *G. arboreum* as our previous work (Sun et al. 2005; Jin et al. 2006). In the winter, the plants were conserved in the green house.

In the study, ten cultures were raised for each treatment, and all treatments were repeated over three times. The statistical significance of the differences was determined using the Student's t-test in Graphpad Prism 8 (Version 8.0.2). Differences between treatments were considered significant when *P < 0.05, **P < 0.01 and ***P < 0.001 in a two-tailed analysis.

# Results

Calli firstly appeared from the two ends of hypocotyls, the edges of cotyledons and roots of *G. arboreum* cv. ZB-1 on $MS_1$ medium. The color and the texture of calli were obviously different on the three types of explants, the initial calli from hypocotyls and roots were compact and light green or light-yellow (Fig. 1A, B), the initial calli from cotyledons were soft and light-green (Fig. 1C). Initial callus from hypocotyls and roots subcultured on $MS_1$ medium for two cycles, proliferated faster and became friable and grayish-white that presented various shapes and irregular states (Fig S1A,B), the proembryo-cultures were not observed in this type of callus. Initial callus from cotyledons gradually became very hard and browning or green, most of the calli could not proliferate on $MS_1$ medium (Fig S1C).

Here the callus from hypocotyls and roots was alternate solid-liquid cultured to induce embryogenic callus. The cell phenotype of initial callus from hypocotyls and roots were diversified and quite irregular, almost no inclusion in cells (Fig S1D, the initial callus was named as C1), initial callus from hypocotyls or roots was suspension cultured in $MS_4$ medium for 2 weeks, the suspension cultures were transferred on the $MS_2$ medium to produce new callus SC1 (Fig. 1D; Fig S1E), the cells in the new callus on $MS_2$ medium about 3 weeks became regular single cell with some inclusions (Fig. 1E; Fig S1F). Proliferated SC1 callus was suspended culture in $MS_4$ medium for 2 weeks again, then transferred onto $MS_3$ medium to form new callus (labeled as SC2, Fig. 1F), the cells of SC2 callus were further spheroidizated, and appeared as single cell with regular borders (Fig S1G), the SC2 callus was proliferated on $MS_3$ medium for 3 weeks, the cells were aggregated and presented various stick shapes (Fig S1H). The proliferated SC2 callus was continued to alternate solid-liquid culture of $MS_3$/$MS_4$ for 2–3 times, the cells of SC3 callus formed from the cultures became more regular and further spherized (Fig S1I), the cells of proliferated SC3 callus on solid $MS_5$ medium gradually became loose and yellow-green (Fig. 1G) and contained more illusions (Fig. 1J). The callus continued subculture on $MS_5$ medium for 2–3 times (3 weeks one time) became light-yellow and the cells appears as regular single cells with distinct nucleus (Fig. 1H,K). The callus of this status was subcultured on $MS_5$ medium for 2–3 times, this step was the first key point of differentiation during the whole somatic embryogenesis. The embryogenic callus gradually formed and appeared as light green and granular (Fig. 1I), the cells of embryogenic callus were compact single or

massive cells, contained rich inclusions (Fig. 1L), or the callus of this status formed soft and rapidly proliferating callus on $MS_5$ medium and almost could not differentiate and further form somatic embryos.

The morphology and growth status of initial callus and new callus formed from the suspension culture were different from the diverse PGRs and the culture modes (Table 1). The initial callus cultured on $MS_1$ medium with 0.09 μM 2, 4-D, 2.32 μM KT, kept fast-growing during the early 14 days, and relatively low-growing during late 14 days, the dry matter weight was very low, the callus contained very few inclusions, which was inconsistent with the cell morphology (Fig S1D). With the increased cycles of alternate solid-liquid cultures, the callus growth ratio was decreased, the growth rate of dry callus was declined, the dry matter weight gradually increased, indicated the callus cells contained more inclusions.

Embryogenic callus was obtained from nonembryogenic callus through several cycles of alternative solid-liquid culture and alternated different PGRs on solid $MS_5$ medium. Embryogenic callus of *G. arboreum* were obtained on $MS_5$ medium with 2.46 μM IBA, 0.93 μM KT (G5) or 0.045 μM 2,4-D + 2.46 μM IBA + 0.465 μM KT (G4) from the suspension cultures after several cycles of alternate solid-liquid culture. The embryogenic callus of *G. arboreum* became loose, granular, and light-yellow on $MS_5$ medium with 2.46 μM IBA and 2.32 μM KT (Fig. 1I,L) after several cycles of subcultures, somatic embryogenesis embodied in embryos and abnormal plantlets were formed from the light-green embryogenic callus during growing on $MS_5$ medium with G5 (Fig. 2A,C). Embryogenic callus was subcultured on the $MS_5$ medium with low concentration of PGRs (IBA 0.984 μM + KT 0.232 μM) to keep the embryogenic status for long time (Fig. 2B).

The culture mode strongly influenced the capacity of somatic embryogenesis in *G. arboreum* (Fig. 3A). The initial callus from hypocotyls on $MS_2$ medium were long-term frequently subcultured on $MS_3$ and $MS_5$ medium with different PGRs for over 15–18 months. The mass calli of over 20 bottles cultured on the different media, almost no embryogenic callus was induced on the single type of medium. Embryogenic calli were sporadically produced and mixed in nonembryogenic callus, and most of them were still friable nonembryogenic callus with fast-growing on $MS_5$ medium with 2.46 μM IBA and 0.93 μM KT. The somatic embryos produced in embryogenic cells, which originated from initial callus cultured on $MS_5$ medium, were significantly fewer than those from SC1, SC2 and SC3 callus, and it took about 2 years. The frequency of somatic embryogenesis was increased with the increasing cycles of solid-liquid culture. The alternate solid-liquid culture accelerated the induction of embryogenic callus and promoted somatic embryogenesis in 12 months (Fig. 3A).

The calli from the suspension cultures were treated with different PGRs to induce embryogenic callus and somatic embryos. The fresh embryogenic callus originated from the SC callus was cultured on $MS_5$ medium with 6 different PGRs combinations (Table S2). The capacity of somatic embryogenesis varied on $MS_5$ media with different PGR treatments after 2 subcultures of 6 weeks (Fig. 3B). Somatic embryos were relatively easily formed on $MS_5$ medium containing G4 and G5 with somatic embryos reaching 140

per g FW (fresh weight), PGRs were at relatively high level. On $MS_5$ medium with G4, the embryogenic calli proliferated quickly and easily re-formed nonembryogenic callus, more abnormal embryos produced (Fig. 2D). On $MS_5$ medium with G5, the embryos formed normally with various shapes (Fig. 2E,F). The frequencies of somatic embryo formation were almost at the same low level with G1, G2 and G3 with massive proliferated callus (Fig S2A,B). The frequency of somatic embryogenesis was only 20 per g FW callus on the $MS_5$ medium without no PGRs (Fig S2C), was also very low on $MS_5$ medium with G1 (Fig S2D), G2 (Fig S2E) and G3 (Fig S2F). Embryogenic cultures contained proembryo structures such as globular-shaped and heart-shaped embryos (Fig. 2F). These cultures gradually developed into somatic embryos of various stages, including tulip-shaped and cotyledonary embryos.

Somatic embryos were transferred onto $MS_5$ medium with G5 PGR and glucose, produced many immature embryos and abnormalities, the frequency of embryo maturation was about 16% (Fig. 3C). Abnormal embryos rarely germinated, further formed callus or abnormal embryos again as explant when subcultured on $MS_5$ medium. Frequencies of embryo maturation in *G. arboreum* were significantly improved on the medium with maltose 3% (w/v) or glucose 1.5% (w/v) + maltose 1.5% (w/v) ranging from 16 to 43.9% and the number of abnormal embryos formation decreased (Fig. 3C). Germinated embryos with an elongated hypocotyl and cotyledons were transferred onto $MS_5$ medium with G5 (2.46 μM IBA, 0.93 μM KT), glucose and maltose (1:1) for further development and maturation (Fig. 2G). Only about 8% of *G. arboreum* mature embryos produced plantlets with roots and/or leaves, many of the regenerated plantlets were subcultured to root (Fig. 2H) or produce new buds (Fig. 2I) on $MS_5$ medium. The plantlets with well rooting were successfully and directly transferred to the soil (Fig. 2J) or grafted onto germinated plants of *G. arboreum* (Fig. 2K). From the whole experiment, over 30 plants regenerated from the embryogenic callus of *G. arboreum* within 18 months (Fig. 2L).

In this study, normal plantlets were obtained in *G. arboreum* within about 18 months. The effective process of embryogenic callus induction and somatic embryo germination should be improved and optimized for the gene functional analysis and gene editing in the diploid cotton species.

# Discussion

The diploid *G. arboreum* or *G. herbaceum* or their other A-genome progenitor was considered to be a putative contributor of the A subgenome for tetraploid cotton ($2n = 4x = 52$) species, where the D subgenome came from *G. raimondii* ($D_5$) (Wendel et al. 2009;Li et al. 2014; Huang et al. 2020). The A-genome species of *G. arboreum* and *G. herbaceum* are cultivated and produce spinnable fiber like the cultivated tetraploid cottons of *G. hirsutum*, whereas the D-genome species only has 1–2 mm fuzzy covered seeds. No somatic embryogenesis was achieved with any accession of *G. herbaceum* (Sakhanokho et al. 2001) until today, the other A-genome species *G. arboreum* became the important choice for gene function analysis better than the allotetraploid cotton species, for the complex allotetraploid nature and the big genome size in *G. hirsutum*, while *G. arboreum* could offer the very good model for the analysis of cotton agronomic traits like fiber quality, disease and pest resistance (Guo et al.

2006; Shi et al. 2006; Qin et al. 2007; Ma et al. 2008; Pang et al. 2010; Qin and Zhu 2011; Pegg and Brady 2002; Khadi et al. 2011; Hande et al. 2017; Erpelding and Stetina 2018), and also provides an essential tool for the identification, isolation and manipulation of important cotton genes conferring agronomic traits for molecular breeding and genetic improvement of the allotetraploid upland cotton, *G. hirsutum*, currently dominates the world's cotton commerce (Li et al. 2014; Du et al. 2018Zhu et al. 2019; Huang et al. 2020).

Plant regeneration through somatic embryogenesis was primarily difficult in cultivated cotton species. Accordingly, cotton genetic improvement were also very difficult using biotechnology such as genetic transformation, protoplast culture, and somatic hybridization, even the coming gene editing technology. Since the complex genome of the allotetraploid *G. hirsutum* and the genotype dependence were strongly limited the highly effective regeneration system via somatic embryogenesis in the elite upland cotton cultivars. The regeneration systems of the wild species were different according to different species (Sun et al. 2003; 2006; Wang et al. 2007). The culture time, capacity and method of callus induction, proliferation, differentiation and somatic embryogenesis were different with various wild species, with the exception that callus-inducing medium was effective for the wild cottons tested. The diploid *G. arboreum* was still different to obtain somatic embryo and regenerated plants via somatic embryogenesis until now.

The selection of potential embryogenic callus was the crucial step in cotton regeneration, while for *G. arboreum*, it is not simple and sufficient to select embryogenic calli from nonembryogenic calli according to the published methods. Many measures were used to induce embryogenic callus from nonembryogenic callus just like in the wild cotton species, such as changing PGR combinations, suspension cultures, inorganic component ($MgCl_2$, $KNO_3$) regulation, and inorganic salts stress (Sun et al. 2006; Wang et al. 2007), amino acids added in medium to improve somatic embryogenesis, somatic embryo maturation and germination (Chia and Saunders 1999; Wu et al. 2004), changing the sugars as carbon source to prevent browning of cells, promote embryo mature and germination, and rooting (Sun et al. 2006; Kumria et al. 2015). The measures used individually or together were effective for wild cotton species, but not all measures were useful for *G. arboreum*. Callus of wild cotton species maintained their embryogenic potential over 48 months, when subcultured on media containing 3% (w/v) glucose with low level of PGR (Sun et al. 2006), glucose was suitable for inducing callus in wild cottons and *G. arboreum*.

Cotton somatic embryo maturation and germination were also another very crucial step to plant regeneration, and regulated by PGRs, environmental stresses including changing the concentrations of the major and minor salts, dehydration on filter paper, adjusting humidity (Firoozabady and DeBoer 1993; Kumria et al. 2003; Sun et al. 2006), perhaps a combination of stresses might be more helpful than a single stress condition for somatic embryogenesis (Kumria et al. 2003). PGRs such as IAA or NAA and $GA_3$ promoted embryo maturation in *G. hirsutum* (Trolinder and Goodin 1987), adding $GA_3$ and changing sugar sources effectively improved wild cotton species somatic embryos maturation and germination into plantlets, and effectively improved *G. arboreum* somatic embryogenesis. The various abnormality often happened in the wild cotton species (Sun et al. 2006), and also appeared in *G. arboreum*. High frequency of somatic embryogenesis in wild cottons and *G. arboreum* was hampered by the low

frequency of embryo maturation and conversion, and high frequency of abnormality (Sun et al. 2006). We have successfully induced callus from *G. arboretum*, regenerated plants obtained from *G. arboreum* through somatic embryogenesis were successfully transferred to the soil. We should continue to optimize the somatic cell culture system of *G. arboreum* to improve the regeneration efficiency and shorten the regeneration time. The effective somatic embryogenesis and plant regeneration in *G. arboreum* provide the essential tool for cotton gene function analysis, molecular breeding and genetic improvement of upland cotton.

# Abbreviations

2,4-D – 2,4-dichlorophenoxyacetic acid; MS – Murashige and Skoog (1962) medium; MSB – MS medium plus B vitamins (Gamborg et al., 1968); KT – kinetin; IBA – indole-3-butyric acid

# Declarations

### Contributions

YS conceived and designed the experiments. QJ, RW and LK performed the research and prepared the figures, DY participated in data analysis. YS and LK wrote and corrected the article. All authors reviewed and approved the manuscript.


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### Conflict of Interest

The authors declare that they have no conflict of interest.

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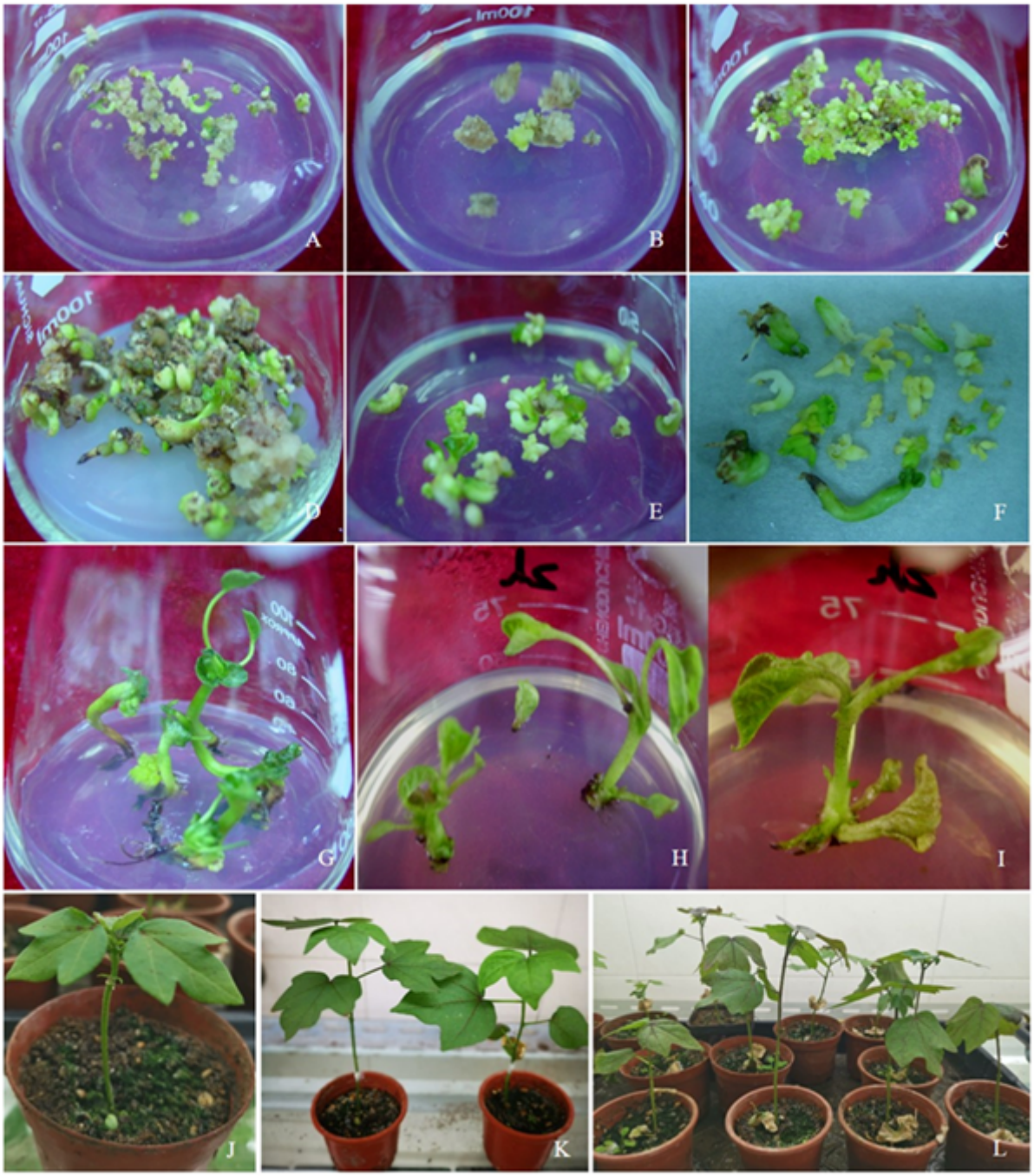
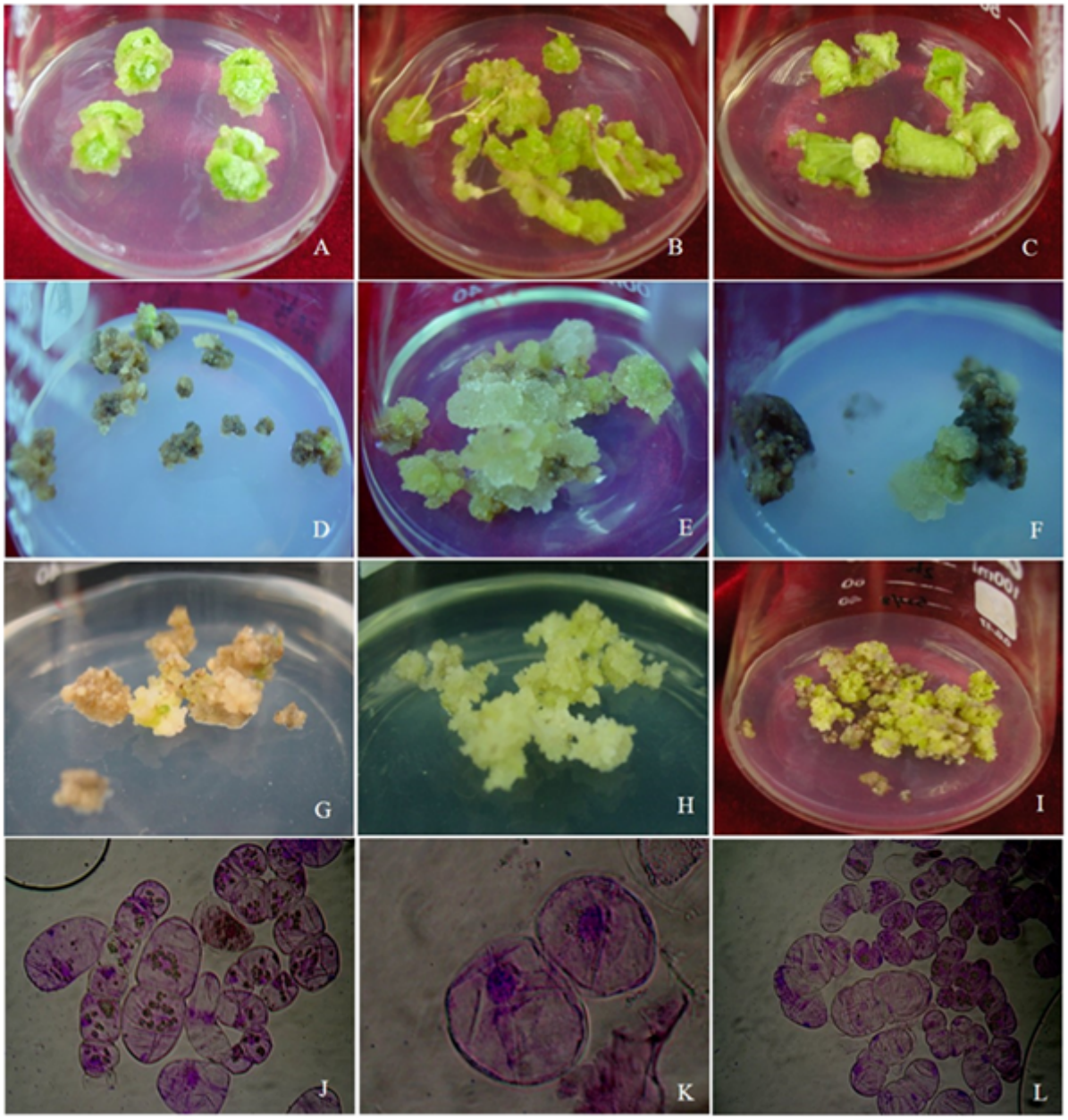
# Figures




## Figure 1

Callus and embryogenic callus induction from G.arboreum and habituated through alternate solid-liquid culture




## Figure 2

Somatic embryogenesis and plant regeneration from the embryogenic callus of G.arboreum

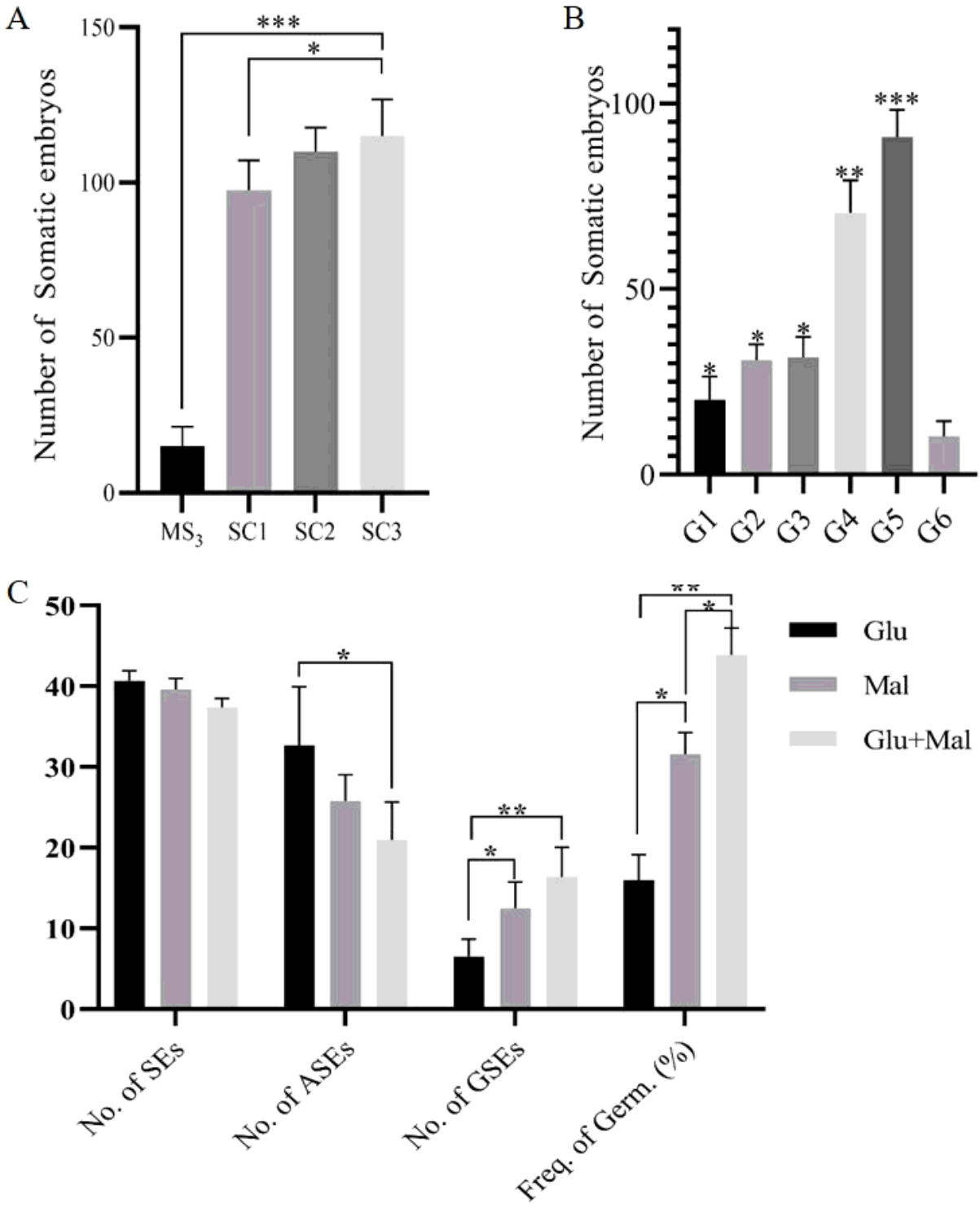



**Figure 3**

The effect of culture method, PGRs and carbon source on somatic embryo formation in G. arboreum

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