Somatic Embryogenesis In Rosa Chinensis CV. ‘Parson’s Pink China’

Yanfang Cai
Hunan Agricultural University https://orcid.org/0000-0001-7365-322X
Lintao Tang
Hunan Agricultural University
Haixia Chen
Hunan Agricultural University
Yufan Li
Hunan Agricultural University
Rong Liu
Hunan Agricultural University
Jiren Chen (279974250@qq.com)
Hunan Agricultural University

Research Article

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Somatic embryogenesis in *Rosa chinensis* cv. 'Parson's Pink China'

Yanfang Cai[1], Lintao Tang[1], Haixia Chen[1,2,3], Yufan Li[1,2,3], Rong Liu[4], Jiren Chen [1,2,3]*

[1]College of Horticulture, Hunan Agricultural University, Changsha 410128, PRC

[2]Hunan Mid-subtropical Quality Plant Breeding and Utilization Engineering Technology Research Center, Changsha 410128, PRC

[3]Engineering Research Center for Horticultural Crop Germplasm Creation and New Variety Breeding, Ministry of Education, PRC

[4]Hunan Biological and Electromechanical Polytechnic, Changsha 410127, PRC

*Corresponding Author: Jiren Chen; E-mail: 279974250@qq.com; Tel: 13787127751; Fax: 0731-84618171

Abstract

This study investigated the effects of explant type, plant growth regulator concentration, callis status, medium conversion time, and medium tilt on the growth of rose somatic embryos. The results showed that *Rosa chinensis* cv. 'Parson's Pink China' leaves could induce normal embryogenic calli but petioles could not. When the 2,4-dichlorophenoxyacetic acid concentration was 3.0 mg/L, the callis induction rate was the highest in the embryo proliferation medium (EP medium) supplemented with 0.5mg/L kinetin, and white and reddish-brown translucent calli were the main type of embryogenic calli induced. As the culture time in EP medium was extended, the relative induction rate for secondary embryos and multicotyledon secondary embryos gradually increased when transferred to embryo maturation medium (EM medium), but the induction rate for somatic embryos decreased. Placing the EM medium at an angle of 45° made the somatic embryos germinate faster and the germination rate was also higher. The germination buds produced by the somatic embryos with two cotyledons showed the fastest germination and greatest survival rates. The results of this experiment will help improve somatic embryo regeneration rates and explore new ways of regeneration, and lays the foundation for further optimization of the somatic embryo genetic

transformation system.

Keywords: Calli induction; somatic embryogenesis; medium; plant growth regulators (PGRs)

Main Conclusion

Extend the embryogenic callus culture time to produce secondary embryos and the secondary embryos can germinate normally. Inclined culture makes the germination rate of somatic embryos higher.

Introduction

Rose is a traditional Chinese flower that has great application value. In addition to being used as fresh cut flowers, it also plays an important role in the food and cosmetic industries. However, it takes considerable manpower and material resources to obtain offspring from Chinese rose plants using conventional cross breeding and other methods. Moreover, some rose varieties are not easy to reproduce, and it is more difficult to use traditional breeding methods to improve varieties. Therefore, molecular plant breeding has gradually entered the field of vision of scientific researchers. Molecular plant breeding is an extension and development of traditional breeding methods. It uses advanced molecular biology techniques to introduce target genes or DNA fragments through vectors or directly into recipient cells to recombine target genes and genetic materials, and new genes are being affected. Expressed in somatic cells, and finally screened out valuable new types from transformed cells to form engineered plants, thereby creating a new technology for targeted breeding of new varieties. Nowadays, the introduction of target genes into recipient cells and their expression through Agrobacterium-mediated is one of the most commonly used molecular plant breeding methods. Although it is convenient to use Agrobacterium-mediated genetic transformation, problems also arise. Many plants transformed by Agrobacterium are chimeras, with poor genetic stability, and the silencing of foreign genes is prominent. The efficiency of high-frequency genetic transformation depends on the high-frequency regenerated plant receptor system. Most somatic embryos originate from single cells. The regenerated plants formed by somatic embryos have relatively stable genetic characteristics and few transgenic plant chimeras

obtained from transformation. And the embryoid body has a strong ability to accept foreign DNA. It is an ideal gene transformation competent cell. Therefore, a stable genetic plant can be obtained through the regeneration of somatic embryos. To make the genetic transformation efficiency of the somatic embryo regeneration system more efficient, it is particularly important to continuously optimize the induction and regeneration process of somatic embryos.

Plant somatic embryogenesis, as the name implies, refers to the process by which plant somatic cells under in vitro conditions develop into new individuals through a pathway similar to that of zygotic embryos. In 1902, the famous German botanist Haberlandt stated that any cell of a plant has the potential to grow into a complete individual, and proposed the concept of plant cell pluripotency. This is the beginning of plant tissue culture. By 1943, White formally put forward the theory of plant cell totipotency. He believed that each plant cell contained a complete set of genome and had the ability to develop into a complete plant. After that, research on plant tissue culture began to develop rapidly. The occurrence of somatic embryos was first obtained by Reinert and Steward et al. in 1958 from the storage roots of carrots, which formed regenerated plants. Therefore, carrots have always been an important model plant for the study of somatic embryos (Steward et al. 1958; Reinert 1959). This major breakthrough not only confirmed the pluripotency of plant cells, but also opened up a new way to study the morphogenesis of plant cells. After that, the factors affecting plant somatic embryogenesis and regeneration began to be studied. According to incomplete statistics, since the 1960s, most rare or important tree species can form regenerated plants through somatic embryogenesis, realizing the industrialization of somatic cell engineering seedling breeding (Shi 2000). Plant somatic embryogenesis has not only become one of the most important means for rapid plant propagation, but also has been applied to genetic transformation in recent years. For example, Liu et al. (2021) used rose leaves as explants to induce somatic embryos with green fluorescent protein as a marker gene and transformed with agrobacterium strain GV3101. They produced transgenic buds after 8 months and the conversion efficiency was as high as 6%. Zakizadeh et al. (2013) transformed rose embryonic calli using agrobacterium strains AGL1, GV3850, and LBA4404 (containing the P35S-INTGUS gene) and successfully obtained transgenic buds with a maximum transformation efficiency of

10%. In addition to rose, somatic embryo induction and regeneration systems have also been applied to coffee (*Coffea arabica* L.) (Ferrari et al. 2021; Valencia-Lozano et al. 2021), tree fern (Anna et al. 2021), *Arabidopsis* (Kamila et al. 2021), *Pinus thunbergii* Parl. (Sun et al. 2021), and other plants.

In general, the number of somatic embryos produced from zygotic embryos is higher than that of other plant tissues and they are easier to induce. For example, Liu et al. (2020) used the cotyledon of a *Fraxinus mandshurica* immature zygotic embryo as the material, first induced the formation of calli, and then formed the somatic embryo. After transplantation and domestication, a completely regenerated plant was obtained and the regeneration rate was 26.4%. Yang et al. (2021) used the immature zygotic embryo of *B. platyphalla* as the material and identified an effective plan for inducing somatic embryogenesis, and Ansari et al. (2021) used young cork oak (*Quercus suber* L.) zygotic embryos and young parietal leaves as materials to induce somatic embryogenesis. Except for zygotic embryos, somatic embryo induction techniques can also use seeds, leaves, petioles, pollen, and radicles as materials. For example, Fawzi et al. (2021) used *Arabidopsis* seeds as materials to establish a simple and efficient somatic embryogenesis induction method and Zakizadeh et al. (2008) used *Rosa hybrida* L. detached leaves for somatic embryogenesis. Different explants have different induction rates for somatic embryos (Siong et al. 2011; Ju et al. 2014). In addition to explants, different plant growth regulators (PGRs) also play an important role. Plant growth regulators, such as 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine, thidiazuron (TDZ), zeatin (ZT), and naphthalene acetic acid (NAA), are widely used in the somatic embryo induction process. Different plant growth regulators and concentrations have different induction effects. For example, when attempting to induce rose somatic embryos, it has been reported that 2,4,5-trichlorophenoxyacetic acid can induce somatic embryos over a larger concentration range than 2,4-D, which means that it can significantly increase the embryo yield (Estabrooks et al. 2007). Furthermore, *Rosa hybrida* L. regeneration frequency is highest on 1 mg/L NAA medium, followed by 4 mg/L ZT medium (Kil et al. 2003). At present, although research on somatic embryos has been gradually perfected, there are still shortcomings and further research is still needed. This study investigated the effects of explant type, plant growth

regulator concentration, callus status, medium conversion time, and medium tilt on the growth of ‘Parson’s Pink China’ (*Rosa chinensis*) somatic embryos. The changes during each period are described in detail and the results provide a reference for further optimization of the plant genetic transformation system and perfecting the influencing factors of somatic embryogenesis.

Materials and methods

Induction of ‘Parson’s Pink’ calli and embryogenic calli

The rose variety ‘Parson’s Pink China’ (*Rosa chinensis*), grown using the aseptic system at the ornamental horticulture tissue culture room of Hunan Agricultural University, Chang Sha, China, was selected as the experimental material. The young leaves and petioles of the rose tissue culture seedlings (6–8 cm) were used as explants. The average leaf size of the rose seedlings was about 0.3 cm^2, with a length of 1 cm and a width of 0.6 cm. The last leaf was cut in half from the middle in a direction perpendicular to the vein to obtain an explant with a length of 0.5 cm and a width of 0.6 cm. The average length of the petiole was about 0.7 cm. The leaves and petioles were placed in embryo proliferation medium (EP medium), which contained Schenk & Hildebrandt basal salt mixture (SH, Phyto Technology Laboratories, Shawnee Mission, KS, United States) + sucrose (3%) + L-proline (300 mg/L) + powdered agar (0.4%, Solarbio) + inositol (0.1 mg/L) + 2,4-D (0, 1.0, 2.5, 3.0, 3.5, or 7.0 mg/L), and either kinetin (KT, 0.5 mg/L) to explore the effect of 2,4-D concentration on leaf and petiole calli induction in EP medium. The pH of the EP medium was adjusted to 5.7 using 2 mg/L NaOH and 5 mg/L hydrochloric acid before sterilization. Then, the mixture was placed in a high-pressure steam sterilizer for sterilization and the temperature and time were set to for 25 min 121 °C. The culture medium was dispensed into petri dishes after sterilization (1 L of culture medium can be divided among 50 culture dishes). After the medium had cooled, the material was laid flat on the medium. A total of five leaves or petioles were placed on each petri dish. There were three repetitions per group. The 2,4-D at 0 mg/L was the control. The other conditions were the same. The leaves and petioles were cultured in the dark, the culture temperature was set to 25 ± 2 °C, the medium was changed every 4 weeks, and the type and

146 growth of the calli were then observed. After all the embryogenic calli grow out, all the
147 embryogenic callis were transferred to EP medium with a 2,4-D concentration of 3 mg/L for
148 culture. The culture period was 13, 17, 21 weeks.
149
150 ***Somatic embryogenesis of 'Parson's Pink China' rose***
151 After culturing in EP medium to the 13th, 17th, and 21st weeks, 1000 mg of white and
152 reddish-brown translucent embryogenic calli induced by leaves were selected. The
153 embryogenic calli were transferred to embryo maturation medium (EM medium) and cultured
154 under red light/dark (16 h/8 h) to promote somatic embryogenesis. The medium contained SH
155 + 2,4-D 1.0 mg/L + TDZ 0.1 mg/L + abscisic acid (ABA, 1.0 mg/L) + gibberellic acid (GA_3, 3.0
156 mg/L) + sucrose 3% + powdered agar 0.4% (Chen et al. 2015). The pH of the EM medium was
157 adjusted to 6.0 using 2 mg/L NaOH and 5 mg/L hydrochloric acid before sterilization. After
158 sterilization in a high-pressure steam sterilizer, the medium was gradually cooled until it was
159 just warm and then 3.0 mg/L of GA_3 and 1.0 mg/L of ABA were added to the medium. The
160 cultures were then shaken well and dispensed into culture flasks (1 L of medium can be
161 dispensed into 25 culture flasks). After the medium had cooled, the embryogenic calli were
162 placed in EM medium and cultured under red light/dark (16 h/8 h) conditions. The red light
163 used in the experiment was provided by 13W Mini Twister Energy Saver Red bulbs (Philips,
164 Guangzhou, China) with a light intensity of about 7.2 μmol/m^2/s. The culture temperature was
165 set to 25 ± 2 °C, the medium was changed every 4 weeks. After culturing to the 11th week, the
166 type and growth of the somatic embryos were observed and recorded.
167
168 ***Plant regeneration from somatic embryos***
169 After the embryos had been cultured in EM medium to the 13th week, the somatic
170 embryos were transferred to the tissue culture room under cool white fluorescent tubes (TLP
171 36W/840; Philips) for culturing. Half of the medium cultures were randomly placed at an angle
172 of 45° and half on a flat table to explore the influence of tilting the medium on the germination
173 of somatic embryos. The initial numbers of the various somatic embryos were counted before
174 germination. The lighting conditions were set to white light for 16 h/dark for 8 h. The light

intensity was about 27 μmol/m^2/s, the culture temperature was 25 ± 2 °C, and the medium was changed every 4 weeks. The somatic embryos were cultured in EM medium to the 20th week. The germination rate for the somatic embryos was measured and then they were transferred to shoot proliferation medium (SP medium) for plant regeneration. The SP medium contained Murashige & Skoog basal medium with vitamins (MS, provided by Phyto Tech Labs, Lenexa, Kansas, USA) + 6-benzylaminopurine 1.0 mg/L + NAA 0.05 mg/L + GA_3 3.0 mg/L + powdered agar 0.6%. The pH of the SP medium was first adjusted to 6.0 using 2 mg/L NaOH and 5 mg/L hydrochloric acid before sterilization. After sterilization in a high-pressure steam sterilizer, the medium was gradually cooled until it was slightly warm and then 3.0 mg/L GA_3 was added to the other culture medium ingredients on a clean workbench. The medium was then shaken well and dispensed into culture flasks. After the medium had cooled, the germinated somatic embryos were transferred to SP medium, the culture temperature was 25 ± 2 °C, and the lighting conditions were set to white light for 16 h/dark for 8 h. The white light was provided by cool white fluorescent tubes (TLP 36W/840; Philips) and the light intensity was about 27 μmol/m^2/s. The regeneration rates of the various germinated somatic embryos were counted at 16 weeks after the start of culture. After 1 week, the regenerated seedlings were placed in the rooting medium (½ MS + 1.0 mg/L 3-indolebutyric acid + 0.5 mg/L NAA + sucrose 3% + agar powder 0.7%) to induce rooting. The roots were allowed to grow to about 2 cm for domestication. Then the plants were transplanted to the greenhouse for cultivation.

Statistical Analysis

The percentage data were arcsine transformed prior to ANOVA to stabilize the variance. Related data were analyzed using SPSS 19.0 (SPSS, Inc., Chicago, IL, USA) and compared using least significant difference tests at the 5 % probability level.

Results and analysis

Induction of 'Parson's Pink China' calli and embryogenic calli using different explant types, concentrations of PGRs, and types of calli

Table 1 shows that during the dark culture of EP medium for 6 weeks, the average calli

induction rates for the leaves (68.89%) were higher than those for the petioles (24.44%), indicating that EP medium is more suitable for the induction of leaf calli. When the 2,4-D concentration was 3 mg/L, the induction rate for leaf calli was the highest at 100%. During the culture process, the leaves and petioles all produced four types of calli. These were white sandy calli, white (Fig. 1A-a) and reddish-brown (Fig. 1A-b) translucent calli, and white flocculent calli. The different types of calli are cultured separately under the original culture conditions and the results showed that the petiole calli types gradually turned brown over the next three weeks, they began to shrink and die, and could not form an embryogenic calli. This shows that although EP medium can induce different types of petioles calli, it is not suitable for the long-term culture of petiole calli, nor can it induce an embryogenic calli. At the same time, the white and reddish-brown translucent calli began to show embryogenicity. At the 9th week, many granular, globular embryos appeared (Fig. 1B) and heart-shaped embryos appeared at 11 weeks (Fig. 1C). However, the white sandy calli could not induce embryogenic calli. Although the white flocculent calli induced radicles, it was unable to induce embryogenic calli. This shows that white and reddish-brown translucent calli are the main types of embryogenic calli induced. Therefore, when the concentration of 2,4-D is 3 mg/L, the white and reddish-brown translucent calli produced by leaves are the most suitable for the induction of embryogenic calli.

Somatic embryo and secondary embryogenesis

After culturing in EM medium for 9 weeks and observation with a microscope, the white and reddish-brown translucent embryogenic calli cultured in EP medium for 13 weeks induced three types of normal somatic embryos. These were somatic embryos without expanded cotyledons (SE0), and somatic embryos with one (SE1) or two (SE2) expanded cotyledons. Embryogenic calli cultured in EP medium for 17 weeks induced three kinds of somatic embryos with secondary embryos during the 9th week in EM medium (Fig. 2A–C), which were cultured in EP medium to the 21th week. This induced the production of multicotyledonous embryos with secondary embryos (Fig. 2D). These secondary embryos were located around the base of the somatic embryos (Fig. 2A–D). The different somatic embryos were counted

after they had been cultured in EM medium to the 11th week. The results showed that, extending the culture time in EP medium led to a gradual increase in the relative induction rate of secondary embryos and multicotyledon secondary embryos when transferred to EM medium, but the induction rate of somatic embryos decreased. This shows that after embryogenic calli are formed, the medium needs to be changed to reduce the concentration of cytokinin so that somatic embryos can be induced normally.

Plant regeneration of Parson's Pink China rose

After culturing in EM medium for 13 weeks, all the somatic embryos were transferred to white light/dark conditions (16 h/8 h) for culturing. The results show that somatic embryos germinated during this part of the culture process (Fig. 3A–C). After calculating the germination rates of the various somatic embryos at the 20th week, it was found that the germination speed of the somatic embryos in the medium placed at an angle of 45° was faster than that of the flat medium, and the germination rate was also higher than that of the flat medium. During the culture process, the secondary embryos of SE0, SE2 and multicotyledonous embryos became the leaflets of somatic embryos and grew alongside the somatic embryo; part of the secondary embryos of SE1 germinated from the base (Fig. 3D–a), and it formed a competitive relationship with the growth of SE1, which inhibited the germination of part of SE1. It shows that the germination ability of SE0, SE1 and multicotyledonous embryos is greater than that of secondary embryos, and is less affected by secondary embryo nutritional competition, while SE1's germination ability is weaker than other types of somatic embryos and is more affected by secondary embryos. The germinated somatic embryos were transferred to SP medium for culture. By the 16th week, healthy seedlings had regenerated (Fig. 3E). The survival rates for the germination buds of the various somatic embryos were calculated and the results showed that the survival rate for buds germinated from SE2 was the highest. After a further week of cultivation, the regenerated plants were transferred to the rooting medium to induce rooting. When the roots were about 2 cm in length, they were domesticated and transplanted into the substrate. After being placed in the greenhouse for cultivation, a total of 89 healthy seedlings (Fig. 3F) grew after 2 months.

Discussion

The regeneration process for rose somatic embryos is affected by many factors. In this process, the formation of embryogenic calli is the key to culturing. This study shows that the leaf can be used to induce embryogenic calli, but the petiole cannot. In addition to the differential expression of genes in different explants, these differences may also be related to the plant genotype and the type and concentration of PGRs. Previous studies have reported that when *Rosa rugosa* is cultivated with 2,4-D and ½ MS medium, 38%, 6.7%, and 8.8% of mature zygotic embryos, cotyledons, and radicle explants, respectively, formed embryogenic calli (Kim et al. 2009). Yang et al. (2020) used the leaf, petiole, and stem axillary buds of rose (*Rosa spp*.) as explants and found that only the stem axillary buds could induce embryogenic calli. This shows that the selection of appropriate explants is critical to the formation of rose embryogenic calli, which is also the case for somatic embryo induction. The explant parts that can induce an embryogenic calli vary for different plants, which shows that the genotypes of related plants also play a decisive role. For example, Zakizadeh et al. (2008) used the detached leaves of eight cultivars (‘Leonie’, ‘Linda’, ‘Sonja’, ‘Toledo’, ‘Tiffany’, ‘Mette’, ‘Etna’, and ‘Andromeda’) of *Rosa hybrida* L. as explants. They found that, except for ‘Tiffany’ and ‘Andromeda’, all cultivars induced an embryogenic calli when cultured with MS medium supplemented with different concentrations of ZT or TDZ. In addition, appropriate hormone types and concentrations are the basis for embryogenic calli induction. Kamo et al. (2005) found that the embryogenic calli of red rose cultivar Kardinal with SH medium supplemented with 13.6 μM 2,4-D was better than that with MS medium supplemented with 18.1 μM dicamba and 0.46 μM kinetin because the number of mature embryos increased by three times. Kim et al. (2003) found that immature zygotic embryos of *Rosa hybrida* L. did not form somatic embryos or embryogenic calli in ½ MS medium supplemented with different concentrations of 2,4-D. Embryogenic calli did form on a medium containing 1.36 μM 2,4-D and 4.44 μM benzylaminopurine at a frequency of 25%. In this experiment, the calli were mainly white and reddish-brown translucent and their texture was very soft when the 2,4-D concentration ranged from 2.5–3.5mg/L. Studies have shown that it is an induced embryogenic callus. The results

291 also show that the concentration of hormones also affects the formation of embryogenic callus
292 by affecting the growth state of the callus. In addition to the above influencing factors, light
293 (Mahmoud and Hassanein 2018), DNA methylation changes and exchange during cell
294 differentiation in vitro (Xu et al. 2004), together with calli status (Kamo et al. 2004), etc. may
295 also be influencing factors.

296 After embryogenic calli are formed, they enter the somatic embryo induction period. In
297 this experiment, somatic embryos with secondary embryos and multicotyledon embryos were
298 obtained by extending the time in EP medium. Ammirato (1987) summarized several stages of
299 somatic embryo development in Gemini plants from the aspect of cytology. Generally,
300 cotyledons are formed by a series of cell divisions of the original embryo, in which a circle of
301 cells forms a cotyledonary collar, and two growth centers appear. From this, two cotyledon
302 primordia develop. If excessive cell division or premature cell enlargement occurs, the
303 structure of the cells in the cotyledonary collar will be affected, and multiple growth centers
304 may be formed and multiple cotyledons may develop. If the cells forming the cotyledonary
305 collar do not divide sufficiently, only one cotyledon will form. For example, after the cotyledon
306 stipule is formed, even after the leaf primordium appears, the cell division activity is still
307 vigorous, and cotyledon development will be connected to each other; if the cell division
308 activity is too low, cell vacuolation will appear prematurely, which will lead to the failure of
309 cotyledon development. This may explain the loss of secondary embryos in this experiment.
310 The EP medium used in this experiment contained cytokinins, such as KT, which have
311 stronger division abilities at slightly higher concentrations. In the early stage of embryonic
312 development, if cell division continues, it will cause the generation of new embryonic growth
313 centers and the formation of multiple or secondary embryos. This suggests that, somatic
314 embryo generation and regeneration efficiency can be improved by reducing the generation of
315 secondary embryos, which means that the point at which the culture medium should be
316 transformed needs to be identified. Previous studies have shown that after the primary somatic
317 embryos are cut into small pieces, secondary embryos can be grown and normal plants can be
318 formed after induction (Zou et al. 2016). This shows that the culture of secondary embryos is
319 also important. This may be achieved by separating and cultivating secondary embryos from

the initial embryos or directly inducing secondary embryos and then separating them. In this way, multiple somatic embryos can be obtained from one somatic embryo. A steady stream of induced or regenerated materials. Using this method can greatly save somatic embryo induction time and improve the efficiency of regeneration and genetic transformation. With regards to other formation factors that affect secondary embryos, Lv and Shi (2006) reported that after being mechanically damaged and separated from the mother, the growth regulators stored in the cells are redistributed to dedifferentiate the cells. During the dedifferentiation process, the factors that control somatic embryogenesis may be activated and show specific gene expressions through signal transduction. They then initiate the somatic embryogenesis program. However, this study did not mechanically damage the base of the somatic embryo, but induced secondary embryos still appeared. It is speculated that the main factor affecting the formation of multiple and secondary embryos is the long culturing time in EP medium, which resulted in prolonged exposure to higher concentrations of cytokinin in the early stage of somatic embryogenesis. Further research is needed on the formation of rose secondary embryos, and its influencing factors and mechanisms, which may be beneficial to the exploration of new rose regeneration pathways and provide information about the exchange of materials and information between cells.

The results showed that tilting the culture medium can induce the germination of somatic embryos and increase their overall germination rate. Too much water on the surface of the medium will affect the germination of somatic embryos and the inclined placement of the medium may have caused a certain amount of drought stress, which is conducive to the germination of somatic embryos. The SE2 somatic embryos had the highest germination rate in this study. To enable embryogenic calli to successfully induce somatic embryos and produce more SE2 type somatic embryos, the following factors may need to be considered. The first is light. Previous studies have shown that when embryogenic calli induced under dark conditions is treated with no light, red light, or white light, the largest number of somatic embryos are produced under red light treatment, mainly with one (SE1) or two (SE2) enlarged cotyledons. However, the largest number of somatic embryos without cotyledons (SE0) were produced by the dark treatment (Chen et al. 2014), which is similar to the results of this experiment. The

second is the composition of the medium. Zakizadeh et al. (2008) found that the frequency of primary somatic embryogenesis was 3.3% on a medium containing 45.6 μM ZT or 45.4 μM TDZ, while the highest proliferation multiple (3.8 times) and the highest embryo maturation frequency (76.6%) were obtained on the ½ MS medium containing 7.57 μM ABA. In addition, the ABA concentration is also important. Chen et al. (2014) found that ABA at a concentration of 9.45 μM was the most effective for the proliferation and germination of rose SE2 embryos. The higher the ABA concentration (from 0–18.90 μM), the higher the percentage of abnormal polycot embryos. Kamo et al. (2005) found that the survival rate for cotyledon stage embryos of the red rose variety Kardinal cultured in MS medium supplemented with ABA (5–20 μM) in the greenhouse was two times higher than that of embryos cultured without ABA, which was significant. These studies show that ABA plays a vital role in the culture of somatic embryos. However, another study found that endogenous ABA, endogenous, biologically active gibberellins, and 1-naphthlcetic acid (IAA) are jointly involved in the acquisition of leaf somatic embryonic capacity by *Medicago truncatula* Gaertn. It is speculated that they may have a similar effect on rose plants (Ewa and Anna 2021). In addition, amino acids also play an important role in the induction of somatic embryos. For example, Das (2010) found that proline stimulates auxin induction during somatic embryogenesis and has a considerable impact on rose somatic embryo development and secondary somatic embryogenesis. In addition to roses, amino acids also play an important role in the induction of somatic embryos in other plants. For example, Maruyama et al. (2021) found that on EM medium supplemented with 175 g/L polyethylene glycol (PEG), 100 μM ABA, 2 g/L glutamine, 1 g/L asparagine, and 0.5 g/L arginine, each gram of Sugi embryogenic cell lines can produce more than 1000 embryos. The above experiments confirmed that complicated mechanisms exist during the development of somatic embryos, and that many factors can affect them. However, to date, it has not been possible to comprehensively study the internal molecular mechanism and this requires further research.

After successfully inducing somatic embryos, plant regeneration becomes the crucial step and the regeneration process for plants is affected by many factors. Mahmoud and Hassanein (2018) in their study on *Rosa hybrida* L. cv 'Eiffel Tower' found that the source of the explants

is very important in the regeneration process. In situ explants produce calli, while in vitro implants regenerate somatic embryos and buds. They also found that gibberellin (GA_3) has a good induction effect on *Rosa hybrida* L. explants and there were no regenerated shoots on the medium containing TDZ, but regenerated shoots were directly obtained on the medium containing GA_3. This shows that GA_3 is a key requirement for successful regeneration. Different plants require different concentrations of GA_3. Generally speaking, low concentrations of GA_3 are not effective, while high concentrations of GA_3 have a counterproductive effect. However, a study by Kim et al. (2003) produced different results. Embryogenic calli were transferred to a medium without growth regulators. The calli subsequently produced a large number of somatic embryos, which then developed into seedlings. Ipekci and Gozukirmizi (2004) also stated that mature somatic embryos were isolated on MS medium without PGRs and then regenerated into somatic embryos. A total of 80% of the seedlings germinated. These results suggest that plant regeneration may also be related to plant genotype. Zakizadeh et al. (2008) investigated eight species of miniature roses and found that although 'Sonja' had the highest embryogenesis frequency (30%), the embryos obtained by 'Sonja' failed to develop into plants. In contrast, the primary somatic embryogenesis frequency for 'Linda' was 3.3%, but only the 'Linda' somatic embryos developed into flowering plants. In this experiment, the survival rate of the germinated SE2 embryos was the highest. However, the seedling mechanism that leads to the production of the three somatic embryos (SE0, SE1, and SE2) and which genes regulate their development is unclear and requires further research.

Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid

EP medium: embryo proliferation medium

KT: kinetin

EM medium: embryo maturation medium

PGRs: plant growth regulators

TDZ: thidiazuron

ZT: zeatin

NAA: naphthalene acetic acid

SH: Schenk & Hildebrandt basal salt mixture

ABA: abscisic acid

GA_3: gibberellic acid

SP medium: shoot proliferation medium

MS: Murashige & Skoog basal medium with vitamins

SE0: somatic embryos without expanded cotyledons

SE1: somatic embryos with one expanded cotyledon

SE2: somatic embryos with two expanded cotyledons

SEp: polycotyledonous embryos

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Conflict of interest

There is no conflict of interest for this study.

Author's contribution

All authors contributed to the study conception and design. Yanfang Cai is the experiment

designer and the executor of the experiment, completing the data collection and writing the first draft of the paper; Lintao Tang is involved in the collection of experimental materials and the result analysis; Haixia Chen and Yufan Li are responsible for the operation of the instrument and discussion of results, and Liu Rong participates in the discussion of results; Jiren Chen is the person in charge of this project, directing experimental design, data analysis and paper revision. All authors have read and agreed to the final text.

Data Availability Statements

All data generated or analysed during this study are included in this published article.

Tables

Table 1. Condition of the 'Parson's Pink China' leaf and petiole calli after dark culture for 6 weeks

2,4-D concentration (mg/L)	[a]Average leaf calli induction rate (%)	[b]Leaf callus type	Average petiole calli induction rate (%)	Petiole callus type
0	0d	/	0c	/
1	73.33 ± 11.55bc	A	33.33 ± 11.55ab	A
2.5	86.67 ± 11.55ab	AB	53.33 ± 11.55a	BC
3	100 ± 0a	BC	33.33 ± 11.55ab	CD
3.5	86.67 ± 11.55ab	BC	20 ± 20bc	CD
7	66.67 ± 11.55c	D	6.67 ± 11.55c	D
Mean	68.89 ± 34.45	/	24.44 ± 21.21	/

[a]Calli induction rate = number of calli in each group with a diameter greater than 2 mm / number of explants in each group.

[b]The types of callis represented by capital letters in the table are: A: white sandy calli, B: white translucent calli, C: reddish-brown translucent calli, D: white flocculent calli.

Means followed by the same lowercase letter within a column were not significantly different at $P < 0.05$.

542

543 **Table 2. Relative induction rates for somatic embryos and secondary embryos of**

544 **embryogenic calli cultured in EM medium for 11 weeks**

[a]Somatic embryo type	[b]Relative induction rates of somatic embryos (%)			Relative induction rates of somatic embryos with secondary embryos (%)		
	[c]13W	17W	21W	13W	17W	21W
SE0	19.9	20.4	18.5	0	2.0	3.7
SE1	12.8	12.5	11.8	0	0.6	1.3
SE2	5.7	4.2	3.8	0	0.4	0.8
SEp	0	0	0.6	0	0	0.6
Mean	9.6	9.275	8.675	0	0.75	1.6

545 [a]Somatic embryo types: somatic embryos without expanded cotyledons (SE0), somatic
546 embryos with one (SE1) or two (SE2) expanded cotyledons, polycotyledonous embryos (SEp).
547 [b]Relative induction rate of somatic embryos = induction number of somatic embryos in each
548 group / relative value. The relative value was uniformly set to 1000.
549 [c]In the table, 13 weeks, 17 weeks, and 21 weeks are the culture times for embryogenic calli in
550 EP medium. The start time was when the initial leaves began to be cultured.

551

552 **Table 3. Germination rates of various somatic embryos in the EM medium at the 20th**

553 **week and the survival rates of the germination buds associated with the various**

554 **somatic embryos in the SP medium at the 16th week**

Somatic embryo type	[a]Germination rates for somatic embryos (%)		[b]Bud survival rates (%)
	Placed at an angle of 45°	Laid flat	
SE0	15.03	11.59	66.20
SE1	9.14	8.16	46.88
SE2	29.85	30.00	82.93
Secondary embryo	12.5	9.375	40.00

SEp	25.00	0	100.00
Mean	18.304	11.825	67.552

[a]Germination rate of somatic embryo = germination number of somatic embryos in each group / initial value of somatic cells in each group. In the inclined medium, the initial value for SE0 was 286, SE1 was 175, SE2 was 67, the secondary embryo was 56 and the polycotyledon was 4. In the medium that was placed flat, the initial value for SE0 was 302, SE1 was 196, SE2 was 70, the secondary embryo was 32 and the polycotyledon was 2.

[b]Bud survival rate = the number of surviving sprouts in each group / the number of sprouting buds in each group.

Figures

Fig. 1 Embryogenic callis type

White (A-a) and reddish-brown (A-b) translucent calli were cultured in EP medium for 6 weeks, granular and spherical embryos (B) were cultured for 9 weeks, and heart-shaped embryos (C) were cultured for 11 weeks. Culture conditions: cultured in the dark at 25 ± 2 °C. Scale bar is 2 mm.

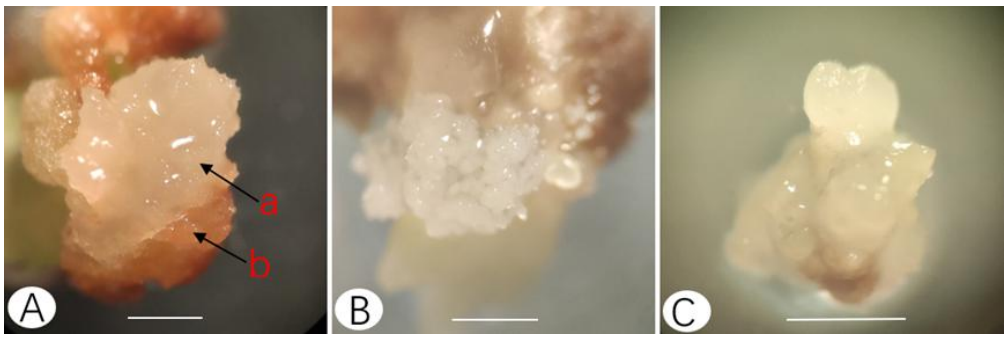

Fig. 2 Different somatic embryo types with secondary embryos

Embryogenic calli cultured in EP medium for 17 weeks and 21 weeks were transferred to EM medium and cultured for 9 weeks. Somatic embryos with secondary embryos were induced and cultured for either 17 weeks (A–C): SE0 (A), SE1 (B), SE2 (C); or cultured for 21 weeks (D): multicotyledonous embryos. Secondary embryos are shown by the arrows in A–D. Culture conditions: 16 hours in red light/8 hours in darkness at 25 ± 2 °C. Scale bar is 2 mm.

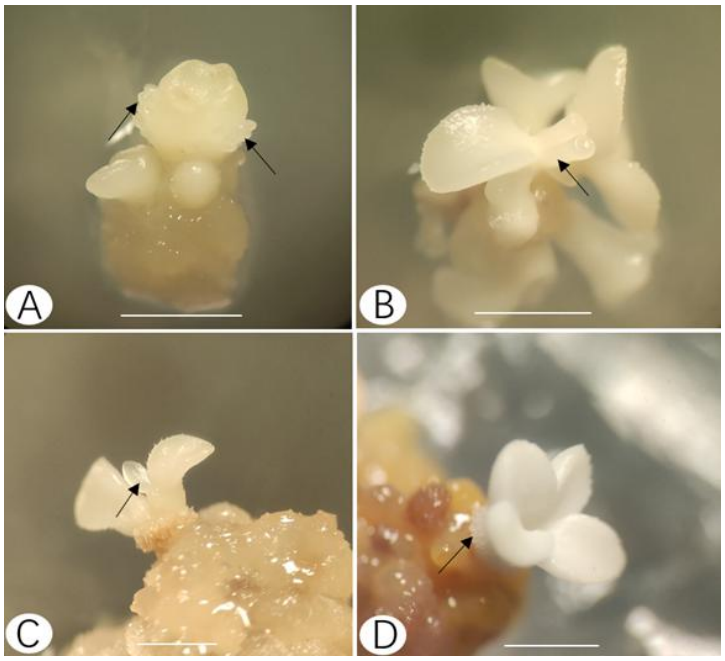

Fig. 3 Germination and seedling process for somatic embryos

Normal somatic embryos (SE0) cultured in EM medium for 13 weeks (A), SE0 sprouting at the 16th week (B), and somatic embryos sprouting at the 20th week (C), the secondary embryo germinates at the base of SE1 (D): germinated secondary embryo (D–a), initial embryo (D–b). Healthy seedlings cultured in SP medium for 16 weeks (E) and rose seedlings transplanted to the greenhouse after rooting (F). Culture conditions: 16 hours for white light/8 hours in darkness at 25 ± 2 °C. Scale bar for A–D is 2 mm, and for E, F it is 2 cm.