Morphological and endogenous phytohormone changes during long-term embryogenic cultures in Korean pine

Yan Liang1 · Xin Xu1 · Hailong Shen2,3 · Meiling Gao1 · Yan Zhao1 · Xue Bai1

Received: 13 April 2022 / Accepted: 24 June 2022 / Published online: 11 July 2022
© The Author(s), under exclusive licence to Springer Nature B.V. 2022

Abstract
In SE of Korean pine, decreasing embryogenic potential was observed in embryogenic callus (EC) with successive subcultures, especially when the cultures were maintained for more than 5–6 months. Thus, aiming to investigate the changes in morphology and the endogenous phytohormone associated with the decreasing somatic embryo differentiation capacity of Korean pine, here, we compared Korean pine calli with different embryogenic potential. It was found that both the external morphological characteristics and internal cell structure were significantly different among the different types of calli. Specifically, similar to zygotic counterparts of conifer trees, the calli with high embryogenic potential were characterized by a higher number of designated proembryogenic masses (PEMs) structure. In addition, surface of this types of callus was covered with a large amount of mucilaginous extracellular matrix (ECM), which could further differentiate into globular embryos containing the embryonal masses (EM) and embryonic tube (ET) structure. In contrast, in those low embryogenic potential calli, we observed disintegrated PEMs structure, a large amount of irregularly shaped cells, and degraded cells, which failed to form somatic embryos. The endogenous hormone results showed that high levels of abscisic acid (ABA) were beneficial for promoting early somatic embryo differentiation in Korean pine, but maintaining high embryogenic potential required moderate levels of indole-3-acetic acid (IAA), and low levels of zeatin-riboside (ZR). Additionally, a high ratio of IAA/ABA, methyl jasmonate (MeJA)/ABA, and gibberellins (GA3)/ABA were conducive to maintaining high embryogenic potential, whereas a low ratio of IAA/ABA and MeJA/ABA were effective for early somatic embryos morphological differentiation. The results can provide morphological and physiological indicators for identifying high embryogenic potential calli suitable for SE and help determine the best protocol on addition of exogenous hormones in the construction of a maintainable system of EC in Korean pine.

Key message
The results provides morphological and endogenous hormones indicators for identifying high embryogenic potential calli in Korean pine, be critical for evaluating calli suitable for SE.

Keywords Pinus koraiensis · Embryogenic callus · Long-term culture · Morphology · Endogenous hormones

Introduction
Korean pine (Pinus koraiensis Sieb. et Zucc.) is a critical coniferous species ecologically and economically in several countries, such as China, South Korea, North Korea, Japan, and the Far Eastern region of Russia (Wang et al. 2018). It is a dominant species of the natural mixed coniferous and broadleaf forests of the temperate forests in Northeast China. In addition, Korean pine is well known for its high economic value, such as producing high-quality timber and foods for humans enriched with numerous nutrient properties, and its seeds being...
used as industrial raw materials in foods, cosmetics, and medicines, etc. (Shpatov et al. 2017; Wang et al. 2018). However, because of over-harvesting timber and pine nuts, the natural populations of Korean pine have drastically declined; Korean pine has been classified as a rare and nationally endangered species in China nowadays (Peng et al. 2021). Hence, to preserve the natural Korean pine resources and ensure a stable and sustainable source of Korean pine, high-quality seedlings and successful planting are imperative.

In forestry, the production of somatic embryogenesis (SE) represents the fastest and most efficient way to obtain unlimited production of clones with elite traits to meet the demands of production (Find et al. 2008; Peng et al. 2020). It is an indispensable tool for biotechnological applications, including production of artificial seeds (Cheruvathur et al. 2013), and also for genetic transformation to obtain multiplication of desired genotypes (Egertsdotter et al. 2019). The plant SE is a complex developmental process in which somatic cells undergo restructuring through a series of morphological, physiological, and molecular changes to form embryogenic cells under in vitro conditions, and then these embryogenic cells gradually grow and specialize into tissues through steps similar to zygotic embryogenesis (Fehér 2015). In recent years, more and more coniferous trees have successfully obtained somatic embryos and commercial production by the technology of SE has been achieved in some tree species (Egertsdotter et al. 2019). However, compared with most angiosperm woody trees, the clonal production of recalcitrance is still a general problem for many conifer species (Lelu-Walter et al. 2013).

For many plant species, the decreasing embryogenic potential in embryogenic callus (EC) with successive subcultures is a common issue and is one of the most significant problems preventing commercial implementation of the SE technique (Breton et al. 2006; Li et al. 2019; Gao et al. 2020). Research on the biological mechanisms of reduced embryogenic potential indicated that embryogenic cultures during an extended period might be subject to an increase in the frequency of somaclonal variations, resulting in some undesirable consequences, including decreasing embryogenic potential in EC (Rival et al. 2013). Findings from those integrated cell biology studies may help overcome the bottlenecks encountered with the SE (Guerra et al. 2016; Pereira-Dias et al. 2020). Based on the observation of histological morphology in conifers trees, the cell proliferations in cultures were generally driven by proembryogenic masses (PEMs), which divided cyclically into PEM I, II, and III, and development of PEMs had substantial impacts on the differentiation capacity of the somatic embryo (Filonova et al. 2000; Stasolla and Yeung 2003; Steiner et al. 2015). In addition, according to Pěnčík et al. (2015) and Weckx et al. (2019), the increased frequency of somaclonal variations during extended culture stages was directly associated with the production and accumulation of hormones.

In our previous research, it was found that EC from the same cell lines of Korean pine could maintain high growth rates and high embryogenic potential during the first two months by repetitive subcultivation, whereas they tended to lose the differentiating potential after extending subculture over prolonged periods such as 5–6 months. Interestingly, we found that different types of calli during this extended subculture process were characterized by friable or compact, white or yellow-green, transparent or opaque, etc. in appearance. Similarly, Gao et al. (2020) also mentioned that the initial white EC of Korean pine gradually changed into yellow or brown during long-term subculture. In addition, Peng et al. (2020) carried out studies on the energy storage and antioxidant enzymes in three types of calli of Korean pine (i.e., EC from immature zygotic embryos, non-embryonic callus (NEC) from mature zygotic embryos, and the calli of lost SE potential, respectively). However, there was no detailed analysis of the reasons for the decreasing embryogenic potential and the differences in the internal and external morphology of these three types of calli. Considering the challenges in maintaining EC for a long term and the extremely low induction rate of EC in Korean pine (Gao et al. 2020) as well as laborious, costly, and time-consuming work to build a new callus induction system needed for a new explant each time, morphological and physiological indicators are urgently needed to evaluate calli suitable for SE as a critical step to solve the problems of loss in the embryogenic potential during subculture in this species. However, to our best knowledge, little knowledge is currently available about the underlying mechanisms of the loss in the embryogenic potential of subcultured EC from Korean pine. More effective and more reliable methods for identifying EC and NEC that are suitable for SE in the early stages are essential for minimizing the errors during selection of EC for further use. In this present study, different types of calli of Korean pine were utilized to investigate morphological modifications and endogenous hormone changes that are associated with different embryogenic potentials. Our first goal was to determine the morphological and physiological indicators for identifying high embryogenic potential calli suitable for SE in the early stages. Secondly, examination of endogenous hormone changes in EC of different embryogenic potentials can help determine the best strategy for adding exogenous hormones in the construction of a maintainable system of EC in Korean pine.
Materials and methods

Preparation of biological materials

The EC from immature seed of Korean pine open-pollinated single plant preferred elite families (L 22) were induced following the procedure described earlier by Peng et al. (2020). Durzan medium (DCR) (Gupta and Durzan 1985) was used as EC induction medium, which contained 2.0 mg·L$^{-1}$ 1-Naphthaleneacetic acid (NAA), 1.5 mg·L$^{-1}$ 6-benzyladenine (6-BA), 0.5 g·L$^{-1}$ casein hydrolysate (CH), 0.5 g·L$^{-1}$ l-glutamine, 3 % (w/v) sucrose, and was solidified with 0.4 % phytagel. After four weeks of culture, the induced EC were transferred to the maintenance medium for 6 months, and then subcultured for 6 months on the same maintenance medium that was replaced by fresh medium every 2 weeks. The maintenance medium was DCR basic medium supplemented with 0.25 mg·L$^{-1}$ 6-BA, and 1 mg·L$^{-1}$ NAA, with the addition of sucrose, phytagel, CH, and L-glutamine being the same as EC induction medium. The calli during the process of subculture were classified to six types according to their external morphological characteristics such as color, texture, shape, and degree of dispersion, which were two-month white calli (C1), six-month white calli (C2), yellow-green calli (C3), water-stained calli (C4), brown calli (C5), and fibrotic calli (C6), respectively. Except for C1 that was maintained for two months, C2–C6 types of calli were all maintained for six months. Subsequently, all the calli were subcultured on a hormone-free DCR medium to promote the differentiation of the embryogenic structures. The addition of sucrose, phytagel, CH, and l-glutamine in the differentiation medium were the same as EC induction medium. After 7 days, we recorded the number of early somatic embryos and those obtained cultures with early globular embryos were defined as the C7-type calli (C7). Ten calli (0.5 g for each callus) were used in this experiment and the treatments were repeated three times. The samples in vitro were all kept in a growth chamber at 24 ± 2 °C and the dark conditions. The pH of all mediums was adjusted to 5.8 with 0.1 M NaOH and 0.1 M HCL before autoclaving. l-Glutamine was sterilized with 0.22 μm filters and supplemented into the autoclaved medium after being cooled to about 50 °C. Other plant growth regulators (PGRs) and additives were added to the medium before sterilization. All products were purchased from Sigma-Aldrich (Madrid, Spain), except sucrose, CH, and L-glutamine from Solarbio (Beijing, China).

The above seven types of cultures were selected for morphological identification. Three samples for each type of calli with each of them being 0.2 g fresh weight (FW), were quickly frozen in liquid nitrogen and stored at −80 °C for determining endogenous hormone content.

Morphology observation of embryogenic cultures

The external morphological characteristics of the different types of embryogenic cultures from Korean pine were observed with a stereomicroscope (Olympus SZX 7). The environmental scanning electron microscope (ESEM) (Phenom Pro) was used to further observe the cell structures of these cultures. The small samples of fresh, uncoated, and wet calli (3 to 10 mm diameter) were mounted on aluminum stubs with double-sided adhesive tapes and directly observed with the ESEM, without prior fixation. The accelerating voltage was changed from 5 to 10 kV, and the air pressure inside the ESEM microscope column was leveled at 60 Pa (Pandey et al. 2017).

Determination of endogenous hormones

Measurement of the endogenous hormones including indole-3-acetic acid (IAA), zeatin–riboside (ZR), gibberellins (GA$_3$), absciscic acid (ABA), brassinosteroids (BR), and methyl jasmonate (MeJA) of different types of cultures in Korean pine were performed using the ELISA kit (Zhao et al. 2006), which was purchased from the Center of Crop Chemical Control, China Agricultural University, China. It measured optical density at 490 nm, drew a calibration curve, and calculated the IAA, GA$_3$, ZR, ABA, BR, and MeJA levels. Each sample assay was repeated three times. After excluding outlier data, the data of the three replicates were averaged for analysis.

The hormone content and ratio data were shown as the mean ± SE in three replicates. Data were analyzed using variance analysis (ANOVA) in SPSS software version 17.0 (SPSS Inc., Chicago, Illinois). Tukey’s new multiple range tests were conducted to detect differences in endogenous hormone contents among samples (p < 0.05). As the samples of ratios of endogenous hormones were limited, it was not possible to execute normality tests; thus, non-parametric analysis of variance (Kruskal–Wallis test) was applied to calculate the significant differences between the comparisons (corrected p < 0.05). In addition, multiple comparisons of mean ranks were conducted to determine the parameters that contributed significantly to the final score.

Results

Morphological characteristics and somatic embryo differentiation of embryogenic cultures from Korean pine

In conifers, to keep the cells alive and prevent from browning, EC during the induction phase usually needs to be subcultured on maintenance medium and the maintenance
medium needs to be replaced with fresh medium at regular intervals (Bonga 2017). In our present study, when EC from the same clone was transferred to the subculture medium with the same basal composition, we observed that the morphology of the initially induced white EC changed during the long-term maintenance process. According to morphological characteristics under the stereomicroscope, the C1-type calli, 2-month old, were white, transparent, loose, and sticky, with filaments on the surface. The vitality of C1 calli was strong and its proliferation speed was fast, with a two-fold increase in proliferation rate within two weeks (Fig. 1A). The C2 calli was the white calli that was maintained for six months. It exhibited milky white color, translucent or opaque, loose, and not sticky. Overall, compared with the 2-month-old C1 calli, the C2-type calli showed the minimum change through the subcultures, retaining their color, softness, and stickiness. In addition, the proliferation rates of C2 calli were slow, the viability during proliferation reduced, and the number of filaments on the surface has decreased (Fig. 1B). The C3-type calli appeared yellow-green in appearance, opaque and loose structure, almost non-sticky, and no filaments, but their multiplication rates were particularly high and the volume increased by 3–4 times in one culture cycle, exhibiting its high vitality (Fig. 3C). The appearance of water-stained calli (C4) were milky white, opaque, and watery (Fig. 1D). They had a tight and smooth texture in general, with a small part being low stickiness. This type of calli could not continue to proliferate after transferred to a fresh medium of the same composition. The fibrous calli (C5) presented a color similar to white snowflake, low stickiness, with filamentous fibrous structure on the loose surface. They maintained a vigorous proliferation rate during repeated subcultures with a fresh medium of the same composition. However, with a longer subculture time, the white fibrous structures on the surface increasingly became denser (Fig. 1E). The browned calli (C6) appeared opaque and had brown spots, but there were almost no filaments on the surface (Fig. 1F). Compared with other types of calli, this type calli hardly kept proliferating after subculturing once or twice because of the loss of cell activities. During this process, the callis became browner gradually until they eventually died, and fully lost the embryogenic potential. Furthermore, we evaluated the embryogenic ability of these calli (C2-C6) by subculturing on a PGR-free medium for 7 days. We observed the differentiated early globular embryos with small globular bulges (Fig. 1G, H). This type calli with the differentiated globular somatic embryos, J C1-type calli with PEM I, K C1-type calli with PEM II and thin membranous ECM structure covering the surface, L C1-type calli with PEM III, M C2-type calli, N C3-type calli, O C4-type calli, P C5-type calli, Q C6-type calli, R C7-type calli, proembryo forming-like cells characterized by small size and round shape. ESEM environmental scanning electron microscope, PEM proembryogenic masses, EH embryonal heads, S suspensor, ECM membranous extracellular matrix, EM early somatic embryos with embryonic mass, ET embryonic tube. * in H–J indicates two-celled PEM I, showing polarized structures.
embryo (C7) were white and translucent, with light yellow granules scattered on surfaces (Fig. 1G), and these early embryos further developed into mature somatic embryos under ABA treatment (Fig. 1I). However, it’s worth noting that these globular embryos were only successfully differentiated from the C1-type calli, with the number of somatic embryos of 30.7 g⁻¹. Different from the C1-type calli, other types of calli (C2–C6), after subculturing on a differentiation medium, gradually turned to dark brown or necrotic, with no somatic embryo differentiation. Taken these results together, we speculated that C1-type calli had the highest embryogenic potential, and the embryogenesis ability of other types of calli (C2–C6) reduced significantly, almost losing the embryogenic potential after six months of subculture.

**Morphological characteristics of embryogenic cultures under the scanning electron microscopy from Korean pine**

It is well known that somatic embryo development in conifer species follows a precise developmental pathway characterized by distinct cell aggregates, the organized mass of proliferating embryogenic tissue is often called EC, and many researchers prefer to use "PEM" to distinguish the proliferation phases of EC. An individual PEM usually passes through a series of defined stages (PEM I, PEM II, and PEM III). PEM is initiated with the formation of PEM I, which is characterized by few meristematic cells subtended by a vacuolated elongated suspensor-like cell, and this structure is formed by asymmetrical cell division. PEM II has a similar structure, but with more than one vacuolated suspensor (S) cells and embryonic head (EH) cells. Both the size and number of PEM II increase and develop further into PEM III. These PEM III structures, however, cannot differentiate into somatic embryos unless they are transferred to the PGR free medium (Filonova et al. 2000; Stasolla and Yeung 2003).

Although observations with the stereo microscope offered some judgment about the high-quality calli, information such as cell composition of EC of Korean pine is still limited. Therefore, in our study, ESEM observations have also been conducted to determine cell composition and structures of these different types of embryogenic cultures of Korean pine. As shown in Fig. 1J–R, the histological observations by ESEM revealed that the surface cell composition and morphology of these types of calli from Korean pine were quite different. Microscopically, the C1-type calli were characterized by designated PEMs-like structure (Fig. 1J–L), and polarized cell structure clusters of different sizes were found. Specifically, a higher number of PEM I (Fig. 1J) was observed on the callus surface, but PEM II (Fig. 1K) and PEM III (Fig. 1L) were sporadically detected in this type of calli. Consistent with the above classical development of conifer PEMs, PEM I occurred in C1-type calli had a polar structure with compact, rounded EH in the apical part and a single, enlarged vacuolated S in the basal part, which were tightly connected. Both PEM II and PEM III possessed more than one vacuolated S cell and EH cell but the number and size of the S cells and EH cells were different between PEM II and PEM III. In contrast to C1-type calli, the number of irregular and unorganized cells in C2-type calli increased significantly. Some degraded cells occurred, to some extent. However, some spherical cells were tightly arranged to form cell clusters and lacked an apparent PEMs-like structure. Interestingly, a large number of disintegrated globular EH cells and elongated S cells from cell clusters showed up and they were scattered (Fig. 1M). Compared with C1-type calli, in C3, C4, and C5 types of calli, the number of irregularly shaped cells significantly increased. Some were long, some were round or oval, and some showed a tadpole-like structure (Fig. 1N–P). Most cells were disorganized, accompanied by larger intercellular space between the cells. Different from C2 types calli, disintegrated cells from those PEMs-like structures were almost invisible in these types calli. Unlike the C1-C5 types of calli, the brown calli (C6) usually contained many cells that were died or close to death (Fig. 1Q). Severe degradation of cells occurred and resulted in gradually losing growth capacity. Different from the first six types of calli, the C7-type calli contained the differentiated globular embryo, these early globular embryos had a polar structure with a compact, round embryonal mass (EM) in the apical part, and attained a distinct round shape, consisting of cells that were tightly connected in the proximity of the EH but typically loosely arranged in the distal part of degenerated long-stalked S, namely embryonic tube (ET) (Fig. 1R). Furthermore, we observed the surface of C1-type calli was covered with mucilaginous and membranous structure, namely, extracellular matrix (ECM) (Fig. 1K). Different from C1-type calli, only a small amount of ECM was observed on the surface of other types of calli.

**The effects of endogenous hormones on the embryogenic ability of embryogenic cultures in Korean pine**

**Changes of single endogenous hormone content**

As shown in Fig. 2, overall, the content of IAA, ABA, and MeJA was significantly higher than those of ZR, GA3, and BR.

Among all the types of calli, the IAA content in browned calli (C6) was highest (43.78 ng·g⁻¹ FW), significantly higher than that of other types of calli (p<0.05), followed by the yellow-green calli (C3) (34.88 ng·g⁻¹ FW). The content of IAA was at a moderate level in the white EC of two months (C1).
Compared with IAA, the content of ZR in all types of calli was low. The higher content of ZR occurred in the yellow-green calli (C3) (5.29 ng·g\(^{-1}\) FW), the fibrous calli (C5) (4.58 ng·g\(^{-1}\) FW), and the calli containing early embryos (C7) (4.87 ng·g\(^{-1}\) FW). The content of ZR was at a lower level in white embryogenic calli of two months (C1). As the primary biological function of cytokinins is to promote cell division (Staden et al. 2008), higher ZR content in C3, C5, and C7-types of calli indicated that cells of these types of calli still maintained a rapid proliferation.

Similar to ZR, the content of GA\(_3\) in different calli was low (2.53–5.09 ng·g\(^{-1}\) FW). The higher content of GA\(_3\) occurred in the yellow-green calli (C3) (5.09 ng·g\(^{-1}\) FW) and the calli containing early embryos (C7) (4.83 ng·g\(^{-1}\) FW), which was significantly higher than the other five types of calli (2.53–3.60 ng·g\(^{-1}\) FW). The content of GA\(_3\) of C1-type callus was at a moderate level, which was only significantly higher than C2 type calli and not significantly different from C4, C5, and C6 types of calli.

As for the ABA content, it was high in all types of calli (13.52–34.20 ng·g\(^{-1}\) FW). The ABA content in C7 calli was the highest (34.20 ng·g\(^{-1}\) FW), significantly higher than the other six types of calli (13.52–25.46 ng·g\(^{-1}\) FW), and the content in C1, C2, C4 and C5 types of calli was lower than that in C3 and C6. This results indicated that high levels of ABA were beneficial to promote early somatic embryo differentiation in Korean pine.

Similar to ZR and GA\(_3\), the content of BR in all types of calli was low (2.96–4.52 ng·g\(^{-1}\) FW). The fibrous calli (C5) had the highest BR content (4.52 ng·g\(^{-1}\) FW). The content of BR in C1 type of calli was relatively low.

The content of MeJA in all types of calli was high (15.15–20.43 ng·g\(^{-1}\) FW). Specifically, the MeJA content in C3, C6, and C7 was higher, while lower content of MeJA appeared in the other four types of calli.

### Changes in the ratio of endogenous hormone content

To clarify the effects of endogenous hormone balance on the decrease of embryogenicity in Korean pine, the ratios of the endogenous hormones were analyzed. As shown in Fig. 3, the ratios of endogenous hormones varied among different types of calli, and variation patterns and ranges differed among different hormone ratios. Specifically, the values of IAA/ABA and MeJA/ABA were significantly higher (0.54–2.09) than the values of GA\(_3\)/ABA, BR/ABA, IAA/ABA, and ZR/IAA (all ≤ 0.30). The lowest values of IAA/ABA (0.54) and MeJA/ABA (0.60) occurred in C7-type calli, whereas the highest values of IAA/ABA (2.09), MeJA/ABA (1.21), and GA\(_3\)/ABA (0.26) were found in C1-type calli. It is worth noting that too high ratios of IAA/ABA can cause browning. In addition, both the values of BR/ABA and ZR/ABA were higher in C1, C2, C4, and C5 (0.21–0.30), C5 and C7 had higher ZR/IAA (0.22–0.26). Therefore, it can be concluded that high IAA/ABA, MeJA/ABA, and GA\(_3\)/ABA ratios were conducive to maintaining a high embryogenic potential of EC. However, low IAA/ABA and MeJA/ABA ratios could benefit morphological differentiation of the early globular embryos. There was no obvious trends in the values of BR/ABA, ZR/ABA, and ZR/IAA among different types of calli, indicating that these ratios had little
relationship with the maintenance of embryonic ability and somatic embryo differentiation.

Discussion

In plant SE, in addition to induction of EC, it is also crucial whether induced EC can maintain embryogenicity for a long time, especially for conifers, long-lived trees (Bonga 2017; Li et al. 2019). A progressive decline of EC growth, an increase in necrosis rate, and a decrease or even loss of the morphogenetic differentiating potential of somatic embryos in EC maintained through repetitive subculturing have all been reported in Pinus pinaster (Breton et al. 2006), Olea europaea (Bradaï et al. 2016), Araucaria angustifolia (Pereira-Dias et al. 2020) and Saccharum sp. (Passamani et al. 2020). Recently, SE from immature zygotic embryo explants of Korean pine was achieved, but this propagation pathway has also been proven to have a problem of reduced embryogenic potential during long-term maintenance of EC (Gao et al. 2020; Peng et al. 2021). Therefore, there are still a few challenges in SE, including the loss of embryogenic potential, the extremely low induction rate of EC in Korean pine (Gao et al. 2020), and the requirement for a new callus induction system of Korean pine for a new explant each time, which is laborious, costly, and time-consuming. To help solve this challenges, early evaluations of whether the callus is suitable for SE are important. In this context, we compared the morphological and endogenous hormone differences among different types of calli from Korean pine to help the early identification of calli with high embryogenic potential and to provide a reference for the addition of exogenous hormones during the long-term maintenance process of EC.

The relationship between morphological characteristics and embryogenic potential of embryogenic cultures in Korean pine

Researches on related biological mechanisms of reduced embryogenic potential showed that the changes in morphological characteristics can be easily detected by comparing morphological structures of different types of calli (Passamani et al. 2020). Based on previous literature, long-term culture can modulate morphological changes in EC cells, which further interferes with the production of somatic embryos (Passamani et al. 2020). The study on sugarcane found that EC was smooth and dense, while NEC was brittle or soft and translucent (Silveira et al. 2013). Furthermore, the color of calli changed from white to yellow, green or brown, etc., which often occurred in the long-term cultured callus of O. europaea (Pires et al. 2020). Similar to what has been reported in these studies, the above-mentioned characteristics of calli was also clearly observed in our present study, and the typical embryogenic cultures of Korean pine were classified into multiple types according to morphological characteristics. Among them, the short-term subcultured embryogenic calli (C1) was different from all other types in terms of color, texture, proliferation speed, and differentiation ability. C1-type calli was white, highly organized, fast-growing, high differentiation ability. In contrast, the calli from the same cell line but maintained for 6 months (C2–C6) exhibited different morphology with C1. The morphology of C2–C6 types of calli was similar to the morphology of NEC and this morphology directly influenced the early somatic embryo differentiation. Thus, it can be concluded that the callus color and texture in Korean pine determine its fate, with or without embryogenesis.
In classical gymnosperm embryology, somatic embryo development follows a precise developmental pathway characterized by distinct cell aggregates (Filonova et al. 2000; Stasolla and Yeung 2003). Not surprisingly, our research also suggested that SE of Korean pine passed through a PEMs stage, followed by the differentiation and maturation of somatic embryos. In this study, the C1-type calli contained a large number of PEM-like structures. We observed the formation of PEM I in C1-type calli, which was characterized by few round dividing cells (two-celled or four-celled proembryo) subtended by an elongated vacuolated suspensor-like cell. Only a small amount of PEM II and PEM III cell aggregates appeared in C1-type calli, which have been developed with a comparable appearance with zygotic embryos. In addition, our results were similar to the reports for other conifers trees, such as Juniperus communis (Helmersson and Von Arnold 2009), Cunninghamia lanceolata (Zhou et al. 2017), and A. angustifolia (Oliveira et al. 2019; Pereira-Dias et al. 2020), in which high embryogenic potential calli during the proliferation phase showed a whitish translucent and mucilaginous cell masses by typically PEMs. In contrast to the C1-type cultures, a clear difference in cell morphology and structure was found between C1-type and other types of cultures (C2-C6). Specifically, degrading cells appeared, the organized PEM-like structures disintegrated, and the number of irregular and unorganized cells increased in C2–C6 types of calli. All the characteristics of cell morphology and structure in C2–C6 types calli were similar to those of nonembryogenic cells found in other studies (Silveira et al. 2013; Gautier et al. 2018; Reeves et al. 2018). The cell morphology and structure in C2-C6 types calli directly influenced the formation of somatic embryo, as confirmed by our results of somatic embryo differentiation in this study, that is, the early somatic embryos were only formed on the surface of C1-type calli. Unfortunately, although mature somatic embryos was observed, the number obtained was not enough to carry out the subsequent experiments on germination/conversion of somatic embryos. Considering the importance of this step for obtaining regenerated plants, relevant germination experiment will be further carried out in the future, and the key technologies for somatic embryo maturation of Korean pine need to be further optimized.

Overall, based on the findings of the cell morphological characteristics in this study, we speculate that the presence of PEMs-like structures would be helpful for maintaining the high embryogenic ability of Korean pine, whereas the failure of many embryogenic cells to form early somatic embryos is caused by disturbed or arrested PEMs structures, as mentioned in previous literatures (Márquez-Martín et al. 2012). Thus, a system that is conducive to maintaining a high level of PEMs under subcultures will help Korean pine obtain high embryogenicity. Furthermore, some features common in EC, such as the most abundant presence of ECM structures, are considered as indicators of the embryogenic and regenerative potential of EC (Binte et al. 2018; Gautier et al. 2018; Ming et al. 2019). Accordingly, we speculate the presence of ECM might be related to the maintenance of embryogenesis potential in Korean pine.

The key role of endogenous hormones in the maintaining embryogenic ability of Korean pine

As we all know, the SE in conifers usually occurs indirectly and this indirect mode requires hormones to induce pre-determinant cells firstly, after which somatic cells transform into embryogenic cells. Among all hormones, auxins are generally considered to be the more important one in the acquisition of embryogenic competence. Although previous research on SE has reported that a low content IAA was beneficial to the maintenance of EC (Klimaszewska et al. 2016), it did not necessarily mean that the lower was the better, too low IAA content will lead to reduced embryogenic potential accompanied by morphological changes in Korean pine, such as prone to differentiation of somatic embryos (Klimaszewska et al. 2016; Yu et al. 2019). Our results also found higher content of IAA in the browned calli and lower level of IAA in the C7-type calli containing early embryos, in addition, moderate IAA content was found in the high embryogenic calli (C1), implying that an appropriate level of auxin is required for the maintenance of Korean pine EC, whereas higher-than-optimal levels will result in browning. Callus browning is a common feature in plant cell culture, which often results in decreased regenerative ability, poor growth, or even death (Zhang et al. 2020). According to previous studies (Bradaï et al. 2019; Sangra et al. 2019), the imbalance of auxin metabolism is one of the leading causes for the appearance of abnormalities in somatic embryos because the accumulation of auxin disrupts the normal genetic and physiological processes in the cells, and as a consequence, this may affect the integrity of the previously acquired developmental pathway. ABA has always been considered to play a critical role in somatic embryo differentiation, an elevated level of ABA is required for somatic embryo maturation in many coniferous species (Pullman et al. 2016; Chen et al. 2018; Vondrakova et al. 2018). Recently, Peng et al. (2020) suggested that increasing ABA concentrations (up to 80 μmol·L⁻¹) in the maturation medium improved Korean pine somatic embryo yield. In line with these studies, our results also suggested that a high level of ABA was conducive to the morphological differentiation of somatic embryos of Korean pine, whereas a low level of ABA was beneficial to the maintenance of embryogenic potential. Kępczyńska and Orłowska (2021) also found high
level of ABA reduced embryogenic competence in *Medicago truncatula*.

As for BR, which is also known to act synergistically with auxins and might be involved in the control of plant SE, enhancing EC initiation in some coniferous species (Pullman et al. 2003, 2016). In contrast, Xu et al. (2020) found that a lower level of BR might promote early somatic embryo development in *Dimocarpus longan*. However, in the present study, the levels of BR did not show any noticeable effect on embryogenic potential and differentiation capacity. In addition, our study confirmed that the low level of ZR in callus with high embryogenic potential, which was in line with the previous report that the decrease of CTKs level was related to the embryogenic potential, and low-level CTKs was the key factor for long-term maintenance calli with thriving (Wu et al. 2020). Until now, there were no reports on the role of GAs during SE. Similarly, our results also suggested that GA\textsubscript{3} did not play a significant role in the process of Korean pine somatic embryo differentiation and embryogenic potential maintenance.

MeJA is an uncommon hormone used in SE. For example, the previous studies on *Daucus carota* (Tokui et al. 1995) and *Medicago sativa* (Ruduś et al. 2006, 2009) found MeJA inhibited somatic embryo formation. Ruduś et al. (2006) found that MeJA did not affect somatic embryo differentiation of *M. sativa*, but it had a negative impact on the maintenance of embryogenic potential. Recently, Morcillo et al. (2020) reported that MeJA application contributed a recovery in the differentiation capacity of an embryogenic line of *Quercus ilex*. In this present study, there was no obvious trend in the content of MeJA. Based on all findings above, we speculate that MeJA may play different roles in somatic embryo formation and embryogenic potential maintenance in different plants, depending on the sensitivity of different species to MeJA, or a balance between hormones in this process plays a key role, rather than a single hormone.

The embryogenic potential maintenance in plant SE is a complex physiological and biochemical process. Therefore, the level of any single hormone cannot fully explain its roles and the balance between hormones is also critical in this process and is often used to evaluate embryogenesis (Pérez-Jiménez et al. 2013; Grzyb et al. 2018; Wu et al. 2020). Our results showed that compared with other endogenous hormones, higher ratios of MeJA/ABA, IAA/ABA, and GA\textsubscript{3}/ABA were favorable for the maintenance of embryogenic potential in Korean pine, but for early somatic embryo differentiation, lower ratios of MeJA/ABA and IAA/ABA were more effective. It is worth mentioning that although the level of each individual phytohormone showed no or small differences among different types of calli, the ratios of both GA\textsubscript{3}/ABA and MeJA/ABA did differ greatly. Thus, the imbalance between hormones might be one of the mechanisms of reduced embryogenic potential and somatic embryo differentiation in Korean pine. Similar results were also found in *Cyathea delgadii* (Grzyb et al. 2017), *Picea balfouriana* (Li et al. 2019), and *Ormosia henryi* (Wu et al. 2020), in which the ratios of IAA/ABA and GA\textsubscript{3}/ABA were significantly higher in EC than NEC. Additionally, too high ratios of IAA/ABA, which mainly resulted from a high level of IAA, can cause browning. García et al. (2019) mentioned that the decreased and/or lost somatic embryo production capacity in long-term cultures may be partly due to the formation and accumulation of auxin and the consequent imbalance of auxin metabolism, and this long-term culture can modulate morphological changes in cells of EC. Our results demonstrates that the mechanism of endogenous hormones regulating the reduction of embryogenicity is different between browned calli and other types of NEC. Overall, these findings together indicate a potentially improved protocol to maintain embryogenicity of EC for a long term.

Morphological modifications and changes in the endogenous hormones have been highlighted as necessary for maintaining embryogenic potential (Passamani et al. 2018). In this study, we observed morphological differences and changes of endogenous hormones in different embryogenesis potential cultures of Korean pine, and thus proposed that the regulation of hormones to maintain embryogenic potential and the ability to form somatic embryos is related to the appearance or disappearance of PEMs, EM and ECM structures. The decrease of embryogenic potential during the maintenance of EC is a complex process, which includes metabolism, gene expression (e.g., the related genes of *SERK, LEC, BBM*), and a series of physiological changes in addition to endogenous hormones (Passamani et al. 2020). Therefore, further studies, including molecular studies, are required to better understand the complex mechanisms for decreasing embryogenic potential in Korean pine SE. Furthermore, although we confirmed that the maintenance of EC in Korean pine requires accumulation of IAA/ABA, MeJA/ABA, and GA\textsubscript{3}/ABA, information about the regulatory role of them in this process is still limited. Thus, it is also necessary to carry out experiments on the effect of IAA, MeJA or GA\textsubscript{3} alone in the maintenance of Korean pine EC.

**Conclusions**

In summary, both morphological responses and changes in endogenous hormones in relation to the decrease of embryogenic potential during the process of long-term maintenance of EC from Korean pine have been examined in the present study. Our results will provide morphological and physiological indicators for the embryogenesis potential of EC from Korean pine. The findings will be helpful for identifying more effective and more reliable EC at early stages and thus, can help minimize the errors during selection of
EC for further use. Moreover, our results are valuable for determine the best protocol for the addition of exogenous hormones in the construction of a maintainable system of EC in Korean pine.

Author contributions YL conceived the study, designed the experiment, and revised the manuscript. XX performed the experiment and wrote the draft of the manuscript. HLS provided technical guidance and contributed to the writing of the manuscript. MLG supervised the experimental process. YZ collected plant materials. XB Proofread the manuscript. All authors participated in the experiment. All authors have read and agreed to the published version of the manuscript.

Funding This research was financially supported by grants from the National Natural Science Funds of China (Grant No. 31800515) and the Foundation of Heilongjiang Province of China Educational Committee (Grant No. 145109137).

Declarations

Conflict of interest The authors have no conflict of interest to report.

References

Binte MS, Wagiran A, Syafiqoh JN, Salia H, Zulkifli N, Ming NJ (2018) The effects of temperature on callus induction and regeneration in selected Malaysian rice cultivar indica. Sains Malays 47:2647–2655. https://doi.org/10.17576/jsm-2018-4711-07

Bonga JM (2017) Can explant choice help resolve recalcitrance problems in in-vitro propagation, a problem still acute, especially for adult conifers? Trees 31(3):781–789. https://doi.org/10.1007/s00468-016-1509-z

Bradaï F, Pliego-Alfaro F, Sánchez-Romero C (2016) Long-term somatic embryogenesis in olive (Olea europaea): Influence on regeneration capability and quality of regenerated plants. Sci Hortic 199:23–31. https://doi.org/10.1016/j.scienta.2015.12.010

Bradaï F, Sánchez-Romero C, Martin C (2019) Somaclonal variation in olive (Olea europaea L.) plants regenerated via somatic embryogenesis: influence of genotype and culture age on genetic stability. Sci Hortic 213:208–215. https://doi.org/10.1016/j.scienta.2016.10.031

Breton D, Harvengt L, Trontin JF, Bouvet A, Favre JM (2006) Longterm subculture randomly affects morphology and subsequent maturation of early somatic embryos in Maritime pine. Plant Cell Tissue Organ Cult 87(1):95–108. https://doi.org/10.1007/s11295-013-0620-1

Chen YK, Xu XP, Chen XH, Chen Y, Zhang ZH, Xu XH, Lin YL, Lai ZX (2018) Seed-specific gene MOTHER OF FT and TFL1 (MFT) involved in embryogenesis, hormones and stress responses in Dimocarpus longan Lour. Int J Mol Sci. https://doi.org/10.3390/ijms19082403

Chevuathur MK, Abraham J, Thomas TD (2013) Plant regeneration through callus organogenesis and true-to-type conformity of plants by rapid amplification in Desmodium gangeticum (Linn.) dc. Appl Biochem Biotechnol 169(6):1799–1810. https://doi.org/10.1007/s12010-013-0171-2

Chertkovskii D, Ahmad L, Clapham D (2019) Automation and scale up of somatic embryogenesis for commercial plant production, with emphasis on Conifers. Front Plant Sci. https://doi.org/10.3389/fpls.2019.00109

Fehér A (2015) Somatic embryogenesis-stress-induced remodeling of plant cell fate. Biochim Biophys Acta 1849(4):385–402. https://doi.org/10.1016/j.bbabio.2014.07.005

Fiolona LH, Bozhkov PV, Von Arnold S (2000) Developmental pathway of somatic embryogenesis in Picea abies as revealed by time-lapse tracking. J Exp Bot 51(343):249–264. https://doi.org/10.1093/jexbot/51.343.249

Find JI, Floto F, Krogstrup P, Moller JD, Norgaard JV, Kristensen MMH (2008) Cryopreservation of an embryogenic suspension culture of Picea sitchensis and subsequent plant regeneration. Scand J for Res 8(2):156–162. https://doi.org/10.1080/02827593.2008.9382765

Gao F, Peng CX, Wang H, Shen HL, Yang L (2020) Selection of culture conditions for callus induction and proliferation by somatic embryogenesis of Pinus koraiensis. J for Res 32(2):483–491. https://doi.org/10.1007/s11738-020-01471-7

Garcia C, Almeida AAF, Costa M, Britto D, Valle R, Royaert S, Marelli JP (2019) Abnormalities in somatic embryogenesis caused by 2,4-d: an overview. Plant Cell Tissue Org Cult 137:193–212. https://doi.org/10.1007/s11240-019-01569-8

Gautier F, Eliasova K, Leple JC, Vondrakova Z, Lomenech AM, Mette LC, Label P, Costa G, Trontin JF, Teyssier C, Lelu-Walter MA (2018) Repetitive somatic embryogenesis induced cytological and proteomic changes in embryogenic lines of Pseudotsuga menziesii {mirb}. BMC Plant Biol. https://doi.org/10.1186/s12870-018-1337-y

Grzyb M, Kalandyk A, Waligórski P, Mikula A (2017) The content of endogenous hormones and sugars in the process of early somatic embryogenesis in the tree fern Cyathea delgadii Sternb. Plant Cell Tissue Organ Cult 129:387–397. https://doi.org/10.1007/s11240-017-1185-8

Grzyb M, Kalandyk A, Mikula A (2018) Effect of TBA, furidone and salicylic acid on somatic embryogenesis and endogenous hormone and sugar contents in the tree fern Cyathea delgadii Sternb. Acta Physiol Plant. https://doi.org/10.1007/s11738-017-2577-4

Guerra MP, Steiner N, Farias-Soares FL, Vieira LDn, Fraga HPF, Rogge-Renner GD, Maldonado SB (2016) Somatic embryogenesis in Araucaria angustifolia (Bertol.) Kunze (Araucariaceae). Methods Mol Biol 1359:439–450. https://doi.org/10.1007/978-1-4939-3061-6_24

Gupta PK, Durzan DJ (1985) Shoot multiplication from mature trees of Douglas-fir (Pseudotsuga menziesii) and sugar pine (Pinus lambertiana). Plant Cell Rep 4:177–179. https://doi.org/10.1007/BF00269828

Helmersson A, Von Arnold SV (2009) Embryogenic cell lines of Juniperus communis: easy establishment and embryo maturation, limited germination. Plant Cell Tissue Organ Cult 96(2):211–217. https://doi.org/10.1007/s11240-008-9477-7

Kępczyńska E, Orłowska A (2021) Profiles of endogenous ABA, bioactive GAs, IAA and their metabolites in medicago truncatula gaertn. non-embryogenic and embryogenic tissues during induction phase in relation to somatic embryo formation. Planta. https://doi.org/10.1007/s00425-021-03582-8

Klimaszewska K, Hargreaves C, Lelu-Walter MA, Trontin JF (2016) Advances in conifer somatic embryogenesis since year 2000. In vitro embryogenesis in higher plants. In: In vitro embryogenesis in higher plants. Methods in molecular biology, vol 1359. Humana Press, New York. https://doi.org/10.1007/978-1-4939-3061-6_7

Lelu-Walter MA, Thompson D, Harvengt L, Sanchez L, Toribio M, Paques LE (2013) Somatic embryogenesis in forestry with a focus on Europe: state-of-the-art, benefits, challenges and future direction. Tree Genet Genomes 9(4):883–899. https://doi.org/10.1007/s11295-013-0620-1

Li QF, Deng C, Zhu TQ, Ling JJ, Zhang HG, Kong LS, Zhang SG, Wang JH, Chen XY (2019) Dynamics of physiological and miRNA changes after long-term proliferation in somatic...
embryogenesis of *Picea balfouriana*. Trees 33(2):469–480. https://doi.org/10.1007/s00468-018-1793-x

Márquez-Martín B, Barceló-Muñoz A, Pliego-Alfaro F, Sánchez-Romero C (2012) Somatic embryogenesis and plant regeneration in avocado ( *Persea americana* Mill.): influence of embryogenic culture type. J Plant Biochem Biotechnol 21(2):180–188. https://doi.org/10.1007/s13562-011-0091-0

Ming NGJ, Mostafiz SB, Johon NS, Zulkifli NSA, Wagiran A (2019) Combination of plant growth regulators, maltose, and partial desiccation treatment enhance somatic embryogenesis in selected Malaysian rice cultivar. Plantas-Basel 8(6):144. https://doi.org/10.3390/plants8060144

Morcillo M, Sales E, Ponce L, Guíllén A, Segura J, Arrillaga I (2020) Effect of elicitors on holm oak somatic embryoid development and efficacy inducing tolerance to *Phytophthora cinnamomi*. Sci Rep. https://doi.org/10.1038/s41598-020-19195-w

Oliveira LF, Santos D, Flohe HE (2019) Polyamine and amino acid profiles in immature *Araucaria angustifolia* seeds and their association with embryogenic culture establishment. Trees 34(3):845–854. https://doi.org/10.1007/s00468-019-01938-y

Pandey M, Jayaramaiah RH, Dholakia BB, Punekar SA, Giri AP (2017) A viable alternative in vitro system and comparative metabolite profiling of different tissues for the conservation of *Ceropogia karadensis*. Plant Cell Tissue Org Cult 131(3):391–405. https://doi.org/10.1007/s11240-017-1292-6

Passamani LZ, Bertolazzi AA, Ramos AC, Santa-Catarina C, Thelen JJ, Silveira V (2018) Embryogenic competence acquisition in sugarcane callus is associated with differential H+ pump abundance and activity. J Proteome Res 17(8):2767–2779. https://doi.org/10.1021/acs.jproteome.8b00213

Passamani LZ, Reis RS, Vale EM, Sousa KR, Aragao VPM, Santa-Catarina C, Silveira V (2020) Long-term culture with 2,4-dichlorophenoxyacetic acid affects embryogenic competence in sugarcane callus via changes in starch, polyamine and protein profiles. J Proteome Res 140(2):415–429. https://doi.org/10.1007/s11240-017-1327-w

Péčnik A, Turečková V, Paulšíš V, Rohlick J, Strnád M, Mihaljević S (2015) Ammonium regulates embryogenic potential in *Cucurbita pepo* through pH-mediated changes in endogenous auxin and abscisic acid. Plant Cell Tissue Org Cult 122(1):89–100. https://doi.org/10.1007/s11240-015-0752-0

Peng CX, Gao F, Wang H, Shen H, Yang L (2020) Physiological and biochemical traits in Korean pine somatic embryogenesis. Forests. https://doi.org/10.3390/f11050577

Peng CX, Gao F, Wang H, Shen H, Yang L (2021) Optimization of maturation process for somatic embryo production and cryopreservation of embryogenic tissue in *Pinus koraiensis*. Plant Cell Tissue Org Cult 144(1):185–194. https://doi.org/10.1007/s11240-020-01918-y

Pereira-Dias F, Steiner N, Cangahuala-Inocente GC, Lando AP, Santos M, Guerra MP (2020) Integrated proteomics and histochemical analysis of *Araucaria angustifolia* (Bertol.) Kuntze (Araucariaceae) in embryogenic suspension culture. Ann for Res 63(2):27–43. https://doi.org/10.15287/afar.2020.1918

Pérez-Jiménez M, Cantero-Navaarro E, Acosta M, Cos-Terrer J (2013) Relationships between endogenous hormonal content and direct somatic embryogenesis in *Prunus persica* L. Batsch cotyledons. Plant Growth Regul 71(3):219–224. https://doi.org/10.1007/s10725-013-9822-7

Pires R, Cardoso H, Ribeiro A, Peixe A, Cordeiro A (2020) Somatic embryogenesis from mature embryos of *Olea europaea* L. cv. “Galega Vulgar” and long-term management of calli morphogenic capacity. Plants. https://doi.org/10.3390/plants9060785

Pullman GS, Zhang Y, Phan BH (2003) Brassinolide improves embryogenic tissue initiation in conifers and rice. Plant Cell Rep 22(2):96–104. https://doi.org/10.1007/s00299-003-0674-x

Pullman GS, Olson K, Fischer T, Egertsdotter U, Frampton J, Bucalo K (2016) Fraser fir somatic embryogenesis: high frequency initiation, maintenance, embryo development, germination and cryopreservation. New for 47(3):453–480. https://doi.org/10.1007/s11056-016-9525-9

Reeves C, Hargreaves C, Trontin JF, Lelu-Walter MA (2018) Simple and efficient protocols for the initiation and proliferation of embryogenic tissue of Douglas-fir. Trees 32(1):175–190. https://doi.org/10.1007/s10656-017-1622-7

Rival A, Ilbert P, Labeyrie A, Torres E, Doubeau S, Personne A, Dussert S, Beule T, Durand-Gasselin T, Tregear JW, Jaielot E (2013) Vibrations in genomic DNA methylation during the long-term in vitro proliferation of oil palm embryogenic suspension cultures. Plant Cell Rep 32(3):359–368. https://doi.org/10.1007/s00292-012-1369-y

Ruduš I, Kępczyńska E, Kępczyński J (2006) Comparative efficiency of abscisic acid and metyl jasmonate for indirect somatic embryogenesis in *Medicago sativa* L. Plant Growth Regul 48(1):1–11. https://doi.org/10.1007/s10725-005-5136-8

Ruduš I, Weiler EW, Kępczyńska E (2009) Do stress-related phytohormones, abscisic acid and jasmonic acid play a role in the regulation of *Medicago sativa* L. somatic embryogenesis? Plant Growth Regul 59(2):159–169. https://doi.org/10.1007/s10725-009-9399-3

Sangra A, Shahin L, Dhir SK (2019) Long-term maintainable somatic embryogenesis system in *Alalfa* (*Medicago sativa*) using leaf explants: embryogenic sustainability approach. Plants-Basel. https://doi.org/10.3390/plants8080278

Shpatov AV, Popov SA, Salnikova OI, Kulina TP, Shmidt EN, Um BH (2017) Composition and bioactivity of lipophilic metabolites from needles and twigs of Korean and Siberian Pines (*Pinus koraiensis Siebold & Zucc.* and *Pinus sibirica Du Tour*). Chem Biodivers. https://doi.org/10.1002/cbdv.201600203

Silveira V, De Vita AM, Macedo AF, Dias MFR, Flohe EIS, Santa-Catarina C (2013) Morphological and polyamine content changes in embryogenic and non-embryogenic callus of sugarcane. Plant Cell Tissue Org Cult 114(3):351–364. https://doi.org/10.1007/s11240-013-0330-2

Staden JV, Zazimalova E, George EF (2008) Plant growth regulators II: cytokinins, their analogues and antagonists. Ann Thorac Surg 82(6):2031–2036. https://doi.org/10.1016/j.athoracsur.2008.06.029

Stasolla C, Yeung EC (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tissue Org Cult 74(1):35–55. https://doi.org/10.1023/A:1023345803336

Steiner N, Farias-Soares FL, Schmidt EC, Pereira MLT, Scheid B, Rogge-Renner GD, Bouzon ZL, Schmidt D, Maldonado S, Guerra MP (2015) Toward establishing a morphological and ultrastructural characterization of proembryogenic masses and early somatic embryos of *Araucaria angustifolia* (Bert.) O. Kuntze. Protoplasma 253(2):487–501. https://doi.org/10.1007/s00709-015-0827-0

Tokuji Y, Mizue Y, Masuda H (1995) Effects of methyl jasmonate and concanavalin A on embryogenesis and the induction of secondary somatic embryos of carrot. Biosci Biotechnol Biochem 59(9):1675–1678. https://doi.org/10.1271/bbb.59.1675

Vondrakova Z, Dobrev P, Pesek B, Fischerova L, Vagner M, Motyka V (2018) Profiles of endogenous phytohormones over the course of norway spruce somatic embryogenesis. Front Plant Sci. https://doi.org/10.3389/fpls.2018.01283

Wang L, Li XY, Wang HC (2018) Physicochemical properties bioaccessibility and antioxidative activity of the polyphenols from pine cones of *Pinus koraiensis*. Int J Biol Macromol 126:385–391. https://doi.org/10.1016/j.ijbiomac.2018.12.145

Weckx S, Inzé D, Maene L (2019) Tissue culture of oil palm: finding the balance between mass propagation and somaclonal variation. Front Plant Sci 10:722. https://doi.org/10.3389/fpls.2019.00722
Wu GY, Wei XL, Wang X, Liang X, Wei Y (2020) Changes in biochemistry and histochemical characteristics during somatic embryogenesis in *Ormosia henryi* Prain. Plant Cell Tiss Org Cult 144(3):505–517. https://doi.org/10.1007/s11240-020-01973-5

Xu XP, Chen XH, Chen Y, Zhang QL, Su LY, Chen X, Chen YK, Zhang ZH, Lai ZX YL (2020) Genome-wide identification of mirnas and their targets during early somatic embryogenesis in *Dimocarpus longan* lour. Sci Rep. https://doi.org/10.1038/s41598-020-60946-y

Yu Y, Qin WQ, Li Y, Zhang CJ, Wang Y, Yang ZE, Ge XY, Li FG (2019) Red light promotes cotton embryogenic callus formation by influencing endogenous hormones, polyamines and antioxidative enzyme activities. Plant Growth Regul 87(2):187–199. https://doi.org/10.1007/s10725-018-0461-x

Zhang K, Su J, Xu M, Zhou Z, Zhu X, MaX HJ, Tan L, Zhu Z, Cai H, Liu F, Sun H, Gu P, Li C, Li Y, Zhao W, Sun C, Fu Y (2020) A common wild rice-derived *BOC1* allele reduces callus browning in indica rice transformation. Nat Commun 11(1):443. https://doi.org/10.1038/s41467-019-14265-0

Zhao J, Li G, Yi GX, Wang BM, Deng AX, Nan TG, Li ZH, Li QX (2006) Comparison between conventional indirect competitive enzyme-linked immunosorbent assay (competitive ELISA) and simplified ELISA for small molecules. Anal Chim Acta 571(1):79–85. https://doi.org/10.1016/j.aca.2006.04.060

Zhou XH, Zheng RH, Liu GX, Xu Y, Zhou YW, Thomas L, Laux T, Zhen Y, Harding SA, Shi JS, Chen JH (2017) Desiccation treatment and endogenous iaa levels are key factors influencing high frequency somatic embryogenesis in *Cunninghamia lanceolata* (lamb.) hook. Front Plant Sci. https://doi.org/10.3389/fpls.2017.02054

**Publisher's Note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.