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

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Abstract

Somatic embryogenesis (SE) is a biotechnological tool with high application potential for *in vitro* propagation and regeneration of conifers species. However, in SE of Korean pine, a dominant species of the natural mixed coniferous and broadleaf forests of the temperate forests in Northeast China, decreasing embryogenic potential was found in embryogenic callus (EC) with successive subcultures, especially when the cultures were maintained for more than 5-6 months, which is one of the most significant problems preventing commercial implementation of the SE technique. Aiming to investigate the morphological and the endogenous phytohormone changes associated with the decreasing embryogenic potential of Korean pine, here, we compared seven types of calli during long-term embryogenic culture. 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 their 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 ABA were beneficial for promoting early somatic embryo differentiation in Korean pine, but maintaining high embryogenic potential required moderate levels of IAA and low levels of ZR. Additionally, a high ratio of IAA / ABA and MeJA / ABA was conducive to maintaining high embryogenic potential, whereas a low ratio of IAA / ABA and MeJA / ABA were effective for early somatic embryos morphological differentiation. This study demonstrates morphological and physiological characteristics of the embryogenesis potential of Korean pine calli.

1. 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 wood 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).

As for Korean pine, despite having a well-established SE regeneration system, embryogenic calli (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 EC tend to lose the differentiating potential after extending subculture over prolonged periods such as 5-6 months, which is one of the most significant problems preventing commercial implementation of the SE technique (Gao *et al.* 2020; Peng *et al.* 2020, 2021). Similar problems have also been found in other pine trees, such as *Pinus pinaster* (Breton *et al.* 2006) and *Picea balfouriana* (Li *et al.* 2019). SE production capacity in Korean pine declined rapidly during long-term culture and this has become a major hindrance towards the research progress and commercial applications of Korean pine somatic embryos. Considering the problem that EC was difficult to maintain for a long term, coupled with the extremely low induction rate of EC in Korean pine (Gao *et al.* 2020), a new callus induction system of Korean pine is needed for a new explant each time, which is laborious, costly, and time-consuming, therefore, morphological or physiological markers are needed to evaluate callus suitable for SE as a critical step towards solving the problems associated with this process in conifers trees.

Research on the biological mechanisms of reduced embryogenic potential during successive subcultures indicated that embryogenic cultures during an extended period with the same culture conditions might be subject to an increase in the frequency of somaclonal variations, resulting in some undesirable consequences, such as decreasing embryogenic potential in EC (Rival *et al.* 2013). Findings from the integrated cell biology studies may contribute to overcome the bottlenecks encountered with the SE. For example, morphological changes observed during EC proliferation resulted from the subsequent differential competence of the somatic embryos (Guerra *et al.* 2016; Pereira-Dias *et al.* 2020). Based on the observation of histological morphology, in conifers trees, the proliferations in cell cultures were generally driven by proembryogenic masses (PEMs), which divided cyclically into PEM I, II, and III, and development of PEMs significantly affected the ability of differentiation of the somatic embryo (Filonova *et al.* 2000; Stasolla and Yeung 2003; Steiner *et al.* 2015). In addition, according to Pěňčík *et al.* (2015), the reduced embryogenic potential during extended culture stages may be partly due to the production and accumulation of hormones. As for Korean pine, although Peng *et al.* (2021) carried out studies on the energy storage and antioxidant enzymes in SE, to our best knowledge, little information is currently available about the underlying mechanisms of the loss in the embryogenic potential of subcultured EC

from Korean pine. Therefore, identifying the morphological and endogenous hormones differences between EC and non-embryonic callus (NEC) is essential for clarifying the mechanism of the decreasing embryogenic potential in the long-term cultured EC of Korean pine.

Consistent with the above reports by Gao *et al.* (2020), in our previous research, it was also found that several different types of calli were frequently produced when EC from same cell lines in Korean pine was subcultured on the same culture media for 5-6 months, some of which had the potential to generate somatic embryos, while others did not. Interestingly, these EC were characterized by friable or compact, white or yellow-green, transparent or opaque, *etc.* in appearance. Given this, in this present study, these different types of calli in Korean pine were utilized to investigate morphological modifications and endogenous hormone changes of calli with different embryogenic potential in long-term culture. The results can provide morphological and physiological characteristics for identifying high embryogenic potential calli, be critical for evaluating calli suitable for SE, and be valuable for constructing a maintainable system of EC in Korean pine.

2. Materials And Methods

2.1. 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 $2.0 \text{ mg} \cdot \text{L}^{-1}$ 1-Naphthaleneacetic acid (NAA), $1.5 \text{ mg} \cdot \text{L}^{-1}$ 6-benzyladenine (6-BA), $0.5 \text{ g} \cdot \text{L}^{-1}$ casein hydrolysate (CH), $0.5 \text{ g} \cdot \text{L}^{-1}$ L-glutamine, and 3 % (w/v) sucrose, and was solidified with 0.4 % Phytigel. After inducing for approximately 4 weeks, the induced EC were transferred to the proliferation and maintenance medium, which consisted of DCR supplemented with $0.25 \text{ mg} \cdot \text{L}^{-1}$ 6-BA, $1 \text{ mg} \cdot \text{L}^{-1}$ NAA, $0.5 \text{ g} \cdot \text{L}^{-1}$ CH, and $0.5 \text{ g} \cdot \text{L}^{-1}$ L-glutamine, 3 % (w/v) sucrose, and was solidified with 0.4 % Phytigel. 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 $0.22 \mu\text{m}$ 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 EC were transferred to the same but fresh subculture medium every 2 weeks. The cultures *in vitro* were all kept in a growth chamber at $24 \pm 2^\circ\text{C}$ and the dark conditions for 6 months. Subsequently, the calli maintained for two months or six months were subcultured on a hormone-free culture medium to promote the differentiation of the embryogenic structures, and then obtained the embryonic cultures containing early globular embryos. During the process of subculture, the calli were classified to seven types according to their external morphological characteristics such as color, texture, shape, and degree of dispersion and the seven types were white calli maintained for two months (C1), white calli maintained for six months (C2), yellow-green calli (C3), water-stained calli (C4), brown Calli (C5), fibrotic calli (C6), and calli with the globular embryo (C7), respectively. The above seven types of

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

2.2. 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). We used an environmental scanning electron microscope (ESEM) (Phenom Pro) further to observe the cell structures of these cultures. The small samples of fresh, uncoated, and wet embryogenic 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).

2.3. Determination of endogenous hormones

Measurement of the endogenous hormones including indole-3-acetic acid (IAA), zeatin-riboside (ZR), gibberellins (GA_3), abscisic acid (ABA), brassinosteroids (BR), and methyl jasmonate (MeJA) of different types of cultures in Korean pine were performed using the ELISA kit (Bollmark *et al.* 1988), 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 $\pm$ 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.

3. Results

3.1. Morphological characteristics 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, as described by Gao *et al.* (2020) and Peng *et al.* (2020), 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, these EC could be divided into 7 types (Fig. 1A-G). 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 (Figure 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 block 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. The C7 type calli were differentiated calli with early embryos. The whole calli were white and translucent, with light yellow granules scattered on their surfaces (Fig. 1G). The globular embryos under development with small globular bulges could be seen. In addition, the early embryonal part was bullet-shaped, opaque and distinguishable from the suspensor, indicating it was in the initial stages of somatic embryo formation.

We initially evaluated the embryogenic ability of these embryogenic cultures by subculturing on a PGR-free medium for 30 days. Sporadically, the differentiated early somatic embryos were formed (Fig. 1G). However, these globular embryos were only successfully differentiated from the C1-type calli, whereas 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.

3.2 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, and the mass of proliferating embryogenic tissue is often called EC during the organized nature of conifer cultures, 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 the embryogenic calli 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 during the long-term subculture of Korean pine. As shown in Fig. 1H-P, the histological observations by ESEM revealed that the surface cell composition and morphology of these seven types of calli from Korean pine were quite different. Microscopically, the C1-type calli of Korean pine were characterized by designated PEMs-like structure (Fig. 1H, 1I, 1J), and polarized cell structure clusters of different sizes were found. Specifically, a higher number of PEM I (Fig. 1H) was observed on the callus surface, but PEM II (Fig. 1I) and PEM III (Fig. 1J) 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.

We found the surface of C1-type calli was covered with mucilaginous and membranous structure, namely, extracellular matrix (ECM). 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 scattered (Fig. 1K). 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. 1L, 1M, 1N). Most cells were disorganized, accompanied by larger intercellular space between the cells. In addition, different from C2 types calli, disintegrated cells from those PEMs-like structures were almost invisible. Unlike the C1-C5 types of calli, the brown calli (C6) usually contained

many cells that were died or close to death (Fig. 1O). Severe degradation of cells occurred and resulted in gradually losing growth capacity. Different from the first six types of calli, the C7-type calli developed a better formed and individualized cell aggregate containing early embryos (Fig. 1P). The 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. 1P). As for ECM structure, different from C1 type calli, only a small amount was observed on the surface of other types of calli.

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

Generally, during the plant SE, high concentration auxin can promote the induction of EC, however, low concentrations of auxin and cytokinin are required in the EC maintenance stage (Yu *et al.* 2019, Ree *et al.* 2019). Exogenous addition of ABA in the medium is beneficial to the differentiation of somatic embryos (Yu *et al.* 2019, Ree *et al.* 2019). In the present study, we found that endogenous hormones differed significantly in different types of embryogenic cultures of Korean pine.

1) Changes of single endogenous hormone content

As shown in Fig 2, overall, the content of IAA, ABA, and MeJA levels 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 embryogenic calli 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), significantly higher than other types of calli. The content of ZR was at a lower level in white embryogenic calli of two months (C1), similar to the trend of IAA content. 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 GA₃ in different calli was low (2.53-5.09 ng·g⁻¹ FW). The higher content of GA₃ 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 GA₃ 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. The results indicated that high levels of ABA were beneficial to promote early somatic embryo differentiation in Korean pine while maintaining a high embryogenic potential required low levels of ABA.

Similar to ZR and GA₃, the content of BR in different types of Korean pine 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 the seven 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 level of MeJA content appeared in the other four types of calli.

2) 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. Furthermore, 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 GA₃ / 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) and MeJA / ABA (1.32) were found in C1-type calli. It is worth noting that too high ratios of IAA / ABA and Me JA / ABA can cause browning.

Unlike the ratios of IAA / ABA and JA-Me / ABA, the GA₃ / ABA ratio was higher in C1, C3, C4, and C5 (0.20-0.26). Both the values of BR / ABA and ZR / ABA were higher in C1, C2, C4, and C5 (0.21-0.30). In addition, C2, C4, C5, and C7 had higher ZR / IAA (0.15-0.26), whereas the ZR / IAA ratio kept at a relatively low level in C1, C3, and C6 (0.08-0.15). Therefore, it can be concluded that high IAA / ABA and MeJA / ABA ratios were conducive to maintaining a high embryogenic potential. However, low IAA / ABA and MeJA / ABA ratios could benefit morphological differentiation of the early globular embryos. There was no obvious changes in the values of BR / ABA, ZR / ABA, GA₃ / 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.

4. Discussion

In plant SE, in addition to induction of EC, the ability of EC to proliferate indefinitely through cyclic embryogenesis is also one of the essential steps, especially for conifers, long-lived recalcitrance trees (Bonga 2017; Li *et al.* 2019). However, a progressive decline of EC growth, an increase in necrosis rate, and a decrease or even loss of the morphogenic differentiating potential of normally-developed somatic embryos in embryogenic cultures that maintained through repetitive subculturing on the same medium have all been reported in *Pinus pinaster* (Breton *et al.* 2006), *Coffea arabica* (Landey *et al.* 2015), *Olea europaea* (Bradaï *et al.* 2016), *Araucaria angustifolia* (Pereira-Dias *et al.* 2020) and *Saccharum* sp. (Passamani *et al.* 2020). Recently, SE from female gametophytes 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). Considering the loss of embryogenic potential as a critical bottleneck in SE, coupled with the extremely low induction rate of EC in Korean pine (Gao *et al.* 2020), a new callus induction system of Korean pine is needed for a new explant each time, which is laborious, costly, and time-consuming, therefore, morphological or physiological markers are needed to evaluate callus suitable for SE as a critical step towards solving the problems associated with this process in conifers trees. Although Peng *et al.* (2021) carried out studies on the energy storage and antioxidant enzymes in Korean pine SE, to our best knowledge, little information is currently available about the underlying mechanisms of the loss in the embryogenic potential of subcultured EC from Korean pine. In this context, to obtain insight into the processes that may lead to loss of embryogenic potential, we compared the morphological and endogenous hormones differences in different types of embryogenic cultures, this study can help improve the maintenance cycle of EC and the optimization of the SE methods in Korean pine.

4.1 The relationship between morphological characteristics and embryonic 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 well-developed somatic embryos (Fu *et al.* 2012; 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 culture callus of *Olea 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 during long-term maintenance 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 overall

different morphology with C1, which was similar to those of NEC. This morphology directly influenced the early somatic embryo formation, as reported in the previously study on Korean pine by Peng *et al.* (2021). Thus, it can be concluded that the EC of Korean pine cannot be maintained for more than two months because a rapid and extensive differentiation of somatic embryos occurs or because EC grows very slowly or turns brown. These morphological changes indicate reduction or loss of its embryogenesis. Most frequently, an essential step during SE is switching from proliferating embryogenic tissue to the development of embryos. In our study, the early somatic embryos were formed on the surface of C7-type calli, which presented typically bipolar structures. The early somatic embryos also had a granular to spiky morphotype due to protruding at their surface, and this was consistent with the morphological characteristics of early somatic embryos in a previous study on *Araucaria angustifolia* (Pereira-Dias *et al.* 2020).

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 formation and development of somatic embryos, similar to the morphological stages of the zygotic counterpart of conifers. In this present study, the C1-type calli contained a greater number of PEMs-like structures. This result demonstrated that somatic embryo development of Korean pine was initiated from the formation of PEM I characterized by few round dividing cells (two-celled or four-celled proembryo) subtended by an elongated vacuolated suspensor-like cell. Simultaneously, a small amount of PEM $\square$ and PEM $\square$ cell aggregates appeared in C1-type calli, which were developmentally equivalent in appearance to zygotic embryos. In addition, our results were similar to the reports of other conifers trees, such as *Juniperus communis* (Helmersson and Von Arnold 2009), *Cunninghamia lanceolata* (Zhou *et al.* 2017), and *Araucaria 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 between cell morphology and structure was found in other types of cultures (C2-C6). In detail, in all other types of calli, degrading cells appeared and the polarity was disturbed to some extent. Moreover, in these types of calli, the organized PEMs-like structures disintegrated and the number of irregular and unorganized cells increased, which led to no formation of somatic embryos. All these characteristics of cells morphology and structure in C2-C6 -type calli were similar to those of nonembryogenic cells found in other studies (Silveira *et al.* 2013; Reeves *et al.* 2018; Gautier *et al.* 2018), which directly influenced the formation of somatic embryo.

To sum up the findings of the morphological characteristics, it was found that calli with high embryogenic potential of long-term maintenance from Korean pine were characterized and selected by the presence and greater degree of organization of PEMs. Oppositely, somatic embryos were hardly observed in some types of cultures in which tissue of PEMs structures disappeared or disintegrated. Thus, we speculated that this cell state in PEMs phase would be helpful for maintaining the high embryogenic ability of Korean pine. The failure of many embryogenic cells to form mature somatic embryos is highly associated with a disturbed or arrested PEMs structure, as mentioned in precious

studies (Von Arnold *et al.* 2002; Márquez-Martín *et al.* 2012). Therefore, 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 to EC, such as the most abundant presence of ECM structure (Reeves *et al.* 2018; Lelu-Walter *et al.* 2017; Gautier *et al.* 2018) and the unique structure of ECM that covered the callus surface, were indicators of the EC formation that plays a fundamental role at the proembryo stage of SE and is considered to be restricted to the cell of embryogenic and regenerative potential (Binte *et al.* 2018; Ming *et al.* 2019). Therefore, we speculate the presence of ECM might be related to the acquisition of embryogenesis potential in Korean pine.

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

Different mechanisms have been proposed for the decline of embryogenic potential during the maintenance of EC, among which plant hormones are well known to play essential roles in this process (Klimaszewska *et al.* 2016; Wu *et al.* 2020). 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 embryonic cells. Auxins are generally considered to be the more important hormone in the acquisition of embryogenic competence. Once somatic embryo development is established and has proceeded to some degree, levels of IAA decrease again (Klimaszewska *et al.* 2016). 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 of somatic embryo and prevent their differentiation. According to previous studies (Fehér 2015; Wojcikowska and Gaj 2017; 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. Additionally, 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.

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) also suggested that increasing ABA concentrations (up to 80 $\mu\text{mol}\cdot\text{L}^{-1}$) in the maturation medium improved Korean pine somatic embryo yield. In parallel to this study, 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 embryonic potential. Kępczyńska and Orłowska (2021) also found a similar trend of the effectiveness of ABA, that is, high level of ABA reduced embryogenic competence in *Medicago truncatula*.

In this present study, a high level of MeJA promoted somatic embryo differentiation, but a low level of MeJA was helpful for sustaining embryogenic potential. MeJA is an uncommon hormone used in SE. For example, Ruduś *et al.* (2006) observed in *Medicago sativa* that MeJA did not affect somatic embryo differentiation, but it had a negative impact on the maintenance of embryogenic potential. Their results were contrary to our study and previous studies with *Daucus carota* (Tokuji *et al.* 1995) and *Medicago sativa* (Ruduś *et al.* 2006, 2009) that all found inhibited somatic embryo formation by MeJA. Recently, Morcillo *et al.* (2020) reported that MeJA application produced a recovery in the differentiation capacity of an embryogenic line of *Quercus ilex*. Based on all findings above, we speculate that MeJA may play different roles in somatic embryo formation and embryogenic potential maintenance of different plants, depending on the sensitivity of different species to MeJA.

Previous research reported that BR was 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 BR might promote early somatic embryo development of calli in *Dimocarpus longan*. However, the levels of BR did not show any noticeable effect on embryogenic potential and differentiation capacity in the present study. Our study confirmed that the low level of ZR in embryogenic tissues of 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 GA₃ did not play a significant role in the process of Korean pine somatic embryo differentiation and embryonic potential maintenance.

Overall, the embryogenic potential maintenance in SE is a complex physiological and biochemical process; therefore, the level of any single hormone cannot fully explain the ability of the embryogenic potential maintenance. The balance among hormones is also critical in this process (Grzyb *et al.* 2018) and often used to evaluate embryogenesis (Pérez-Jiménez *et al.* 2013; Wu *et al.* 2020). Therefore, we analyzed the ratios of some hormones that might have significant effects. Our results showed that compared with other endogenous hormones, higher MeJA / ABA and IAA / ABA were favorable for the maintenance of embryogenic potential in Korean pine. However, for early somatic embryo morphogenic differentiation, lower ratios of MeJA / ABA and IAA / ABA were more effective and the imbalance between hormones might be one of the mechanisms of reduced embryogenic potential. Similar results were also found in *Corylus avellana*, in which the IAA / ABA ratio was significantly higher in EC than NEC (Wu *et al.* 2020). Together with the morphological results, we can speculate that the regulation of hormones on the maintenance of embryogenic potential and the somatic embryo formation ability is associated with the morphological changes of cell aggregates, such as PEMs and EM, and with the presence of ECM structure.

Morphological modifications and changes in the endogenous content have been highlighted as necessary for maintaining embryogenic potential (Passamani *et al.* 2018). Although to some extent, we observed morphological differences in different embryogenesis potential cultures of Korean pine, the

control of endogenous hormones is likely through the appearance or disappearance of PEMs. The decrease in embryogenic potential during SE is a complex developmental process, which includes metabolism, gene expression, and a series of physiological changes (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.

Although it has been confirmed that the maintenance of embryogenic calli of Korean pine requires accumulation of MeJA / ABA, information about the regulatory role of MeJA in different aspects of SE is still limited. Thus, it is also necessary to carry out experiments on the effect of MeJA alone and together with ABA in the maintenance of Korean pine EC.

5. Conclusions

The coniferous SE *in vitro* proved to be very efficient for large-scale propagation of many tree species, providing a more valuable and rapid way than the traditional cutting procedure. Nevertheless, as we all know, the SE protocols of coniferous species, e.g., Korean pine, is still challenging because of many factors, such as recalcitrance. The most common problem encountered in SE is not only the difficulty in the induction of embryogenic callus but also the accompanied gradual loss of embryogenesis capacity during the long-term subculture, hindering the applications of these techniques to large scale micropropagation. Both morphological responses and changes in endogenous hormones in relation to the reduction of embryogenic competence during the process of long-term maintenance of Korean pine have been examined in the present study. This study provides morphological and physiological indicators for the embryogenesis potential of 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 embryogenic callus for further use, which is valuable for clarifying the mechanism of the decreasing embryogenic potential in the long-term cultured EC of Korean pine.

Declarations

Author Contributions: Y.L. conceived the study, designed the experiment, and revised the manuscript. X.X. performed the experiment and wrote the draft of the manuscript. H.L.S provided technical guidance and contributed to the writing of the manuscript. M.L.G supervised the experimental process. Y.Z. collected plant materials. X.B. Proofread the manuscript. All authors participated in the experiment. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

The morphological features of different types of embryogenic cultures during the long-term subculture of Korean pine. A-G represents the calli morphology under the stereo microscope (bar = 5 mm) and H-P represents the morphological features under ESEM (bar = 100 μ m). (A) white embryogenic calli for two months (C1-type), (B) white calli for six months (C2-type), (C) yellow-green calli (C3-type), (D) water-stained calli (C4-type), (E) fibrous calli (C5-type), (F) brown calli (C6-type), (G) calli with the differentiated early globular embryos (in the yellow circle) when C1-type calli subcultured to a PGR-free medium for 30 days (C7-type), (H) C1-type calli with PEM I, (I) C1-type calli with PEM II and thin membranous ECM structure covering the surface, (J) C1-type calli with PEM III, (K) C2-type calli, (L) C3-type calli, (M) C4-type calli, (N) C5-type calli, (O) C6-type calli, (P) 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.

Figure 2

Changes in the content of endogenous hormones in different types of embryogenic cultures of Korean pine. The data are expressed as the means $\pm$ SD, and means followed by the same letters are not significantly different by Tukey's multiple tests ($p < 0.05$).

Figure 3

Changes in the ratio of endogenous hormones in different types of embryogenic cultures of Korean pine. The data are expressed as the means $\pm$ SD. According to Kruskal-Wallis ANOVA, the mean values marked with the same letters do not differ significantly at the 0.05 confidence level.