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Formamide deionized accelerates the somatic embryogenesis of *Cunninghamia lanceolata*

Shichan He[1], Zhaodong Hao[1], Dandan Wang[1], Yulin Guo[1], Hua Wu[1], Asif Ali[1], Renhua Zheng[2*], Xueyan, Zheng[3], Jinhui Chen[1], Jisen Shi[1*]

[1]*Key Laboratory of Forest Genetics & Biotechnology of Ministry of Education of China, Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing 210037, China;* [2]*Key Laboratory of Timber Forest Breeding and Cultivation for Mountainous Areas in Southern China, Fujian Academy of Forestry, Fuzhou, 350012, China;* [3]*National Germplasm Bank of Chinese fir at Fujian Yangkou Forest Farm, Shunchang 353211, China*

Abstract

Aim of study: To improve the efficiency of the somatic embryogenesis (SE) in *Cunninghamia lanceolata.*

Area of study: The study was conducted at Nanjing Forestry University (Nanjing, China).

Materials and methods: Immature cones of *C. lanceolata,* genotype 01A1 which was planted in Yangkou State-owned Forest Farm (Fujian, China), were used to induced callus. These calli were used to induce SE, concentration gradients of 0 g/L, 0.01134 g/L, 0.1134 g/L, 1.1134 g/L and 11.34 g/L of FD was added, to explore the optimal concentration for promoting SE of *C. lanceolata.*

Main results: Low concentration of FD promoted the maturation of somatic embryos, while high concentration of FD lead to browning of embryogenic callus. The seedling rate and rooting number of seedlings induced by different concentrations of FD were significantly different.

Research highlights: This study may aid in the rapid maturation of *C. lanceolata* somatic embryos and is useful for accelerated *C. lanceolata* breeding.

Keywords: *C. lanceolata*; Formamide Deionized; Somatic embryogenesis; Seedling rate.

Abbreviations used: FD (Formamide Deionized), FD_0 (the concentration of 0 g/L FD), $FD_{0.01134}$ (the concentration of 0.01134 g/L FD), $FD_{0.1134}$ (the concentration of 0.1134 g/L FD), $FD_{1.134}$ (the concentration of 1.134 g/L FD), $FD_{11.34}$ (the concentration of 11.34 g/L FD).

Authors' contributions: SJS, CJH and ZRH conceived and planned the experiments. ZRH and ZXY are responsible for field tests, sample collection, etc. WH, GYL induced embryogenic callus. The HSC performed somatic embryo induction, analyzed the data, and wrote this manuscript. WDD, AA, HZD and CJH provided critical feedback. CJH and SJS give administrative, technical, or material support.

Introduction

Cunninghamia lanceolata (Lamb.) Hook, belonging to the Taxodiaceae family, is the principal indigenous tree species in subtropical Southern China (Duan *et al.*, 2017) and has long been cultivated since more than one thousand years ago (Zhao *et al.*, 2009; Chen *et al.*, 2013; Tang *et al.*, 2016). As one of the most popular plantation timber species in China, *C. lanceolata* is used extensively in construction, railroad ties, mine timber, furniture and wood pulp, etc. (Li *et al.*, 2015), because of its good quality timber, rapid growth, straight stem, and highly resistance to bending (Mei *et al.*, 2017). At present, the *C. lanceolata* plantation area is approximately 9 million ha, accounting for 30% of the national afforestation area, which ensures that it occupies an important position in the forest resources of China (Lu *et al.*, 2015; Hu *et al.*, 2017).

For woody plants, conventional breeding is inefficient, expensive, and time-consuming due to its inherently longer

life cycle and the inevitable dilution of desirable traits resulting from genetic separation and gene flow (John *et al.*, 1999; Zhou *et al.*, 2017). More specifically, *C. lanceolata* produces a high content of astringent seeds (seeds filled with tannin-like substance, it does not have the ability to germinate), leading to the unstable yield and low sowing quality. In the past 60 years, breeding workers have made a lot of efforts on genetic improvement of *C. lanceolata* (Shi *et al.*, 2010; Zheng *et al.*, 2015a; Zheng *et al.*, 2015b), such as mass production of cuttings (Li & Gary, 1999), application of tissue culture for propagation (Zhu *et al.*, 2007), and genetic engineering (Gao *et al.*, 2013). However, these genetic improvements are still subject to various restrictions, such as the number of mother tree cuttings, site space, and rooting rate. For the traditional tissue culture, root induction is especially difficult, as well as the induced roots are small and weak, finally affecting the quality of seedlings and the success rate of afforestation (Wen, 2011). In addition, *C. lanceolata* has always lacked a stable genetic transformation system. These limitations make it difficult for the production of improved *C. lanceolata* seedlings to meet the large demands of modern forestry production.

For coniferous species, somatic embryogenesis (SE) is the most effective biotechnology for asexual propagation (Olarieta *et al.*, 2020). The unique developmental potential of SE represents a very powerful biotechnological tool for regenerating plants from cell culture systems, as well as a potential model for investigating the structure, physiological, and molecular events occurring during plant embryogenesis (Hu *et al.*, 2017). Since the first report of SE in Norway spruce in 1985 (Hakman & Von Arnold, 1985), this in vitro process has been initiated for a number of coniferous species (Hu *et al.*, 2017), such as *Pinus taeda* (John *et al.*, 1999), Picea abies (Hakman *et al.*, 1985), Pinus massoniana (Zhang *et al.*, 2006), and Pinus radiata (Cerda *et al.*, 2002).

Our laboratory had successfully established the SE system of *C. lanceolate* (Zhou *et al.*, 2017). However, SE of *C. lanceolate* still has some complications needed to be addressed, such as the minimal induction rate, serious browning, difficult callus differentiation, and weak growth of regenerated plants. Formamide is a transparent oily liquid with a slight ammonia odor. It is hygroscopic and miscible with water and ethanol, slightly soluble in benzene, chloroform, and ether. It has two active functional groups, carbonyl and amide, which are easy to react. Formamide was found to be able to increase the specificity of PCR reaction (Sarkar *et al.*, 1990) and inhibit the activity of RNase and prevent the degradation of RNA (Chomczynski, 1992). It can inhibit the formation of the secondary structure of the DNA molecule. Thus, formamide is the most commonly used denaturant in DNA detection (Han *et al.*, 2010). Formamide Deionized (FD) is deionized formamide, which is more stable than formamide. Now, FD is widely used in biochemistry and molecular biology, especially in nucleic acid research. Our previous studies have shown that low concentration of FD can promote SE of *Liriodendron* hybrids (unpublished data). Besides, there are no other reports on the application of FD in SE of any species. Here, we found that low concentration of FD promoted the somatic embryo maturation of *C. lanceolata*.

Materials and Methods

Plant Materials

The genotype of *C. lanceolata* callus was 01A1, which were induced from immature cones (the zygotic embryo formed after pollination) that were collected from Yangkou State-owned Forest Farm (Fujian, China) (Fig. S1 [suppl.]). Generally, the callus with good genotype can maintain a good growth state after subculture for about 20 days (Fig. 1). At this point of time, the calli look white, transparent, bright, and glossy on the surface, soft and conglutinated in texture, with obvious early proembryo on the surface (Fig. 1A). When observed under an inverted microscope, the
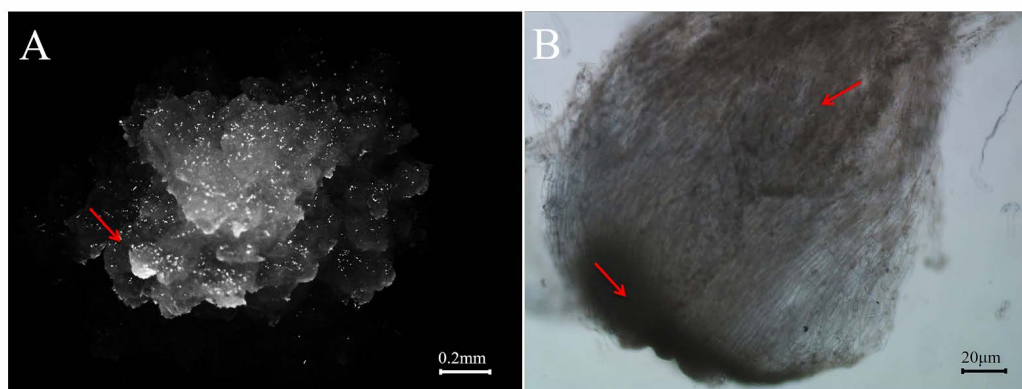

Figure 1. Experimental materials (embryogenic callus) used in the experiment.
A) Embryogenic callus of 01A1, the arrow is the early proembryo.
B) ESM structure of 01A1 embryogenic callus, the arrowheads represent dense embryonic head structure and loose embryonic stem structure, respectively.

embryogenesis suspensor mass (ESM) has obvious dense embryo head and loose suspensors (Fig. 1B).

Callus Culture

DCR (Gupta & Durzan, 1985) is the basic medium for induction and culture of *C. Lanceolata* callus, supplement with 10 mg/L Vitamin C (VC), 0.5 mg/L 6-Benzylaminopurine (6-BA), 0.5 mg/L Kinetin (KT), 450 mg/L Glutamine, 100 mg/L Inositol, 500 mg/L Casein hydrolysate (CH), 2.5 g/L Activated carbon, 2.4 g/L Gelrite, 20 g/L Maltose, and according to different cultivation conditions, a certain amount of Auxin was added. For the callus induction medium of *C. lanceolata*, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) was added. And, after the callus was successfully induced, the amount of 2,4-D will be halved for subculture. The callus induction and culture conditions were kept in dark at 23 °C.

Somatic Embryo Induction with Different Concentration of FD

DCR is the basic medium for SE induction of *C. lanceolata,* too. Here, we used 1/2 DCR (Only macrosalts were reduced by half, and the other nutrients were consistent with the basic medium of DCR) medium, supplement with 10 mg/L VC, 5 mg/L gibberellic acid (GA_3), 6 mg/L abscisic acid (ABA), 450 mg/L Glutamine, 200 mg/L Aspartic acid, 200 mg/L Proline, 5 g/L Inositol, 500 mg/L CH, 170 g/L Polyethylene glycol (PEG), 2 g/L Activated carbon, 2.4 g/L Gelrite, 25 g/L Maltose, pH 6.0. Then, FD's treatment with concentration gradient of 0 g/L (FD_0), 0.01134 g/L ($FD_{0.01134}$), 0.1134 g/L ($FD_{0.1134}$), 1.1134 g/L ($FD_{1.134}$) and 11.34 g/L ($FD_{11.34}$) were added to the medium. Repeated at three to five times (3~5 dishes) for each concentration and 6 pieces of callus (each piece of callus is about 0.06 g) were placed per dish, cultured in dark condition at 23 °C. The FD we used is produced from Amresco.

Growth of Somatic Seedlings

Using DCR as a basic medium with 10 mg/L VC, 20 g/L sucrose, 6.8 g/L Agar powder, pH 5.8. The induced cotyledons were then transferred to the growth medium and cultured at 16:8 h (light: dark) photoperiod.

Data Statistics and Analysis

We used graphpad prism version 6.0 (graphpad software, La Jolla, CA, USA) to analyze the number of embryos in each dish. One-way ANOVA was used to test the difference between each treatment group and the control group. Duncan multiple comparison test was used to compare the mean value with the significance level being set as α equaling to 0.05.

Results

Low Concentration of FD Accelerates SE of *C. lanceolata*

In order to explore the effect of FD on SE of *C. lanceolata,* we inoculated the callus of *C. lanceolata* on somatic embryo induction medium, containing 0 g/L, 0.01134 g/L, 0.1134 g/L, 1.1134 g/L, and 11.34 g/L FD respectively. In general, the cotyledonary embryos can form from the calli of FD_0, $FD_{0.01134}$, and $FD_{0.1134}$ treatments, while the callus treated with higher concentration (1.1134 g/L and 11.34 g/L) of FD would turn brown after 3 weeks, thus cannot form cotyledon embryos. We found that the proembryogenic masses (PEMs) turned yellow slightly on the seventh day (Fig. 2A, 2E, 2I), but the structure of ESM had no obvious change (Fig. 3A, 3E, 3I). Then, two weeks later, the appearance characteristics of PEMs changed greatly with the color turned yellow (Fig. 2B, 2F, 2J). Furthermore, PEMs entered early embryogeny stage, accompanied by the elongation and condensation of the embryo head (Fig. 3B, 3F, 3J). More specifically, the density of embryo head cells under $FD_{0.01134}$ and $FD_{0.1134}$ were much higher compared to the treatment of FD_0 and embryo head cells under $FD_{0.1134}$ had the highest density. However, with the further increase of FD concentration, callus browning was obviously observed and there were no any changes in its ESM structure (Fig. S2 [suppl.]). On the 35th day, somatic embryos under the $FD_{0.01134}$ and $FD_{0.1134}$ treatments both entered the late embryogeny stage, owning a dense embryonic head and highly vacuolated long polarized cells (Fig. 2G, 2K, red arrows), while control (FD_0) did not. In ESM structure, the somatic embryo under the treatment of $FD_{0.1134}$ is much longer and denser than that under the $FD_{0.01134}$ treatment (Fig. 3G, 3K). Finally, somatic embryos under the $FD_{0.01134}$ treatment entered the early stage of cotyledon embryo on the 49th day, with an indentation separating the developing cotyledon structures (Fig. 2H). Meanwhile, two cotyledons of somatic embryos under the $FD_{0.1134}$ treatment expanded and entered the cotyledon embryo stage (Fig. 2L). Somatic embryos of *C. lanceolata* has developed into the mature cotyledon embryo stage on the 56th day (Fig. 4B, 4C, 4E, 4F), whereas the early cotyledon embryos appeared under control (Fig. 4A, 4D).

The average number of cotyledons per culture dish induced by FD_0, $FD_{0.01134}$, and $FD_{0.1134}$ treatments was 20, 16.5, and 10.8, respectively. There was no significant difference in the number of cotyledon embryos induced by FD_0 and $FD_{0.01134}$ treatments (Fig. 5), although $FD_{0.01134}$

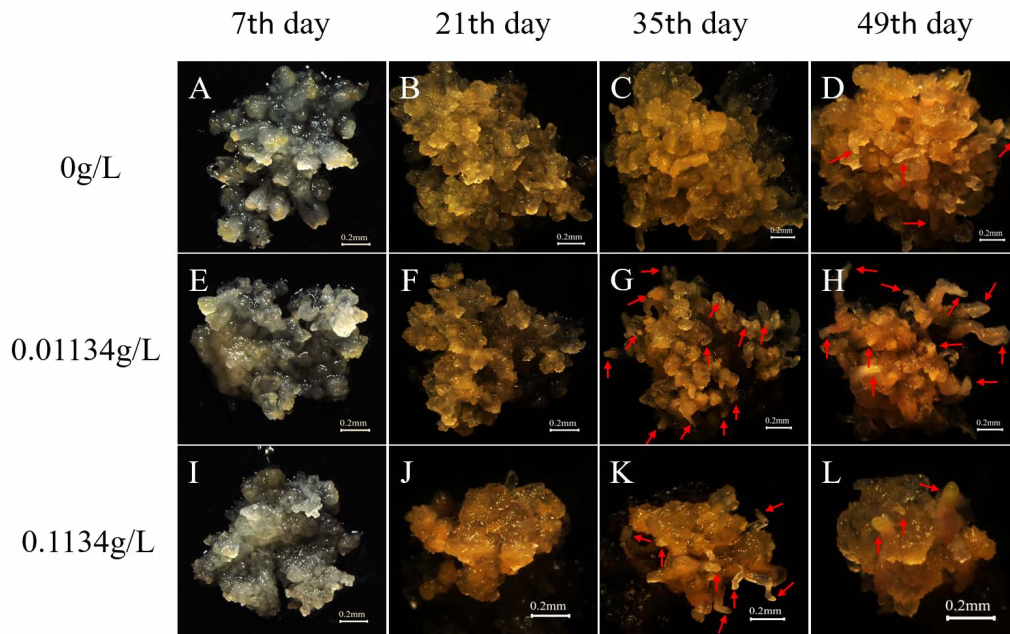|	7th day	21th day	35th day	49th day
0g/L	A	B	C	D
0.01134g/L	E	F	G	H
0.1134g/L	I	J	K	L

Figure 2. The PEM state of FD_0, $FD_{0.01134}$ and $FD_{0.1134}$ at day 7, 21, 35 and 49th, respectively. The red arrows indicate the induction of somatic embryos.

treatments shortened the maturation time of somatic embryos by nearly three weeks (Fig. 2G, 4A). In addition, although the maturation time of somatic embryos was shortened under the $FD_{0.1134}$ treatment (Fig. 2K), even faster than $FD_{0.01134}$ treatment (Fig. 3K), the number of cotyledon embryos induced from the $FD_{0.1134}$ treatment was significantly reduced compared with the FD_0 treatment (Fig. 5). Therefore, the optimal FD concentration for accelerating the maturation of *C. lanceolata* somatic embryos here is 0.01134 g/L.

Low Concentration of FD Improves Seedling Rate of *C. lanceolata.*

We then transferred the induced cotyledons to the basic medium, after a month or so of subculture, most cotyledon embryos grew into small seedlings of *C. lanceolata*, and a few of them had taken root (Fig. 6A). Cotyledon embryos that did not grow into small seedlings turned brown and still remained two cotyledons (Fig. 6B). For the treatments of FD_0, $FD_{0.01134}$, and $FD_{0.1134}$, we obtained 51, 59, and 32
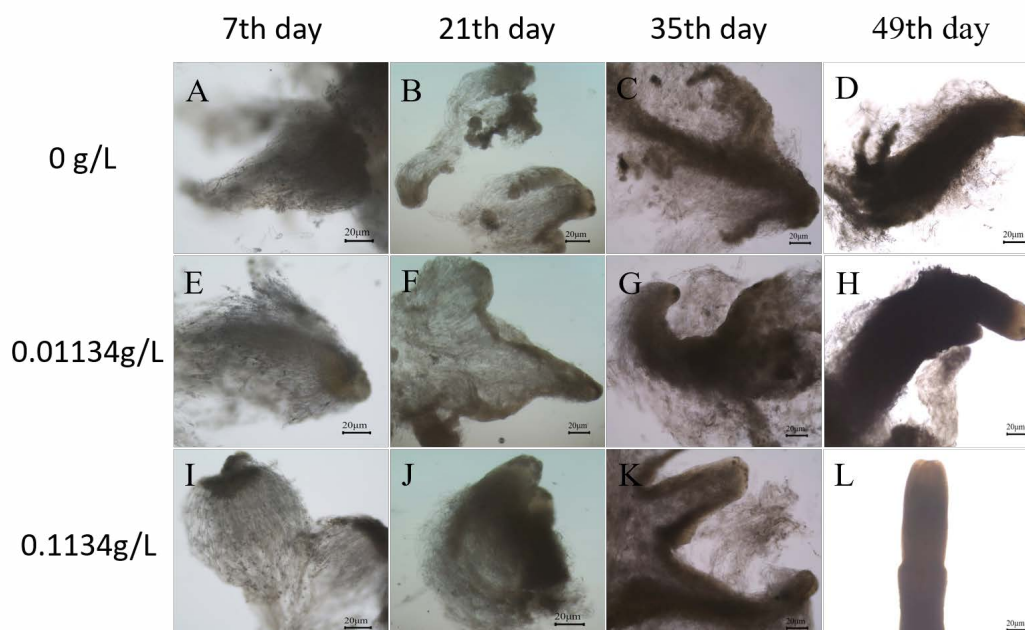
	7th day	21th day	35th day	49th day
0 g/L	A	B	C	D
0.01134g/L	E	F	G	H
0.1134g/L	I	J	K	L

Figure 3. ESM structure of FD_0, $FD_{0.01134}$ and $FD_{0.1134}$ at day 7, 21, 35 and 49th, respectively.
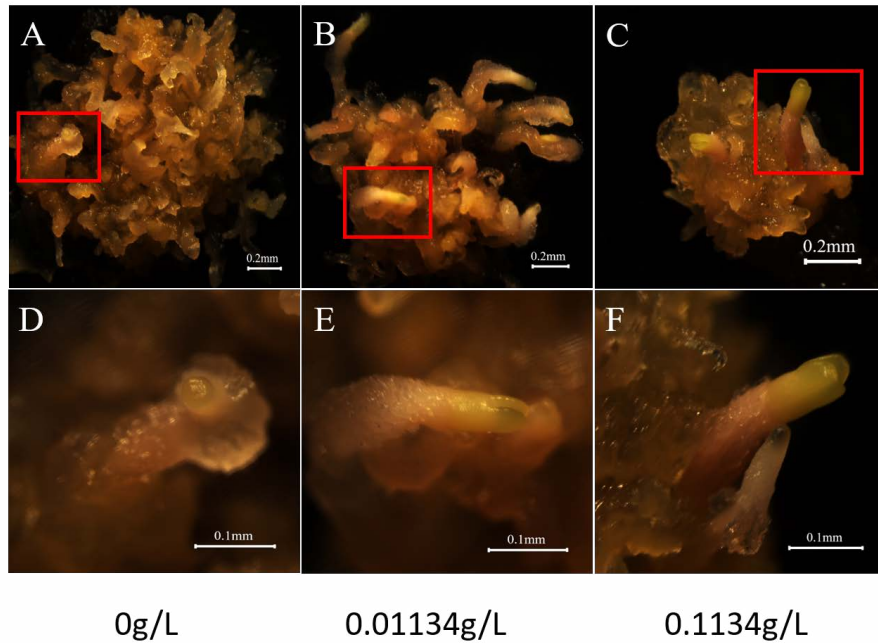
0g/L   0.01134g/L   0.1134g/L

Figure 4. The PEM state of FD_0, $FD_{0.01134}$ and $FD_{0.1134}$ at day 56th, respectively (56 days).

seedlings, respectively, according to a seedling rate of 85.0%, 89.4%, and 59.3%, respectively. These results showed that $FD_{0.01134}$ treatment had the highest seedling rate, followed by the control treatment. For the $FD_{0.1134}$ treatment, ~40% co-tyledon embryos could not grow into seedlings. Therefore, FD at low concentration has a certain promotion effect on the formation of somatic embryo seedlings.

High Concentration of FD Inhibits the Rooting of *C. lanceolata.*

We then observed the rooting of these seedlings on the basic medium, after subculture for about a month. For the treatments of FD_0, $FD_{0.01134}$, and $FD_{0.1134}$, 15, 17, and 8 soma-tic embryo seedlings were rooting, corresponding to a roo-ting rate of 29.4%, 28.8%, and 25.0%, respectively. In terms of rooting rate of somatic embryo seedlings, there was little difference between the treatments of FD_0 and $FD_{0.01134}$, while the rooting rate of the treatment of $FD_{0.1134}$ was significantly lower than the former two. These results indicated that the rooting of somatic embryo seedlings induced by high con-centration of FD was inhibited to some extent.
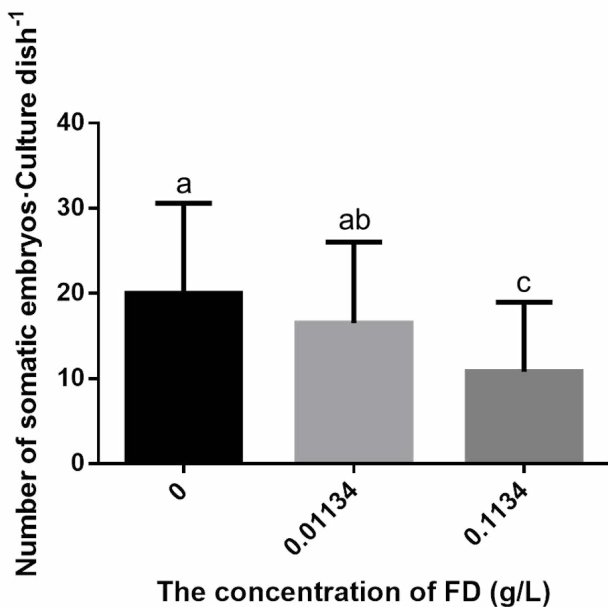
Discussion

Plant growth regulators play important roles in the ini-tiation, maintenance, and maturation of somatic embryos (Jimenez, 2005). Previous studies have shown that ABA (Filonova *et al.*, 2000; von Arnold *et al.*, 2005; Vales *et al.*, 2007), PEG (Linossier *et al.*, 1997; Stasolla *et al.*, 2003), polyamines (Wang *et al.*, 2020) and amino acids (Aleith & Richter, 1991; Mohd Din *et al.*, 2016; Carlsson *et al.*, 2017; Solanki *et al.*, 2019) all play important roles in SE. Here, FD is applied to SE of *C. lanceolata* as a new plant growth

Figure 5. The number of somatic embryos induced by diffe-rent concentrations of FD.
One-way ANOVA was used to test the difference between the data of each treatment group and the control group. Duncan multiple comparison test was used to compare the mean va-lues. The data shown in the chart were the average values and standard errors of repeated measurements. Different letters in-dicate significant difference in the level of α =0.05.
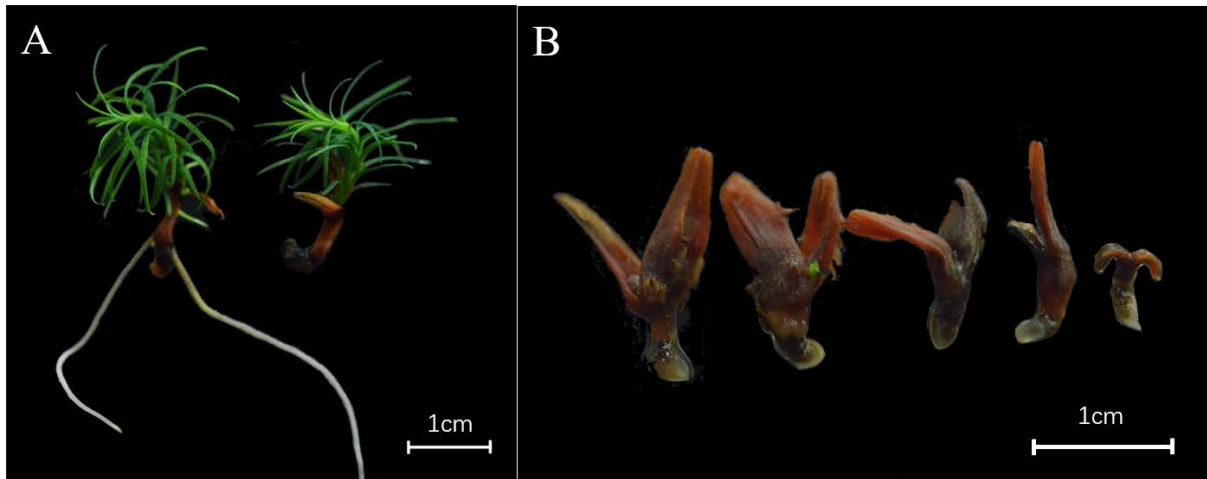
Figure 6. Somatic embryo seedling status. A represents the seedling forming state of somatic embryo seedlings, both rooting and not rooting; B represents the status of five kinds of somatic embryo seedlings that failed to form seedlings.

regulator. We found that 0.01134 g/L FD could shorten the maturation time of somatic embryos by nearly three weeks compared with the control group, while ensuring no significant difference in the number of somatic embryos. However, the mechanism by which FD promotes somatic embryo maturation in *C. lanceolata* is still unclear. As far as we know, there is no published report showing the role of FD in somatic embryo development. Because FD has certain toxicity, it can promote DNA denaturation (Han *et al.*, 2010), we suspect that it produces a certain stress response to the system during the SE of *C. lanceolata*, which leads to the shortening of the SE of *C. lanceolata*. However, the use of higher concentration leads to the decrease of SE or even the failure of SE due to its toxic effect. On the other hand, FD can promote SE of *C. lanceolata*, possibly because it can inhibit the activity of RNA enzyme (Chomczynski, 1992) and protect the mRNA expression of genes related to SE, so as to achieve the function of promoting SE.

In conclusion, this study provides new insights for accelerating SE of gymnosperms. By adding different plant growth regulators to the medium, SE can be accelerated. In our study, FD can significantly promote the maturation of somatic embryos of *C. lanceolata*, which is of great significance for shortening the breeding cycle of *C. lanceolata*.

Acknowledgements

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