



# Indirect somatic embryogenesis and regeneration of *Fraxinus mandshurica* plants via callus tissue

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**Abstract** Somatic embryogenesis of *Fraxinus mandshurica* has the problems of low somatic embryo (SE) yield, unsynchronized SE development, and a high percentage of deformed SEs. We aimed to improve *F. mandshurica* SE production by synchronizing SE development, improving SE quality, and inducing root formation to obtain complete regenerated plants. Cotyledons of immature zygotic embryos of *F. mandshurica* were induced to form callus and then SEs. The SE induction percentage from explants differed among 32 mother trees, and the one with the highest SE induction percentage (29.8%) was used for further experiments. The highest callus induction percentage was 94.2% on ½-strength Murashige and Skoog medium (MS½)

supplemented with 0.15 mg·L<sup>-1</sup> naphthalene acetic acid. The highest callus proliferation coefficient (240.5) was obtained on McCown's Woody Plant Medium containing 0.1 mg·L<sup>-1</sup> 6-benzyl adenine and 0.15 mg·L<sup>-1</sup> 2, 4-dichlorophenoxyacetic acid. The highest number of SEs (1020.5 g<sup>-1</sup> fresh weight) was obtained on MS½ medium supplemented with 1 mg·L<sup>-1</sup> 6-benzyladenine. The highest number of cotyledon embryos (397/g fresh weight) was obtained by incubating materials on medium containing 1 mg·L<sup>-1</sup> abscisic acid and then applying a drying treatment. The cotyledon embryos were milky white, uniformly sized (average length 4.7 mm), and 80% of them were normal. The SE rooting percentage on ½MS medium containing 0.01 mg·L<sup>-1</sup> NAA was 37.5%. Overall, the germination percentage of SEs was 26.4%, and complete regenerated plants were obtained after transplanting and acclimation. These results provide more possibilities for the preservation and breeding of *F. mandshurica*.

Yang Liu and Cheng Wei contributed equally to this work.

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**Keywords** *Fraxinus mandshurica* · Somatic embryogenesis · Callus induction · Cell differentiation · Plant regeneration

## Abbreviations

½MS	Medium with one-third strength of the macroelements of murashige and skoog (1962)
2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
BA	6-Benzyladenine
CH	Casein hydrolysate
IAA	Indoleacetic acid
IBA	Indole-3-butyric acid
MS	Medium of murashige and skoog (1962)
MS½	Medium with one-half-strength of all elements of MS
NAA	Naphthaleneacetic acid

SE(s) Somatic embryo(s)  
WPM Woody plant medium

## Introduction

Somatic embryogenesis is not only a valuable model for embryo cell biology and molecular biology research (Lelu-Walter et al. 2013; Us-Camas et al. 2014), but also an effective system for plant germplasm innovation and large-scale propagation of excellent germplasm resources (Park 2014). The complex process of somatic embryogenesis depends on genotype and is influenced and regulated by many other factors. The difficulty in inducing SE varies among different species (Khan et al. 2010). Somatic embryogenesis can be direct or indirect (Guan et al. 2016). Under special conditions, explants directly induce somatic embryos (SEs) as direct somatic embryogenesis, and explants form SEs as indirect somatic embryogenesis by forming callus. Most species undergo somatic embryogenesis indirectly (Corredoira et al. 2013, 2015). As long as there are appropriate explants, culture conditions, and culture environment, most plant species can be induced to form callus and SEs. An indirect somatic embryogenesis system has been established for *Castanea mollissima*, *Carica papaya*, and *Medicago truncatula*, among others (Lu et al. 2017; Solórzano-Cascante et al. 2018; Orłowska and Kępczyńska 2020). For other plants, such as *Liriodendron hybrida* and *Catalpa fargesii*, direct and indirect somatic embryogenesis systems produce SEs that can grow into complete plants (Chen et al. 2012; Jiang et al. 2014).

Manchurian ash (*Fraxinus mandshurica* Rupr.) is a precious broad-leaved tree species in northeastern China. It is cold tolerant, drought resistant, grows rapidly, has a well-developed root system, and produces excellent wood with a beautiful texture. *F. mandshurica* is mainly propagated via seeds, but the seeds have deep dormancy characteristics (Yang et al. 2017). Therefore, the application of asexual reproduction and biotechnology for *F. mandshurica* has great potential. Asexual reproduction of individuals who have been selected, improved, and genetically manipulated can accelerate the breeding process (Lelu-Walter et al. 2013). Over the last 17 years, studies on the somatic embryogenesis of *F. mandshurica* have identified good explant sources for somatic embryogenesis and the optimum period for explant harvesting (Sun et al. 2010), described the physiological and biochemical changes in somatic embryogenesis (Cong et al. 2012), SE maturation, and germination (Yang et al. 2013), documented the proteomic profile of SEs (Liu et al. 2015). In our research, we have found that SEs of *F. mandshurica* can form directly or indirectly via callus. That is, direct somatic embryogenesis and indirect somatic embryogenesis occur on the same explant (Horstman et al. 2017). However, due

to the low percentage of callus emergence (6.5%) (Zhang et al. 2015), callus culture of *F. mandshurica* has received little attention in the past.

The main problems with direct somatic embryogenesis of *F. mandshurica* are the low SE yield (Yang et al. 2013), unsynchronized SE development (Zhang and Shen 2007; Yang et al. 2013), and genetic instability, all of which restrict large-scale production. In this study, we devised a strategy to increase callus proliferation, improve embryo differentiation, and synchronize embryo development, which ultimately increased the number of *F. mandshurica* SEs and complete regenerated plants. These results lay the foundation for the preservation of excellent germplasm resources of *F. mandshurica*, and for its molecular breeding and large-scale industrial breeding.

## Materials and methods

### Plant materials

Immature (undehydrated, green) seeds of *F. mandshurica* were collected in early August 2017 from fifteen 60-year-old free-pollinated parent trees growing at the University Forest of Northeast Forestry University, Harbin, Heilongjiang Province, China (126°37'55" E, 45°43'16" N), and from seventeen 40-year-old free-pollinated parent trees growing at Hongguang Forest Farm, Jilin Province, China (127°45'81" E, 42°30'41" N).

### Explant preparation

According to the method of Liu et al. (2015), the seeds were soaked for 12 h, disinfected in 75% alcohol for 10 s, and treated with 2% (v/v) sodium hypochlorite solution with continuous agitation for 10 min. The immature embryos were squeezed out with tweezers, then each single cotyledon was cut and placed onto induction medium (10 cotyledons per dish, 5 dishes per treatment). The induction medium (Yang et al. 2013) was ½-strength Murashige and Skoog medium (MS½) supplemented with 5 mg L<sup>-1</sup> naphthalene acetic acid (NAA), 2 mg L<sup>-1</sup> benzyl adenine (BA), 400 mg L<sup>-1</sup> casein hydrolysate (CH), 75 g L<sup>-1</sup> sucrose, and 3 g L<sup>-1</sup> gellan gum (Gelrite, G1910, Sigma-Aldrich Co., St Louis, MO, USA). The pH of the medium was adjusted to 5.8 before high-temperature and high-pressure steam sterilization. The cotyledons were cultured at 25 ± 2 °C in the dark, and subcultured every 30 days. The culture status and number of SEs were recorded at 60 d of culture.

### Callus induction experiment

After 60-day cultivation, yellowish-brown cell mass (Fig. 1a) and SEs at different developmental stages were carefully removed from the explant surface, then the cell masses and SEs were cut into small pieces and inoculated onto callus induction media (0.3 g of material per dish and 20 replicates per treatment). Two types of callus induction media were tested:

Induction medium I: MS½ containing NAA at different concentrations (0, 0.05, 0.1, 0.15, 0.20 mg L<sup>-1</sup>), 400 mg L<sup>-1</sup> CH, 25 g L<sup>-1</sup> sucrose, and 3 g L<sup>-1</sup> gellan gum, pH=5.8.

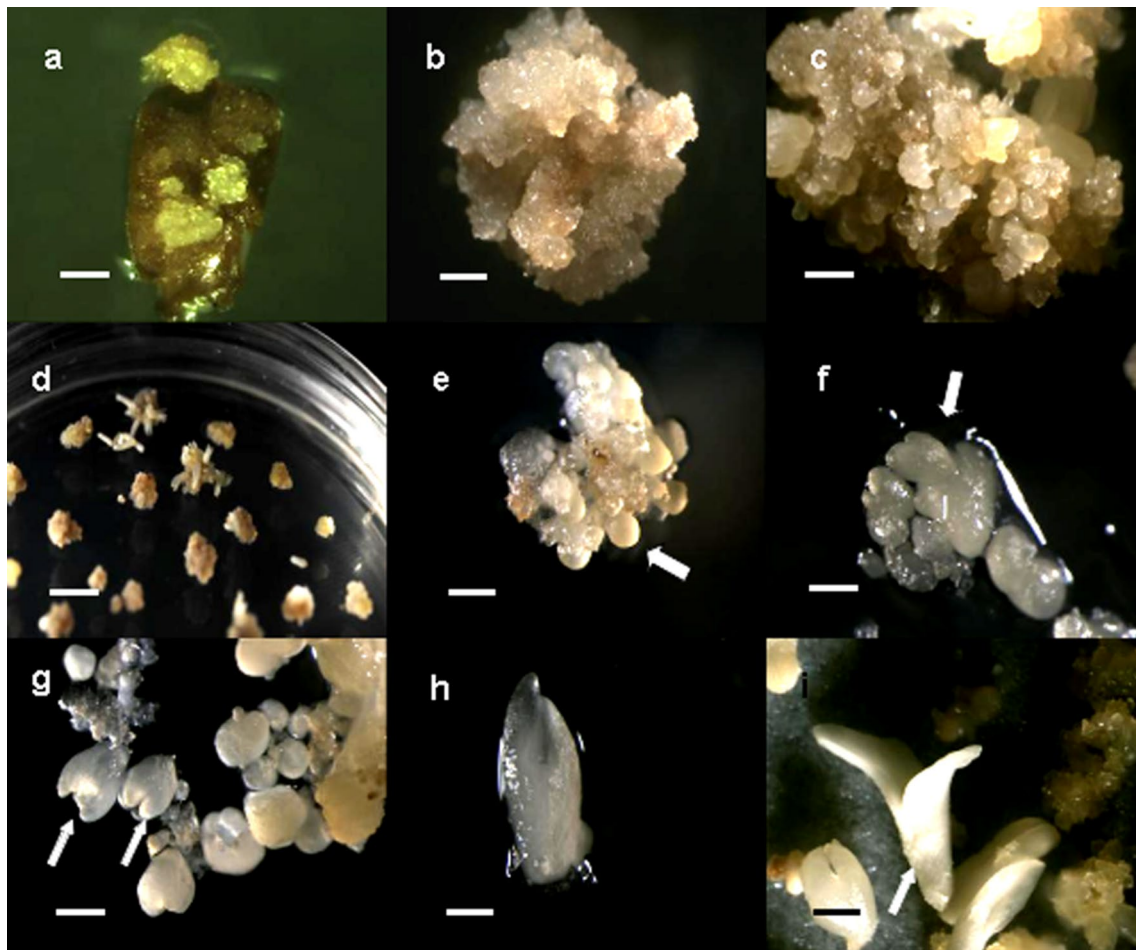
Induction medium II: MS½ containing 0.05 mg L<sup>-1</sup> NAA, different concentrations of BA (0, 1, 2 mg L<sup>-1</sup>), 400 mg L<sup>-1</sup> CH, 25 g L<sup>-1</sup> sucrose, and 3 g L<sup>-1</sup> gellan gum, pH=5.8.

The callus induction rate was counted after culturing in the dark at 25 ± 2 °C for 1 month.

### Callus proliferation experiment

#### Experiment 1: cell line selection

Explants derived from the No. 2 tree at the University Forest of Northeast Forestry University, P. R. China were used in this experiment. Yellowish-brown, translucent, loosely structured callus was removed from different cell lines after 1 month of induction culture. Old and browning cells were removed from the surface of the callus in a clean bench, and then the callus was transferred onto proliferation medium McCown's Woody Plant Medium (WPM) supplemented with 0.15 mg L<sup>-1</sup> 2, 4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg L<sup>-1</sup> BA, 20 g L<sup>-1</sup> sucrose, and 3.5 g L<sup>-1</sup> gellan gum, pH=5.8). The callus proliferation coefficient was calculated after culture in the dark at 25 ± 2 °C for 1 month.



**Fig. 1** Indirect somatic embryogenesis of *Fraxinus mandshurica*. **a.** Callus induction; **b.** Embryogenic callus at proliferation stage; **c.** Early stage of callus differentiation; **d.** Late stage of callus differentiation; **e.** Globular embryo differentiated from callus; **f.** Heart-shaped

embryo differentiated from callus; **g.** Torpedo-shaped embryo differentiated from callus; **h.** Cotyledon embryo differentiated from callus; **i.** Cotyledon-shaped embryos after maturing for 30 days. Scale bars: 1 mm (**a–c, i**); 1 cm (**d**); 1.2 mm (**e–h**)

### Experiment 2: growth regulator selection

Experiment 1 showed that cell line 2–1 had a strong proliferation ability, so this cell line was used in Experiment 2. Cells of cell line 2–1 were inoculated onto WPM media supplemented with different concentrations of BA and 2,4-D as described below. The callus proliferation coefficient was calculated after culture in the dark with  $25 \pm 2$  °C for 1 month.

Callus proliferation medium I: WPM supplemented with different concentrations of BA (0, 0.1 and 0.2 mg L<sup>-1</sup>), 0.15 mg L<sup>-1</sup> 2,4-D, 20 g L<sup>-1</sup> sucrose, and 3.5 g L<sup>-1</sup> gellan gum, pH=5.8.

Callus proliferation medium II: WPM supplemented with different concentrations of 2,4-D (0, 0.15, and 0.3 mg L<sup>-1</sup>), 0.1 mg L<sup>-1</sup> BA, 20 g L<sup>-1</sup> sucrose, and 3.5 g L<sup>-1</sup> gellan gum, pH=5.8.

### Callus differentiation experiment

Using callus from cell line 2-1 after 1 year of proliferation as the experimental material, we collected embryogenic callus (beige, translucent, granular loose callus) in the clean bench and transferred it onto differentiation media (MS½ medium supplemented with different concentrations of NAA and BA). The cells were cultured in the dark at  $25 \pm 2$  °C and subcultured every 30 days.

#### Differentiation medium I

MS½ medium containing different concentrations of NAA (0, 1, and 2 mg·L<sup>-1</sup>), 1 mg·L<sup>-1</sup> BA, 400 mg·L<sup>-1</sup> CH, 20 g·L<sup>-1</sup> sucrose, and 3.5 g·L<sup>-1</sup> gellan gum, pH=5.8.

#### Differentiation medium II

MS½ medium containing different concentrations of BA (0, 1, and 2 mg·L<sup>-1</sup>), 1 mg·L<sup>-1</sup> NAA, 400 mg·L<sup>-1</sup> CH, 20 g·L<sup>-1</sup> sucrose, and 3.5 g·L<sup>-1</sup> gellan gum, pH=5.8.

### Somatic embryo maturation experiment

#### Drying treatment

Using the method of (Lelu-Walter et al. 2018), 3 g of SEs from cell line 2–1 was added to liquid culture medium (MS½ + 20 g·L<sup>-1</sup> sucrose) in the clean bench. The mixture was shaken, then 0.3 g of the mixture was poured onto sterilized filter paper in a Buchner funnel. Excess liquid was removed by gentle vacuum, and then the filter paper was spread onto the surface of the maturation culture medium.

### Maturation medium

MS½ medium containing different concentrations of ABA (0, 1, 1.5, and 2 mg·L<sup>-1</sup>), 400 mg·L<sup>-1</sup> CH, 20 g·L<sup>-1</sup> sucrose, 1 g·L<sup>-1</sup> activated carbon, and 3.5 g·L<sup>-1</sup> gellan gum, pH=5.8. The same materials cultured on ABA-free medium without drying were used as the control (CK), and the other conditions were the same as above.

After culture in the dark at  $25 \pm 2$  °C for 30 days, the materials were cultured for 2 weeks in light conditions (40 μmol m<sup>-2</sup> s<sup>-1</sup>; 16-h light/8-h dark photoperiod), and then transferred to MS½ medium for a further 30-day culture in the dark (Chen et al. 2019).

### Somatic embryo germination and rooting experiment

The white, elongated cotyledon-shaped embryos obtained from the maturation culture were used as materials for root culture. The basic medium was 1/3-strength MS, and four different germination media were produced by adding different concentrations of NAA, indole butyric acid (IBA), and IAA, as follows:

Germination medium I: 1/3MS + 0.01 mg·L<sup>-1</sup> NAA (Yang et al. 2013);

Germination medium II: 1/3MS + 0.01 mg·L<sup>-1</sup> NAA + 2 g·L<sup>-1</sup> activated carbon;

Germination medium III: 1/3MS + 1.0 mg·L<sup>-1</sup> IBA + 1.0 mg·L<sup>-1</sup> IAA (Du and Pijut 2008);

Germination medium IV: 1/3MS + 0.5 mg·L<sup>-1</sup> IBA + 0.5 mg·L<sup>-1</sup> IAA.

All germination media contained 20 g·L<sup>-1</sup> sucrose and 3.5 g·L<sup>-1</sup> gellan gum, pH=5.8.

The embryos were cultured at  $25 \pm 2$  °C under a 16-h light/8-h dark photoperiod with a light intensity of 40 μmol m<sup>-2</sup> s<sup>-1</sup>. The germination and rooting of SE seedlings were observed and recorded.

### Plant regeneration and acclimatization

Rooted, well-developed SEs were transplanted into a plastic container filled with substrate (peat soil: vermiculite: perlite (v:v:v)=5:3:2). The substrate was mixed with MS liquid medium, autoclaved, and then allowed to cool. The culture medium was washed from the roots of the SEs before transplanting. Immediately after transplanting, the SEs were covered with plastic wrap and cultivated in a culture room at  $25 \pm 2$  °C under natural light, with daily irrigation to maintain high air humidity. After 15-day culture, the plastic wrap was gradually removed and materials were transferred to  $25 \pm 2$  °C under light at 40 μmol m<sup>-2</sup> s<sup>-1</sup>. The plantlets were watered daily during transplanting and acclimatization.

## Statistical analysis

Data were collated with Microsoft Excel 2007 (USA). We used SPSS software (2015, v.23, SPSS Inc., Chicago, IL, USA) for one-way analysis of variance of the SE induction rate, callus state coefficient, callus induction percentage, fresh weight multiplication factor, proliferation coefficient, SE induction percentage, number of SEs, SE rooting percentage, and SE sprouting percentage. Sigmaplot (2011, v.12.5, SYSTAT, USA) software was used to draw graphs. We used the following calculations to obtain various rates and indexes:

$$\text{SEinduction percentage(\%)} = \frac{\text{Number of explants with somatic embryogenesis}}{\text{Number of surviving explants inoculated}} \times 100 \quad (1)$$

$$\text{Callus state coefficient(\%)} = \frac{\text{Number of callus in good condition}}{\text{Number of callus inoculated}} \times 100 \quad (2)$$

$$\text{Callus induction percentage(\%)} = \frac{\text{Number of explants producing callus}}{\text{Number of surviving explants inoculated}} \times 100 \quad (3)$$

$$\text{Fresh weight multiplication factor(\%)} = \frac{\text{Weight of callus after proliferation}}{\text{Weight of callus during inoculation}} \times 100 \quad (4)$$

$$\text{Number of SEs(a/g)} = \frac{\text{Number of embryos induced}}{\text{Weight of callus}} \quad (5)$$

$$\text{Rooting percentage (\%)} = \frac{\text{Number of SEs rooting}}{\text{Number of SEs inoculated}} \times 100 \quad (6)$$

$$\text{Germination percentage (\%)} = \frac{\text{Number of SEs with new shoots}}{\text{Number of SEs inoculated}} \times 100 \quad (7)$$

## Results

### Explant preculture results

After 60-day pre-cultivation, we calculated the frequency of SEs formed from explants from 32 different *F. mandshurica* mother trees from two forest farms (Table 1). The frequency of SEs differed significantly among the mother trees ( $P < 0.05$ ). SEs formed from explants from 7 out of 15 mother trees growing at the University Forest, Northeast Forestry University, China. The highest SE induction rate (29.8%) was from mother tree No.2, and this rate was significantly higher than those for the other mother trees

( $P < 0.05$ ). SEs formed explants from 7 out of 17 mother trees growing at Hongguang Forest Farm, and the highest SE induction percentage (16%) was from mother tree No.21. The SE induction percentage was higher for trees growing at University Forest than for trees growing at Hongguang Forest Farm, but the difference was not significant ( $P > 0.05$ ). Thus, the SE induction percentage from immature zygotic embryos of *F. mandshurica* was not related to region, but was related to the genotype of the mother tree. We selected materials from mother tree No. 2 for subsequent experiments (Table 1).

### Callus induction

When the concentration of NAA remained constant and the BA concentration increased, the callus induction percentage decreased (Fig. 2a). The highest callus induction percentage (9.9%) was on medium containing only 0.05 mg L<sup>-1</sup>

NAA, and the lowest (1.5%) was on medium containing 0.05 mg L<sup>-1</sup> NAA and 2 mg L<sup>-1</sup> BA. Thus, BA inhibited callus induction.

On medium containing only NAA, in the concentration range of 0 to 0.15 mg L<sup>-1</sup>, the callus induction percentage increased with the increase of NAA concentration ( $P < 0.05$ ) (Fig. 2b). The highest callus induction percentage (94.2%) was on medium containing 0.15 mg L<sup>-1</sup> NAA and the lowest (76.7%) was on medium 0.2 mg L<sup>-1</sup> NAA ( $P < 0.05$ ). Thus, an appropriate concentration of NAA was beneficial for *F. mandshurica* callus induction.

**Table 1** Somatic embryo induction from different mother trees of *Fraxinus mandshurica*

Mother trees of Northeast Forestry University	Somatic embryo induction (%)	Mother trees of Hongguang forest farm	Somatic embryo induction (%)
2	29.8±5.8a	L1	0a
4	2.0±2.0c	L2	0a
10	0c	L3	0a
11	0c	L4	0a
12	0c	L5	10.0±5.5bc
13	0c	L6	0a
14	2.5±2.5c	L7	0a
15	6.0±4.0c	L9	0a
16	0c	L10	2.0±2.0ab
17	0c	L12	0a
18	6.0±2.5c	L13	0a
19	0c	L14	8.0±4.9ab
20	15.0±6.5b	L15	10.0±3.2bc
21	4.0±4.0c	L16	2.0±2.0ab
22	0c	L17	0a
		L18	3.3±3.3ab
		L21	16.0±6.8c

Note: Data are mean ± standard deviation. Different lowercase letters in the same column numbers indicate significant differences ( $P=0.05$ )

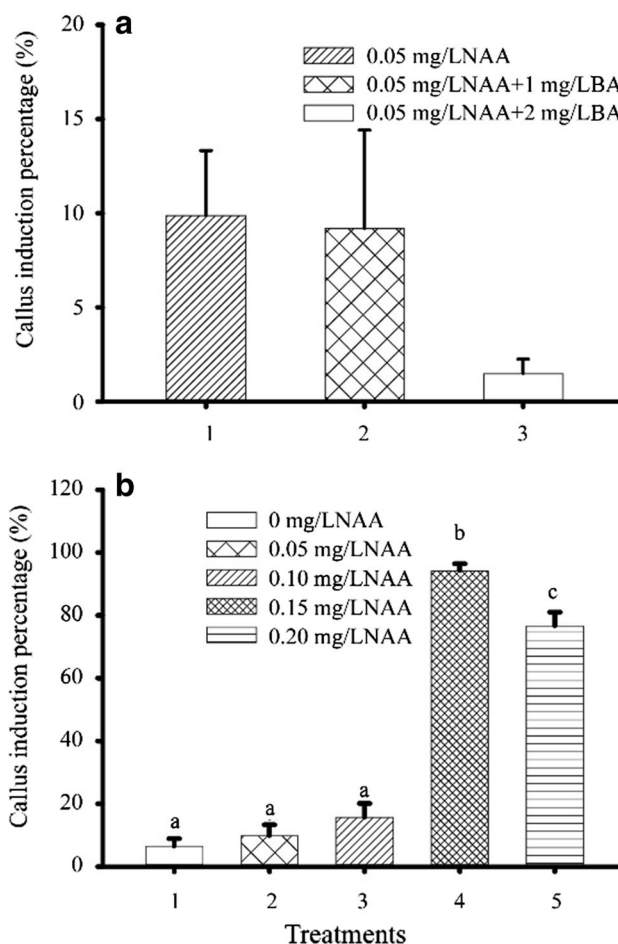
## Callus proliferation

### Cell line selection

A comparative analysis of the state coefficients of callus from 40 cell lines is shown in Table 2. The coefficient of callus state differed significantly among different cell lines. The highest callus state coefficient (100%) was for cell line 2-1 (the No. 1 genotype of the No. 2 tree from University Forest, Northeast Forestry University, China). Therefore, cell line 2-1 was used for subsequent experiments.

### Plant growth regulator selection

Different plant growth regulators significantly affected callus proliferation of *F. mandshurica* (Table 3). (1) The highest fresh weight proliferation coefficient of callus (240.5%) was on medium containing 0.1 mg L<sup>-1</sup> BA and 0.15 mg L<sup>-1</sup> 2,4-D. On that medium, the callus was yellowish-brown and loose. In addition, granular embryogenic callus formed, from which SEs differentiated later (Fig. 1b). (2) The fresh weight proliferation coefficient of callus was also high (228.7%) on medium without BA, but the callus was soft, excessively wet, and non-granular (no SEs differentiated later). (3) On medium without 2,4-D, callus showed poor proliferation,



**Fig. 2** Effects of NAA and BA on embryogenic callus induction of *Fraxinus mandshurica*. **a** Callus induction percentage on medium containing 0.05 mg·L<sup>-1</sup> NAA and increasing concentrations of BA. **b** Callus induction percentage on medium containing increasing concentrations of NAA only

severe browning, a hard texture, and a block shape. On that medium, the fresh weight proliferation coefficient of callus was 111.3% after 30-day culture, and the callus showed little growth.

The callus differentiation process is shown in Fig. 1c–h. Callus differentiation of *F. mandshurica* was positively affected by BA, but not by NAA (Table 4). As the BA concentration in the medium increased, the percentage of callus differentiation into SEs first increased and then decreased. At 30 days of culture, the highest induction percentage of SEs (118.8 g<sup>-1</sup>; 5.9 SEs per callus) was on medium containing 1 mg·L<sup>-1</sup> BA, and after 90 days of culture, these values had increased to 1025.5·g<sup>-1</sup> and 51 SEs per callus. These values were significantly higher than those in the other treatments ( $P < 0.05$ ). The lowest induction percentage of SEs (17.7 g<sup>-1</sup>) at 30 days of culture was on medium without BA. The lowest number of SEs per callus (0.9) was on medium containing 1 mg·L<sup>-1</sup> NAA.

The frequency of globular embryos was the highest (73%) in the early stage of differentiation culture (Table 5). As the culture time extended to 90 days, the frequency of heart-shaped and cotyledon-shaped embryos increased. When only 1 mg L<sup>-1</sup> NAA was added to the medium, the synchronization of SE development was best, and the frequency of cotyledon-shaped embryos was the highest. The frequencies of torpedo-shaped and cotyledon-shaped embryos decreased, and cotyledon-shaped embryos were significantly lower on medium containing 2 mg L<sup>-1</sup> NAA and 1 mg L<sup>-1</sup> BA ( $P < 0.05$ ) than on the other types of media.

#### Somatic embryo maturation

A white-opaque appearance was the criterion for maturation of *F. mandshurica* SEs. The effect of ABA on SE maturation is shown in Figs. 3a–e and Table 6. Undried cotyledon-shaped embryos cultured on maturation medium without ABA (CK) for 30 days (Fig. 3a) formed abundant cotyledon embryos (320.7 g<sup>-1</sup>), but the cotyledons were translucent, curled, and stunted, with a high rate of malformation and browning. At 30 days after the drying treatment, the number of cotyledon embryos (Fig. 3c) on medium containing 1 mg L<sup>-1</sup> ABA was 397 g<sup>-1</sup>, and the cotyledons developed well, with the cotyledons accounting for 51% of the total embryo length (average length, 4.7 mm). The cotyledons were healthy, milky white, stretched, and elongated, with a uniform size and a low rate of malformation. The lowest number of cotyledon embryos (189.6 g<sup>-1</sup>) was medium containing 2 mg L<sup>-1</sup> ABA (Fig. 3e). Their average length was 3.31 mm, and the cotyledon embryos were translucent and stunted with a high rate of malformation.

Transfer the cotyledon embryos to PGR-free medium in the dark culture for 30 days. During this time, the materials that had been cultured on medium containing higher concentrations of ABA showed significantly inhibited SE maturation (Figs. 3f–j, Table 7). During the 30 days of culture in the dark, the number of cotyledon embryos (Fig. 3h) treated with drying and 1 mg·L<sup>-1</sup> ABA increased to 624 g<sup>-1</sup> (1.57 times higher than before); the average length was 9.60 mm (significantly higher than in other treatments,  $P < 0.05$ ); and the proportion of cotyledon length out of total embryo length decreased by 11% (significantly lower than in other treatments,  $P < 0.05$ ). The embryos in this treatment showed the lowest browning percentage (0.2%); the highest rooting percentage (37.99%,  $P < 0.05$ ); and the lowest percentage of malformation (10%). However, for the materials that had been cultured on medium containing a higher concentration of ABA (2 mg·L<sup>-1</sup>) (Fig. 3j), the number of cotyledon embryos was only 176.7 g<sup>-1</sup> (about 1/4 of that formed in the 1 mg·L<sup>-1</sup> ABA treatment); and the proportion of cotyledon length to total embryo length was increased (74%). The embryos in this treatment showed the lowest rooting

**Table 2** Callus state coefficients of different cell lines of *Fraxinus mandshurica*

Cell line	Callus in good condition	Total callus	Callus state coefficient (%)
1	25.0	25.0	100.0
2	7.0	16.0	43.8
3	6.0	8.0	75.0
4	3.0	15.0	20.0
5	0	3.0	0
6	18.0	20.0	90.0
7	6.0	11.0	54.5
8	0	7.0	0
9	3.0	10.0	30.0
10	1.0	17.0	5.9
11	10.0	16.0	62.5
12	1.0	9.0	11.1
13	0	7.0	0
14	0	5.0	0
15	0	6.0	0
16	5.0	20.0	25.0
17	0	10.0	0
18	0	7.0	0
19	13.0	18.0	72.2
20	11.0	15.0	73.3
21	0	10.0	0
22	1.0	11.0	9.1
23	0	8.0	0
24	0	8.0	0
25	0	8.0	0
26	0	7.0	0
27	0	9.0	0
28	0	4.0	0
29	1.0	8.0	12.5
30	0	9.0	0
31	5	15.0	33.3
32	8.0	14.0	57.1
33	9.0	16.0	56.3
34	13.0	16.0	81.3
35	10.0	16.0	62.5
36	6.0	11.0	54.5
37	0	8.0	0
38	11.0	15.0	73.3
39	6.0	13.0	46.2
40	8.0	14.0	57.1

percentage (8.2%) and the highest percentage of malformation (85%). Undried cotyledon embryos cultured without ABA (CK) (Fig. 3f) formed a large number of cotyledon embryos (961.7 g<sup>-1</sup>), but the browning percentage (24.9%) was significantly higher than that in other treatments, and the

embryos were curled and stunted, which was not conducive to later SE development.

#### Somatic embryo germination and rooting

Next, the SEs were transferred to fresh media for germination and rooting (Fig. 4a–b). The culture conditions significantly affected the germination of SEs ( $P < 0.05$ , Table 8). Low concentrations of auxin were beneficial for the rooting of SEs (Table 8). The highest rooting and germination percentage of SEs (37.5% and 26.4%, respectively) were on GM I medium (1/3 MS + 0.01 mg·L<sup>-1</sup> NAA). However, the rooting and germination percentage of SEs were inhibited on GM II medium (GM I with the addition of activated carbon). The lowest rooting and germination percentages of SEs (0% and 5.6%, respectively) were on GM III medium, which had a high concentration of auxins (1.0 mg L<sup>-1</sup> IBA + 1.0 mg L<sup>-1</sup> IAA). On GM III medium, the SEs did not take root and barely grew, but the hypocotyl elongated. On GM IV medium, which had half the concentrations of IBA and IAA in GM III, the rooting and germination rates of SEs were significantly increased.

#### Plant regeneration and acclimatization

Before transplanting, the SE seedlings were acclimated for 3 days in a domestication room. At 15 days after transplanting, the survival percentage was 100%. Seedlings showed strong growth with extended leaves, new pinnate compound leaves, and an average seedling height of 3.75 cm. At 30 days after transplanting, the average seedling height was 6.29 cm. At 60 days after transplanting, the survival percentage was 90.9% and the average seedling height was 9.26 cm (Fig. 4c).

## Discussion

### Induction of embryogenic callus

In this study, an appropriate concentration of auxin positively affected callus induction from *F. mandshurica*. In the range of 0.1 to 0.15 mg L<sup>-1</sup> NAA, the callus induction percentage increased significantly with increasing NAA concentrations (Fig. 2b). In *Fraxinus excelsior*, the embryogenic callus induction needed the combination of 2,4-D and 6-BA (Ozudogru et al. 2010). Previous studies have demonstrated that the induction of plant callus by plant growth regulators

**Table 3** Fresh weight multiplication coefficient of *Fraxinus mandshurica* callus (%)

PGR (mg L <sup>-1</sup> )		Culture time (d)				Callus status
BA	2,4-D	3	9	15	30	
0	0.15	173.1 ± 44.3ab	491.8 ± 131.6a	191.8 ± 21.5a	228.7 ± 20.1a	Soft, non-granular
0.1	0.15	261.6 ± 49.9a	287.0 ± 45.4ab	181.1 ± 20.2a	240.5 ± 32.5a	loose, granular
0.2	0.15	161.4 ± 29.5ab	227.8 ± 56.7b	126.4 ± 4.0b	178.2 ± 12.7ab	Slightly hard, granular
0.1	0	106.2 ± 25.5b	147.1 ± 29.6b	101.9 ± 7.2b	111.3 ± 14.5b	Severe browning, lumpy
0.1	0.15	261.6 ± 49.9a	287.0 ± 45.4ab	181.1 ± 20.2a	240.5 ± 32.5a	loose, granular
0.1	0.3	128.2 ± 8.3b	148.4 ± 8.2b	137.3 ± 21.7ab	175.3 ± 40.4ab	hard, granular

Note: Data are mean ± standard deviation. Different lowercase letters in the same column numbers indicate significant differences ( $P = 0.05$ )

**Table 4** Callus differentiation in *Fraxinus mandshurica*

PGR (mg L <sup>-1</sup> )		Cultured 30 d		Cultured 90 d	
NAA	BA	Number of somatic embryos·(g <sup>-1</sup> )	Number of somatic embryos per callus	Number of somatic embryos (g <sup>-1</sup> )	Number of somatic embryos per callus
0	1	118.8 ± 26.5a	5.9 ± 1.3a	1020.5 ± 231.4a	51.0 ± 11.6a
1	1	37.2 ± 21.1b	1.9 ± 1.1b	829.4 ± 99.7ab	41.5 ± 5.0ab
2	1	32.3 ± 10.6b	1.6 ± 0.5b	366.6 ± 80.0b	18.3 ± 4.0b
1	0	17.7 ± 7.4b	0.9 ± 0.4b	616.7 ± 123.7ab	30.8 ± 6.2ab
1	1	37.2 ± 21.1b	1.9 ± 1.1b	829.4 ± 99.7ab	41.5 ± 5.0ab
1	2	22.3 ± 7.6b	1.1 ± 0.4b	758.7 ± 158.3ab	37.9 ± 7.9ab

Note: Data are mean ± standard deviation. Different lowercase letters in the same column indicate significant differences ( $P = 0.05$ )



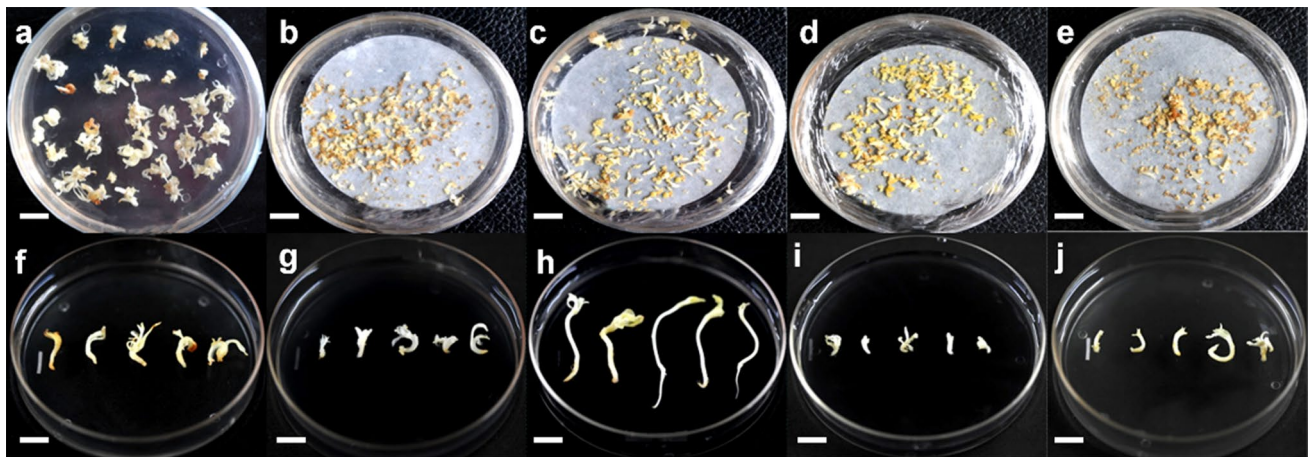
**Table 5** Influence of plant growth regulators on somatic embryo development of *Fraxinus mandshurica*

PGR (mg L <sup>-1</sup> )		Culture for 30 d		Culture for 90 d					
NAA	BA	Globular embryo (%)	Heart shaped embryo (%)	Torpedo embryo (%)	Cotyledon embryo (%)	Globular embryo (%)	Heart shaped embryo (%)	Torpedo embryo (%)	Cotyledon embryo (%)
0	1	73.2±3.1	8.4±1.7a	10.4±2.0	8.0±2.8ab	61.9±4.2	11.7±1.6ab	12.7±1.8	13.7±2.2a
1	1	76.4±6.7	3.9±1.5b	15.5±5.5	4.2±3.0ab	54.6±7.8	14.5±2.2a	13.6±3.7	17.2±3.8ab
2	1	65.6±8.3	2.4±1.2b	16.1±4.5	15.9±6.6a	66.4±5.6	10.1±1.9ab	14.0±2.3	9.5±3.0a
1	0	65.4±10.9	0.2±0.2b	26.3±10.6	8.1±6.6ab	47.3±4.3	13.6±1.2ab	13.0±1.0	26.1±4.9b
1	1	76.4±6.7	3.9±1.5b	15.5±5.5	4.2±3.0ab	54.6±7.8	14.5±2.2a	13.6±3.7	17.2±3.8ab
1	2	69.7±8.7	3.6±1.5b	25.0±8.8	1.8±1.0b	63.1±9.6	8.9±1.4b	12.8±4.4	15.2±4.6ab

Note: Data are mean ± standard deviation. Different lowercase letters in the same column numbers indicate significant differences ( $P=0.05$ )

is affected by many factors such as plant species, culture conditions, explants age, and the location of explants (Shin et al. 2019). The positive effect of auxin on callus induction may be because the endogenous auxin levels were low in the

*F. mandshurica* explants. By affecting a variety of auxin-related enzymes, exogenous auxin can regulate the content of endogenous auxin (Machakova et al. 2008). A study on the *Arabidopsis* transcriptome showed that the leaf-to-callus



**Fig. 3** Somatic embryo maturation process of *Fraxinus mandshurica*. **a**: SEs after 30 days of culture on CK; **b–e**: SEs after 30 days of culture on media containing different concentrations of ABA (0, 1, 1.5, and 2.0 mg·L<sup>-1</sup>); **f**: Cotyledon-shaped embryos after 30 days of

culture on CK and 30 days of culture on basic medium; **g–j**: Cotyledon-shaped embryos after 30 days of culture on media containing different concentrations of ABA (0, 1, 1.5, and 2.0 mg·L<sup>-1</sup>) and 30 days of culture on basic medium. Scale bars: 1.1 cm (**a**); 1.0 cm (**b–j**)

**Table 6** Properties of somatic embryos after drying treatment and culture on media containing ABA for 30 days

PGR (mg L <sup>-1</sup> ) ABA	Cotyledon Embryos (g <sup>-1</sup> )	Ratio of cotyledon to embryo length (%)	Average length (mm)	Malformed embryo percentage (%)	Developmental morphology
0	248.2±16.0bc	32.2±1.5a	3.7±0.2a	70.0	Translucent; unstretched; different sizes
1.0	397.0±27.7a	51.0±1.8b	4.7±0.2b	20.0	Milky white; cotyledons stretch out; same size
1.5	225.7±16.3bc	35.0±2.2a	3.4±0.2a	60.0	Translucent; unstretched;
2.0	189.6±32.7b	46.3±2.6b	3.3±0.2a	80.0	Translucent; unstretched;
CK	320.7±72.1ac	52.0±7.4b	3.2±0.3a	80.0	Translucent; curly; individual Browning

Note: CK, materials cultured on ABA-free medium without drying. Malformed embryos are multi-cotyledonary embryos and incompletely differentiated SEs. Data are mean ± standard deviation. Different lowercase letters in the same column indicate significant differences ( $P=0.05$ )

process involved the stage of auxin response gene upregulation (He et al. 2012). Wójcikowska et al. (2013) found that an auxin treatment promoted somatic embryogenesis by activating transcription factors, including LEAFY COTYLEDON2 (LEC2), which controls IAA synthesis in explants. In that study, an auxin treatment led increased LEC2 activity, and subsequently activates the YUCCA (YUC) genes, increasing the content of endogenous auxin Perez-Perez et al. (2019). found that the induction of auxin synthesis genes and the accumulation of auxin in cells are related to the requirements of auxin in the initiation and development of somatic embryogenesis.

### Proliferation and browning of embryogenic callus

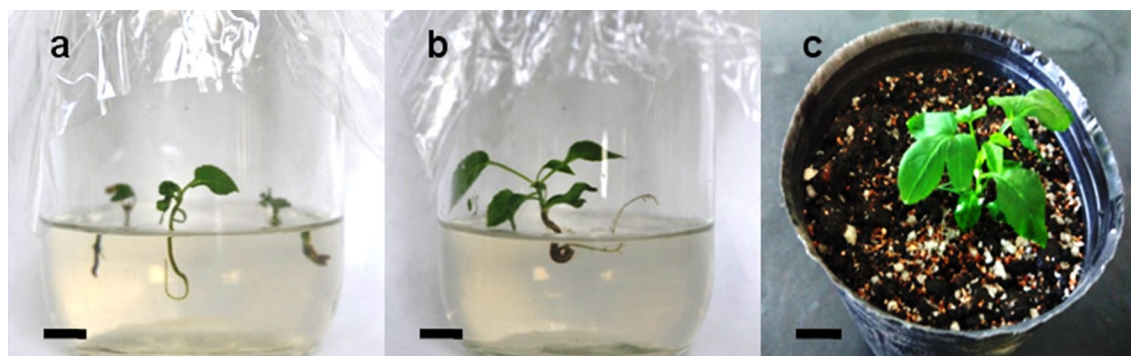
In the process of callus proliferation, we often choose some plant growth regulators, such as the auxin 2,4-D can not only induce direct somatic embryogenesis, but also is necessary for the process of callus proliferation in indirect somatic embryogenesis (Pasternak et al. 2002). In addition to stimulating auxin responses, 2,4-D can also increase the endogenous IAA level (Li et al. 2011). However, 2,4-D should not be used during the subsequent development and maturation

of SEs (Zavattieri et al. 2010). The removal of exogenous 2,4-D was found to trigger IAA polar transport and the formation of an auxin gradient in embryonic callus (Su et al. 2009). In the present study, we found that serious callus browning occurred on medium containing 2,4-D, and this became more serious with longer times between subculturing. The degree of browning could be reduced by shortening the subculture period, or by adding anti-browning agents such as ascorbic acid, citric acid, and polyvinylpyrrolidone (data not shown). In olive (*Olea europaea*) callus, as the length of time between subculture extended, the embryo quality decreased (Bradai et al. 2016). Similarly, the callus quality began to decrease after 9-year subculture, but some cell lines remained embryogenic after 20 years of subculture in oil palm (*Elaeis guineensis* Jacq.) (Konan et al. 2010). In that case, the excessive nitrogen demand for polyamine synthesis was one of the most likely causes of the decline in callus quality with extended culture time (Konan et al. 2010). Culture conditions and genotypes are two important factors that affect the embryogenic maintenance of callus (Bradai et al. 2016). In this study, the embryogenic ability of *F. mandshurica* embryogenic callus did not change significantly after 2-year subculturing. However, further research

**Table 7** Properties of embryos after drying treatment, culture on ABA-containing medium, and then culture on PGR-free medium for 30 days

ABA (mg·L <sup>-1</sup> )	Cotyledon embryo number (g <sup>-1</sup> )	Ratio of cotyledon to embryo length (%)	Average length (mm)	Browning percentage (%)	Rooting percentage (%)	Malformed embryo percentage (%)
0	558.0 ± 78.0a	46.0 ± 2.5ab	6.0 ± 0.3bc	3.0 ± 0.9a	18.5 ± 3.8a	30
1.0	624.0 ± 113.8a	40.0 ± 4.5a	9.6 ± 0.8a	0.2 ± 0.2a	38.0 ± 5.1b	10
1.5	269.3 ± 31.5b	52.0 ± 3.7b	5.6 ± 0.4b	0.9 ± 0.5a	16.6 ± 5.3a	60
2.0	176.7 ± 8.5b	74.0 ± 2.5c	7.2 ± 0.9bc	3.1 ± 0.9a	8.2 ± 2.8a	85
CK	961.7 ± 123.7c	66.7 ± 4.2c	7.8 ± 0.8ac	24.9 ± 3.8b	12.8 ± 4.8a	30

Note: CK, material was cultured on ABA-free medium without drying. Malformed embryos are multi-cotyledonary embryos. Data are mean ± standard deviation. Different lowercase letters in the same column numbers indicate significant differences ( $P=0.05$ )



**Fig. 4** Germination, acclimatization, and transplanting of *Fraxinus mandshurica* emblings. **a.** Plantlet after rooting for 30 days; **b.** SEs acclimated before transplanting; **c.** SE seedlings after 60-day transplantation. Scale bars: 1 cm (**a**, **b**); 5 cm (**c**)

**Table 8** Germination and rooting of somatic embryos of *Fraxinus mandshurica*

Culture medium	Rooting percentage (%)	Germination percentage (%)
GM I: 1/3MS + 0.01 mg·L <sup>-1</sup> NAA	37.5 ± 7.2a	26.4 ± 6.1a
GM II: 1/3MS + 0.01 mg·L <sup>-1</sup> NAA + 2 g·L <sup>-1</sup> activated carbon	25.0 ± 4.8a	13.9 ± 7.4ab
GM III: 1/3MS + 1.0 mg·L <sup>-1</sup> IBA + 1.0 mg·L <sup>-1</sup> IAA	0b	5.6 ± 5.6b
GM IV: 1/3MS + 0.5 mg·L <sup>-1</sup> IBA + 0.5 mg·L <sup>-1</sup> IAA	27.8 ± 11.1a	16.7 ± 0ab

is required to determine whether the embryogenic ability of *F. mandshurica* embryogenic callus changes during long-term preservation, and the key factors affecting any changes.

### Differentiation of embryogenic callus

In plant cell division and differentiation, auxin and cytokinin are key regulators, and the balance between them plays an important role in SE development (Binte and Wagiran 2018; Ming et al. 2019). Although the existence of 2,4-D induced the embryogenic potential of callus, cytokinin was required for the development of SEs during subculturing (Wang et al. 2014). Our results showed that, at the early stage of callus differentiation, *F. mandshurica* callus differentiation was promoted by BA but inhibited by NAA. In the growth and development of *Arabidopsis*, the polar transport of endogenous auxin is the key factor (Liu et al. 2017). The gradient of endogenous auxin and polar auxin transport influenced by PINFORMED1 (PIN1) were identified as the key factors for WUSCHEL (WUS)-induced somatic embryogenesis (Fehér 2019). In *Arabidopsis*, a short-term low concentration of cytokinin can promote somatic embryogenesis, but if the concentration of cytokinin is too high, it can inhibit polar auxin transport, thus inhibiting somatic embryogenesis (Bernula et al. 2020). Similarly, in our study, when the concentration of BA in the medium was increased, callus differentiation of *F. mandshurica* was inhibited. Cytokinin inhibits the expression of LEC2 and FUSCA 3 (FUS3), the key transcription factors in somatic embryogenesis (Horstman et al. 2017; Bernula et al. 2020).

### Maturation of somatic embryo

SE maturation is the key step of plant transformation during somatic embryogenesis. In the process of SE maturation, the ecology and morphology have changed. With the production and accumulation of storage materials, SE changes from transparent to milky white (The standard of different species maturity; Márquez-Martín et al. 2011). In banana (*Musa* spp.), drying at 25 ± 1 °C for 2 h was shown to significantly improve the SE induction rate and maturation rate (Natarajan et al. 2020). Similarly, a partial drying treatment was shown to significantly improve the regeneration ability of a Malaysian rice cultivar (*Oryza sativa*; Ming et al. 2019). In

our study, the immature embryos placed onto MS½ medium supplemented with 1 mg L<sup>-1</sup> ABA after drying showed the best maturation rate, and the cotyledons were elongated and milky white. In the future, the differentiation of embryogenic callus may be further improved by optimizing the period of culture on ABA-containing medium, as well as the osmotic pressure of the medium and the photoperiod.

### Somatic embryo rooting and plant regeneration

The formation of roots from embryos is an important step in a somatic embryogenesis system, as it determines whether the production of SE seedlings can be industrialized and commercialized. In general, reduced-strength WPM or MS media (1/2 or 1/3 strength) can increase the rooting capacity of most plants (Du and Pijut 2008). Plant growth regulators affect the germination of SEs. For example, the regeneration of plants of a Malaysian rice cultivar were weak without NAA (Binte and Wagiran 2018). The type of sugar and the concentration of auxin were found to significantly affect the number of roots formed from SEs of date palm (*Phoenix dactylifera*; Ibrahim et al. 2009). In this study, the highest SE germination rate and rooting percentage (26.4% and 37.5%, respectively) were on medium containing 0.01 mg·L<sup>-1</sup> NAA. Most SE seedlings grew normally, and the rooting rate achieved in this study was higher than that achieved previously (Yang et al. 2013, rooting percentage 27.1%). Further research is needed to improve the quality of SEs, the rate of SE formation, and the quality of SE seedlings.

### Conclusion

Immature cotyledons of *F. mandshurica* were used as explants to establish an indirect somatic embryogenesis system through callus. The callus proliferated and differentiated into SEs. Compared with previous methods, the methods used in our study resulted in increased yield of SEs and better synchronization of SE development. Using these methods, complete regenerated plants were obtained. These results lay the foundation for the preservation of germplasm resources, and for the molecular and large-scale breeding of *F. mandshurica*.

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