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Optimization of Somatic Embryo Regeneration of *Larix olgensis* Through Multiple Media

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Abstract To establish a stable and efficient *Larix olgensis* somatic embryo regeneration system. In this study, immature zygotic embryos of *Larix olgensis* were used as explant materials for embryogenic callus induction, and embryogenic callus with good growth were selected to induce somatic embryo formation by liquid medium A, semi-solid medium B, and solid medium C. The callus induction medium is S medium+2,4-D 1.0 mg/L + 6-BA 0.5 mg/L + KT 0.3 mg/L, sucrose 20 g/L, agar 3 g/L; the subculture medium is S medium+2, 4-D 0.15 mg/L + 6-BA 0.05 mg/L + KT 0.05 mg/L, sucrose 30 g/L, agar 6 g/L. Mature medium A is S medium + PEG4000 100 g/L + ABA 15 mg/L + $AgNO_3$ 10 mg/L, sucrose 45 g/L liquid medium. Mature medium B and C are mature medium A supplemented with 2 g/L and 10 g/L agar, respectively. The maturation time of this method is about 32 days, the synchronization rate is 78.0%, and the proportion of somatic embryos in normal morphology is 83.0%. Somatic embryo formation efficiency in normal morphology can reach 223.0 embryos/g embryogenic callus, somatic embryo germination rate can reach 47.2%. Compared with a single maturation medium, the maturation time is shortened, somatic embryo generation is more synchronized, and high somatic embryo yield and quality are improved. It provides a way for the commercialization of somatic embryo regeneration of *Larix olgensis*, brings great convenience to large-scale breeding and reproduction, physiological and biochemical research, and genetic modification, and has great practical application value.

Keywords *Larix olgensis*; Embryogenic callus; Somatic embryogenesis

There are about 18 species of *Larix* genus. *Larix olgensis* is an important coniferous fast-growing afforestation tree species in the warm temperate zone of northeast and northern China with the characteristics of wide distribution, strong adaptability, less diseases and insect pests, and excellent material quality. The early fast-growing is the top among all coniferous trees, and it is an afforestation tree species that is highly valued by state-owned forest farms and farmers. The afforestation area is expanding (Zhu et al., 2013). *Larix olgensis* breeding research is an important field of coniferous tree improvement in China. The conventional propagation method of *Larix olgensis* is seed propagation or asexual (cuttage) propagation, but the propagation cycle is long, and the propagation efficiency is low, which is difficult to meet the requirements of large-scale planting (Zhao et al., 2014). Compared with the traditional breeding methods, somatic embryogenesis has the advantages of high propagation efficiency and free from seasonal restrictions. It can overcome the problems of unstable seed yield, large progeny variation and low rooting rate of cuttage propagation in actual production of *Larix olgensis*, which is helpful for the rapid propagation of excellent varieties of *Larix olgensis* and lays the foundation for the physiological and biochemical research and genetic transformation (Song et al., 2018).

Studies on somatic embryogenesis of *Larix olgensis* have begun since the 1980s (Guo et al., 2003, Plant Physiology Communications, 39(5): 531-535). Larch species and hybrids such as *Larix decidua*×*Larix kaempferi* (Klimaszewska, 1989), *Larix occidentalis* (Thompson and Von Aderkas, 1992), *Larix kaempferi* (Kim et al., 1998; Lü et al., 2005), *Larix kaempferi*×*Larix olgensis* (Wang, 2008) and *Larix olgensis* var. *principis-rupprechtii* (Qi, 2000) have successively established corresponding somatic embryogenesis systems. In recent years, somatic embryogenesis methods have been continuously optimized (Klimaszewska et al., 2016; Rocha et al., 2015;

Abrahamsson et al., 2017; Nunes et al., 2018; Song et al., 2018). However, there are still some disadvantages in somatic embryogenesis of *Larix olgensis* such as difficult embryogenic callus induction, easy embryo loss, low incidence of somatic embryos, long formation time, high malformation rate, asynchronous occurrence and low germination rate. The lack of efficient and stable somatic embryo regeneration system can not meet the requirements of commercial production of *Larix olgensis* somatic embryos. No matter for fine breeding or genetic improvement of *Larix olgensis*, more efficient somatic embryogenesis methods are urgently needed.

Based on the classification criteria of embryos, the embryogenesis of conifers can be divided into three stages according to the time sequence: proembryonic mass (PEM) stage, early embryonic formation (single embryo) and late embryonic formation (cotyledon embryo). In the process of somatic embryogenesis of *Larix olgensis*, the embryogenic callus was induced to obtain the callus at the PEM stage, and the embryogenic callus was proliferated and maintained in subculture medium, and then transferred into somatic embryo maturation medium to complete the development of the latter two stages.

At present, the somatic embryogenesis of *Larix olgensis* is completed in a single solid medium with specific components. In this study, three kinds of somatic embryogenesis maturation medium were used to optimize somatic embryogenesis, and an efficient and stable somatic embryogenesis regeneration solution of *Larix olgensis* was established. It is possible for large-scale commercial somatic embryogenesis breeding, and it is also convenient for physiological and biochemical research and genetic transformation.

1 Results and Analysis

1.1 Acquisition of embryogenic callus

The immature embryos collected on June 15 and 25 were in different developmental stages. The immature zygotic embryo collected on June 15 were in the early or middle stage of single embryo (Figure 1A; Figure 1B). Polyembryony is common in this period, that is, more than one zygotic embryo exists in one seed (Figure 1B). Immature zygotic embryos in seeds collected on June 25 (Figure 1C; Figure 1D) were in the late stage of single embryo or the early stage of cotyledon embryo, the volume of zygotic embryo in this period was significantly larger than that of the previous batch.

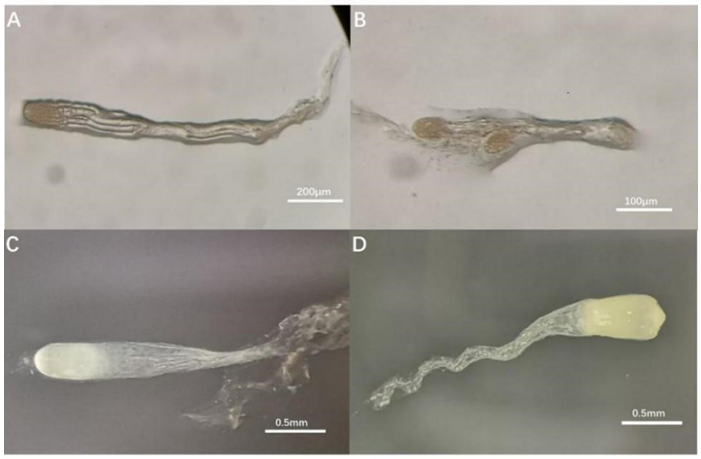

Figure 1 Immature zygotic embryo of *Larix olgensis*
Note: A,B: June 15; C, D: June 25

Embryogenic callus could be induced from immature zygotic embryos collected at different dates and treated with different inoculation methods (Table 1; Figure 2). Embryogenic callus of *Larix olgensis* is light white transparent filament, and the structure of embryogenic suspensor mass (ESM) can be observed under high power objective (Figure 2E), which is mainly composed of two types of cells, one is embryo proper cells with dense cytoplasm and small cell volume. The other type is embryo suspensor cells, which is highly vacuolated and extended more or less. Embryogenic suspensor mass (ESM) is the early proembryogenic mass (PEM).

Table 1 Callus induction rates of immature zygotes treated with different inoculation methods and collected at different dates

Sampling time	With endosperm (%)	Without endosperm (%)
June 15	33.0±2.7	35.0±5.0
June 25	25.0±6.1	29.0±4.2

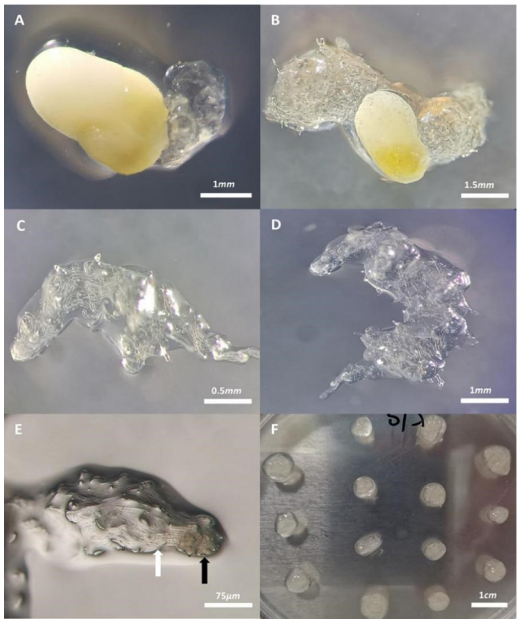

Figure 2 Embryogenic callus induction of immature zygote of *Larix olgensis*

Note: A: With endosperm induction day 4; B: With endosperm induction day 10; C: Without endosperm induction day 4; D: Without endosperm induction day 10; E: ESM structure in embryogenic callus (the embryo proper cells are indicated by black arrows and the embryo suspensor cells are indicated by white arrows); F: The embryogenic callus induced is selected and transferred to the subculture medium

1.2 Embryogenic callus under different subculture conditions

ESM of embryogenic callus in subculture medium with relatively high available water concentration (low agar content) and higher plant growth regulator (auxin, cytokinin) divides vigorously, the number of cells is less, and most of them are in PEM I or PEM II. With the decrease of available water concentration (agar content increased) and the decrease of growth regulator concentration, the number and volume of ESM cells increased, and most of them were in the stage III of the PEM (Figure 3). PEM I and PEM II usually died under ABA, and only PEM III formed somatic embryos under ABA after embryogenic callus was transferred into mature medium. Therefore, subculture under relatively low available water concentration (high osmotic pressure) and low growth regulator concentration is conducive to the formation of late somatic embryos.

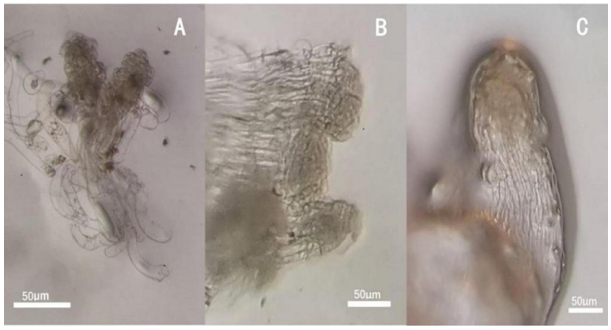

Figure 3 The status of ESM in subculture medium with different available water concentration and plant growth regulator concentration

Note: A~C: ESM in the first, second, and third subculture, the water concentration and plant growth regulator concentration in the

medium reduced from left to right

1.3 Acquisition of mature somatic embryos

Embryogenic callus is at the PEM stage of embryo development. After transferred into mature medium, mature somatic embryos were developed through the early stage of embryonic formation and the late stage of embryonic formation under the combined action of ABA, water stress and other factors. ABA stimulation and the decrease of available water concentration in the environment are the key factors to promote somatic cell maturation.

The effects of ABA at different concentrations on the maturation of somatic embryos in the maturation medium (Table 2) showed that there was no normal somatic embryogenesis in the experimental group without ABA, indicating that ABA was necessary for somatic embryogenesis. With the increase of ABA concentration in mature medium, the yield and quality of somatic embryos were improved. When the concentration was 15~20 mg/L, the optimal value was reached, and the yield of somatic embryos decreased significantly with the increase of ABA concentration. At the same time, too high ABA concentration had adverse effects on the germination of somatic embryos after maturation. The experimental results showed that the optimal ABA concentration in mature media A, B and C was 15 mg/L.

Table 2 Effect of different ABA concentrations in the mature medium on somatic embryogenesis

ABA (mg/L)	Somatic embryo number (Number/g)	Synchronization rate (%)	Germination rate (%)
0	0	0	0
5	65.7±10.5	35.5±5.6	33.4±3.2
10	93.5±23.6	46.2±3.2	44.1±2.5
15	223.0±21.8	78.0±3.1	47.2±3.4
20	209.4±15.7	80.3±2.7	37.6±2.9
40	79.5±5.3	82.1±2.3	23.4±1.8

Water stress also plays a key role in somatic embryogenesis. In the natural environment, during the maturation of zygotic embryos, the available water concentration in the surrounding environment (endosperm) is gradually reduced. In this study, the effects of different sucrose concentration and PEG4000 concentration in the 3 mature media on somatic embryogenesis were analyzed. The results (Table 3) showed that with the increase of sucrose and PEG4000 concentrations, the osmotic pressure of the medium increased, and the cells were dehydrated. Sucrose could be absorbed and utilized by cells, while PEG is a polymer compound that does not penetrate cells. The sucrose concentration in mature medium is higher than that in subculture medium, which is beneficial to the maturation of somatic embryos. However, increasing sucrose concentration will not cause further water stress, which may be due to the regulation of osmotic balance between cells and the environment by absorbing sucrose. PEG4000 can maintain the relatively high osmotic pressure environment in the medium, and the increase of PEG4000 concentration promotes the formation of somatic embryos. When the concentration reaches 100 g/L, it is most beneficial to the development of somatic embryos. The experimental results showed that when sucrose concentration was 45 g/L and PEG4000 concentration was 100 g/L in mature medium, the optimal somatic embryogenesis effect could be achieved.

Table 3 Effects of different sucrose concentration and PEG4000 concentration in the mature medium on Somatic Embryogenesis

Sucrose concentration (g/L)	PEG4000 concentration (g/L)	Somatic embryo No. (No./g)	Germination rate (%)
30	0	0	0
30	50	7.3±5.3	10.9±5.6
30	100	109.6±25.2	49.2±3.8
45	0	34.3±12.1	7.8±4.1
45	50	127.6±17.5	45.7±2.4
45	100	223.0±21.8	47.2±3.4
60	0	18.0±7.7	7.2±6.9
60	50	54.5±9.4	28.6±3.0
60	100	159.4±22.8	25.0±2.3
90	0	14.7±7.7	0

90	50	35.7±11.0	3.3±1.9
90	100	43.6±9.7	6.8±2.2

Hardener agents lock water in physical way to reduce the available water concentration. In the mature solution of this study, somatic embryos were finally matured in medium C. The variety and concentration of hardener agents in medium C affected the available water concentration in the final maturation environment of somatic embryos and had an important impact on the formation of somatic embryos. In medium C, the water stress increased with the increase of hardener agent concentration. When the agar concentration was 10 g/L or the gellan gum concentration was 7 g/L, the somatic embryogenesis was optimal (Table 4). Continue to increase the concentration of hardener agent will cause excessive dehydration, and adverse effects on somatic embryogenesis.

Table 4 Effect of different hardener agents in C medium on somatic embryogenesis

Agar concentration (g/L)	Gellan gum concentration (g/L)	Somatic embryo No. (No./g)	Germination rate (%)
5	-	34.5±7.1	12.4±2.7
8	-	156.9±10.8	42.3±2.3
10	-	223.0±21.8	47.2±3.4
15	-	52.1±14.6	28.4±2.1
-	5	73.2±8.9	40.6±4.5
-	7	202.5±11.6	50.1±2.7
-	10	112.3±9.4	33.7±1.2

The experimental results showed that the optimal mature medium formula and operation mode were as follows: medium A was S medium supplemented with 45 g/L sucrose, 100 g/L PEG4000, 15 mg/L ABA, and 10 mg/L $AgNO_3$. Semi-solid medium B was medium A supplemented with 2 g/L agar. Solid medium C was medium A supplemented with 10 g/L agar (or 7 g/L gellan gum). The embryogenic calli with good growth cultured on the new subculture medium for 10 d were selected, shaking in liquid medium A at 100 r/min, and cultured in the dark under suspension condition for 4 d at 20°C~25°C. The culture in medium A was filtered out with a fine filter and transferred into semi-solid medium B in an erlenmeyer flask. The medium was shaking at 75 r/min, and then cultured in dark for 5 d under the conditions of suspension. The culture medium B was filtered out by fine filter, dried with filter paper, and then placed on the surface of medium C in a petri dish, cultured until late cotyledon embryo formation at 20°C~25°C. The mature somatic embryo was gradually formed after being transferred to medium C (Figure 4). Single embryo formation at 7 days later in medium C. Early cotyledon embryo formation about two weeks in medium C, and cotyledons growth (Figure 5). The late cotyledon embryo was formed in 23 d (Figure 6). The collective embryos were observed and collected (Figure 7), and the mature late cotyledon embryos were transferred to the germination medium for germination (Figure 8). The maturation time of this method is about 32 days, the synchronization rate is 78.0%, and the proportion of somatic embryos in normal morphology is 83.0%. Somatic embryo formation efficiency in normal morphology can reach 223.0 embryos/g embryogenic callus, somatic embryo germination rate can reach 47.2%.

The embryogenic callus was directly transferred into a single solid mature medium, that is, only a single component of solid medium was used to induce somatic embryos of embryogenic callus, and the available water concentration in the medium was reduced at one time instead of gradient continuous reduction (Table 5).

Single solid mature medium was used to induce somatic embryogenesis. Under the optimal conditions, the earliest mature somatic embryogenesis time was 40 d, and the number of normal somatic embryogenesis was 105.4 embryogenic callus/g, the deformity rate was about 35.0%, and the synchronization rate was 62.3%. Under the optimal conditions, the comparative analysis of somatic embryogenesis between three kinds of mature medium and single mature medium showed that (Table 6) the mature time, somatic embryo number, normal morphology rate, synchronization rate and germination rate of three kinds of mature medium were better than those of single mature medium. Analysis of variance showed that the data were significantly different (p<0.01). It was proved that this liquid-solid somatic embryogenesis method optimized the formation of somatic embryos, shortened the

maturation time, promoted the synchronization of somatic embryogenesis, and improved the yield and quality of somatic embryos compared with the single solid maturation medium.

Figure 4 Mature somatic embryos gradually formed after transfer to C medium

Note: A: 5 days after transfer to C medium; B: 10 days after transfer to C medium; C: 16 days after transfer to C medium; D: Single embryo formation at 7 days later in medium C; E: Early cotyledon embryo formation at 14 days in medium C; F: Late cotyledon embryo formation at 23 days in medium C

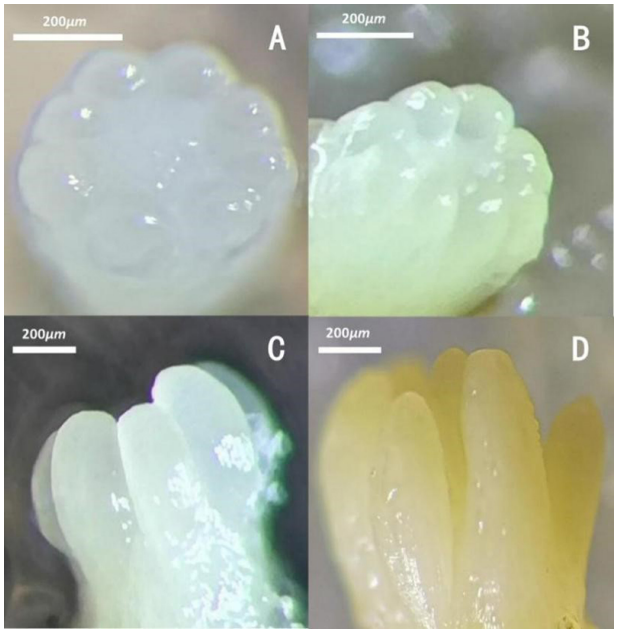

Figure 5 Development of somatic embryo cotyledons in medium C

Note: A,B: The cotyledons of the early cotyledon embryos (transferred to C for 14 days); C,D: The cotyledons of the later cotyledon

embryos (transferred to C for 23 days)

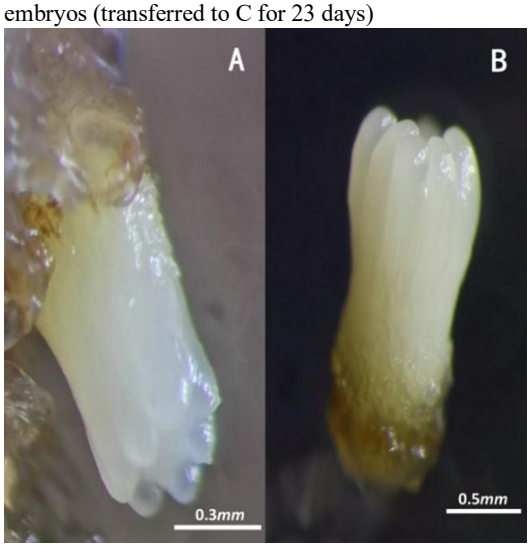

Figure 6 Somatic embryos at the cotyledon stage

Note: A: The early cotyledon embryo (transferred to C for 14 days); B: The late cotyledon embryo (transferred to C for 23 days)

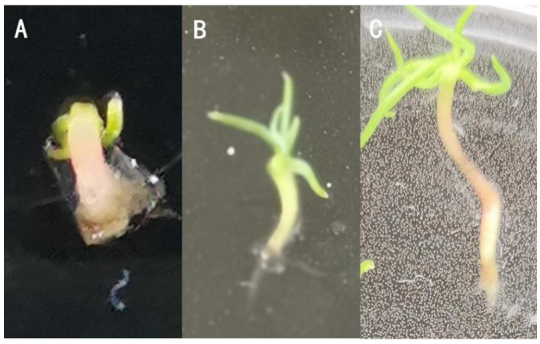

Figure 7 Collected Larix olgensis Somatic embryos

Figure 8 Germination of somatic embryos in germination medium

Note: A,B,C: Germinating cells at 5 d, 10 d and 15 d in medium

Table 5 Somatic embryo generation in a single mature medium

Agar concentration (g/L)	PEG4000 concentration (g/L)	Somatic embryo No. (No./g)	Deformity rate (%)	Synchronization rate (%)	Days for somatic embryo maturation (d)
10	100	105.4±15.7	35±3.1	62.3±6.6	40.0±1.9
7	100	76.8±9.4	35.9±3.4	57.5±5.3	42.0±1.7
10	50	56.4±7.9	38.2±4.8	52.6±4.9	45.0±1.6

Table 6 Comparison of somatic embryogenesis between three mature media and single mature medium

Mature solution	Somatic embryo No. (No./g)	Deformity rate (%)	Synchronization rate (%)	Days for somatic embryo maturation (d)	Germination rate (%)
Three mature media	223.0±21.8 a	17±1.1 a	78.0±3.1 a	32±1.2 a	47.2±3.4 a
Single mature medium	105.4±15.7 b	35±3.1 b	62.3±6.6 b	40±1.9 b	39.5±3.5 b

Note: Different letters indicate significant difference at 0.01 level

2 Discussion

When zygotic embryos were used as explants to induce embryogenic callus of *Larix olgensis*, the induction rate of embryogenic callus was low, and a large number of zygotic embryos were often needed to induce embryogenic callus, and the collection of explants had 'window phase' (Stasolla and Yeung, 2003; Klimaszewska et al., 2016; Song et al., 2016). In this study, immature zygotic embryos of *Larix olgensis* were collected in the middle and late June. During this period, zygotic embryos were easily dedifferentiated in the induction medium to form embryogenic callus. The key requirements of medium for embryogenic callus induction are low osmotic pressure and high plant growth regulator concentration. No matter with or without endosperm induction, ideal embryogenic callus induction rate could be obtained. The induction rate of embryogenic callus was slightly higher when the endosperm was removed, and the immature embryos were stripped. However, because the immature embryos were too small, it was difficult to operate when the endosperm was removed, and the immature embryos were easy to be lost in the process, while the operation was more convenient when the endosperm was stripped. The gradual decrease of hormone concentration and available water concentration during the subculture of embryogenic callus can increase the EMS, the number of cells, which is basically in PEM III and conducive to the maturation of subsequent somatic embryos.

The somatic embryo mature solution of gymnospermae is to put embryogenic callus on a single component of solid mature medium to complete the whole process of somatic embryo maturation (Klimaszewska et al., 2016; Nunes et al., 2018; Song et al., 2018). The somatic embryogenesis of *Larix olgensis* generally needs more than 40 days, and the number of normal somatic embryos is generally not more than 100/g. The deformity rate is generally high, the occurrence is asynchronous, and the repeatability is poor. The somatic embryogenesis is unstable, and the plant transformation rate of somatic embryos is poor. The optimized mature solution of somatic embryogenesis adopted three mature media A, B and C to gradually reduce the available water concentration, which was closer to the natural environment during the development of zygotic embryos, and the available water in endosperm gradually decreased. Embryo maturation in liquid medium can make embryogenic callus fully and evenly contact with medium material, accelerate material transportation, and exchange, and improve the efficiency of embryo maturation. In the study, the somatic embryogenesis of this solution was compared with that of the single mature medium solution. This solution shortened the mature time of somatic embryos by more than one week, more than doubled the number of somatic embryos formed, halved the deformity rate, increased the synchronization rate by about 15%, and increased the germination rate by about 10%. The somatic embryogenesis of *Larix olgensis* is relatively difficult, and there is no efficient and stable commercial model at present. The somatic embryogenesis of *Larix olgensis* widely distributed in Northeast China is rarely reported. This study provides a way for the commercialization of somatic embryo regeneration of *Larix olgensis*, brings great convenience to large-scale breeding and reproduction, physiological and biochemical research, and genetic

modification, and has great practical application value.

3 Materials and Methods

3.1 Experimental materials

Immature seeds of *Larix olgensis* were collected from Dagujia Forest Well-bred Base in Fushun City, Liaoning Province (42.38°N, 124.86°E) in 2018. The seeds were collected in two batches on June 15 and June 25, and inoculated in callus induction medium within 3 d after collection.

3.2 Embryogenic callus induction

The full seeds of *Larix olgensis* were selected and washed with running water for 4 h. Disinfected 1 min with 75% alcohol and sterilized 10 min at 4.5% NaClO. Peeled off the seed coat, cut the endosperm to the embryo (with endosperm) or directly removed the immature embryo (without endosperm), inoculated on the embryogenic callus induction medium at 20°C~25°C, dark cultured for 30~45 d, and induced the embryogenic callus of *Larix olgensis*. The induction medium is S medium (1/2 SPE medium, Gupta and Durzan, 1987) +2,4-D 1.0 mg/L+6-BA 0.5 mg/L+KT 0.3 mg/L, sucrose 20 g/L, agar 3 g/L, glutamine 0.5 g/L, lactium 0.5 g/L, pH=5.8. Statistics of different explants collection time and different inoculation methods of embryogenic callus induction rate was carried out, and analyzed with the help of variance.

3.3 Embryogenic callus subculture

The induction medium was induced for about 30 d, and the selected embryogenic callus was transferred to the embryogenic callus subculture medium at 20°C~25°C under dark conditions, 15 d was a cycle of subculture. The first subculture component is S medium +2,4-D 0.5 mg/L +6-BA 0.25 mg/L+KT 0.15 mg/L, source 30 g/L, agar 4 g/L. The second subculture component is S medium+2,4-D 0.25 mg/L+6-BA 0.12 mg/L+KT 0.8 mg/L, source 30 g/L, agar 5 g/L. After that, it can be subcultured on the subculture medium with S medium+2,4-D 0.15 mg/L (or NAA 0.3 mg/L)+6-BA 0.05 mg/L+KT 0.05 mg/L, source 30 g/L, agar 6 g/L for a long time. The above subculture media are supplemented with glutamine 0.5 g/L and lactium 0.5 g/L, pH=5.8.

3.4 Somatic embryo maturation

Liquid maturation medium A, semi-solid maturation medium B and solid maturation medium C were successively used to induce somatic embryogenesis. Medium A is S medium supplemented with 30~90 g/L source, 0~100 g/L PEG4000, 5~40 mg/L ABA, and 10 mg/L $AgNO_3$, 0.5 g/L glutamine, 0.5 g/L lactium, pH is 5.8. Medium B is medium A supplemented with 2 g/L agar. Medium C is medium A supplemented with 5~15 g/L agar or 5~10 g/L gellan gum.

Embryogenic calli with good growth were cultured on the new subculture medium for 10 d. Each 1 g was torn with tweezers and inoculated into 50 mL liquid medium A in a conical flask. The calli were cultured in dark for 4 d under the conditions of shaking at 100 r/min, 20°C~25°C and suspension. The medium A was filtered out with a fine filter and transferred into semi-solid medium B with medium volume in a conical flask, cultured in dark for 5 d under the conditions of shaking at 75 r/min, 20°C~25°C and suspension. The medium B was filtered out by fine filter, and the moisture was absorbed by filter paper. It was inoculated on the surface of medium C (about 0.2 g per dish, 5 groups per dish) in culture dish (with diameter of 9 cm, 15~20 mL medium per dish), 20°C~25°C, dark culture for 23~30 d, and the formation of somatic embryos was observed. The mature late cotyledon embryos were transferred to WPM medium supplemented with sucrose 25 g/L, agar 4 g/L, activated carbon 0.2%, 20°C~25°C, light culture germination.

Observed the somatic embryogenesis time and recorded the number of somatic embryos per gram callus (only normal somatic embryos were calculated), synchronization rate of somatic embryos (mature somatic embryos after 5 d of maturation of the fastest cotyledon embryos/normal somatic embryos×100%), deformity rate (abnormal somatic embryos/total embryos×100%), and germination rate of somatic embryos (germinated somatic embryos/inoculated somatic embryos×100%). Data were analyzed by variance analysis.

In the process of method optimization, the following comparative experiments were carried out to verify the effect of the solution and the optimal formula of mature medium A, B and C was as follows: Medium A, B and C supplemented with 100 g/L PEG4000, 45 g/L sucrose, respectively, and medium C supplemented with 10 g/L agar. The medium was supplemented with 0 mg/L, 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L and 40 mg/L ABA, respectively, and their effects on somatic embryogenesis were compared. The medium A, B, C was supplemented with 15 mg/L ABA, medium C was supplemented with 10 g/L agar. The effects of sucrose concentrations of 30 g/L, 45 g/L, 60 g/L and 90 g/L and PEG4000 concentrations of 0 g/L, 50 g/L and 100 g/L on somatic embryogenesis were compared in mature medium. The medium A, B, C was supplemented with 15 mg/L ABA, 100 g/L PEG4000, 45 g/L source, and the medium C was supplemented with 5 g/L, 8 g/L, 10 g/L, 15 g/L agar or 5 g/L, 7 g/L, 10 g/L gellan gum, respectively, and their effects on somatic embryogenesis were compared.

The embryogenic calli cultured on the new subculture medium for 10 d were transferred into the S medium, which was a single solid mature medium, supplemented with 45 g/L sucrose, 15 mg/L ABA, 10 mg/L $AgNO_3$, and added with 50 g/L, 100 g/L PEG, and 7 g/L, 10 g/L agar, respectively, and cultured in dark at 20°C~25°C to induce somatic embryos and compare the formation of somatic embryos.

Authors' contributions

LWF was the experimental designers and executor of this study, who completed the data analysis and wrote the first draft of paper. HJY participated in the experimental design and the analysis of experimental results. JXN was the project designer and director, guiding experimental design, data analysis, writing and revision. All authors read and approved the final manuscript.

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