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A Preliminary Study on Somatic Cell Embryogenesis of *Quercus mongolica*

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Abstract In order to establish a callus induction and somatic embryogenesis system of *Quercus mongolica*, and provide an effective culture system for molecular mechanism research of somatic embryogenesis, the leaves of annual seedlings of *Quercus mongolica* were used as explants to study the main influencing factors of callus induction and somatic embryogenesis. The results show that the best way to disinfect the leaves was as follows: 75% alcohol 30 s + 0.1% $HgCl_2$ 4 min; The best medium combination for callus induction is: MS+0.5 mg/L 2,4-D+0.5 mg/L 6-BA, the induction rate is 96.7%; The best medium combination for somatic embryogenesis was MS + 0.1 mg/L 2,4-D + 1 mg/L 6-BA, the induction rate was 58.0%. In this study, the best way of callus induction from *Quercus mongolica* leaves was selected and spherical embryos were induced, which laid a foundation for further research on the establishment of somatic embryogenesis system of *Quercus mongolica.*

Keywords *Quercus mongolica*; Somatic embryogenesis; Callus; Leaf

Quercus mongolica, a deciduous tree of *Quercus* L. in Fagaceae, is the main secondary forest species in northeast China (Gao et al., 2013). The original natural forest of *Quercus mongolica* has been seriously damaged, the original germplasm resources have been greatly reduced, and some groups are in endangered state for a long time. And there was a serious shortage of good species with long growth cycle, late seed setting rate, low yield. Therefore, effective methods should be taken to improve the breeding efficiency of *Quercus mongolica*.

Somatic embryogenesis is the best method of asexual propagation tissue culture for most species (coniferous tree and broad leaved tree) (Stasolla and Yeung, 2003; Merkle and Nairn, 2005; Oh et al., 2010). In recent decades, following the somatic embryogenesis reported by Q.lebani in 1982, studies on plant regeneration of *Quercus* by somatic embryogenesis have increased year by year (Zhang and Yao, 2004). The somatic embryogenesis systems of Quercus genus have been mature, including Spanish summer oak (*Quercus robur* L.) (Sánchez et al., 2003), European cork oak (*Quercus variabilis*) (Pinto et al., 2002), holly oak (*Quercus ilex*) (Martinez et al., 2017), and Czech durmast (Qurcus sessiliflora Salisb.) (Cvikrová et al., 2003). However, there are relatively few researches on somatic embryogenesis of oak tree in China. At present, only cork oak (*Quercus variabilis*) (Zhang et al., 2007), Liaodong oak (*Quercus liaotungensis* Koidz) (Wei, 2001), low-level oak (*Quercus acutissima Carr.*) (Liao, 2012), Suma oak (*Quercus shumardii*) (Lv et al., 2018) have successfully realized plant regeneration through somatic embryogenesis in China.

Selecting appropriate medium is one of the key steps for somatic embryo induction, proliferation, and maturation. The somatic embryo induction of *Querces* was mostly based on MS and WPM (Valladares et al., 2006). There were also some experiments in which 1/2 MS was used as the medium, but the induction effect was general (Sasaki et al., 1988). Many experiments have shown that plant growth regulators are an essential factor for somatic embryogenesis (Zhai et al., 2004), and proper regulation of auxin and cytokinin concentrations also has a very important effect on inducing embryogenic callus culture. For most woody plants, auxin alone is difficult to induce somatic embryos or the induction rate of somatic embryos is very low. However, the combination of auxin and cytokinin can significantly increase the incidence of somatic embryos (Yao, 2004). 2,4-D is the most important component of exogenous plant hormones in embryogenic callus culture (Li et al., 2013), which is more

obvious in young tissue. The higher concentration of 2,4-D should be added in the primary induction (Ji et al., 2002), but the concentration of 2,4-D could be reduced or replaced by NAA in subculture (Jia et al., 2004).

In order to obtain high frequency callus and somatic embryos of *Quercus mongolica*, and provide an effective callus and somatic embryogenesis system for next molecular mechanism research of somatic embryogenesis, the buds were selected as explants for *in vitro* induction in this study.

1 Results and Analysis

1.1 Effect of different disinfection methods on disinfection effect

Effects of different disinfection treatments on the disinfection effect of leaves (Table 1).

It can be seen that the disinfection time significantly affects the disinfection effect of the leaves (Table 1). With the increase of $HgCl_2$ disinfection time, the infection rate of explants decreased gradually, and the survival rate increased first and then decreased with the increase of disinfection time, which indicated that too long disinfection time would affect the activity of leaves. The infection point occurred at the disinfected leaf for 4 min during disinfection, and the survival rate reached 65.3%. Although the infection rate was lower when the $HgCl_2$ disinfection time was 5 min, the mortality rate was much higher than that of 4 min. Therefore, in combination with the infection rate, mortality rate and survival rate, the best way to disinfect the leaves was as follows: 75% alcohol 30 s + 0.1% $HgCl_2$ 4 min.

Table 1 Effect of disinfection treatment on disinfection effect

Disinfection reagents and time	Infection rate (%)	Mortality rate (%)	Survival rate (%)
75% alcohol+0.1% $HgCl_2$ 3 min	46.7±3.5a	8.0±1.7c	45.3±5.0b
75% alcohol+0.1% $HgCl_2$ 4 min	25.7±8.1b	9.0±7.2c	65.3±15.0a
75% alcohol+0.1% $HgCl_2$ 5 min	21.0±7.2b	27.7±5.0b	51.3±2.3ab
75% alcohol+0.1% $HgCl_2$ 6 min	20.3±15.3b	34.3±5.1b	45.3±13.7b
75% alcohol+0.1% $HgCl_2$ 7 min	15.7±5.1b	58.7±5.1a	25.7±5.1c

Note: Different lowercase letters indicated that there was significant difference in p <0.05 level among different treatments

1.2 Effects of medium types on callus induction

Here are the effects of different medium types on callus induction (Table 2).

The results of callus induction rate of different medium types were as follows: MS>WPM>1/2MS. It can be seen that different medium types significantly affect the callus induction rate. In MS medium, the callus induction rate is as high as 96.7%, which is significantly higher than the other two medium. The callus induction rate of WPM medium was higher than that of 1/2MS medium, but there was no significant difference between them. After 14 d of culture in MS medium, leaf notch and midrib would be significantly thickened and slightly callus would occur. After 30 d of culture, it was observed that the induced callus were large and mostly milky white or yellow. Explants cultured in WPM medium developed callus relatively late. Callus formation occurred around 21 d after culture. Explants cultured in 1/2MS medium began to show a small amount of callus around 30 d after culture, and the induced callus developed slowly and small. Compared with WPM and 1/2MS, MS medium had the highest callus induction rate, and the callus appeared earlier and developed better texture. Therefore, MS was the optimal medium for callus induction.

Table 2 Effects of medium types on callus induction

Medium types	Induction rate (%)	Callus state
MS	96.7±3.5a	The callus appeared earlier, with larger area and faster growth
WPM	74.4±8.1b	The callus appeared later, with smaller area and slower growth
1/2MS	64.5±10.8b	The callus appeared late, with small area and slow growth

Note: Different lowercase letters indicated that there was significant difference in p<0.05 level among different treatments

1.3 Effects of different growth regulator combinations on callus induction

Here are the specific results of different combinations and ratios of growth regulators on callus induction (Table 3).

Different hormone combinations and ratios had significant differences in callus induction in leaves (Table 3). In the combination of 2,4-D+6-BA, when the concentration of 2,4-D was constant, the induction rate decreased with the increase of 6-BA concentration. When the concentration of 6-BA was constant, the induction rate decreased with the increase of 2,4-D concentration. And the different concentration ratio of these two hormones combined will significantly affect the induction rate of callus. It can be seen that 0.5 mg/L 2,4-D+0.5 mg/L 6-BA are the concentration combinations with the highest induction rate, up to 96.7%. Callus appeared around 14 d after induction. Callus grew quickly and had a large area. At around 28 d, callus covered the surface of leaf explants, and the surface of callus was shiny, mostly fresh milky white, yellow, or green. In the combination of NAA+6-BA, when the concentration of NAA was constant, the induction first increased and then decreased with the increase of 6-BA concentration. When the concentration of 6-BA was constant, the induction rate decreased with the increase of NAA concentration. The induction rates of the two hormones were significantly different under different concentration combinations. It can be seen that 0.5 mg/L NAA+1 mg/L 6-BA are the concentration combinations with the highest induction rate, up to 88.7%. Callus appeared around 20 d after induction. In the process of subculture, the callus grew faster and the individual was larger. At 30 d, callus covered the surface of leaf explants, and the surface of callus was milky yellow and dark green (Figure 1). In a word, in the callus induction stage of leaves, the most appropriate hormone ratio is 0.5 mg/L 2,4-D+0.5 mg/L 6-BA.

Table 3 Effects of different hormone combinations on callus induction

Treatment	2,4-D(mg/L)	NAA(mg/L)	6-BA(mg/L)	Induction rate (%)
1	0.5		0.5	96.7±3.5a
2	0.5		1	80.0±10.0b
3	0.5		2	83.3±6.5ab
4	1		0.5	74.3±5.1bcd
5	1		1	61.0±8.5d
6	1		2	33.3±8.5f
7		0.5	0.5	77.7±5.0bc
8		0.5	1	88.7±5.1ab
9		0.5	2	74.3±5.1bcd
10		1	0.5	64.7±6.8cd
11		1	1	65.3±14.0cd
12		1	2	34.3±14.0e

Note: Different lowercase letters indicated that there was significant difference in $p < 0.05$ level among different treatments

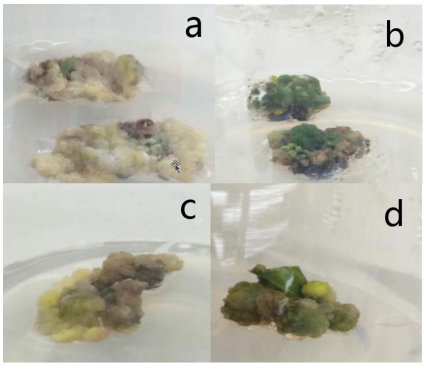

Figure 1 Callus induced by *Quercus mongolica* leaves

Note: a: white, milky yellow callus induced by 2,4-D, 6-BA combination treatment; b: green callus induced by 2,4-D, 6-BA combination treatment; c: milky yellow callus induced by NAA, 6-BA combination treatment; d: dark green callus induced by NAA, 6-BA combination treatment

1.4 Effects of growth regulator combination on somatic embryo induction

(1) Effects of 2,4-D and 6-BA combinations on somatic embryo induction

The combination of 2,4-D and 6-BA can significantly affect the induction rate of somatic embryos. After 30 d of culture, the maximum somatic embryo induction rate was 58.0% under the hormone combination of 0.1 mg/L 2,4-d +1 mg/L 6-BA, and there was no significant difference between the induction rates of 0.1 mg/L 2,4-D+1 mg/L 6-BA and 0.1 mg/L 2,4-D+0.5 mg/L 6-BA. At the hormone level of 0.1 mg/L 2,4-D+1 mg/L 6-BA, somatic embryos still maintained a strong embryogenic ability in the process of subculture proliferation, and some globular embryos were differentiated and developed in the next step. Induced globular embryos (Figure 2a) were found on the surface of white callus and brown callus after 15 d of culture with 2,4-D, 6-BA combination treatment. Globular embryos of different sizes were gradually developed and clustered together. After 25 d of culture, the globular embryos would further develop into torpedo-shape embryos and heart-shape embryos (Figure 2b; Figure 2c). The non-embryogenic callus induced during culture showed a brown compact structure and could not continue to differentiate (Figure 2d).

(2) Effects of NAA and 6-BA combinations on somatic embryo induction

NAA and 6-BA also significantly affect the induction rate of somatic embryos (Table 4). After 40 d of culture, the maximum somatic embryo induction rate was 50.0% under the hormone combination of 0.5 mg/LNAA＋0.5 mg/L6-BA. Under the combined use of NAA and 6-BA, about 15 d of culture, it was found that small amount of globular embryos (Figure 2e) were formed in both pale yellow loose and white loose granular callus on the subculture medium, and globular embryos also occurred aggregation phenomenon. After 30 d of culture, globular embryos were further differentiated into heart-shape embryos (Figure 2f). New globular embryos could still be induced after 40 d of culture. Some somatic embryos further developed to torpedo-shape embryos (Figure 2g; Figure 2h), and the color gradually changed from transparent to white.

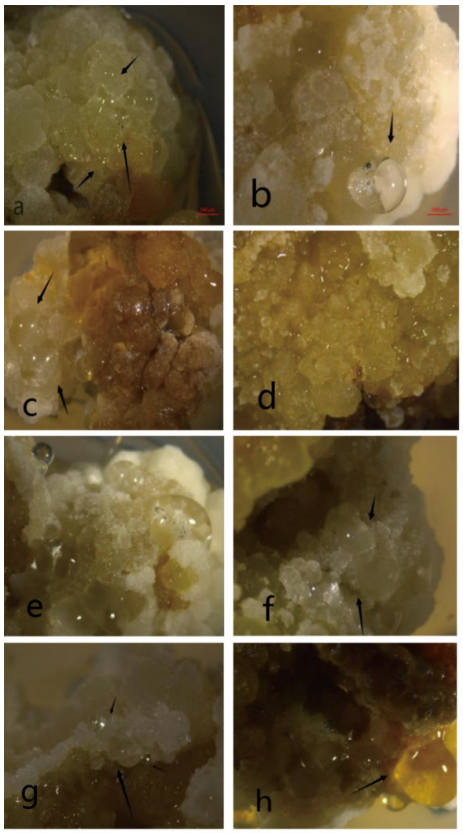

Figure 2 Induced somatic embryos

Note: a: globular embryos; b: heart-shape embryos; c: torpedo-shape embryos; d: callus; e: globular embryos; f: heart-shape embryos; g, h: torpedo-shape embryos

Table 4 Effects of growth regulators on somatic embryo induction

Treatment	2,4-D(mg/L)	6-BA(mg/L)	NAA(mg/L)	Somatic embryo induction rate (%)
1	0.05	0.5		14.3±5.1e
2	0.05	1		35.7±5.1bc
3	0.05	2		32.0±8.5bcd
4	0.1	0.5		55.3±4.0a
5	0.1	1		58.0±11.5a
6	0.1	2		19.0±3.5e
7	0.2	0.5		42.0±10.1b
8	0.2	1		26.7±3.5cde
9	0.2	2		21.3±5.1de
10		0.5	0.1	12.3±8.1e
11		1	0.1	17.7±6.8cde
12		2	0.1	19.0±7.2cde
13		0.5	0.2	28.0±8.5bcde
14		1	0.2	29.0±10.1bcd
15		2	0.2	25.7±10.3bcde
16		0.5	0.5	50.0±7.0a
17		1	0.5	41.0±7.2ab
18		2	0.5	32.3±5.0bc
19		0.5	1	39.7±5.8ab
20		1	1	35.7±14.0ab
21		2	1	14.3±8.1de

Note: Different lowercase letters indicated that there was significant difference in $p < 0.05$ level among different treatments

2 Discussion

The leaves of annual seedlings of *Quercus mongolica* were used as explants to study the main influencing factors of callus induction and somatic embryogenesis in this study. The results showed that the best way to disinfect the leaves was as follows: 75% alcohol 30 s + 0.1% $HgCl_2$ 4 min. At this time, the infection rate was 25.7%, the mortality rate was 9.0%, and the survival rate was 65.3%.

In this experiment, the influence of MS, WPM and 1/2MS on callus induction rate was studied. It was found that MS medium had the highest induction rate and the fastest induction rate when young leaves were used as explants, followed by WPM medium. It may be because MS medium contains high content of inorganic salts, complete types of microelement, and relatively high concentration (Li, 2002). Especially, MS medium contains heavy metal Co^{2+}, which can not only improve the induction rate of callus, but also promote the occurrence of somatic embryos. The callus induced by different media were also different in time and texture. The callus induced by MS medium with short time, soft texture and large volume. The type and concentration of plant growth regulators also significantly affected the callus induction rate. 2,4-D is often added as auxin in the induction phase of the younger tissue. When auxin and cytokinin are used in combination, the induction rate will be significantly increased. Some tree species such as North American red oak (*Quercus rubra*), in the leaf as explants for somatic embryo induction, the ratio of hormone requirements are more strict, and the ratio of cytokinin and auxin concentration should not be less than 1 (Rancillac et al., 1996). In this experiment, the highest callus induction rate of 96.7% was obtained by the combination of 0.5 mg/L 2,4-D+1 mg/L 6-BA.

Plant growth regulator is also one of the key factors in the process of subgeneration proliferation. Generally, auxin and cytokinin are used in combination. After somatic embryos are induced, the concentration of auxin will be reduced or 2,4-D will be replaced by NAA. Too high concentration of auxin will inhibit the further growth and development of somatic embryos, and some plants can proliferate without hormone (Zhang, 2008). In this experiment, auxin 2,4-D and NAA, and cytokinin 6-BA were selected to induce somatic embryogenesis. The results showed that the callus induction rate was significantly increased when 2,4-D was combined with 6-BA. In

order to promote the redifferentiation of embryogenic callus, the concentration of 2,4-D was reduced in time and 2,4-D was replaced by NAA at the same time. And globular embryos were successfully induced in the subculture. The best medium combination for somatic embryogenesis was as follows: MS+0.1 mg/L 2,4-D+0.5 mg/L 6-BA, the induction rate was 58.0%. The induction rate of somatic embryos in 0.5 mg/L NAA+0.5 mg/L 6-BA treatment was up to 50.0%. There were obvious differences in appearance and structure between embryogenic callus and non-embryogenic callus. The cells of embryogenic callus with smaller volume, dense cytoplasm, large and clear nucleus, while the cells of non-embryogenic callus with larger volume, sparse cytoplasm.

3 Materials and Methods

3.1 Experimental materials

The experimental materials were taken from annual seedlings of *Quercus mongolica*. In mid-January 2018, *Quercus mongolica* seeds stored in the library (0~4℃) were planted indoors (natural light, room temperature 16℃). Germination began about 20 d, and leaves were collected after 60 d for the experiment.

3.2 Explants treatment

Washed the removed leaves under running water for 2 h and then dried. First, 75% alcohol was used for disinfection for 30 s, and then 0.1% $HgCl_2$ was used for disinfection for 3 min, 4 min, 5 min, 6 min and 7 min, respectively. The above steps were cleaned with sterile water for 3 times before treatment. Drained the leaves with sterilized filter paper, cut them into squares of the same size, and placed them forward in the induction medium. Each treatment was repeated in 16 petri dishes, with 5 explants in each dish. Counted the infection rate, mortality rate and survival rate of explants after 15 d of culture.

3.3 Callus induction

3.3.1 Screening of basic medium

Three basic medium (MS, WPM, 1/2MS) were prepared and the disinfected leaves were inoculated on the induction medium in equal amounts. The components of the medium were as follows: different medium +0.5 mg/L 2,4-D+0.5 mg/L 6-BA+6 g/L agar +30 g/L sucrose +0.5 g/L hydrolyzed casein, and the PH was adjusted to 5.8. Each treatment was repeated in 18 petri dishes, with 5 explants in each dish.

The culture conditions were as follows: (23±2)℃ for 16 h/d light culture. Calculated the induction rate of callus after 30 d, meanwhile, observed and recorded. the morphology of callus in different medium.

3.3.2 Screening of plant growth regulators

The optimal medium was selected as the basic medium, and 2,4-D, NAA and 6-BA of different concentrations were added, respectively, a total of 12 treatments. The other components of the medium were as follows: MS+6 g/L agar+30 g/L sucrose+0.5 g/L hydrolyzed casein, and the PH was adjusted to 5.8. Each treatment was repeated in 18 petri dishes, with 5 explants in each dish.

The culture conditions were as follows: (23±2)℃ for 16 h/d light culture. Subculture every 14 days. Recorded and observed callus morphology everyday. Calculated the induction rate of embryonal callus after 30 d. After two times of subculture of callus, white or yellowish white, soft and viscous embryogenic callus were selected for proliferation culture.

3.4 Screening of somatic embryo-induced growth regulators

Selected the embryogenic callus with vigorous growth, white or yellowish white, globular soft, and viscosity from above culture progress. After being removed from the culture, they were inoculated to the medium that induced somatic embryogenesis, in which 2,4-D, NAA and 6-BA of different concentrations were added, respectively. A total of 21 treatments were performed, 30 g/L sucrose, 0.5 g/L hydrolyzed casein and 6 g/L agar were added at the same time, and the PH was adjusted to 5.8. The culture conditions were as follows: (23±2)℃ in darkness. Calculated the induction rate of somatic embryos after 30 d.

3.5 Data statistics and analysis

In this experiment, the infection rate, mortality rate and survival rate were calculated to reflect the best disinfection method of explants, and the induction rate was used to reflect the induction of explants callus and somatic embryos.

Infection rate=(Number of explants infected/Number of explants inoculated)×100%;
Mortality rate=(Number of explants dead/Number of explants inoculated)×100%;
Survival rate=(Number of explants alive/Number of explants inoculated)×100%;
Callus induction rate=(Number of explants that induce callus/Number of explants inoculated)×100%;
Somatic embryo induction rate=(Number of explants that induce somatic embryos/Number of explants inoculated)×100%.

Excel was used to record and calculate the obtained data, SPSS Statistics 17.0 was used to process the data, and Duncan multiple comparison method was used to analyze the difference significance.

Authors' contributions

JZH and LQ designed and carried out the study. JZH, LQ, and LMY performed the statistical analysis. JZH drafted the manuscript. LCP participated in the design of the study and performed the statistical analysis. LXJ conceived of the project, directed the design of the study, data analysis, draft and revision. All authors read and approved the final manuscript.

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