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Research Article

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Posted Date: February 21st, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2588759/v1

Induction of embryonic callus from hypocotyl and needle of *Pinus thunbergii* Parl.

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Abstract: To lay the foundation for the establishment of somatic embryogenesis system from the vegetative organs of *Pinus thunbergii* Parl, the induction of somatic embryogenic callus(EC) was studied using hypocotyls as explants to find out the key factors affecting EC induction, and investigate the effects of medium type, concentration of auxin (2,4-D) and cytokinin (6-BA) on the EC induction. Orthogonal experiment design with three factors and four levels was adopted. MS, WPM, B_5 and DCR were used as the basic medium, respectively, with different concentrations of 2,4-D (0.5 mg • L^{-1}, 1.5 mg • L^{-1}, 2.0 mg • L^{-1}, 2.5 mg • L^{-1}) and 6-BA (0.5 mg • L^{-1}, 1.0 mg • L^{-1}, 1.5 mg • L^{-1}, 2.0 mg • L^{-1}), with additional 0.2 mg • L^{-1} polyvinylpyrrolidone(PVP), 6 g • L^{-1} agar and 20 g • L^{-1} sucrose to induce callus. The interactions between the factors, the optimal combination and the best hormone concentration ratio were investigated by statistical methods. Callus were sampled for morphological observation under a stereoscopic microscope, followed by cytological observation under a biological microscope using a double staining method of acetate magenta and Evans blue to identify whether they were embryonic callus. The polyphenol oxidase (PPO) contents in the callus of different succession cultures were measured to test whether embryonic stage of the induced callus could be maintained. The results showed that the optimal combination was DCR + 2.5 mg • L^{-1} 2,4-D + 1.0 mg • L^{-1} 6-BA, and this protocol resulted in 98.7% (±1.9%) induction of callus. Using this protocol, callus of needle explants was also successfully induced, but the callus induction was slower. Microscopic observation reveals a distinct embryonic callus structure, an embryonic head with several suspensor cells. The analysis of variance showed that among the three factors, 2,4-D concentration significantly affected the callus induction rate ($p<0.05$), while the medium type and the 6-BA concentration had nonsignificant effect. Even so, the analysis of interaction showed that there was a significant interaction between the combination of 2,4-D and 6-BA ($p<0.05$). The PPO viability was significantly higher in the callus grown for 3 weeks, while it decreased after first and second succession. The above results inferred that 2,4-D plays an important role in embryotic callus induction process in the hypocotyl of *P. thunbergii* Parl. Although 6-BA concentration level by itself had no significant effect on callus induction rate, there may be complex links between the 6-BA and 2,4-D in the signal transduction pathways.

Key words: Somatic embryogenesis; Embryogenic callus; *Pinus thunbergii* Parl; Hypocotyl; Needle

Introduction

Pinus thunbergii Parl is an evergreen tree of the *Pinaceae*, resistant to drought and barrenness, with strong resistance to diseases and insects, and is a famous coastal greening species, as well as a preferred species for barren mountain afforestation, landscaping and roadside trees, with high ecological value and economic effect (Tsuyoshi *et al.*, 2016). At present, the main propagation method of *P. thunbergii* Parl is seeding, which has the disadvantage that the excellent traits of the parent are more differentiated or even disappeared. In addition, the propagation by grafting also limits the rapid propagation and popularization application of superior clones to a certain extent (Yan *et al.*, 2022). To efficiently propagate superior genotypes (especially pine nematode-resistant genotypes) of *P. thunbergii* Parl, and to achieve massive proliferation in a short cycle, it is particularly important to conduct research on *in vitro* culture technology.

Some studies have been done previously on the technical system of somatic embryogenesis in *P. thunbergii* Parl,

but all of them have used immature zygotic embryos as explants (Li *et al.*, 2012; Sun *et al.*, 2015), and studies on hypocotyl or needle as explants have not been reported. The time taken from somatic embryogenesis to completion of new cultivar breeding in different explants of *P. pinaster* was previously compared, and showed that the time when using immature zygotic as explant was 3-4 times longer than that when using vegetative organs (Lelu-Walter *et al.*, 2016). Moreover, the use of immature zygotic embryos explants has the disadvantages of limited collection period, unstable growth traits and easy mutation, as well as the inability to fully retain the excellent traits of the parent, while the use of vegetative organ explants can compensate for these disadvantages (Malabadi *et al.*, 2003). Fan *et al.* (2020) investigated the effects of 6-BA, IBA and NAA hormone combinations and ratios on callus induction from needles, epicotyls and young roots of *P. tabulaeformis* Carr, and all these three explants successfully produced callus, but whether they were embryonic was not specified. Wang *et al.* (2022) used *P. densiflora* var. *zhangwuensis* needles as explants and induced callus successfully in DCR medium containing different concentrations of 2,4-D and NAA, but the obtained callus was proved non-embryonic. Pereira *et al.* (2021) used root tip of *P. halepensis* as explants, and eventually obtained positively proliferating non-embryonic callus (NEC) by regulating the composition of the medium (including the use of auxin and cytokinins). Malabadi *et al.* (2003) obtained embryonic callus (EC) of *P. patula* using vegetative stem tip slices explants in DCR medium supplemented with gellan gum, maltose and hormones (2,4-D, NAA, BA), but the callus induction rate was lower than 45%. Another study in *P. virginiana* showed that some induced EC showed slight browning after a period of incubation, inhibiting subsequent proliferation (Tang *et al.*, 2004). Above studies showed that somatic embryogenesis from the vegetative organs of *Pinaceae* plants was more difficult, and most of the induced callus were NEC, and they were difficult to proliferate further (Lelu-Walter *et al.*, 2016).

Somatic embryogenesis using conifer hypocotyls and other vegetative organs as explants includes four stages: induction of EC, identification and proliferation of EC, maturation and germination of somatic embryos (Tretyakova *et al.*, 2021). One of the most fundamental steps is the EC induction from vegetative organs. Although there are fewer studies in this area, some regularities can still be preliminarily summarized. The basic medium for conifer somatic embryogenesis is often composed by a combination of macroelements, microelements and organic matters(e.g. PVP), in which the content of NH^{+4} and NO^{-3} plays an important role (Wang *et al.*, 2021). The types and concentrations of exogenous hormones usually including auxin and cytokinin play an important role in the EC induction in *Pinaceae* species, and those hormones can promote EC induction by regulating the cell cycle and triggering cell division (Francis *et al.*, 2001). Usually, high concentrations of auxin and low concentrations of cytokinin are beneficial to callus induction (Wang *et al.*, 2006). Auxin is considered as the most important hormone during somatic embryogenesis due to its role in cell division and differentiation (Cooke *et al.*, 1993). The synthetic auxin 2,4-D is chemically more stable and 10-1000 times active than natural growth hormones, such as indole-3-acetic acid (IAA) (Phillips *et al.*, 2019), and is involved in the process of EC induction for many plants (Litz *et al.*, 1998; Raghavan, 2004; Gaj, 2004). Research on cell physiology and gene expression changes induced by 2,4-D suggests that it may act as a stress factor to trigger embryonic developmental patterns in cultured plant cells (Song, 2013), prompting the transformation of somatic cells to embryonic cells. Peng *et al.* (2020) analyzed the physiological differences between EC and NEC of Korean pine, and found that low levels of polyphenol oxidase (PPO) was one of the more easily determined EC markers. Based on the above studies, four basic media including MS, WPM, B_5, and DCR, which differed greatly in nitrogen content, were used with four concentrations of 6-BA

and 2,4-D. The effects of basic medium types, different concentrations of 2,4-D and 6-BA on hypocotyl callus induction in *P. thunbergii* Parl were investigated by orthogonal tests, post hoc multiple comparisons and interaction analysis of various factors, and the morphological and cytological observations of the induced callus were carried out, at the same time PPO activity in callus were also assayed. The study aims to provide feasible technical support for the mass expansion of high-quality *P. thunbergii* Parl.

Materials and Methods

Treatment of explants

The *P. thunbergii* Parl seeds were obtained from the excellent resistant provenance of Kunyushan mountain in Shandong Province, China. The cultured hypocotyls and needles were used as explants, which were first disinfected with 75% alcohol for 30 s and rinsed 3-4 times with sterile water, then disinfected with 2% NaClO for 10min and rinsed 4-5 times with sterile water.

Effect of different factors on callus induction

The hypocotyl was cut into 5-7 mm segments and inoculated onto different media to induce callus in a clean bench (vertical flow). A three-factor, four-level orthogonal experiment was used for screening the best protocol, with the three factors being medium type, 2,4-D concentration and 6-BA concentration, respectively. The four base media were B_5, DCR, MS and WPM, with added hormones and concentrations of 2,4-D (0.5 mg • L^{-1}, 1.5 mg • L^{-1}, 2.0 mg • L^{-1}, 2.5 mg • L^{-1}) and 6-BA (0.5 mg • L^{-1}, 1.0 mg • L^{-1}, 1.5 mg • L^{-1}, 2.0 mg • L^{-1}), as detailed in Table 1. Each treatment was inoculated with 40 explants and replicated three times. Incubation in dark conditions at 23±2℃. The growth rate and status of the callus were observed, and the induction rate was calculated. Based on the above experiments, the best medium type and the optimal hormone concentration ratio were found.

Table 1 Factors and levels in orthogonal experiment design

Levels	Factors		
	A: Culture medium types	B: 2,4-D concentration/ (mg • L^{-1})	C: 6-BA concentration/ (mg • L^{-1})
1	WPM	1.5	2.0
2	MS	2.0	1.0
3	B5	0.5	0.5
4	DCR	2.5	1.5

Microscopic observation

Callus were sampled for morphological observation under a stereoscopic microscope (Stemi 305, Germany), and then it was double stained with acetate magenta and Evans blue and cytologically observed under a biological microscope (DM500 + ICC50W, China) to identify whether it was embryonic. The specific operation was as follows: a small amount of callus was taken on a slide, and broken up with forceps after putting a drop of water, then first stain with 1% acetate magenta and second stain with 0.05% Evans blue was carried out, and the excess stain was aspirated by covering the coverslip, and the stained callus was observed under the microscope (Xu *et al.*, 2017).

Determination of PPO content

To test whether the induced EC could maintain embryonic state during the proliferation stage, the PPO assay was performed. PPO is a copper-containing oxidase that is present in both plants and cultured cells. It can oxidize mono- and di-phenols to produce quinone, which causes browning. Quinone has characteristic light absorption at 420 nm which can be measured by spectrophotometric method.

Extraction of crude enzyme solution was shown as follows: The ratio of the callus weight (g) to the volume of the extraction solution (L) was 1:10, which was weighed proportionally and then homogenized in an ice bath (centrifuged at 8000 r/min for 10 min). After the crude enzyme solution was extracted, a portion was aspirated and boiled in a water bath above 90℃ for 5 min, removed and cooled under running water, which was used as the supernatant of the control tube boiling.

The assay tube (600 μL buffer + 150 μL matrix solution + 150 μL crude enzyme solution supernatant) and the control tube(600 μL buffer + 150 μL matrix solution + 150 μL supernatant of boiling treated crude enzyme solution) were incubated accurately at 37℃ for 10 min and then removed, immediately transferred to a boiling water bath above 90℃ for 5 min, then cooled with running water and centrifuged at 1000 r/min for 10 min at room temperature. Using a sample of 1 cm optical diameter cuvette, distilled water was used for zeroing, and the supernatant was taken at a wavelength of 420 nm to determine the absorbance value of each tube ($\Delta A = A_{assay} - A_{control}$). At last, PPO vitality was calculated using the formula below:

$$\text{PPO Vitality}\left(U/g\text{Fresh weight}\right)=\frac{\Delta A\times V_1\times V_2\times 1mL}{0.01\times W\times V_3\times V_2}\div T$$

where W is fresh weight of the sample (g), V_1 is the total volume of extraction solution (mL), V_2 is the total volume of reaction system (0.9 mL), V_3 is volume of sample taken (0.15 mL), and T is the reaction time (10 min).

Data Processing

IBM SPSS statistic 26 and Excel software were used for analysis of extreme difference, variance and interaction. The analysis of extreme difference can visually analyze the data in the Table 2, where k is the average value of the factors at each level, and the optimal level of each factor can be judged by the k-value to derive the optimal combination of the test. R is the difference between the maximum and minimum values of the factors at each level, and the magnitude of the R-value can reflect the magnitude at which the test factors contribute to the EC induction rate, allowing the factors to be ranked in order of priority. A large R-value of a factor means that it has a large impact the test and is usually the main factor; conversely, a factor with a small R-value is usually a minor factor. Analysis of variance (ANOVA) for orthogonal experiment can investigate the influence of each factor (medium type, 2,4-D concentration and 6-BA concentration) and the influence of the interaction between the factors and determine which factor make a significant difference ($p<0.05$). For the factor with a significant effect, post hoc multiple comparison was performed using LSD method to understand the significant difference between different levels of this factor.

Results and Analysis

Morphology and cytology of callus

Hypocotyl explants were cultured on induction medium for 7 d, and yellowish callus began to produce at both ends of the incision. With the increase of time, the number of callus gradually increased and their size also gradually increased, as well as the overall expansion and dehiscence of cut segments (Fig. 1a) or the formation of a dumbbell shape (Fig. 1b). The presence of EC could be clearly observed under the stereoscopic microscope (Fig. 1c, d). After double staining with aceto magenta and Evans blue, the callus was observed under biological microscope, and embryonic head with several suspensor cells could be observed (Fig. 1e), which was character of EC; the other type of mostly round cells with darker granular color (Fig. 1f), which was NEC.

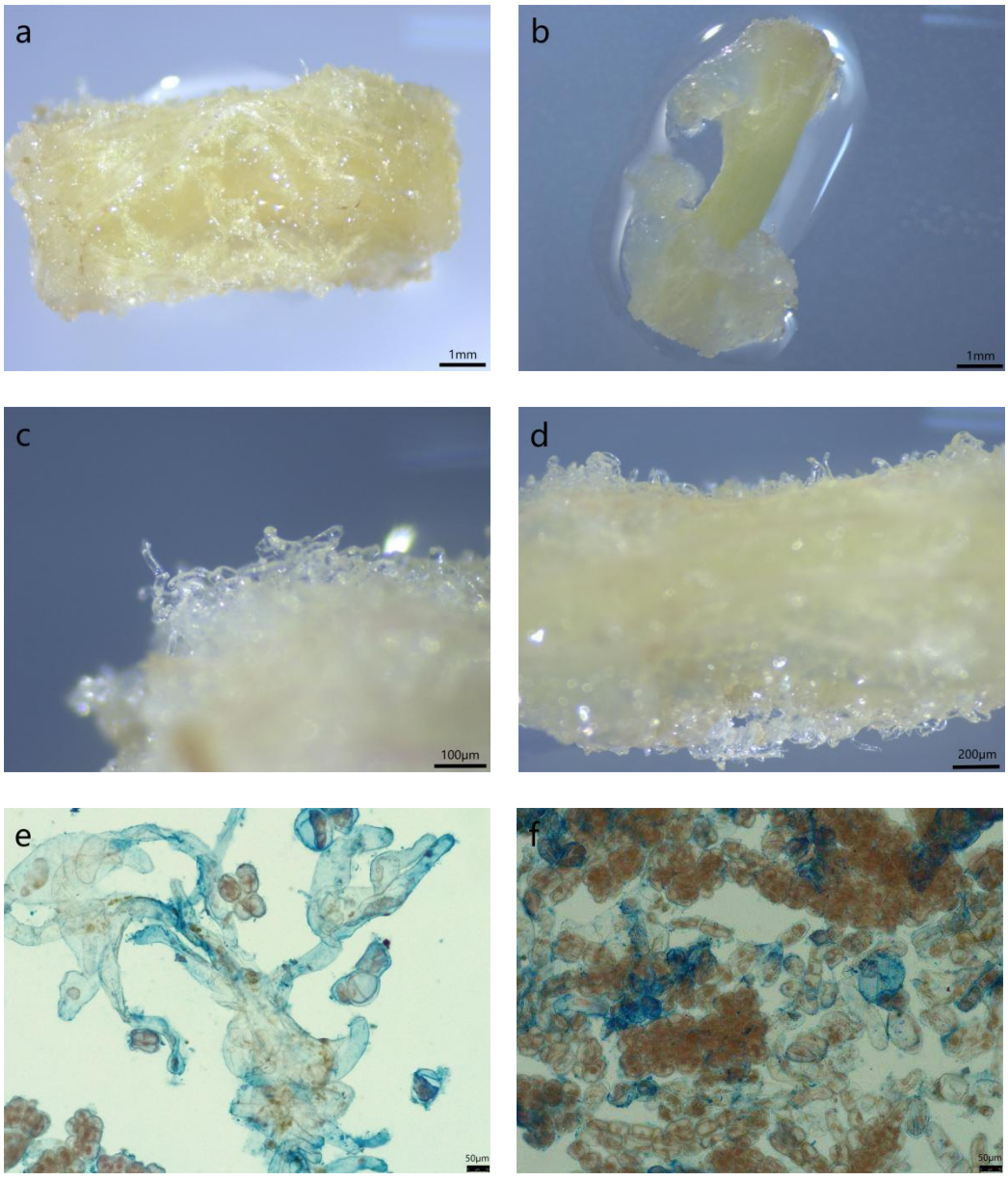

Fig. 1 Observation of hypocotyl callus (a-d: Observation with stereoscopic microscope. e-f: Staining observation with biological microscope)

The results of orthogonal experiment and statistical analysis

The induction rates of the 16 protocols were compared after 14 d (Table 2) and extreme difference analysis was performed(Table 3). It can be seen that the order of the magnitude was $R_B > R_C > R_A$, which meant the order of factor effects as 2,4-D concentration > 6-BA concentration > medium type, and $A_4B_4C_2$ was the optimal combination for this experiment based on the k-value, that was DCR + 2.5 mg • L^{-1} 2,4-D + 1.0 mg • L^{-1} 6-BA.

In this study, R^2 value in ANOVA was 0.944 (Table 4), which implied that these three factors could explain 94.4% of the variation in the data. According to the P-value, 2,4-D concentration had a significant effect on the callus induction rate($p<0.01$), while medium type and 6-BA concentration had no significant effects ($p>0.05$). The result of post hoc multiple comparison showed that there were significant differences between the levels of 2,4-D concentration, the mean callus induction rates in 1.5, 2.0, 2.5 levels were all significantly higher than that of the 0.5 level, although there were no significant differences among these three levels ($p<0.01$, Table 6).

As shown in Table 5, there was no significant effects of interaction between the medium type and 2,4-D concentration or between the medium type and 6-BA concentration on the callus induction rate. But for the BC

interaction, F_{BC} was 47.285, far greater than F_{crit}, which fell in the rejecting domain, indicating that the interaction between 2,4-D concentration and 6-BA concentration had a significant effect on the callus induction rate. It can be seen that the concentration level of 6-BA by itself had no effect on the experimental results (Table 4), but when it was combined with 2,4-D there was an interactive effect.

Table 2 Statistics of experimental results on the induction rate of callus

Number	Culture medium types	2,4-D concentration/ (mg • L^{-1})	6-BA concentration/ (mg • L^{-1})	Average induction rate	Standard deviation	Coefficient of variation
1	WPM	1.5	2.0	0.921	0.0284	0.0309
2	WPM	2.0	1.0	0.892	0.0543	0.0608
3	WPM	0.5	0.5	0.597	0.0905	0.1515
4	WPM	2.5	1.5	0.914	0.0374	0.0409
5	MS	1.5	1.0	0.953	0.0359	0.0376
6	MS	2.0	2.0	0.939	0.0601	0.0641
7	MS	0.5	1.5	0.715	0.1049	0.1468
8	MS	2.5	0.5	0.863	0.0574	0.0666
9	B5	1.5	0.5	0.870	0.0252	0.0289
10	B5	2.0	1.5	0.879	0.0471	0.0536
11	B5	0.5	2.0	0.597	0.0520	0.0871
12	B5	2.5	1.0	0.968	0.0237	0.0245
13	DCR	1.5	1.5	0.898	0.0147	0.0164
14	DCR	2.0	0.5	0.952	0.0159	0.0167
15	DCR	0.5	1.0	0.660	0.0362	0.0549
16	DCR	2.5	2.0	0.986	0.0203	0.0206

Table 3 Analysis of extreme difference of orthogonal tests

K	A	B	C
	Culture medium types	2,4-D concentration/ (mg • L^{-1})	6-BA concentration/ (mg • L^{-1})
K1	3.324	3.642	3.442
K2	3.469	3.662	3.472
K3	3.314	2.569	3.282
K4	3.496	3.730	3.406
k1	0.831	0.911	0.861
k2	0.867	0.915	0.868
k3	0.828	0.642	0.821
k4	0.874	0.933	0.852
R	0.046	0.290	0.048

Table 4 Analysis of variance for orthogonal experiment

Factors	Sum of squares of deviations	Degree of freedom	Mean square	F	P
Culture medium types	0.007	3	0.002	0.949	0.474
2,4-D concentration/ (mg • L^{-1})	0.232	3	0.077	32.255	0.000**
6-BA concentration/ (mg • L^{-1})	0.005	3	0.002	0.735	0.568
Error	0.014	6	0.002		

$R^2 = 0.944$; *: $p<0.05$; **: $p<0.01$.

Table 5 Interaction analysis

Source of difference	Sum of squares of deviations	Degree of freedom	Mean Square	F	P-value	F_{crit}
A*B Interaction	0.061	3	0.020	0.071	0.974	3.239
A*C Interaction	0.643	3	0.214	0.755	0.535	3.239
B*C Interaction	0.993	3	0.331	47.285	0.000	3.239
A*B*C Interaction	1.132	6	0.189	0.985	0.457	2.508

F: Calculated F-value; P-Value: Probability value corresponding to F-value on the cumulative probability curve; F_{crit}: Critical value of F corresponding to the significant level. A, B and C represented medium type, 2,4-D concentration and 6-BA concentration, respectively.

Table 6 Results of post hoc multiple comparisons of 2,4-D concentrations

	(I)Name	(J)Name	(I)Average value	(J)Average value	Difference value(I-J)	p
2,4-D concentrations	1	2	0.911	0.915	-0.005	0.883
	1	3	0.911	0.642	0.268	0.000**
	1	4	0.911	0.933	-0.022	0.516
	2	3	0.915	0.642	0.273	0.000**
	2	4	0.915	0.933	-0.017	0.613
	3	4	0.642	0.933	-0.290	0.000**

**: p<0.01. The meaning of number 1-4 was the same as in Table 1.

Validation of the optimal induction protocol

The orthogonal experiment also has some limitations, for example, the level of factors examined is only limited to the selected range and cannot reflect the overall situation comprehensively (Wang *et al.*, 2003). The optimal concentration of 2,4-D was the maximum of the experimental design, so it was assumed that the concentration of 2,4-D could be increased further. A protocol of DCR + (2.5, 3.0, 4.0) mg • L^{-1} 2,4-D + 1.0 mg • L^{-1} 6-BA + 0.2 mg • L^{-1} PVP + 6 g • L^{-1} agar + 20 g • L^{-1} sucrose was designed and experimentally verified. The results in Table 7 show that the callus induction rate could reach 98.7% (±1.9%) at the 2,4-D concentration of 2.5 mg • L^{-1}, but it decreased significantly with increased concentrations above 2.5 mg • L^{-1}, so the optimal 2,4-D concentration was determined at 2.5 mg • L^{-1}.

Table 7 Validation of optimal concentration of auxin

Test protocols	Culture medium	2,4-D concentration/ (mg • L^{-1})	6-BA concentration/ (mg • L^{-1})	Number of tests1	Number of tests2	Number of tests3	Average value	Standard deviation
1	DCR	2.5	1.0	1.000	0.960	1.000	0.987a	0.019
2		3.0		0.900	0.950	0.933	0.928b	0.021
3		4.0		0.942	0.913	0.927	0.927b	0.012

Notes: Different lowercase letters after values indicate a significant difference ($p < 0.05$).

On this basis, experiments with *P. thunbergii* Parl needles explant have been successfully induced (Fig. 2b), but callus production was slower than that of hypocotyls (Fig. 2a).

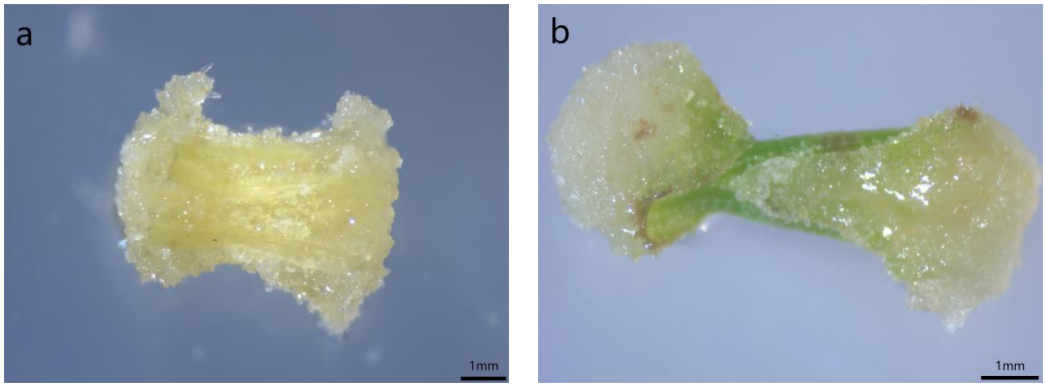

Fig. 2 Comparative observation of callus (a: Hypocotyl callus grown for 21 days, b: Needle leaf callus grown for 35 days)

Analysis of the PPO content

The hypocotyl calluses were induced with the best protocol obtained, and they were sampled after 21 days of induction and at proliferation culture stages for the determination of PPO content, respectively. As shown in Table 8, the PPO viability was significantly higher in the 21-day-induction callus, while it decreased at proliferation culture stage, and it was consistent with the conclusion obtained by Tang W *et al.* (2004) in studying the molecular mechanism of *P. virginiana* Mill. calllus. The decrease of PPO activity facilitated the maintenance of embryonic state in the callus, and the induced EC was able to maintain the embryonic state during the proliferation stage.

Table 8 Polyphenol oxidase (PPO) activity

Samples	Determination group	Control group	PPO Vitality
a	0.392	0.213	119.33
b	0.271	0.183	58.67
c	0.253	0.184	46.00

a: 21 days of induction growth; b: primary succession culture; c: secondary succession culture.

Discussion

Exploration of optimal culture media

The induction of EC is crucial for somatic embryogenesis, and the basic medium type is one of the many factors that affect the callus induction (Isah, 2016). The basic media currently used for forest tree tissue culture are MS, WPM, SH, CM and GD, among which MS and modified MS are most commonly used, and WPM is often used as a basal medium for *Pinaceae* plants (Phillips *et al.*, 2019). Li *et al.* (2006) screened three media, BM, DCR and modified MS, concluded that DCR was the most suitable medium for EC induction in *P. bungeana*. Wang *et al.* (2010) compared DCR and MS media in a somatic embryogenesis assay of immature zygotic embryos of *Larix kaempferi*, and found that the callus induction rate was higher in DCR than MS. Hu *et al.* (2022) used immature zygotic embryos as the material and tested them on B_5, DCR, MLP, MLV, WPM and WV media, the results showed that the highest EC induction rate of 40.0% was achieved on DCR media. In this study, we concluded that the best basal medium suitable for hypocotyl EC induction in *P. thunbergii* Parl was also DCR, which may be related to the content of NO^{3-} and NH^{4+}. The basic medium for conifer somatic embryogenesis consists of a combination of macronutrients, calcium salts, iron salts and organic substances, in which the NO^{3-} and NH^{4+} content plays an important role in somatic embryogenesis (Wang *et al.*, 2021).

Exploration of optimal hormones and hormone concentrations

Through the use of polar auxin transport inhibitors and substances with auxin-resistant properties, the necessity of auxin for somatic embryogenesis has been demonstrated in several plants (Jiménez, 2005). For *P. thunbergii* Parl, the optimum hormone for the induction of EC using immature zygotic embryos explants was 4 mg • L^{-1} 2,4-D + 2 mg • L^{-1} 6-BA, and the callus induction rate was only 3.33% when cultured on hormone-free medium (Sun *et al.*, 2015). In our study, both 2,4-D and 6-BA concentrations used for hypocotyl of *P. thunbergii* Parl were lower than that of immature zygotic embryos, but the EC induction rate was higher. Among the other *Pinaceae* plants, the EC induction rate of *Larix olgensis* increased with increasing 2,4-D concentration within a certain range, with the highest induction rate of 11.1% at a concentration of 1.5 mg • L^{-1}, and began to decrease when the 2,4-D concentration exceeded 1.5 mg • L^{-1} (Song *et al.*, 2016). The results of the study on somatic embryogenesis of nematode-resistant *P. massoniana* Lamb. showed that the combination of 2,4-D and 6-BA was used for better EC

induction rate, and the highest induction rate of 27.8% was achieved by adding 2.0 mg • L^{-1} 2, 4-D and 1.0 mg • L^{-1} 6-BA (Chen *et al.*, 2019). Both 2,4-D and 6-BA play an important role in the EC induction in most *Pinaceae* plants. However, there are exceptions, for *P. elliottii* E., kinetin had a significant effect on the EC induction rate with an average induction rate of up to 22.2%, while the addition of different concentrations of 2,4-D and 6-BA did not have a significant difference (Cheng *et al.*, 2021). Therefore, the suitable hormones and concentrations for different *Pinaceae* plants would be different, and the concentration of hormones is not the higher the better, but needs to be tested to find the best value.

Our study showed that 2,4-D had a significant effect on the induction of callus, and the induction rate reached the maximum when 2,4-D concentration was 2.5 mg • L^{-1}, and it decreased significantly after increasing the concentration further, which is consistent with the previous study that 2,4-D can act as a growth factor to promote cell division and elongation at low concentrations, while higher concentrations act as an herbicide to inhibit plant growth instead (Song, 2013). It was found that the EC induction rate was significantly lower with only 2,4-D than with both 2,4-D and 6-BA (Li *et al.* 2006), and the interaction analysis of orthogonal test also showed that there was a significant interaction between 2,4-D and 6-BA, suggesting that the effect of 2,4-D would enhanced with 6-BA, and the combination of both was more favorable for somatic embryogenesis, and there may be complex links between the two hormones in the signal transduction pathways (Phillips *et al.*, 2019).

Study prospect

In this study, the hypocotyl of *P. thunbergii* Parl was used as the explant material, and the best medium for callus induction was initially explored. On this basis, experiments were conducted with needles explants, and callus has been successfully induced, but the callus production was slower than that of hypocotyl explants. Subsequent experiments need to be done to explore the role of the PVP in EC identification and maintenance of hypocotyl and needle explants, to enrich propagation materials such as young shoot tips or root tips, and to establish a complete system of somatic embryogenesis of vegetative organs in *P. thunbergii* Parl.

Conclusions

The best basal medium for the EC induction of hypocotyls was DCR, and the best hormone concentration ratio was 2.5 mg • L^{-1} 2,4-D + 1.0 mg • L^{-1} 6-BA, which resulted in 98.7% (±1.9%) callus induction. Among the three factors of the experimental design, only 2,4-D concentration had a significant effect on the induction rate of callus ($p<0.05$), while the results of interaction analysis showed that there was a significant interaction effect ($p<0.05$) between the combination of both 2,4-D and 6-BA, which indicated that the concentration level of 6-BA itself had no effect on the experimental results, but there was an interactive effect when it was combined with 2,4-D. The structure of EC was clearly observed, and an embryonic head with several suspensor cells were observed by acetate magenta and Evans blue staining.

Author contributions P.M conceived and planned the paper; J.D wrote and C.G edited the manuscript. J.L provided insight and suggestions to improve the manuscript. All authors read and approved the manuscript.

Funding This study is funded by National Natural Science Foundation of China (31770675) and Qingdao Agricultural University Doctoral Start-Up Fund (20210048)

Data availability Data available upon reasonable request to the corresponding author.

Declarations

Conflict of interest The authors have no confict of interest to disclose

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