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High-Efficiency Somatic Embryogenesis from Seedlings of *Koelreuteria paniculata* Laxm.

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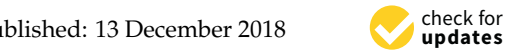

Abstract: Research Highlights: In the current study, we established a method for plant regeneration via somatic embryogenesis (SE) in *Koelreuteria paniculata* Laxm. for the first time. Background and Objectives: *K. paniculata* is an important ornamental and medicinal plant in China. However, the plant has difficulty with asexual reproduction, which imposes a limitation on large-scale propagation. Materials and Methods: Embryogenic calluses were induced from stems of aseptic seedlings on induction media. The effects of different media types and concentrations of N6-benzyladenine (BA), α-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) on callus induction were examined. Embryogenic calluses were then transferred to Driver-Kuniyuki Walnut (DKW) media containing NAA (0.1–0.2 mg L^{-1}) or 2,4-D (0.5–2.0 mg L^{-1}) to develop somatic embryos. Cotyledon embryos were cultured on DKW media containing NAA (0.1–0.2 mg L^{-1}) until maturation, and were then transferred to 1/2 DKW medium supplemented with 1.0 mg L^{-1} indole-3-butyric acid (IBA) to produce complete plants. The effects of IBA and NAA on rhizogenesis were then examined by clonal culture. Results: The maximum callus induction frequency (80.25%) was obtained on DKW medium supplemented by 0.5 mg L^{-1} BA, 0.25 mg L^{-1} NAA, and 1.5 mg L^{-1} 2,4-D. NAA had a more pronounced effect on somatic embryo growth than did 2,4-D, with a maximum SE frequency (54.75%) observed with 0.1 mg L^{-1} NAA added to DKW medium. For clonal culture, the highest rooting rate (52%) was observed on 1/4 DKW medium containing 1.5 mg L^{-1} IBA. Histology studies confirmed the presence of embryogenic calluses and somatic embryos in different stages. Conclusions: This protocol provides a novel method for large-scale propagation of *K. paniculata*, and creates opportunities for genetic engineering in this species.

Keywords: clonal culture; germination; histology; plant growth regulators; somatic embryos

1. Introduction

Koelreuteria paniculata Laxm. (family Sapindaceae) is an important ornamental plant [1,2] with pale yellow flowers and green leaves; it is often cultivated as street and park vegetation in Northern China [3]. *K. paniculata* also has important medicinal properties because it is rich in secondary metabolites, including gallate derivatives, cyanolipids, and flavonoids [3–5]. Secondary metabolites, which play an important role in plant growth, have been shown to affect plant defenses [6,7]. The seeds and leaves of *K. paniculata* are often used as antifungal and antibacterial agents [3]. *K. paniculata*

also has an important environmental protection value acting as a heavy metal accumulator in phytoremediation [8].

K. paniculata is difficult to propagate because of hard seed shells and a long dormancy period. The seeds have low and variable germination rates, even within populations [9]. Although much research has been conducted in an attempt to improve germination rates [2,10,11], it has proved insufficient for the development of *K. paniculata* breeding programs. Conventional hybridization in this species is a difficult and time-consuming process due to its small flowers. Cutting and grafting are also difficult to apply to *K. paniculata* because the high levels of flavones cause the wounds to brown easily. These difficulties in obtaining and maintaining *K. paniculata* propagules limit large-scale propagation and genetic transformation in this species. Hence, it is important to develop an efficient approach to the clonal propagation of elite *K. paniculata* lines.

Plant tissue culture technology is a rapid and efficient way to obtain clonal plants in plant breeding. Somatic embryogenesis (SE) and organogenesis are the two dominant methods in tissue culture [12], offering advantages including large-scale regeneration, germplasm conservation, and genetic transformation [13–15]. On the other hand, SE may produce unicellular somatic embryos [16], and it can reduce the production of chimeras of single-cell origin [12,17]. Since somatic embryos are similar to zygotic embryos, SE can be used in seed synthesis, which is favorable for the handing, storage, shipping, and commercial application of elites [16]. Similarly, somatic embryos also can be used as models to study the development of zygotic embryos. Through comparative genomics and morphological development research, it has been shown that somatic and zygotic embryos have similar developmental features and gene expression patterns [18]. Similar results were also observed in a walnut study, in which somatic and zygotic embryos shared similar developmental patterns in protein profiling [19].

The main objective of this study was to develop an effective approach to *K. paniculata* regeneration though SE. Therefore, we investigated the potential of different media types and different concentrations of plant growth regulators (PGRs) to initiate embryogenic calluses. We examined the effects of 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthaleneacetic acid (NAA) as PGRs as stimulating factors on SE induction and the influence of indole-3-butyric acid (IBA) and NAA on rooting in clones.

2. Materials and Methods

2.1. Plant Materials and Culture Initiation

Immature *K. paniculata* seeds were collected from Beijing Forestry University, Beijing, China during November 2016, approximately 60-days post-anthesis. Seed collections were performed when the seed diameter reached 5–6 mm, the seed coat became soft green or slightly brown, and embryo development was completed (Figure 1a).

To initiate culture, immature seeds were collected and rinsed with running water for 2 h. The seeds were then surface decontaminated in 75% alcohol (v/v = Alcohol volume/Total volume) for 30 s and rinsed twice with sterile water, followed by soaking with sodium hypochlorite (Analytical reagent, available chlorine concentrations $\geq$7%) for 5 min. Finally, the seeds were thoroughly rinsed five times with sterile water, and the seed coats were peeled off using scissors and tweezers. Sterile seeds were inoculated on 1/2 Driver-Kuniyuki Walnut (DKW) medium [20] supplemented with 20 g L^{-1} sucrose, 5.5 g L^{-1} agar (Biodee, Beijing, China), and 1.0 mg L^{-1} IBA. The pH was adjusted to 5.8 in all media, followed by autoclaving at 121 °C for 15 min. Sterile plants were cultured under a 16-h photoperiod provided by cool white fluorescent lamps (40–60 µmol m^{-2} s^{-1}), at a temperature of 25 $\pm$ 2 °C.

Figure 1. *Koelreuteria paniculata* Laxm. plant regeneration from in vitro culture of zygotes and from somatic embryogenesis (SE). (**a**) Immature seeds. (**b**) Aerial roots (arrow in **b**) incubated on I1 medium. (**c**) Browning stem segment incubated on I3 medium. (**d**) Calluses incubated on I6 medium. (**e**) Embryogenic calluses incubated on DKW (Driver-Kuniyuki Walnut) medium. (**f**) Aerial roots (arrow in **f**) on IEM3 (IEM, Induction Medium of Embryogenic Calluses). (**g**) Callus cultured for more than three months. **(h)**. (**k**) Globular and cotyledon embryos, respectively, under light. (**i**) Plantlet derived via SE. (**j**) Plantlet derived via zygotic embryo. (**l**) White, translucent embryogenic calluses (arrow in **l**) and cotyledon embryos cultured in darkness. (**m**) Brown, hard embryogenic calluses cultured in light. (**n**) Calluses (arrow in **n**) produced in clonal culture of stem sections. (**o**) Plantlets from zygotic embryos. Scale bar: **a**–**r** = 1 cm, **s** = 10 cm.

2.2. Embryogenic Callus Induction and Proliferation

After two months of culture, the well-developed seedlings (Figure 1o) were used as the material for callus induction. The stem tips of seedlings were removed, and the stem segments (1–2 cm) without leaf primordia were used as explants. The explants were inoculated on nine different callus induction media in the pre-experiment (Table 1). Based on the results of the preliminary experiment, new stem explants were inoculated on four improved callus induction media, DKW media with a combination of N^6-benzyladenine (BA), NAA, and 2,4-D (Table 2). After 2 months, embryogenic calluses, friable and yellowish calluses, were obtained on improved induction media. To further improve the frequency of embryogenic callus induction, they were sub-cultured every month on the four improved callus induction media. All callus induction media were supplemented with 5.5 g L^{-1} agar and 30 g L^{-1} sucrose.

Table 1. Effects of medium types and plant growth regulator (PGR) concentrations on callus induction in *K. paniculata*.

Number	Basic Medium	PGR (mg L^{-1})			Calluses Induction Frequency + SE (%)	Cultured Conditions
		BA	NAA	2,4-D		
I1	MS	0.25	0.25	0.50	40.47 + 6.47 ^{bc}	rhizogenesis
I2	MS	0.50	0.50	1.00	51.87 + 6.47 ^{b}	non-embryogenic callus
I3	MS	0.75	0.75	1.50	0.00 + 00.00 ^{d}	browning
I4	B5	0.25	0.50	1.50	25.90 + 6.41 ^{c}	rhizogenesis
I5	B5	0.50	0.75	0.50	40.70 + 6.41 ^{bc}	rhizogenesis
I6	B5	0.75	0.25	1.00	29.60 + 6.41 ^{c}	non-embryogenic callus
I7	DKW	0.25	0.75	1.00	85.20 + 6.41 ^{a}	rhizogenesis
I8	DKW	0.50	0.25	1.50	81.50 + 6.41 ^{a}	embryogenic callus
I9	DKW	0.75	0.50	0.50	77.80 + 11.10 ^{a}	embryogenic callus

Date represented mean ± SE of all replicates, each with nine cultures. Lowercase letters represent the result of multiple comparisons, means having the same letter were not significantly different by Tukey's multiple range test ($p \leq 0.05$). PGR, plant growth regulator; BA, N6-benzyladenine; NAA, α-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog medium; B5, Gamborg medium.

Table 2. Effects of 6-benzylaminopurine (6-BA), 1-naphthaleneacetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) on callus induction in *K. paniculata*.

Number	PGR (mg L^{-1})			Calluses Induction Frequency ± SE (%)
	BA	NAA	2,4-D	
IEM1	0.50	0.25	1.50	80.25 ± 0.08
IEM2	0.75	0.25	1.50	70.25 ± 0.09
IEM3	0.50	0.50	0.50	69.50 ± 0.04
IEM4	0.75	0.50	0.50	74.25 ± 0.05

Date represented mean ± SE of all replicates, each with nine cultures, and there was no significant difference between dates by Tukey's multiple range test ($p \leq 0.05$).

2.3. Somatic Embryogenesis and Maturation

Subsequently, embryogenic calluses were transferred to regeneration media, i.e., DKW medium supplemented with 2,4-D (0.5–1.5 mg L^{-1}) or NAA (0.1–0.2 mg L^{-1}) for SE induction. Medium without PGRs was used as the control. Embryos (globular, reniform, torpedo, and cotyledon embryo) obtained from the regeneration media were sub cultured on maturation media, i.e., DKW medium supplemented with NAA (0.1–0.2 mg L^{-1}) under light (16-h photoperiod) or dark conditions. All regeneration and maturation media were supplemented with 5.5 g L^{-1} agar and 30 g L^{-1} sucrose. Finally, mature embryos (Figure 2h), which are dark green and elongated radicles, were transferred to 1/2 DKW medium supplemented with 20 g L^{-1} sucrose, 5.5 g L^{-1} agar, and 1.0 mg L^{-1} IBA for better growth.

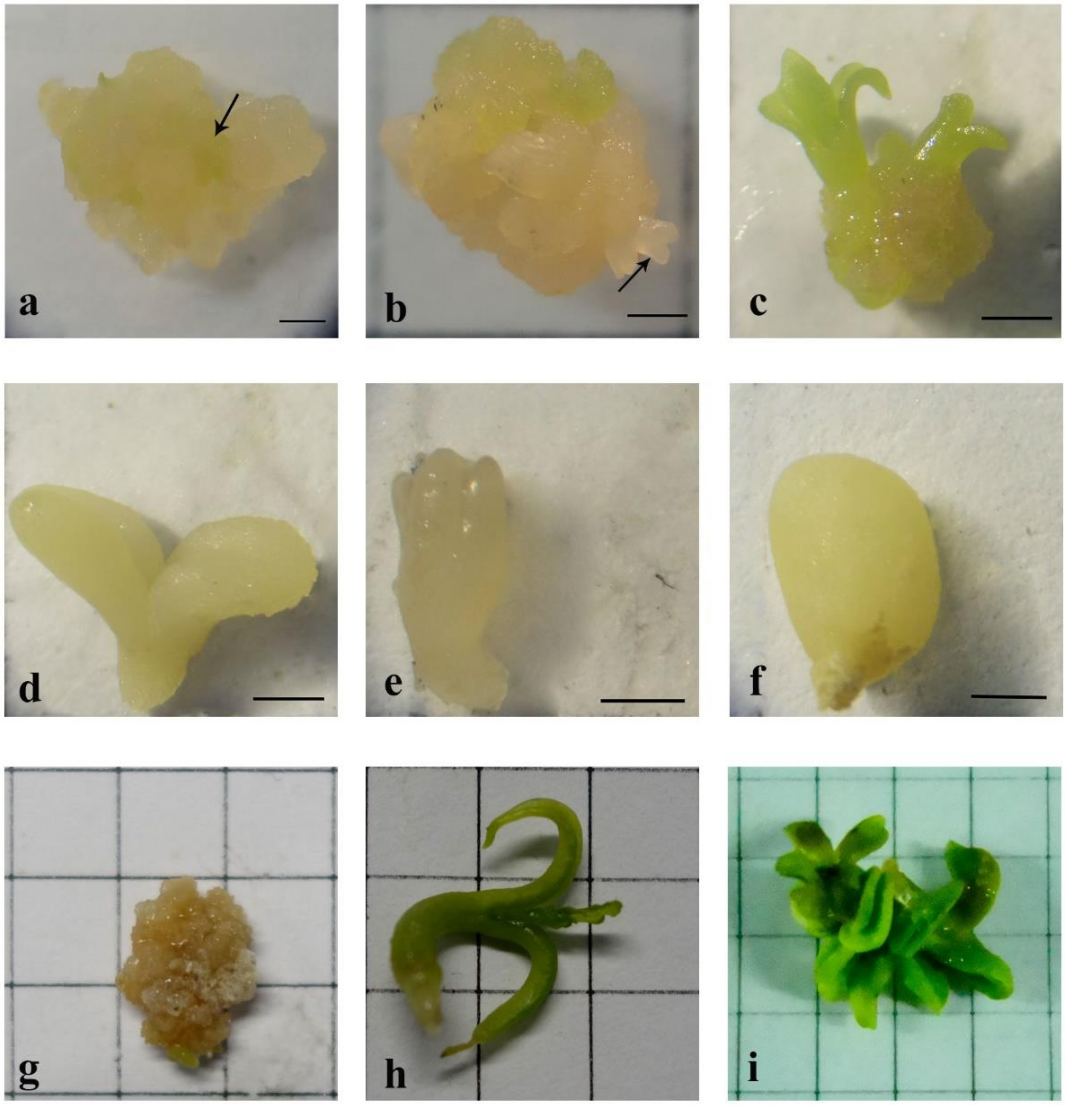

Figure 2. Characterization of in vitro morphogenic responses of *K. paniculata* embryogenic calluses. (**a**) Embryogenic callus with smooth surfaces, green embellishments (arrow in **a**) and nodular protrusions. (**b**–**c**) Cotyledon embryo (arrow in **b**) derived via embryogenic callus. (**d**) Normal cotyledon embryo. (**e**) Fused cotyledon embryo. (**f**) Monocotyledonous embryo. (**g**) Sub-cultured embryogenic callus. (**h**) Mature cotyledon embryo. (**i**) Mature fused cotyledon embryo. Bars in **a**–**f** represent 0.2 cm. Size of squares in **g**–**i** are 1 cm × 1 cm.

2.4. Histology

Histological analyses of embryogenic calluses and somatic embryos were performed according to [21]. Plant materials were fixed in a fixative solution (formaldehyde:glacial acetic acid:alcohol, 8:1:1), and then dehydrated using graded ethanol (50, 70, 85, 95, and 100% v/v). Processed materials were embedded in paraffin wax and cut using a microtome device (model no. RM2235, Leica, Frankfurt, Germany) to obtain thin sections (8 µm). Aniline blue was used for staining. The stained sections were observed by fluorescence microscopy (BX61, Olympus, Tokyo, Japan) and photographed using a camera (DP72, Olympus, Tokyo, Japan).

2.5. Clonal Culture

Plantlets obtained via SE were prepared for clonal rooting culture after about 2 months of culture. We inoculated shoot segments that were 2–3 cm in length on 1/4 DKW medium supplemented with

various concentrations of IBA (0.5–2.5 mg L^{-1}). To optimize the rooting rate, we also analyzed the combined effect of IBA and NAA. All media were supplemented with 5.5 g L^{-1} agar and 20 g L^{-1} sucrose.

2.6. Statistical Analysis

All experiments were performed with three replicates, and each experiment contained nine cultures. Rates of callus induction were calculated after 2 months of incubation in darkness. Frequency of SE was analyzed after calluses had been cultured for 45 days on DKW media supplemented with NAA or 2,4-D. Numbers of somatic embryos were confirmed by classification (Table 3) because they were difficult to count. The effect of the culture condition on somatic embryo maturation was confirmed by measuring the length of hypocotyl and cotyledon, and somatic embryo germination rates under light were calculated after 2 weeks. Rooting rates were calculated to assess root induction after 2 months of incubation. All data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test using the SPSS software (version 19.0 for Windows, SPSS Inc., IBM, Armonk, NY, USA). Significance was determined at a level of $p \leq 0.05$. All frequencies were calculated using the below formulas:

$$\text{Calluses induction rate} = \frac{\text{Number of explants producing calluses}}{\text{Total number of explants}} \times 100\% \quad (1)$$

$$\text{Frequency of SE} = \frac{\text{Number of explants producing somatic embryos}}{\text{Total number of explants}} \times 100\% \quad (2)$$

$$\text{Germination rate} = \frac{\text{Number of germinated cotyledon embryos}}{\text{Total number of cotyledon embryos}} \times 100\% \quad (3)$$

$$\text{Rooting rate} = \frac{\text{Number of rooting explants}}{\text{Total number of explants}} \times 100\% \quad (4)$$

Table 3. Effects of 2,4-D or NAA on SE frequency in *K. paniculata*.

Plant Growth Regulators (mg L^{-1})	Frequency of Somatic Embryogenesis ± SE (%)	Number of Somatic Embryos
2,4-D		
0	25.50 ± 0.04 ^{bc}	*
0.50	23.25 ± 0.08 ^{c}	**
1.00	46.75 ± 0.05 ^{ab}	***
1.50	32.50 ± 0.12 ^{abc}	**
NAA		
0.10	54.75 ± 0.16 ^{a}	***
0.20	37.75 + 0.09 ^{abc}	***

Date represented mean ± SE of all replicates, each with nine cultures. Means having the same letter were not significantly different by Tukey's multiple range test ($p \leq 0.05$). *, represents the number of SEs per culture less than 10. **, represents the number of SEs per culture between 10 and 30. ***, represents the number of SEs per culture more than 30.

3. Results

3.1. Embryogenic Callus Induction and Proliferation

The types of basic culture media and the concentrations of PGRs significantly affected the formation of embryogenic callus in *K. paniculata* (Table 1). The calluses cultured on I7, I8, and I9 media obtained the highest induction rates. The embryogenic callus was obtained only on I8 and I9 media. Different concentrations of PGRs also affected the formation of the callus in *K. paniculata*. Inappropriate concentrations of PGRs even led to rhizogenesis (Figure 1b) and browning (Figure 1c),

which were not suitable for subsequent experiments. The callus (Figure 1d) obtained from the I6 medium was too small and hardly survived in subculture, similar results were also obtained on the I2 medium. In the improved induction media, there were no significant differences in callus induction rates among IEM1, IEM2, IEM3, and IEM4 (IEM, Induction Medium of Embryogenic Calluses) (Table 2) ($p \leq 0.05$). The maximum induction rate was achieved on IEM1 (80.25%) after 2 months of culture. Friable, yellowish, embryogenic calluses were obtained on all improved induction media (Figure 1e). However, there were differences between the improved induction media during callus proliferation. Calluses obtained from IEM3 produced aerial roots when sub-cultured two or three times (Figure 1f). Calluses incubated on IEM4 grew slowly; some became brown and died. IEM1 and IEM2 were the better culture conditions for callus proliferation. The concentrations of 2,4-D and NAA played a role in the process of callus proliferation. A high concentration of 2,4-D and low concentration of NAA were more beneficial than other concentrations for the proliferation of embryogenic callus. In addition, the culture time also had an effect on the callus proliferation. Calluses (Figure 1g) that were sub cultured on induction media for more than three months were found to be darker in color than those obtained under primary induction, but all had embryogenetic potential.

3.2. Somatic Embryogenesis and Maturation

The concentration of PGRs significantly affected the frequency of somatic embryo formation. Both high (1.5 mg L^{-1}) and low (0.5 mg L^{-1}) concentrations of 2,4-D failed to promote the SE. The highest frequency of somatic embryo formation was 54.75%, obtained on DKW medium supplemented with 0.1 mg L^{-1} NAA, indicating that low concentrations of NAA had an important effect on SE in *K. paniculata* (Table 3). Interestingly, two different types of embryogenetic calluses, which produced somatic embryos, were obtained in regeneration media: white, translucent calluses (Figure 1h) and brown, hard calluses (Figure 1k).

The cotyledon embryos were transferred to fresh medium (DKW + NAA) to improve growth during maturation. In the process, light had a beneficial effect on the growth of somatic embryos cultured with NAA. Somatic embryos obtained from media containing NAA were bright green when cultured under light (Figure 1l,m), whereas, somatic embryos incubated in the dark were blanched (Figure 1h), and when exposed to light they turned green. The culture condition also affected the growth of cotyledon embryos. Hypocotyl length of cotyledon embryos cultured under light was longer than that in the dark condition, while the length of cotyledon had no significant difference between the two culture conditions (Table 4). The concentration of NAA also had an effect on the growth of cotyledon embryos, but there was no significant impact on germination rates due to different concentrations of NAA (90.83%, 92.81%) under light. However, germination rates tailed off to below 30% when embryogenic calluses were sub-cultured five times. Cotyledonary embryos incubated on media supplemented with NAA under light for about 2 weeks developed a dark green and elongated white radicle (Figure 2h). The plantlets were subsequently transferred to flasks containing 1/2 DKW medium supplemented with 1.0 mg L^{-1} IBA to obtain complete plants. Compared to the seedlings (Figure 1j), sturdy plants (Figure 1i) obtained via SE exhibited no obvious morphological differences.

Table 4. Effects of NAA and culture condition on cotyledon embryo growth in *K. paniculata*.

NAA (mg L^{-1})	Condition of Culture	Length of Hypocotyl ± SE (cm)	Length of Cotyledon ± SE (cm)
0.1	Light	1.19 ± 0.12 [b]	0.53 ± 0.10
0.2	Light	1.50 ± 0.13 [a]	0.58 ± 0.08
0.1	Dark	0.44 ± 0.10 [d]	0.49 ± 0.08
0.2	Dark	0.64 ± 0.09 [c]	0.51 ± 0.09

Date represented mean ± SE of all replicates, each with nine cultures. Means having the same letter were not significantly different by Tukey's multiple range test ($p \leq 0.05$).

3.3. Morphology and Histology Analysis

During callus induction, procambium was produced from non-embryogenic calluses in *K. paniculata* (Figure 3a). Compared to non-embryogenic calluses, cells of embryogenic calluses had clear boundaries and were mainly square or oval in shape (Figure 3b). Non-embryogenic calluses exhibited a disorderly appearance (Figure 3a). Primary embryogenic calluses (Figure 2a) and sub-cultured calluses (Figure 2g) all had smooth surfaces and nodular protrusions. The nodular calluses gradually evolved into globular or cotyledonary embryos as development progressed (Figure 2b,c). The appearance of globular embryos was accompanied by the formation of protoderm, and globular embryos gradually separated from embryogenic calluses (Figure 3c,d). The globular embryos then evolved into kidney-shaped embryos, with polar elongation at both ends (Figure 3e). The differentiation of the shoot apical meristem then resulted in the separation of two cotyledon embryos, and the polar growth of the root meristem led to hypocotyl elongation. During this process, heart-shaped (Figure 3g) and torpedo-shaped embryo (Figure 3h,j) morphologies appeared successively. After hypocotyl growth was completed, the two cotyledons continued to develop, eventually forming cotyledonary embryos (Figures 2d and 3i). In mature cotyledon embryos, tight border tissue (Figure 3k,m) and vascular tissue (Figure 3l) was also observed. Similarly, deformed embryos were observed, including fused cotyledons (Figure 2e,i) and monocotyledons (Figures 2f and 3f).

3.4. Clonal Culture

Different concentrations of IBA had a significant impact on rhizogenesis in *K. paniculata*. Higher rooting rates were observed when clones were cultured with 1.0–1.5 mg L^{-1} IBA (Figure 4). Stem elongation was inhibited as IBA concentration increased, especially when they were cultured with 2.5 mg L^{-1} IBA. The combined application of NAA and IBA failed to improve the rooting rate (Table 5) and even extended the plantlet response time. Stem segments incubated with NAA and IBA produced calluses (Figure 1n) while preventing root production.

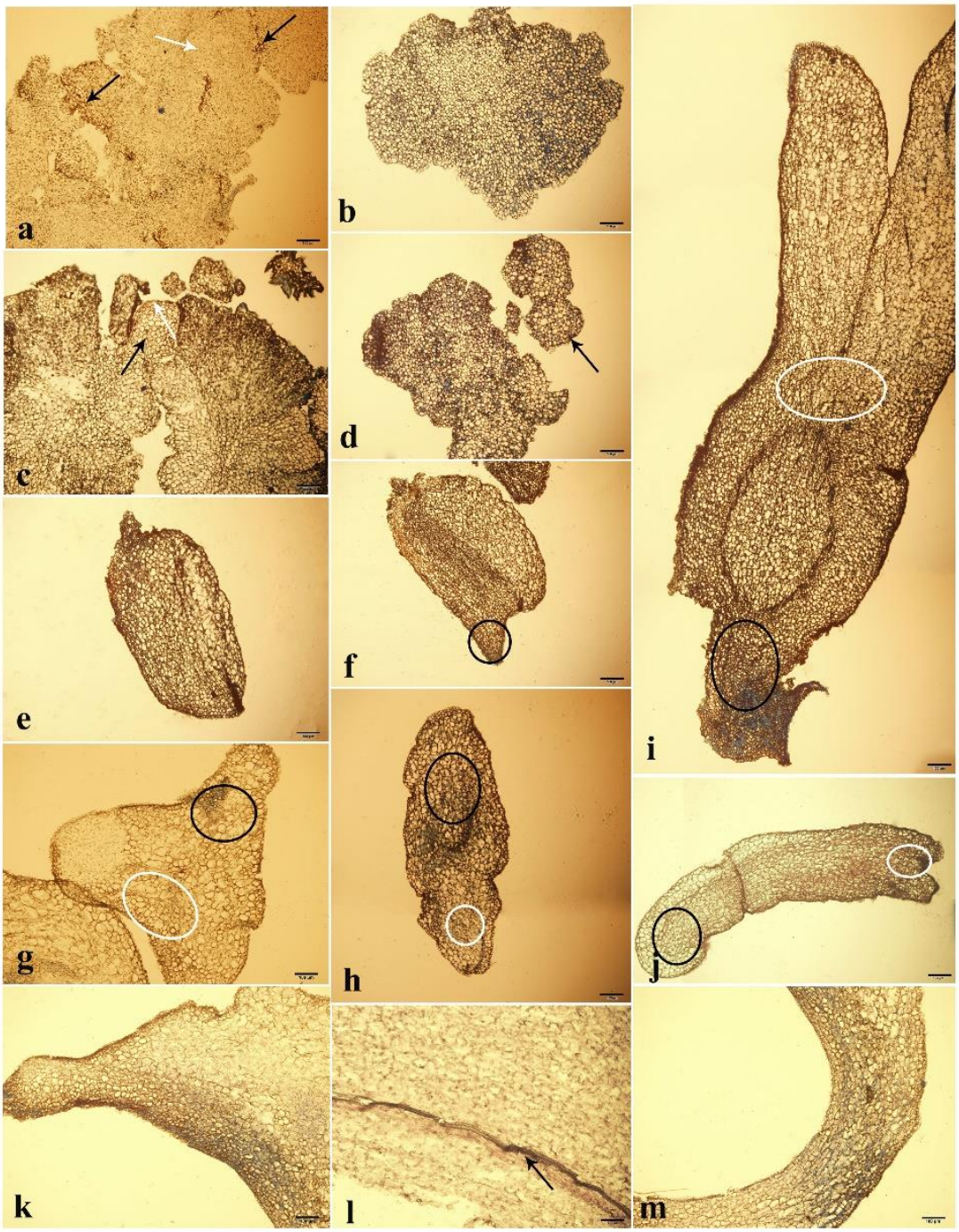

Figure 3. Various stages of somatic embryo induction and development in *K. paniculata*. **a** Procambia (black arrow in (**a**) in non-embryogenic calluses (white arrow in **a**). (**b**) Embryogenic calluses. (**c**–**d**) Globular embryos (black arrow in **c** and **d**) separated from calluses, protoderm (white arrow in **c**) formation in globular embryo. (**e**) Kidney-shaped embryos. (**f**) Root meristem (black circle in **f**) in monocotyledonous embryo. (**g**) Shoot apical meristem (white circle in **g**) and root meristem (black circle in **g**) in heart-shaped embryo. (**h**), (**j**) Shoot apical meristem (white circle in **h**, **j**) and root meristem (black circle in **h**, **j**) in torpedo-shaped embryos. (**i**) Shoot apical meristem (white circle in **i**) and root meristem (black circle in **i**) in cotyledon embryo. (**k**) Cotyledon longitudinal section in mature cotyledon embryo. (**l**) Vascular tissue (black arrow in **l**) in mature cotyledon embryo. (**m**) Hypocotyl longitudinal section in mature cotyledon embryo. Scale bar: **a**–**k**, **m** = 100 µm; **l** = 50 µm.

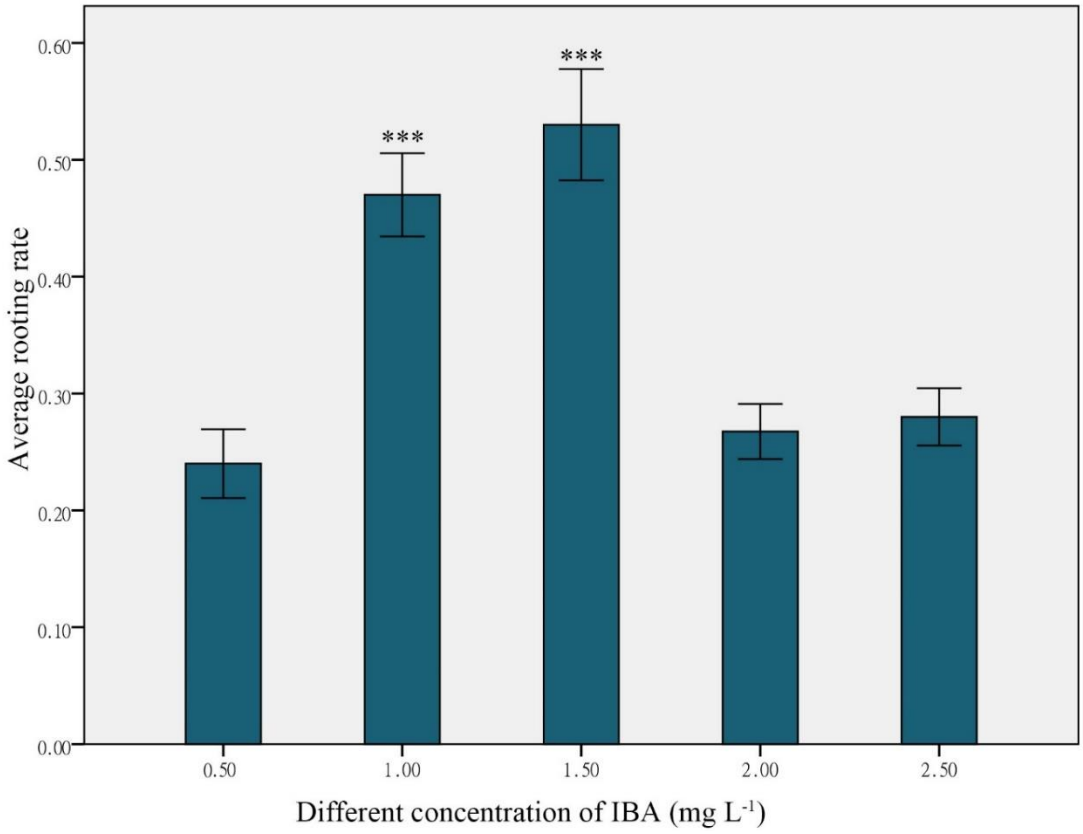

Figure 4. Average rooting rates for cultures supplemented with various concentrations of indole-3-butyric acid (IBA), bars represent standard error. ***, there was a significant difference by Tukey's multiple range test ($p \leq 0.01$).

Table 5. Rooting rates on different concentrations of indole-3-butyric acid (IBA), alone or in combination with NAA.

IBA (mg L^{-1})	NAA (mg L^{-1})	Rooting Rate ± SE (%)
1.0	0	47.67 ± 0.10
1.5	0	52.00 ± 0.13
1.0	0.1	42.67 ± 0.09
1.0	0.2	37.33 ± 0.03
1.0	0.3	33.17 ± 0.04
1.5	0.1	49.67 ± 0.05
1.5	0.2	37.67 ± 0.04
1.5	0.3	39.67 ± 0.05

Date represented mean ± SE of all replicates, each with nine cultures, and there was no significant difference between dates by Tukey's multiple range test ($p \leq 0.05$).

4. Discussion

4.1. Effect of Plant Growth Regulators on Somatic Embryogenesis

SE is an important method of plant regeneration, and several researchers have applied this technology to various plant species in recent years [21–24]. However, the process of SE is affected by many factors. PGRs, which are important factors in plant development, play a crucial role in SE [25,26]. In this study, combinations of 2,4-D, NAA, and BA were used to induce calluses in *K. paniculata*. There were obvious differences in the characteristics of induced calluses between PGR concentration levels. The media containing low concentrations (0.25 mg L^{-1}) of BA all failed to produce normal calluses. The highest induction rate (80.25%) was obtained on DKW medium

supplemented by 0.5 mg L^{-1} BA, 0.25 mg L^{-1} NAA, and 1.5 mg L^{-1} 2,4-D, which was similar to the study performed in *Elaeis guineensis* Jacq. [27]. Inappropriate PGR concentrations led to browning and rhizogenesis. In addition, media supplemented with 0.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} NAA performed poorly in callus propagation. Aerial roots and slow proliferation were observed in treatments IEM3 and IEM4, respectively, indicating a relationship between PGR concentration and propagation rate. The combination of 2,4-D and other PGRs during SE has been reported for many woody plants [22,28–30], and 2,4-D was proved to be an important induction factor in embryogenic callus induction. In addition, studies about the effects of 2,4-D and other PGRs on embryogenic callus induction were reported. In the study about *Elaeis guineensis*, 2,4-D was proved to have similar effects on embryogenic callus induction to picloram [27], and similar results were also obtained in oil palm [31]. For SE, some studies showed that high 2,4-D concentrations are conducive to embryogenic callus induction and proliferation, and that the reduction or removal of 2,4-D promotes SE [32,33]; however, conflicting results were reported. In the current study, low (0.5 mg L^{-1}) and high (1.5 mg L^{-1}) 2,4-D concentrations were inappropriate for somatic embryo development, which indicated that suitable concentrations of 2,4-D was also important for the development of SE in *K. paniculate*.

We also examined the effect of NAA on SE in this study. Although the concentrations of NAA had no significant effect on the frequency of SE, higher frequency (54.75%) was obtained on DKW medium supplemented by 0.1 mg L^{-1} NAA. This result may indicate that low concentrations of NAA were suitable for SE in *K. paniculate*. In the studies of other woody plants, the frequency of somatic embryogenesis varied from 4.9% to 63% [27–29,34], and the frequency of SE in *K. paniculate* was at a high level in the research. Similarly, NAA has been reported to influence somatic embryo development in holm oak and *Eucalyptus camaldulensis* Dehn. [28,35]. In the study about *Elaeis guineensis*, the ability of NAA to promote somatic embryo development is poor compared with other PGRs [27]. Moreover, other PGRs, such as strigolactone and picloram, have been used to enhance SE in other plants [21,36–38], and picloram performed better than NAA in *Eucalyptus* species [17]. However, in a study of *Chamaecyparis thyoides* L., SE was achieved without the help of plant growth regulators, and the authors speculated that PGRs have no effect on SE in some conifers including *Chamaecyparis* species due to their high endogenous indole-3-acetic acid (IAA) levels [22]. A similar result was also reported in ferns [39]. In addition, NAA was also used to promote somatic embryo maturation. In the study about date palm, NAA was proved to have an effect on somatic embryo germination [40]. In the study, despite the concentration of NAA not having a significant effect on somatic maturation, the date plams all obtained higher germination rates. Therefore, the selection of PGR types and concentrations is an important condition for the development of somatic embryos in woody plants.

4.2. Effect of Basic Media on Somatic Embryogenesis

PGRs are thought by some researchers to play a role in growth regulation and as a source of stress stimulation [30]. It is believed that stress stimulation is an essential promoter of SE. There are several other methods to apply stress stimulation during SE, including inorganic nutrients, hydraulic pressure, and oxidation-reduction potential application [30,41,42]. Basic media and nutrient elements used in plant growth have been reported to affect SE in many species. A study of mango ginger indicated that 1/2 Murashige and Skoog (MS) liquid medium and an optimal concentration of sucrose were two important factors for SE induction, and that sugar provided the required carbon and affected the osmotic potential of the medium [43]. Similar results were also obtained in a study on *Rhinacanthus nasutus* (L.) Kurz., in which SE occurred on 1/2 MS medium [44]. In the current study, three basic media, DKW, MS, and B5 (Gamborg medium) media, were used for callus induction in the preliminary experiment, and embryogenic calluses were only obtained on DKW medium. Calluses obtained from MS (Figure 1b,c) and B5 (Figure 1d) failed to produce somatic embryos, which may have been caused by the difference in the three media. Compared with B5 and DKW medium, MS medium contains higher nitrate components, and the concentrations of inorganic salts

and ions in the medium is higher than that of the other media [45]. In the B5 medium, the content of ammonium is lower, which was considered to be suitable for the culture of dicotyledonous plants [46]. Unlike MS and B5, the DKW medium has a high concentration of calcium, and replaces cobalt with nickel. In addition, the content of organic elements in DKW medium was much lower than that in the other two media [20]. In this study, the explants cultured on MS and B5 media also produced calluses, but hardly survived in sub-culture. Thus, the comprehensive selection of basic medium and PGRs concentration will be an important research hotspot in future research.

4.3. Other Factors Influencing Somatic Embryogenesis

The choice of plant materials also plays a crucial role in plant regeneration [47]. Past studies about SE have revealed that the development stage, explant site, and the genotype of explants affect SE [12,35,48]. In the current study, we obtained aseptic seedlings from immature seeds, and plantlet stems were then used to induce embryogenic calluses. Young *K. paniculata* seeds (Figure 1a) used as initial material have great potential for the induction of embryogenic calluses. Similar studies of other species have indicated that seeds are a good choice for embryogenic callus induction [29,34,49,50]. In addition, the genotype also has an effect on embryogenic callus induction rates. Park et al. [35] determined that SE induction was under strong genetic control in white spruce, similar results were also obtained in the study about holm oak. Although the current study, involving seeds from a half-sibling family, attempted to eliminate the influence of genotypes, the effect of different seeds could not be ignored during the induction and development of somatic embryos. Thus, future *K. paniculata* breeding work should prioritize the selection of plant material. Furthermore, in an ongoing study, we investigated light as a factor affecting somatic embryos maturation, with results showing that light promotes cotyledon formation, color, and morphology; these findings are consistent with those of a study on *Alnus glutinosa* L. [51]. However, light exposure was detrimental during early somatic embryo development. It may be that light exposure is beneficial to SE only after spherical embryo formation. Similarly, in a study of Sengon (*Falcataria moluccana* (Miquel) Barneby & Grimes), dark treatment followed by light treatment was reported to be beneficial to SE [52]. However, different results were reported in a study on strawberry trees [53], in which light inhibited callus formation and SE, and similar results were also obtained in *Larix kaempferi* Sarg. [34]. Thus, light is a factor that should be considered in future SE studies.

4.4. Morphology and Histology Analysis

Our observations of morphology and histology revealed processes in the course of *K. paniculata* somatic embryo development. Embryogenic calluses obtained in this study had nodular protrusions and smooth surfaces, which is similar to the findings of studies of SE in other species [16,21,54]. Primary embryogenic calluses obtained from induction medium were yellow-white with green embellishments (Figure 2a). Following transfer to regeneration medium, two different forms of embryogenic calluses were observed: white, translucent calluses (Figure 1h) and brown, hard calluses (Figure 1k). These different callus forms may have been caused by culture conditions. White, translucent calluses were obtained under dark conditions and long breeding periods, which is consistent with the results of SE studies of other species [43,44,54]. However, brown, hard calluses were achieved when primary calluses were exposed to light for almost 1 month. Quiroz-Figueroa et al. [16] reported a similar result; they observed that non-embryogenic calluses were rough, friable, and translucent, and that embryogenic calluses were brown and hard, similar to the second type of calluses observed in the current study. Similarly, there were also two types of calluses obtained in both white cedar and oil palm study cases. In the study of white cedar, masses of translucent suspensors were obtained without PGRs, while compact, yellow-brown calluses were obtained with 2,4-D and BA [22]. In a study of oil palm, two types of calluses were identified, consisting of partial and complete meristematic cell clusters [55]. Thus, detailed observation of the two types of calluses observed in this study should be conducted.

Different types of somatic embryos were also observed by morphology and histology analysis in the current study. The formation of epidermal protoplasts and vascular bundles are two important events in SE, the former indicating the beginning of SE [56], and the latter possibly indicating the maturation of somatic embryos. All of these events were observed in the current study. Similar to the study in *Petiveria alliacea* L. [57], procambium (Figure 3a) produced from the calluses firstly in *K. paniculata* and then propagated to form embryogenic calluses (Figure 3b). Unlike the non-embryogenic calluses (Figure 3a), embryogenic calluses have clear cell boundaries. Subsequently, the epidermal (Figure 3c) was formed, and global embryos were isolated from embryogenic calluses, followed by the development of kidney-shaped (Figure 3e), heart-shaped (Figure 3g), torpedo-shaped (Figure 3h,j), and cotyledonary embryos (Figure 3i). In the process, the existence of shoot apical meristems (Figure 3g–j) and root meristems (Figure 3g–j) can be clearly observed, which are similar to other studies [17,51]. In addition, vascular tissue (Figure 3l) was also observed in mature cotyledon embryos, which was also observed in the study about *Eucalyptus globulus* Labill. [17].

4.5. Clonal Culture

Clonal culture is the basis of clone establishment and mass propagation of sterile seedlings in tissue culture. In this study, we examined the influence of different concentrations of NAA and IBA. The combined use of NAA and IBA produced poor results; however, NAA appeared to promote callus production. In a preliminary experiment, the concentration of major elements was also tested (Table S1). Compared to DKW and 1/2 DKW, plantlets cultured on 1/4 DKW exhibited a higher rooting frequency. Moreover, the physiological condition of the plants appeared to have the greatest effect on rooting rates. Long culture periods limited rooting rates through wound browning, which may have been caused by abundant flavonoids in *K. paniculata*. Our findings suggest that browning may be a limitation on the improvement of rooting rates. A study of black walnut reported that the combined application of meta-topolin, cytokinins, and liquid medium was effective in promoting shoot multiplication [58]; we believe that this finding could be combined with our own to provide a path for future *K. paniculata* research.

Despite its many advantages, SE has several limitations in woody plants, especially in the induction phase and later stages [24]. In a study of *Pinus radiata* D. Don, long incubation periods were required during the induction phase [59], and similar results were obtained in the current study. Although we were able to harvest regenerated plants through SE, a long culture cycle is a limitation of SE in *K. paniculata*. Moreover, as observed in other studies, the embryogenic potential of calluses declined with incubation time. The number of somatic embryos obtained from calluses and germination rates were inversely related to the length of the culture period, which is consistent with the findings of conifer and holm oak SE studies [30,35,54]. Thus, maintaining embryogenic potential and shortening the culture cycle remain key issues in *K. paniculata* tissue culture.

5. Conclusions

We proposed the first regeneration system for large-scale production of *K. paniculata* through SE. Different media types and different concentrations of PGRs were tested during embryogenic callus induction and somatic embryo development, and the effects of 2,4-D and NAA application on SE were observed. Clonal culture was then achieved on 1/4 DKW media supplemented with IBA, which provides the foundation for the establishment of a clone in *K. paniculata*. This protocol provides a novel method for large-scale propagation of *K. paniculata* and creates opportunities for genetic engineering in this species.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/9/12/769/s1, Table S1: The effect of basic media on rooting rates in *Koelreuteria paniculata* Laxm.

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carried out by T.G. and T.Z.; X.Y. (18811378046@163.com) and X.Y. (xiaomoziyu@126.com) drafted the manuscript; and Z.C. and X.A. revised the manuscript.

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