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Yan Liang (✉ liangyanyanliang@126.com)

Qiqihar University

Xue Bai

Qiqihar University

Xin Xu

Qiqihar University

Hongguo Xu

Qiqihar University

Jing Wang

Heilongjiang Academy of Agricultural Sciences

Peng Pan

Qiqihar University

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Direct in vitro organogenesis from sprouted seeds of a highly economical and ecological valued tree, Korean pine

Yan Liang^{1,2,*}, Xue Bai¹, Xin Xu¹, Hongguo Xu¹, Jing Wang³ and Peng Pan¹

¹ School of Life Sciences and A & F, Qiqihar University, Qiqihar, 161006, China; liangyanyanliang@126.com

² Provincial Key Laboratory of Resistance Gene Engineering and Protection of Biodiversity in Cold Areas, Qiqihar, 161006, China

³ Quality and Safety Institute of Agricultural Products, Heilongjiang Academy of Agricultural Sciences, Harbin 150086, China

* Correspondence: liangyanyanliang@126.com

Abstract: The de novo organogenesis system for Korean pine of this economically and ecologically coniferous species was successfully established using sprouted seeds as the original explants. After 30 days of incubation, 92.67% of explants produced direct shoots on Gupta and Durzan(DCR) medium containing $2 \text{ mg} \cdot \text{L}^{-1}$ kinetin (KT) in combination $0.5 \text{ mg} \cdot \text{L}^{-1}$ thidiazuron (TDZ) with a maximum of about 15 shoots per explant respectively. We also confirmed the organogenic regeneration pattern by scanning electron microscopy (SEM) observation. For shoot elongation and growth after 60 days of culture, we obtained the highest mean length of 34.99 mm from DCR basal media supplemented with 6-benzyladenine (6-BA; $0.2 \text{ mg} \cdot \text{L}^{-1}$), 1-Naphthaleneacetic acid (NAA; $0.1 \text{ mg} \cdot \text{L}^{-1}$), and activated charcoal (AC; $1 \text{ g} \cdot \text{L}^{-1}$). The highest rooting percentages of 20.74-21.48% were observed within two months in the 1/2 DCR medium (major elements halved) enriched with $0.05 \text{ mg} \cdot \text{L}^{-1}$ NAA and 0.5 or $1 \text{ mg} \cdot \text{L}^{-1}$ indole-3-butyric acid (IBA). Rooted plants showed a survival rate of 90.28% in perlite: peat: vermiculite = 1:1:1 after acclimatization. This protocol is a successful and efficient biotechnological approach to the micropropagation of Korean pine, and these data will be helpful to the clonal propagation and conservation of Korean pine.

Keywords: *Pinus koraiensis*; in vitro propagation; Shoot organogenesis; Plant growth regulators

1. Introduction

Korean pine (*Pinus koraiensis* Sieb. et Zucc.) is an evergreen coniferous tree species belonging to the *Pinus* genus and Pinaceae family, a dominant species of the natural mixed coniferous and broadleaf forests in the temperate forests of Northeast China. There are few natural populations of *Korean pine* sporadically distributed in South Korea, North Korea, Japan, and the Far Eastern region of Russia, where it plays a critical ecological role (Wang *et al.* 2018). Korean pine is famous for its high economic value, such as producing good quality timber and food for humans due to its riching in unsaturated fatty acids, proteins, carbohydrates, vitamin E, and other mineral nutrients. In addition, its seeds can also be used as industrial raw materials in food, cosmetics, and medicine (Wang *et al.* 2018; Shpatov *et al.* 2017; Fan *et al.* 2019). However, in recent decades, Korean pine in primary forests has been affected by over-harvesting timber and pine nuts. As a result, the natural populations of Korean pine have drastically declined, and the genetic resources of the species have been threatened by deforestation. Korean pine has been classified as a rare and nationally endangered species in China (Yu *et al.* 2011; Sun *et al.* 2016). Therefore, determining methods to increase the number of Korean pine trees in forests successfully is essential for improving qualified seedlings. However, under natural conditions, culturing Korean pine excellent seedlings to the desired size by conventional seed propagation approach usually needs 20 years because of their prolonged growing rate and late sexual maturity of this species; besides, the methods of grafted seedlings have also unacceptable mortality rates (Wei *et al.* 2020). Hence, to preserve the natural resources and ensure a stable and renewable source of Korean pine for ecological and economic purposes, successful and high-quality cultivation of seedlings and planting is imperative.

Fortunately, supplying a large number, high-quality and genetically superior korean pine seedlings by micropropagation technology is generally considered a highly promising approach. The advantage of micropropagation lies in its potential to eventually produce superior mass genotypes rapidly, and it has been used recently for tree improvement and clonal propagation (Haissig *et al.* 1987; Zhu *et al.* 2019). However, compared with most angiosperm woody trees, the clonal production of recalcitrance is still a general problem for many conifer species. Nowadays, nearly 40 species from the genus *Pinus* have been micropropagated (Kalia *et al.* 2007; Lelu-Walter *et al.* 2008; Alvarez *et al.* 2009; Leandro *et al.* 2013; Zhu *et al.* 2019). For example, *P. radiata* has been commercially propagated using organogenesis (Menzies *et al.* 2000). Direct organogenesis from explants is the best choice for rapid multiplication as it leads to the generation of true-to-type strains. Plantlets develop directly without the callus phase, so the chances of somaclonal variation are minimal in the regenerated plants, better maintaining its elite properties than indirect morphogenesis (Verma *et al.* 2021).

But unfortunately, to our knowledge, thus far, there are no efficient reproducible protocols for Korean pine in vitro direct shoot organogenesis technique. The in vitro propagation technology of Korean pine is challenging and has low

efficiency, which significantly limiting asexual propagation. Given this, in this context, Korean pine sprout seed explants were utilized to establish an effective regeneration system through direct adventitious bud organogenesis. The factors influencing adventitious buds formation, elongation, and rooting of Korean pine in vitro were determined. This study can provide clues for constructing a micropropagation system of Korean pine and artificially regulating organogenesis, facilitating propagation of elite seedlings for reforestation and monocultures in this species' production, and laying a foundation for the realization of industrial seedlings of coniferous species.

2. Materials and Methods

2.1. Plant materials and Explant sterilization

Mature seeds of Korean pine were collected from a single plant of open-pollinated preferred elite families (WH135) in October 2018. The families grew in the Weihe Korean pine Seed Orchard of Heilongjiang Province, northeastern China. These Korean pine seeds harvested in the previous year were stored with moisture sand at 4 °C until they were used for tissue culture in April 2019. Those healthy Korean pine seeds with cracked seed coats were selected for this experiment (Figure 1 a). Firstly, the sprouted buds from Korean pine seeds were taken out of the outer seed coat, removed the endosperm, washed with detergent for 2–3 min, then transferred into running water for 30 min. Secondly, they were then immersed in 70% (v/v) ethanol for 1 min, rinsed with sterile water five times, and then treated with 0.1% (w/v) HgCl₂ for 10 min by five successive washes with sterile distilled water. The radicle of the sterilized sprouted buds (1–1.5 cm) was cut off and then placed vertically in the Durzan medium (DCR) medium to induce adventitious shoots according to the morphological direction. All operations after sterilization were carried out on a clean bench.

2.2. Medium and culture conditions of adventitious bud induction

The explants (Figure 1 b) were inoculated onto DCR basal medium (Gupta and Durzan 1985), which supplemented with different plant growth regulators (PGRs) at different concentrations: thidiazuron (TDZ; 0.2 or 0.5 mg · L⁻¹), kinetin (KT; 1, 2, or 3 mg · L⁻¹) and 1-Naphthaleneacetic acid (NAA; 0 or 0.1 mg · L⁻¹), respectively (Table 1). In addition, all the mediums were supplemented with 500 mg · L⁻¹ casein hydrolysate (CH), 500 mg · L⁻¹ L-glutamine, 3% (w/v) sucrose, and 0.7% (w/v) agar. Thirty tissue materials were used for each treatment, and the experiments were repeated three times. After 30 days of culture, the efficiency of PGRs on adventitious bud induction was determined by recording the frequency of responding explants and the mean number of shoots per explant. In addition, the response changes of explants throughout the induction process were also recorded.

2.3. Scanning electron microscopy (SEM) observation of adventitious bud induction

For morphological observations of the different stages of adventitious shoot differentiation, SEM was employed. The materials at different developmental stages were fixed with FAA fixative (formaldehyde: glacial acetic acid: 70% ethanol = 5:5:90) for more than 48 h and then washed in distilled water for 30 min. Samples were dehydrated in a graded ethanol series (10% increments every 20 min), then soaked in a graded amyl acetate series followed by drying on filter paper at room temperature. They were then sputter-coated with gold in a cold-sputtering system. Samples were examined directly and photographed using a scanning electron microscope (Hitachi S-4300N) at 20 kV.

2.4. Axillary bud growth and elongation

After an initial induction period of 30 days, when induced axillary bud growth was evident (Figure 1 f), transferred to elongation medium and cultured for an additional 60 days, subcultured to fresh medium every 30 days of culture. The medium was also DCR basic medium supplemented with different concentrations 6-benzyladenine 6-BA (0, 0.2, 0.5 or 1 mg · L⁻¹), NAA (0.1 mg · L⁻¹), and activated charcoal (AC; 0 or 1 g · L⁻¹) (Table 2). The addition of sucrose, agar, CH, and L-glutamine were the same as adventitious bud induction treatment. The best medium for shoot elongation was determined based on shoot length.

2.5. Rooting and domestication of shoots

After two months of culture, until formed adventitious buds were 1.5–2.0 cm long (Figure 1 m), individual shoots were separated from the multiple shoots and then transferred onto a fresh root-induction medium to promote the root growth. The rooting medium contained different concentrations and combinations of NAA (0, 0.05, 0.1 or 0.2 mg · L⁻¹) and indole-3-butyric acid (IBA; 0.5, 1 or 1 mg · L⁻¹) or no PGRs, medium without any PGRs was used as a control (CK) (Table 3). In addition, medium also supplemented with 1 g · L⁻¹ AC, 500 mg · L⁻¹ CH, 2 % (w/v) sucrose, and 0.7 % (w/v) agar. Two months later, the rooting rate and root number ≥ 1 cm per rooted shoot were individually recorded. After rooting culture, plantlets with visible roots (≥ 1 cm) were transferred to plastic pots containing sterile peat: perlite: vermiculite (1:1:1; v/v) mixture located in a greenhouse. For acclimatization, plantlets were covered with glass beakers for one week before being exposed to greenhouse conditions by removing the cover. A total of 10 subcultured plantlets (×3 replicates) were collected for rooting induction and domestication.

The basal medium used in this study was the DCR medium. The pH of all mediums was adjusted to 5.8 with 0.1 M NaOH and 0.1 M HCL before autoclaving. L-Glutamine was filter sterilized with 0.22 μm filters and supplemented into the autoclaved medium when it had cooled to about 50°C. Other PGRs and additives were added to the medium before sterilization. Cultures for induction of adventitious bud, elongation, and rooting in vitro were all kept in a growth chamber at $22 \pm 2^\circ\text{C}$, with a 16 h photoperiod of fluorescent light ($60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) provided by cool white fluorescent tubes. Acclimatization was kept in a greenhouse at $22 \pm 2^\circ\text{C}$, with 70–80% relative humidity, and 16 h photoperiod of $80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light. All products were purchased from Sigma-Aldrich (Madrid, Spain), except sucrose and AC from Solarbio (Beijing, China).

2.6. Statistical analysis

All the experiments were conducted thrice with randomized design settings. Data were analyzed by using analysis of variance (ANOVA) in SPSS ver. 17.0 software (SPSS Inc., Chicago, Illinois). Significant differences among the means of the treatments were evaluated using Duncan's new multiple range test ($p \leq 0.05$).

3. Results

3.1. Effects of PGRs on in vitro de novo organogenesis of Pinus koraiensis

Organogenesis is the de novo production of plant organs from organized tissues or callus (Thakur and Kanwar 2018). In this study, direct organogenesis occurred in Korean pine was observed (Figure 1). When supplemented with different cytokinins (CKs) and auxins, the differential response towards multiple adventitious bud induction was recorded. The results of shoot induction varied with the type and concentration of PGRs, as depicted in Table 1. On the whole, the addition of CKs supplemented with lower concentrations auxins (or no addition) to the medium promoted the morphogenic response. Compared with CKs alone in the medium, the exogenous application of high concentration CKs along with low concentration auxin did not significantly improve the adventitious bud induction parameters. Therefore, it can be concluded that adding CKs alone was beneficial for the bud formation of Korean pine, and the addition of auxin was not necessary. Among them, the medium supplementing $2 \text{ mg} \cdot \text{L}^{-1}$ KT in combination with $0.5 \text{ mg} \cdot \text{L}^{-1}$ TDZ, 0 or $0.1 \text{ mg} \cdot \text{L}^{-1}$ NAA was proved to be most effective in terms of yielding average 14.67 and 15.33 shoots per explant with adventitious bud induction rate were 92.67% and 90.66%, respectively after 30 days of incubation, significantly higher than other treatments ($p < 0.05$) (Table 1). Otherwise, compared with TDZ and NAA, suitable KT concentration showed more shoots per explants and adventitious bud induction rate. Of the various treatment tested, with the increase of the KT treatment concentration, Korean pine adventitious buds' induction rate increased first and then decreased. The medium containing $1 \text{ mg} \cdot \text{L}^{-1}$ KT, combined with TDZ (0.2 or $0.5 \text{ mg} \cdot \text{L}^{-1}$) and NAA (0 or $0.1 \text{ mg} \cdot \text{L}^{-1}$), only produced 3.67–6.67 shoots shoot induction rate of 27.4–43.2%. The concentration above or below the optimal dose of KT did not improve the parameters of adventitious buds. Compared with $2 \text{ mg} \cdot \text{L}^{-1}$, when KT concentration increased to $3 \text{ mg} \cdot \text{L}^{-1}$, both the induction rate of adventitious buds and the number of induced buds decreased significantly, in addition, higher KT dose ($3 \text{ mg} \cdot \text{L}^{-1}$) in the medium also led to the development of stunted and clustered buds.

Table 1. Effect of hormone treatment on adventitious bud induction of Korean pine after 30 days of cultures

Number	KT $\text{mg} \cdot \text{L}^{-1}$	TDZ $\text{mg} \cdot \text{L}^{-1}$	NAA $\text{mg} \cdot \text{L}^{-1}$	Adventitious bud induction rate (%) mean	Number of shoots per explant mean
1	1	0.2	0	$27.4 \pm 1.73\text{f}$	$3.67 \pm 0.88\text{f}$
2	1	0.2	0.1	$31.75 \pm 2.12\text{f}$	$5.33 \pm 0.88\text{def}$
3	1	0.5	0	$46.51 \pm 2.06\text{de}$	$5.67 \pm 0.88\text{def}$
4	1	0.5	0.1	$43.2 \pm 0.64\text{e}$	$6.67 \pm 0.67\text{cde}$
5	2	0.2	0	$51.9 \pm 1.81\text{cd}$	$8 \pm 0.58\text{bcd}$
6	2	0.2	0.1	$53 \pm 0.57\text{c}$	$9 \pm 0.58\text{bc}$
7	2	0.5	0	$92.67 \pm 1.98\text{a}$	$14.67 \pm 0.67\text{a}$
8	2	0.5	0.1	$90.66 \pm 0.83\text{a}$	$15.33 \pm 0.88\text{a}$
9	3	0.2	0	$73.24 \pm 2.26\text{b}$	$9.67 \pm 0.88\text{b}$
10	3	0.2	0.1	$53.41 \pm 1.93\text{c}$	$8 \pm 1.15\text{bcd}$
11	3	0.5	0	$51.73 \pm 1.72\text{cd}$	$3.67 \pm 0.88\text{f}$
12	3	0.5	0.1	$47.28 \pm 3.68\text{cde}$	$4.33 \pm 0.88\text{ef}$

Means followed by the same letter in each column are not significantly different by Duncan's multiple tests ($p \leq 0.05$)

Morphological differences under the induction of Korean pine organogenesis were shown in Figure 1. The first morphological changes were recorded during the 7–10 days of culture when the cotyledons were splayed and turned green, the color of the cotyledon base changed to transparent from slightly green and dilated in the region around the bottom of the cotyledons, where observed multiple small protrusions invisible to the naked eye by stereomicroscopic, indicated that the bud primordium and needle primordia initiated and formed (Figure 1 c, g). Otherwise, a small amount of callus was also seen at the location of the hypocotyl. After another ten days of culture, those protrusions showed a further continuous increase in volume and number, just as shown in Figure 1d, which were dense buds cluster of original needles visible to the naked eye. We also observed adventitious shoots at an early development stage, needle primordia with transparent color, and a more evident structure from the cotyledon base by SEM (Figure 1 h). On the 30th day of culture, adventitious shoots were formed completely (Figure 1 e, f). Those protruded bud spots became more prominent, and the color gradually changed to yellowish. As described in Figure 1i, SEM revealed the leaf sheath-like structure was protecting the shoot meristem (asterisk), and clusters of adventitious shoots formed. Otherwise, we also observed numerous adventitious buds at different development stages on the explant simultaneously, and no vitrification was detected (Figure 1 j).

Figure 1. Establishment of the in vitro regeneration system for sprouted seeds explants of Korean pine. (a) the sprouted seeds of Korean pine (bar = 5 mm); (b) explants inoculated into adventitious bud induction medium (0d) (bar = 10 mm); (c) adventitious buds of Korean pine obtained by culturing for 10 days (bar = 2 mm); (d) adventitious buds of Korean pine obtained by culturing for 20 days (bar = 5 mm); (e-f) adventitious buds of Korean pine obtained by culturing for 30 days (bar = 5 mm); (g-j) scanning electron microscopy of adventitious shoot formation from cotyledons 10 (g), 20 (h), and 30 (i, j) days culture, respectively; (g) adventitious bud primordium and needle primordia just also formed after 7–10 days culture (bar = 2 mm). (h) adventitious shoot primordia at early stage of development after 20 days culture (bar = 2 mm); (i) clusters of adventitious shoots formed and the leaf sheath-like structure protecting the shoot meristem (*) after 30 days culture (bar = 2 mm); (j) numerous adventitious buds at different development stages simultaneously after 30 days culture (bar = 3 mm); (k) adventitious buds of Korean pine after elongation for 20 days (bar = 5 mm); (l) adventitious buds of Korean pine after elongation for 40 days (bar = 5 mm); (m) adventitious buds of Korean pine after elongation for 60 days (bar = 10 mm); (n) isolated individual adventitious buds that were used for rooting (bar = 10 mm); (o) the deformed adventitious roots were produced (bar = 8 mm); (p) a large amount of callus formed during the rooting process (bar = 10 mm); (q)

roots were formed from elongated adventitious shoots after 60 days (bar = 15 mm); (r) plantlets established in potting substrate for 30 days (bar = 25 mm)

3.2. Effects of PGRs on Shoot elongation and growth of Korean pine

Once those in vitro-established tender buds were transferred to shoot elongation media, they showed faster growth than the adventitious bud induction stage. According to the results of means comparison analysis, the adventitious buds showed different responses on various shoot elongation media. In general, under eight treatments, with the concentration of 6-BA treatment increased, the length of adventitious buds first increased and then decreased. When 6-BA is not added, regardless of whether AC is added, the adventitious buds elongation was not very obvious, and the average bud length was < 5 mm in 60 days. After 60 days of culture, the highest mean length (34.99 mm) of Korean pine was obtained from DCR basal media supplemented with 6-BA (0.2 mg · L⁻¹), NAA (0.1 mg · L⁻¹), and AC (1 g · L⁻¹) (Figure 2 m), significantly higher than other treatments ($p < 0.05$), followed by AC-free treatment (26.54 mm) (Table 2). Meanwhile, these in vitro seedlings thrived. We also found that appropriate 6-BA addition concentration was more efficient for bud elongation when the 6-BA concentration increased to 0.5 mg · L⁻¹ or 1 mg · L⁻¹, regardless of whether it contained AC, the average shoot length decreased again (< 18 mm), and the needles were tiny and grew slowly. Our results indicate that the complementary of low concentration of 6-BA and NAA was efficient for promoting rapid growth and elongation of shoots in Korean pine. Figure 1 k、1 l and 1 m showed the adventitious buds after elongation treatment for 20, 40, and 60 days, respectively. It can be seen that the adventitious buds of 20 days were slightly elongated, some meristems further developed into shoot buds, and the buds of 40 days were more obvious; at 60 days, the bud length reached its maximum (Table 2).

Table 2. Effect of hormone treatment on adventitious bud elongation of Korean pine after 60 days of cultures

Number	6-BA mg · L ⁻¹	NAA mg · L ⁻¹	AC g · L ⁻¹	Mean shoots length (mm)
1	0	0.1	0	4.74 ± 0.13f
2	0	0.1	1	4.92 ± 0.11f
3	0.2	0.1	0	26.54 ± 0.51b
4	0.2	0.1	1	34.99 ± 0.42a
5	0.5	0.1	0	16.81 ± 0.46d
6	0.5	0.1	1	17.64 ± 0.24c
7	1	0.1	0	5.6 ± 0.06e
8	1	0.1	1	4.43 ± 0.15g

Means followed by the same letter in each column are not significantly different by Duncan's multiple tests ($p \leq 0.05$)

3.3. Ex vitro rooting and acclimatization of the shoots

Induction of roots in vitro is an essential step to establish tissue culture derived from plantlets to the soil. Application of exogenous auxin usually can solve the problem of root induction in rooting recalcitrance species (Wen *et al.* 2020). Generally, the most effective auxins for in vitro rooting are IBA and NAA. In addition, differences in species and genotypes also affect the rooting effect (Hesami and Daneshvar 2019). In our experiments, to obtain hardened plantlets, isolated shoots (1.5 to 2.0 cm long) were excised from adventitious shoots after 60 days of elongation culture and then cultured in the rooting medium (Figure 1 n). Here, the rooting rate was individually recorded. Of the 13 tested treatments, the average rooting percentages vary from 0 to 21.48%, and only one true root ≥ 1 cm per rooted shoot is formed. The highest rooting percentages was 20.74–21.48% when 0.05 mg · L⁻¹ NAA and 0.5 or 1 mg · L⁻¹ IBA were used, significantly higher than other treatments (Table 3). In addition, the medium without any PGRs (CK) did not induce roots from elongated shoots. When NAA treatment concentration increased to higher than 0.1 mg · L⁻¹, it was observed that a large amount of callus was formed at the base of adventitious buds, and many deformed adventitious roots were produced from these calli (Figure 1 o), especially in treatments with NAA at 0.2 mg · L⁻¹ and IBA at 0.5, 1 or 2 mg · L⁻¹, respectively, where no roots were produced, but a lot of calli was produced at the location where the roots were formed (Figure 1 p), in vitro rooting percentages were only between 0–6.67% treated with IBA alone; also, a small amount of callus appeared on their base. Figure 1 q are the rooted shoots after rooting treatment 60d, and the rooted shoots were acclimatized in the sterilized matrix. After one month of growth in the greenhouse, the survival of the weaned plants was 90.28%, and all regenerated plantlets showed normal growth and morphology under greenhouse conditions (Figure 1 r).

Table 3. Effect of auxin treatment on rooting capacity of Korean pine after 60 days of cultures

Number	NAA mg · L ⁻¹	IBA mg · L ⁻¹	Rooting rate explant mean (%)
CK	0	0	0.00 ± 0c
1	0	0.5	0.00 ± 0c
2	0	1	6.67 ± 2.17b
3	0	2	6.67 ± 2.38b
4	0.05	0.5	20.74 ± 0.52a
5	0.05	1	21.48 ± 0.52a
6	0.05	2	6.67 ± 2.17b
7	0.1	0.5	10.37 ± 0.35b
8	0.1	1	11.11 ± 2.72b
9	0.1	2	0.00 ± 0.00c
10	0.2	0.5	0.00 ± 0.00c
11	0.2	1	0.00 ± 0.00c
12	0.2	2	0.00 ± 0.00c

Initiation percentages for individual replicates were transformed by $\arcsin \sqrt{x} / 100$ for ANOVA.

Means followed by the same letter in each column are not significantly different by Duncan's multiple tests ($p \leq 0.05$)

4. Discussion

4.1. De novo shoot organogenesis from Korean pine sprouted seeds explants

In long-lived trees such as conifers, despite considerable amounts of research-derived plantlets by indirect or direct organogenesis, most coniferous species are still regarded as extremely difficult to regenerate (Bonga 2017; Sarmast 2017). Nevertheless, essential steps in establishing in vitro micropropagation are the induction and multiplication of shoots (Li *et al.* 2018). Being a commercially and economically important tree species, developing a robust regeneration protocol for Korean pine is urgently needed. In the present study, the direct organogenesis of Korean pine from sprouted seed explants was achieved, and confirmed the organogenic regeneration pattern by SEM observation. This propagation pathway by direct organogenesis has also been proven to have less risk of somaclonal variations than indirect organogenesis (Verma *et al.* 2017; Zayova *et al.* 2010).

The formation of adventitious shoots is a complex process that involves the participation of PGRs, mainly auxins and CKs, with multiple signaling pathways (Sang *et al.* 2018; Tian *et al.* 2018; Ikeuchi *et al.* 2019). Compared with auxins, CKs are involved in bud breakage, cell division, shoot initiation, and multiplication from explants, which are more frequently applied to induce in vitro shoot organogenesis (Mazri 2015; Zhang *et al.* 2018; Ayala *et al.* 2019). The exogenous CKs application facilitates the explant to reach an optimum cytokinin: auxin ratio for de novo meristem formation (Alvarez *et al.* 2020). Among them, 6-BA, KT, and TDZ are routinely used CKs most efficiently (Khanam *et al.* 2020).

Our results were similar to reports of other woody trees. A combination of a higher cytokinin-to-auxin ratio to the medium was beneficial to induce multiple shoots (Zhu *et al.* 2019; Zhu *et al.* 2010; Tippani *et al.* 2013; Ahmad *et al.* 2020). In our study, compared with CKs alone in the medium, exogenous application of high concentration CKs (KT and TDZ) along with a low dose of auxins did not significantly improve the adventitious bud induction parameters of Korean pine. Therefore, it can be concluded that adding CKs alone was considered essential to sustain the organogenic response of Korean pine explants, whereas the addition of auxin was not necessary. In addition, compared with TDZ and NAA, suitable KT concentration showed more shoots per explants during the adventitious buds induction of Korean pine, and above or below the optimal dose of KT did not show any improvement in parameters of adventitious buds, which is in line with previous studies that exceeding optimal KT levels inhibits bud proliferation (Akbaş *et al.* 2009; Arab *et al.* 2014). TDZ is a phenyl urea-type cytokinin that mimics the activity of both auxin and cytokinin, which are the most preferably used cytokinins in some woody plant regeneration systems (Dewir *et al.* 2018; Rukaya *et al.* 2021). Previous studies described TDZ was used solely for indirect organogenesis and proved more effective for the shoot induction process in a range of plants in vitro (Khanam *et al.* 2018; Wu *et al.* 2020; Syeed *et al.* 2021). However, in our study, TDZ in combination with KT was more conducive to the direct adventitious buds formation of Korean pine, which can be explained by the fact that TDZ is highly stable and regulates endogenous hormones, and shows the activity of both

auxins as well as cytokinin, similarly mentioned in the literature (Dewir *et al.* 2018; Dey *et al.* 2012) also found that TDZ appears to stimulate cells in the apical meristem to divide, multiply, and develop so that bud differentiation occurred. SEM results also confirmed the direct organogenesis pathway in our study, showed shoots arising from cotyledons base of sprouted bud explants.

Nowadays, successful micropropagation has been reported in many pine species using various explants, such as mature zygotic embryos (Cortizo *et al.* 2009; Montalbán *et al.* 2011; Stojičić *et al.* 2012), cotyledons (Alvarez *et al.* 2009), seedling explants (Zhu *et al.* 2010; Hargreaves *et al.* 2005; Nunes *et al.* 2018), and in some cases, explants from mature trees (Cortizo *et al.* 2009; Parasharami *et al.* 2003). Besides, the choice of explant is believed to affect in vitro response differently. In 2018–2019, we also took mature zygotic embryos of Korean pine as explants to induce adventitious bud, but the effect is significantly lower than that of sprouted seeds.

4.2. Shoot elongation and growth of in vitro *Pinus koraiensis*

In the second step of the culture procedure, adventitious buds of Korean pine were transferred onto the shoot elongation medium. After 60 days of culture, our data gave better results regarding growth parameters (the length and number of the shoots). Overall, PGRs had a significant effect on the length and number of the shoots. The in vitro-established buds elongation growth was significantly accelerated, and low concentration combinations of 6-BA and NAA were found to be efficient for promoting rapid growth and elongation of Korean pine adventitious buds; among them, the most extended shoots were generated on the DCR basal media containing 6-BA ($0.2 \text{ mg} \cdot \text{L}^{-1}$), NAA ($0.1 \text{ mg} \cdot \text{L}^{-1}$), and AC ($1 \text{ g} \cdot \text{L}^{-1}$). Therefore, our results agreed with a combination of a higher cytokinin-to-auxin ratio in medium be efficient for successful shoot inducing morphogenic response and stimulating shoot proliferation; however, for shoot elongation in Korean pine, lower concentrations of the same PGRs is more appropriate, which was also in agreement with those plant micropropagation studies that have demonstrated for promoting bud elongation, medium without plant growth regulators (Zhu *et al.* 2019; Stojičić *et al.* 2012), or with a lower level of cytokinin with auxin (Tanf *et al.* 2004; Zhang *et al.* 2006; Zhu *et al.* 2007; Schaller *et al.* 2015) or 0.1–0.5% AC (Zhu *et al.* 2019; Stojičić *et al.* 2012) was usually were proven to be effective. It is worth noting that the rate of shoot multiplication and extent of shoot elongation observed here are better than previously published reports on other pine micropropagation (Zhu *et al.* 2019; Montalbán *et al.* 2011; Stojičić *et al.* 2012).

4.3. Rooting and acclimatization of *Pinus koraiensis* microshoots

As we all know, many coniferous species have a low capacity for rooting in vitro, and the most common problem encountered in the micropropagation of pines is adventitious root formation, and also because of this, reducing the possibilities of applying these techniques for large scale micropropagation (Bonga 2017). Generally, auxins play an essential role in regulating adventitious root formation, and IBA or NAA are often used to promote in vitro rooting (Kalia *et al.* 2007; Alvarez *et al.* 2009; Wang and Yao 2019a; Wang and Yao 2019b). After two months of cultivation on root-promoting medium, the highest rooting percentages were < 30% in our present study. However, NAA concentrations to higher-than-optimal levels (0.1 or $0.2 \text{ mg} \cdot \text{L}^{-1}$) induced a large amount of undesirable callus, and a few deformed adventitious roots were produced at the basal end of the microshoot, indicated that the appearance of callus led to poor rooting. Our findings were consistent with the previous report when high concentration NAA was used to induce adventitious roots in *Pinus massoniana*, a mass of callus from which root primordia differentiated was produced on the base of most shoots (Zhang *et al.* 2006). In addition, when treated with IBA singly, a small amount of callus appeared and resulted in poor rooting (very few or no roots). Similarly, the recent study of in vitro rooting from *Pterocarpus marsupium* also found that beyond the optimum dose of IBA induced undesirable callus at the basal end of microshoot, and no rooting was observed (Ahmad *et al.* 2020). Otherwise, for applied PGRs in vitro rooting, there are some different results, supplementation with 1.2 uM NAA in the medium was effective for rooting induction in *P. massoniana* (Wang and Yao 2019b), most of the clones from *Pinus densiflora* rooted on medium supplemented with $0.2 \text{ mg} \cdot \text{L}^{-1}$ NAA (Zhu *et al.* 2019). IBA was also reported to be better than with NAA or a mixture of both auxins in vitro rooting (Zhu *et al.* 2007), in contrast to this, IBA ($2 \text{ mg} \cdot \text{L}^{-1}$) application with NAA ($0.1 \text{ mg} \cdot \text{L}^{-1}$) was a preferable alternative than IBA only for the *Ficus religiosa* root induction, subsequent development of the induced roots usually proceeded (Hesami and Daneshvar 2019). Our study is consistent with the *F. religiosa* research using hormone types; whereas, the concentration combination is different, which can be explained due to differences in coniferous species that lead to different auxins response for in vitro rooting. In addition, those plantlets in the present study showed a high capacity for transference to the ex vitro environment.

However, unlike pine species, some other tree species are prone to rooting and occasionally show spontaneous in vitro rooting of elongated microshoots (Sarkar and Jha 2017). Medium devoid of PGRs or hormone-free AC produce

higher roots than with PGRs addition. The stimulatory effect of AC on rooting may involve providing a dark environment conducive for the accumulation of auxins or cofactors and / or adsorption of inhibitory substances (e.g., phenolics) and excess hormones (auxins or CKs) carried over from the previous media (Verma *et al.* 2021). Therefore, in our study, 0.2% AC is added to all rooting treatments. However, contrary to earlier reports, our results indicated that culturing on a medium containing 0.2% AC hormone-free did not significantly promote the formation of adventitious roots.

In addition to PGRs type and concentration, other parameters such as plant species, genotype also affect the in vitro root induction procedure. Our results indicated that although a small part of the plants was rooted in the tested medium combinations, root formation was occasionally observed and rarely. Compared with *Pinus peuce* (Stojičić *et al.* 2012), *Pinus densiflora* (Zhu *et al.* 2019), and *P. massoniana* (Wang and Yao 2019b), Korean pine may be a difficult coniferous species for in vitro rooting in micropropagation. Therefore, further studies on in vitro rooting are necessary.

5. Conclusions

Plant multiplication in vitro proved to be very efficient for large-scale propagation of many tree species, providing a more valuable and rapid way than the traditional cutting procedure. Nevertheless, the development of in vitro protocols for coniferous species is still challenging because of such factors as recalcitrance. This study shows that the de novo organogenesis system for Korean pine by adventitious buds formation was successfully established using sprouted seeds as the original explants, constituting the first report for this economically and ecologically recalcitrant coniferous species with a high-frequency and cost-effective regeneration technique. Importantly, direct organogenesis leads to the generation of true-to-type strains to avoid callus phase organization and have fewer chances of somaclonal variation than indirect organogenesis. We believe that these data will be helpful in future research for rapid micropropagation and conservation of Korean pine, and they might also facilitate the development of biotechnological tools to study such coniferous species.

Author Contributions: L.Y. designed the experiment and performed technical guidance. B.X. and X.X. wrote the manuscript and assisted in the data analysis. X. H.G. and W.J. supervised the experimental process. P.P. collected plant materials and investigation. All authors participated in the experimental process. All authors have read and agreed to the published version of the manuscript.

Compliance with ethical standards: We confirm that this manuscript has not been published elsewhere and is not being considered elsewhere. All co-authors have contributed to the work, seen and agreed with the contents of the manuscript. No conflict of interest exists in the submission of this manuscript.

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Figures

Figure 1

Establishment of the in vitro regeneration system for sprouted seeds explants of Korean pine. (a) the sprouted seeds of Korean pine (bar = 5 mm); (b) explants inoculated into adventitious bud induction medium (0d) (bar = 10 mm); (c) adventitious buds of Korean pine obtained by culturing for 10 days (bar = 2 mm); (d) adventitious buds of Korean pine obtained by culturing for 20 days (bar = 5 mm); (e-f) adventitious buds of Korean pine obtained by culturing for 30 days (bar = 5 mm); (g-j) scanning electron microscopy of adventitious shoot formation from cotyledons 10 (g), 20 (h), and 30 (i, j) days culture, respectively; (g) adventitious bud primordium and needle primordia just also formed after 7-10 days culture (bar = 2mm). (h) adventitious shoot primordia at early stage of development after 20 days culture (bar = 2 mm); (i) clusters of adventitious shoots formed and the leaf sheath-like structure protecting the shoot meristem (X) after 30 days culture (bar = 2 mm); (j) numerous adventitious buds at different development stages simultaneously after 30 days culture (bar = 3 mm); (k) adventitious buds of Korean pine after elongation for 20 days (bar = 5 mm); (l) adventitious buds of Korean pine after elongation for 40 days (bar = 5 mm); (m) adventitious buds of Korean pine after elongation for 60 days (bar = 10 mm); (n) isolated individual adventitious buds that were used for rooting (bar = 10 mm); (o) the deformed

adventitious roots were produced (bar = 8 mm); (p) a large amount of callus formed during the rooting process (bar = 10 mm); (q) roots were formed from elongated adventitious shoots after 60 days (bar = 15 mm); (r) plantlets established in potting substrate for 30 days (bar = 25 mm)