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

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Abstract
Korean pine (Pinus koraiensis Sieb. et Zucc.) is a critical coniferous species ecologically. In addition, its fruits have numerous nutraceutical properties and its wood is high quality timber. However, this plant has a low propagation rate and is under risk of extinction. To accelerate breeding of the excellent seedlings and selection from Korean pine, determining methods to increase the number of Korean pine trees successfully is imperative. In this present study, using sprouted seeds as the initial explants, the de novo organogenesis system for this coniferous species has been successfully developed. After 30 days of incubation on Gupta and Durzan (DCR) medium containing 2 mg L⁻¹ kinetin (KT) and 0.5 mg L⁻¹ thidiazuron (TDZ), 92.67% of explants produced direct buds with an average of about 15 buds per explant. We confirmed the organogenic regeneration pattern by a scanning electron microscopy (SEM). For bud elongation after 60 days of culture, we obtained the highest mean length of 34.99 mm on DCR basal media supplemented with 6-benzyladenine (6-BA, 0.2 mg L⁻¹), 1-naphthaleneacetic acid (NAA, 0.1 mg L⁻¹), and activated charcoal (AC, 1 g L⁻¹). The highest rooting rates of 20.74 and 21.48% were obtained within two months of culturing on 1/2 DCR medium (halved major elements) supplemented with 0.05 mg·L⁻¹ NAA and 0.5 or 1 mg ·L⁻¹ indole-3-butyric acid (IBA), respectively. Rooted shoots showed a survival rate of 90.28% after acclimatization in the substrate consisting of perlite, peat, and vermiculite (1:1:1). This protocol is a successful and efficient biotechnological approach to the micropropagation of Korean pine, and the results will be helpful to the clonal propagation and conservation of Korean pine.

Key message
This study developed a useful protocol for multiplying plantlets from sprouted seed explants of Korean pine, an economically and ecologically important coniferous species.

Keywords Pinus koraiensis · In vitro propagation · Shoot organogenesis · Plant growth regulators

Introduction
Korean pine (Pinus koraiensis Sieb. et Zucc.) is an evergreen coniferous tree species belonging to the Pinus genus and the Pinaceae family and it is a dominant species of the natural mixed coniferous and broadleaf forests of the temperate forests in 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 well known for its high economic value, such as producing high quality timber and foods for humans enriched with 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 (Shpatov et al. 2017; Wang et al. 2018; 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 consequence, 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 is essential for improving quality of seedlings. However, under natural conditions, culturing excellent Korean pine seedlings to the maturity stage by conventional seed propagation approach usually needs 20 years because of the 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 Korean pine resources and ensure a stable and sustainable source of Korean pine for ecological and economic purposes, successful and high-quality cultivation of seedlings and planting is imperative.

Fortunately, obtaining a large number of high-quality and genetically superior Korean pine seedlings by the micropropagation technique is generally considered as a highly promising approach. The advantage of micropropagation lies in its potential to produce superior mass genotypes rapidly, and this method has been used for tree improvement and clonal propagation (Haissig et al. 1987; Zhu et al. 2019). Generally, 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). 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, Pinus radiata has been commercialized using organogenesis (Menzies et al. 2000). So far, 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), coryledons (Alvarez et al. 2009), seedling explants (Hargreaves et al. 2005; Zhu et al. 2010, 2019; Nunes et al. 2018), and in some cases, explants from mature trees (Parasharami et al. 2003; Cortizo et al. 2009).

However, compared with most angiosperm woody trees, the recalcitrance during clonal production remains a general problem for many coniferous species. For Korean pine, researchers initiated the studies on the technique of in vitro organogenesis as early as the 1990s (Liu et al. 1991), but unfortunately, little progress has been made. Recently, somatic embryogenesis from immature zygotic embryos of Korean pine was reported (Gao et al. 2020; Peng et al. 2021), but few regenerated plantlets were obtained due to the low induction rates of embryogenic callus and a too long growth subculture cycle. In 2018–2019, we also tried mature zygotic embryos of Korean pine as explants to induce adventitious bud, but both the induction rates of adventitious buds and numbers were very low (unpublished data).

To our best knowledge so far, the in vitro propagation technique of Korean pine is challenging and has low efficiency, there are no efficient reproducible protocols for in vitro direct shoot organogenesis technique for Korean pine. Therefore, finding an efficient method for the propagation of this coniferous species is urgent for forest breeding and afforestation programs. In this context, sprouted seed explants of Korean pine were utilized to establish an effective regeneration system through direct adventitious bud organogenesis. The factors influencing adventitious bud formation, elongation, and rooting of in vitro of Korean pine were determined. This study can provide implications for constructing a micropropagation system of Korean pine, facilitating propagation of elite seedlings for reforestation and monocultures in this species’ production.

Materials and methods

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 (Fig. 1a). 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 water five times, and then treated with HgCl₂ for 10 min followed by five successive washes with sterile distilled water. To induce adventitious shoots, the sterilized sprouted buds (1–1.5 cm) were placed vertically in the DCR medium after cutting its radicle. All operations after sterilization were carried out on a clean bench.

Medium and culture conditions for adventitious bud induction and proliferation

The explants (Fig. 1b) 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. 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 explants were used for each treatment, and the experiments were repeated three times. After 30 days of culture, the effect of PGRs on adventitious bud induction and proliferation was determined by recording the frequency of responding explants and the mean number of buds per explant. In addition, the response changes of explants throughout the induction process were also recorded.

**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 solution (formaldehyde:glacial acetic acid:70% ethanol = 5:5:90) for more than 48 h and then washed in

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**Fig. 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 (0 day) (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), and *r* plantlets established in potting substrate for 30 days (bar = 25 mm).
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.

**Axillary bud growth and elongation**

When induced axillary bud growth was evident (Fig. 1f), they were transferred to elongation medium and cultured for an additional 60 days (subcultured to fresh medium every 30 days). The used medium was 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). The addition of sucrose, agar, CH, and l-glutamine were the same as adventitious bud induction treatment. Ten explants were used for each treatment, and the experiments were repeated three times. The best medium for shoot elongation was determined based on shoot length.

**Rooting and acclimatization of plantlets**

After two months of culture, until formed adventitious buds were 1.5–2.0 cm long (Fig. 1m), 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 containing 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), and supplemented with 1 g·L⁻¹ AC, 500 mg·L⁻¹ CH, 2% (w/v) sucrose, and 0.7% (w/v) agar was tested. Medium without PGRs was used as a control. 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 at 22 ± 2 °C, with 70–80% relative humidity, and 16 h photoperiod of 80 µmol m⁻² s⁻¹ light intensity provided by cool white fluorescent tubes. Acclimatization was carried out in a greenhouse at 22 ± 2 °C, with 70–80% relative humidity, and 16 h photoperiod of 80 µmol m⁻² s⁻¹ light. All products were purchased from Sigma-Aldrich (Madrid, Spain), except sucrose and AC from Solarbio (Beijing, China).

**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 Tukey’s HDS test (p < 0.05).

**Results**

**Effects of plant growth regulators 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 of Korean pine was observed (Fig. 1). When supplemented with different cytokinins (CKs) and auxins, differential responses towards multiple adventitious bud induction occurred. Morphological differences under the induction of Korean pine organogenesis were shown in Fig. 1. The first morphological changes were recorded during the 7th–10th days of culture when the cotyledons were splayed and turned green. Then, the color of the cotyledon base changed from slightly green to transparent and dilated in the region around the bottom of the cotyledons, where multiple small protrusions were observed under stereomicroscopic, indicating that the bud primordium and needle primordia have initiated and formed (Fig. 1c, g). Meanwhile, a small amount of callus was also observed in the location of the hypocotyl. After another ten days of culture, those protrusions showed a further continuous increase in both volume and number as shown in Fig. 1d, which were dense bud clusters of original needles visible to the naked eyes. 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 (Fig. 1h). On the 30th day of culture, adventitious buds were formed completely (Fig. 1e, f). Those protruded bud spots became more prominent, and their color gradually changed to yellowish. As shown in Fig. 1l, observations by SEM revealed that the leaf sheath-like structure was protecting the shoot meristem (asterisk), and clusters of adventitious buds have formed. In addition, we also observed numerous
adventitious buds at different development stages on the explants simultaneously, and no vitrification was detected (Fig. 1j).

The results of bud induction and proliferation varied with the type and concentration of PGRs, as shown in Fig. 2. In general, the addition of CKs supplemented with lower concentrations (or no addition) of auxins to the medium promoted the morphogenic responses. 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 and proliferation 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 supplemented with 2 mg L\(^{-1}\) KT in combination with 0.5 mg L\(^{-1}\) TDZ, 0 or 0.1 mg L\(^{-1}\) NAA was proved to be most effective, yielding average 14.67 and 15.33 buds per explant, with adventitious bud induction rates being 92.67% and 90.66%, respectively after 30 days of incubation, significantly higher than other treatments \((p<0.05, \text{Fig. 2})\). Moreover, compared with TDZ and NAA, treatments with suitable KT concentration showed more buds per explants and adventitious bud induction rates. Of the various treatments tested, with the increase of the KT concentration, both Korean pine adventitious bud induction rates and number of shoots per explant increased first and then decreased. The medium containing 1 mg L\(^{-1}\) KT combined with TDZ (0.2 or 0.5 mg L\(^{-1}\)) and NAA (0 or 0.1 mg L\(^{-1}\)), only produced 3.67–6.67 buds and gained bud induction rates of 27.4–43.2%. The concentration above or below the optimal concentration of KT did not improve these two parameters of adventitious buds. Compared with 2 mg L\(^{-1}\) of KT, when KT concentration increased to 3 mg L\(^{-1}\), both the induction rates of adventitious buds and the number of induced buds decreased significantly. In addition, higher KT dose (3 mg L\(^{-1}\)) in the medium also led to the development of stunted and clustered buds.

**Effects of plant growth regulators 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. Figure 1k, l and 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.

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 < 4 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\(^{-1}\)) (Fig. 3m), significantly higher than other treatments \((p<0.05)\), followed by AC-free treatment (26.54 mm) (Fig. 3). 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 indicated that the
complementary of low concentration of 6-BA and NAA was efficient for promoting rapid growth and elongation of shoots in Korean pine.

**Rooting and acclimatization of the shoots**

Induction of roots in vitro is an essential step to establish tissue culture derived 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 et al. 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 (Fig. 1n). Of the 13 tested treatments, while the average rooting percentages varied from 0 to 21.48%, only one true root was formed per rooted shoot. The highest rooting percentages were 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 (Fig. 4). In addition, the medium without any PGRs (control) did not gain any roots from elongated shoots. When NAA concentration increased to higher than 0.1 mg L⁻¹, it was observed that a large amount of calli were formed at the base of shoots, and many deformed adventitious roots were produced from these calli (Fig. 1o), especially under the treatments with NAA of 0.2 mg L⁻¹ and IBA of 0.5, 1 or 2 mg L⁻¹, respectively, where no roots were produced, but a lot of calli were produced at the location where the roots were formed (Fig. 1p). In vitro rooting percentages were only between 0 and 6.67% when treated with IBA alone with a small amount of calli appearing on their base. Figure 1q showed the rooted shoots after 60-day rooting treatment, and the rooted shoots have acclimatized in the sterilized matrix. After 1 month of growth in the greenhouse, the survival rate of acclimatized plants was 90.28%, and all regenerated plantlets showed...
normal growth and morphology under greenhouse conditions (Fig. 1r).

Discussion

De novo shoot organogenesis from sprouted seed explants of Korean pine

In long-lived trees such as conifers, despite considerable amount of research-derived plantlets from indirect or direct organogenesis, most coniferous species are still regarded as extremely difficult to regenerate under natural conditions (Bonga 2017; Sarmast 2018). For Korean pine, although the in vitro organogenesis technique was first studied in the 1990s (Liu et al. 1991), and recently, in vitro propagation of this species through somatic embryogenesis from callus tissue derived from immature zygotic embryos has also been reported (Gao et al. 2020; Peng et al. 2021), the overall regeneration frequency of Korean pine through either organogenesis or embryogenesis is still low. Thus, the in vitro propagation technique for Korean pine remains challenging with a low efficiency and there are no efficient in vitro reproducible protocols for Korean pine until now. Therefore, as a commercially and economically important tree species, developing a robust regeneration protocol for Korean pine is urgently needed. In several pine species, it is a common practice to induce adventitious buds from whole germinating embryos or cotyledons for a certain time before applying any induction treatments (Valdés et al. 2001; Villalobos-Amador et al. 2002; Hargreaves et al. 2005; Montalbán et al. 2011). Therefore, in this study, we used sprouted seeds as the initial explants to overcome the low frequency of shoot organogenesis that has been encountered in Korean pine. The direct organogenesis of Korean pine from sprouted seed explants was achieved, and SEM observations also confirmed the direct organogenesis method in our study, showing buds arising from cotyledons base of sprouted bud explants. This propagation way by direct organogenesis has also been proven to have less risk of somaclonal variations than indirect organogenesis (Zayova et al. 2010; Verma et al. 2021).

Overall, in our experiment, 92.67% of explants produced direct buds with an average of 15 buds per explant in the best treatment after 30 days of incubation, which was much higher than a previous report in which the induction rate of adventitious buds of Korean pine with mature zygotic embryos as explants was only 30% (Liu et al. 1991). Moreover, it is worth noting that our adventitious bud induction rates were higher than previously reported results of other pine micropropagation (Stojičić and Budimir 2004; Diego et al. 2010; Montalbán et al. 2011; Stojičić et al. 2012; Wang and Yao 2019a; Zarei et al. 2020). However, the adventitious bud induction rates in this study were lower than that of *Pinus massoniana* (99.6%, Zhang et al. 2006) and *Pinus densiflora* (100%, Zhu et al. 2019). And the number of buds inducted in in this study was higher than that of *P. massa- niana* (11.6, Zhang et al. 2006), but was lower than a maximum number of 18 buds in *P. densiflora* (Zhu et al. 2019).

Generally, essential steps in establishing in vitro micropropagation are the induction and multiplication of shoots (Li et al. 2018). The formation and multiplication of adventitious buds 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 and thus 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 explants to reach an optimum cytokinin:auxin ratio for de novo meristem formation (Alvarez et al. 2020). Among them, the most efficient CKs that have been routinely used are 6-BA, KT, and TDZ (Khanam et al. 2020). Our results were similar to those previously reported in other woody trees. A higher cytokinin:auxin ratio in the medium was beneficial to induce multiple shoots (Zhu et al. 2010, 2019; Tippani et al. 2013; Ahmad et al. 2020). In our study, compared with CKs alone in the medium, application of high concentration CKs (i.e., 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 responses of Korean pine explants, whereas the addition of auxin was not necessary. In addition, compared with TDZ and NAA, the treatment with suitable KT concentration showed more buds per explants during the adventitious bud induction of Korean pine, and the concentration 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 inhibited 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; Syeed et al. 2021). Similarly, Dey et al. (2012) and Dewir et al. (2018) both mentioned that TDZ could stimulate cells in the apical meristem to divide, multiply, and develop so that bud differentiation occurred. 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 and Anis 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 bud formation and multiplication of...
Korean pine, which could be explained by that the appropriate concentration of TDZ and whether it was added solely or combined with other hormones are species-specific.

**Shoot elongation and growth of in vitro Korean pine**

The subculture procedure is very important in the long-term culturing and elongation of shoots. In the second step of our experiment, adventitious buds of Korean pine were transferred onto the shoot elongation medium. The results showed that the maximum bud length after 60 days of culture was 34.99 mm and our results were better in terms of shoots length compared to other pine micropropagation, such as *Pinus elliottii × Pinus caribaea* (27.6 mm, Meyer 1998), *Cunninghamia lanceolata* (28.2 mm, Zhu et al. 2007), *Pinus pinaster* (30 mm, Humánez et al. 2011), but the bud length in this study was lower than that of *Pinus sylvestris* (40 mm, Sul and Korban 1998) and *Pinus peuce* (57.5 mm, Stojičić et al. 2012).

Overall, PGRs had significant effects on the length and number of the shoots. The elongation growth of the in vitro-established buds was significantly accelerated. Specifically, low concentration combinations of 6-BA and NAA were found to be efficient for promoting rapid growth and elongation of Korean pine adventitious buds with the most extended shoots being generated on the DCR basal media containing 6-BA (0.2 mg L⁻¹), NAA (0.1 mg L⁻¹), and AC (1 g L⁻¹). Therefore, our results suggested that a combination of a higher cytokinin:auxin ratio in medium was efficient for successful shoot inducing morphogenic responses and stimulating proliferation. In contrast, for shoot elongation of Korean pine, lower concentration of the same PGRs was more appropriate, which was also in agreement with those plant micropropagation studies that have demonstrated that for promoting bud elongation, medium without plant growth regulators (Stojičić et al. 2012; Zhu et al. 2019) or with a lower level of cytokinin with auxin (Zhang et al. 2006; Zhu et al. 2007; Schaller et al. 2015) or 0.1–0.5% AC (Stojičić et al. 2012; Zhu et al. 2019) was usually proven to be effective.

**Rooting and acclimatization of Korean pine microshoots**

It is well known that 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 this has largely limited the applications of these techniques to large-scale micropropagation (Stojičić et al. 2012; Bonga 2017). Generally, auxins play an essential role in regulating adventitious root formation, and IBA and NAA are often used to promote in vitro rooting (Kalia et al. 2007; Alvarez et al. 2009; Wang and Yao 2019a, b). For PGRs applied to in vitro rooting of pine species, there are different results. For example, for *P. radiata*, in vitro organogenesis was a common practice using NAA singly or the mixture of IBA and NAA for rooting (Hargreaves et al. 2005; Montalbán et al. 2012). Most frequently, IBA application was a preferable alternative for in vitro rooting of *P. massoniana* (Zhang et al. 2006), *C. lanceolata* (Zhu et al. 2007), and *Picea abies* (Zarei et al. 2020), which was better than a combination with NAA or a mixture of both auxins. In contrast, supplementation with 0.27 mg L⁻¹ NAA in the medium was effective for rooting induction of *P. massoniana* (Wang and Yao 2019b), and most of the clones from *P. densiflora* also rooted on medium supplemented with 0.2 mg L⁻¹ NAA (Zhu et al. 2019). After 2 months of culture on root-promoting medium, the highest rooting percentages were < 30% in our present study when 0.05 mg L⁻¹ NAA and 0.5 or 1 mg L⁻¹ IBA were used. Our results were consistent with previous studies using hormone types (NAA and IBA), but the concentration combination was different, which can be attributed to differences in coniferous species that lead to different auxins responses of in vitro rooting.

Furthermore, in our study, we observed that NAA concentrations above the optimal levels (0.1 or 0.2 mg L⁻¹) induced a large amount of undesirable calli and also resulted in a few deformed adventitious roots at the basal end of the microshoot. Our findings were consistent with a previous report that when high concentration NAA was used to induce adventitious roots in *P. massoniana* (Zhang et al. 2006), a mass of calli from which root primordia differentiated was produced on the base of most shoots, leading to poor rooting. In contrast to this, rooting in *P. densiflora* was related to the genotypes under the treatment of 0.2 mg L⁻¹ NAA, with the rooting percentages varying from 8.0 to 87.5% among the test clones and no callus being observed at the base of the shoots (Zhu et al. 2019). In addition, in our study, when treated with IBA alone, a small amount of calli appeared and resulted in poor rooting of Korean pine. Similarly, in the study of in vitro rooting from *Pterocarpus marsupium*, it was also found that when the concentration of IBA was beyond the optimum dose, it induced undesirable calli at the basal end of microshoot and no rooting was observed (Ahmad et al. 2020). However, in the study of *P. elliottii* (Nunes et al. 2018), media containing only IBA induced less rooting with no callus formation, which may be due to differences in tree species. In addition, 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 without PGRs or hormone-free AC would 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 was added to all rooting treatments. However, contrary to earlier reports, our results indicated that cultivating 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 factors, 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 rarely. Compared with P. peuce (Stojičić et al. 2012), P. massoniana (Wang and Yao 2019b), and P. densiflora (Zhu et al. 2019), Korean pine may be more difficult for in vitro rooting in micropropagation. Therefore, further improvement of in vitro rooting percentage is necessary for this species, and this can be achieved by optimizing induction conditions such as the medium formula and PGRs combination, light regime and source of carbohydrate.

Conclusions

Plant multiplication in vitro has been proved to be very efficient for large-scale propagation of many tree species, providing a more valuable and faster way than the traditional cutting procedure. Nevertheless, developing in vitro protocols for coniferous species is still challenging because of such factors as recalcitrance. In this study, we developed a useful protocol for multiplying plantlets from sprouted seed explants of Korean pine, an economically and ecologically important coniferous species, although the problem of low rooting rate still needs further optimization. The de novo direct organogenesis system for Korean pine leads to the generation of true-to-type strains to avoid callus phase organization and have fewer chances of somaclonal variation than indirect organogenesis. Nevertheless, this method is not applicable for propagating selected trees in field, and therefore, it is still essential to develop propagation techniques using adult vegetative explants. In summary, our results will be helpful in future research for rapid micropropagation and conservation of Korean pine, and might also facilitate the development of biotechnological tools to study such coniferous species.

Author contributions LY designed the experiment and provided technical guidance. BX and XX wrote the manuscript and conducted the data analysis. XHG and WJ supervised the experimental process. PP 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.

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