Promoting the application of pinus thunbergii Parl. toward enhancing the growth and survival rates of post-germination somatic plantlets

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Abstract

There is a growing need for nematode-resistant Pinaceae species seedlings to cope with the degradation of global coniferous forests due to the prevalence of pine wilt disease. One of obstacle of Pinaceae species plantlets commercialization is the regeneration plantlets from a controlled, sterile environment to the field while maintaining a high survival rate. The low survival rate of plantlets transferred from a lab to soil is mainly because of the quality of plantlet which makes commercialization challenging. For this paper, we investigated the factors that influence the growth of post-germination somatic regeneration plants (SRPs) to promote the application of nematode-resistant of *Pinus thunbergii*. The results indicated that, for rooted plantlet, a suitable liquid medium (1/2 WPM), transplantation stroma (perlite and vermiculite =1:1), and carbohydrate (20 g/L sucrose) were effective for promoting the growth of SRPs. While, for unrooted plantlets, 1 ug/L of brassinolide enhanced plantlets growth and rooting. In the laboratory domestication stage, under light spectrum treatments, blue light (B) significantly promoted the longitudinal growth of shoots, while red light (R) was of benefit for the improvement of root growth. The optimal combination of R/B was the 8:2. After this domestication culture, SRPs of *Pinus thunbergii* could be directly transplanted to the field with a higher survival rate (85.2%). This is the first report on domestication of conifer regeneration plants, that will increase the possibility of afforestation of Pinaceae or other woody plants. In addition, it contributes to breeding project of pine resistance to pine wilt disease.

Introduction

Pinus thunbergii (an evergreen tree species) is resistant to sea mist and wind; thus, it is employed extensively in coastal urban hill greening and as a coastal windbreak in China, Japan, and Southern Korea (Han et al., 2008; Zhang et al., 2009; Mao et al., 2019). However, it is susceptible to pine wilt disease caused by the *Bursaphelenchus xylophilus* nematode, which has propagated worldwide. The epidemic of pine wilt disease has led to a massive decline of *Pinus thunbergii* trees, and threatened entire*Pinus* ecosystems (Hussain et al., 2021). The deployment of PWN-resistant genotypes is an important strategy to control the disease (Li et al. 2022). Thus, a long-term breeding project was undertaken to select resistant clones for pine trees in Japan (Futai K and Furuno T, 1979; Endo R and Fukuhara K, 2017; Fujimoto et al., 1989). Somatic embryogenesis has proved to be one of the most promising techniques for the mass propagation of conifers. Our group had reported the somatic embryogenesis and plant regeneration of nematode-resistant *Pinus thunbergii* (Sun et al., 2019; Wu et al., 2008; Li et al., 2013; Zhu et al., 2019). Besides, many regenerated plants had been obtained from*Pinus* species via somatic embryogenesis, including *Pinus pinea* (Carneros et al., 2009), *Pinus elliottii* (Yang et al., 2020), *Pinus thunbergii* (Maruyama et al., 2005; 2012) and*Pinus radiata* (Montalban et al., 2012). However, reports on the application in afforestation of SRPs were rare, where low survival rate was the primary challenges.

The transition from lab- to field-seedlings is a major obstacle in plant breed technology. The difference of microenvironment between seedlings cultured in lab and in field made the regeneration plant delicate and weakened root system which resulted in a survival rate (Zhou et al., 2022). Transplantation survival directly determines whether tissue-cultured regeneration plant can be utilized in field production. An effective acclimation cultivation project could generate high-quality seedlings to promote their survival rate (Matin et al., 2000). As a facilitator, sugar is essential for plant growth and mediating effective molecular signaling processes for plant development (Smeekens et al., 2010). During micropropagation, sucrose-cleaving enzymes could rapidly promote leaf growth. Moreover, the transport of sugars in the phloem influenced the development of aboveground organs and roots (Pantin et al. 2012; Rottmann et al. 2018; Wang et al. 2013). Therefore, the application of sugar in vitro was key toward enhancing the growth and development of plantlets (Rolland et al. 2006; Zhu et al. 2018). Culture medium and transplantation stroma provide essential nutrients, and possess good water retention, air permeability, and acid-base buffering capacities for plant regeneration. The selection of the transplantation stroma is one of the key factors determining the survival rate of transplants. The survival rate of *P. densiflora* plantlets was up to 60% in mixture of transplantation stroma containing vermiculite-perlite-sand (1:1:1) (Zhu et al., 2010). The was approximate. Brassinosteroids (BRs) have been extensively applied in plant growth studies, as it is considered to be an effective and ecofriendly phytohormone that regulates the differentiation of root epidermal cells and root hairs, while facilitating the formation of lateral roots (Wang et al., 2009; Nolan et al., 2020). Further, the beneficial effects of spectral treatments on plant growth have been widely observed (Ranade et al. 2016; Li et al. 2013). These factors justify the enormous research efforts that have been undertaken to improve nursery protocols for the generation of highly quality plantlets. However, these studies, particularly the application of BRs and spectral treatments for the regulation of plant growth, have been less frequent for pine tree species (Lazzarini et al. 2018). Previously, our group reported the plant regeneration of nematode-resistant *Pinus thunbergii* through somatic embryogenesis (Sun et al., 2019). To further promote the application in afforestation of SRPs, we studied the growth factors of sucrose, medium types, BRs, and spectral treatments on the of nematode-resistant *Pinus thunbergii* SRPs. In addition, their survival rates were continuously monitored. The aim of this study was to develop a reliable acclimation cultivation project that would improve the quality of *pinus* species plantlet and increase the transplanted survival rate in field.

Materials and methods

Plant material

The SRPs were obtained from 1539-1 and 1637-2 cell lines through somatic embryogenesis. The 1539-1 and 1637-2 cell lines were initiated in nematode-resistant *P. thunbergii* families 39 and 37, respectively. The protocols for somatic embryogenesis in nematode-resistant *P. thunbergii* was conducted according to our previous group report (Sun et al. 2019). Briefly, embryogenic cells were isolated from immature cones and cultured on a maintenance medium for proliferation. Subsequently, the embryogenic cells (fresh weight 1g) were transferred to Erlenmeyer flasks (including 30 mL of liquid proliferation medium) and placed on an orbital shaker at 90 rpm for 1 week. The 2 mL suspension was transferred to a maturation medium for 10 weeks in the dark at 23 ± 1. After 10 weeks of culturing (without intervals), the cotyledon somatic embryos were taken from the maturation medium and transferred to the germination medium for four weeks under cool white fluorescent light (16/8 h) at 23+-1. The post-germination SRPs were selected as the experimental samples.

Determination of root and shoot lengths after sucrose, liquid medium, and culture stroma treatments

The SRPs of 1539-1 were transferred to the growth medium.**Sucrose treatment** : the growth medium was a 1/2 WPM liquid medium (30 mL), which was supplemented with culture stroma vermiculite: perlite =1:1 (1/5 volume of flask, approximately) and sucrose (10, 20, 30, and 40 g/L). Each SRP was transferred into a dedicated flask, and each group was treated with 15 plantlets and repeated three times. After one-month of culturing under cool white fluorescent light (16/8h) at 23+-1, the regenerated plantlets were removed from the flasks and the roots were rinsed. Next, the roots and root shoot lengths (total lengths of the aboveground

parts) of the regenerated plants were measured with the scale.

Liquid medium treatment: the growth medium was supplemented with sucrose (20 g/L), culture stroma vermiculite: perlite =1:1 (˜1/5 volume of flask) and the liquid medium (DCR, LP, GD, and 1/2WPM) (30 mL). The other conditions were the same as the sucrose treatments.

Transplantation stroma treatment: The growth medium contained 30 mL of a 1/2WPM liquid medium, which was supplemented with 20 g/L sucrose, and a 1/5 flask volume of transplantation stroma (perlite, vermiculite, perlite and vermiculite = 1:1, respectively). The other conditions were identical to sucrose treatments. The transplantation stroma was bottled for sterilization and cooled prior to adding the sterilized liquid medium. Following growth enhancement, the plantlets were transplanted into a field for domestication.

Determination of root and shoot length following brassinolide treatments

The SRPs were obtained from 1637-2 cell lines, and the SRPs with shoots but no radicles were transferred to the rooting medium. The rooting medium was supplemented with 1/4 WPM (large element reduction), brassinolide (0, 10, and 1 μg/L), 20 g/L sucrose, 0.5g/L activated carbon, and 6 g/L agar. Each SRP was transferred into a dedicated flask. Each group was treated with 15 plants, which was repeated three times. The plantlets were cultured on the rooting medium for one-month under cool white fluorescent light (16/8h) at 25±1. The plantlets were then removed from the rooting medium and the roots were rinsed, after which the roots and shoot lengths (total lengths of the aboveground parts) were measured with a scale.

Determination of root and shoot lengths after red and blue LED light treatments

Experiments that investigated the impacts of different light wavelengths on SRP growth were conducted with the 1637-2 cell lines. The SRP was transferred to the growth medium which was supplemented with 30 mL of a 1/2WPM liquid medium, 20 g/L sucrose, and 1/5 flask volume of culture stroma (perlite and vermiculite = 1:1, respectively). Each SRP was transferred into a dedicated flask. The plantlets were cultured for one-month under LED treatments (16/8h) at 25+-1c. LEDs with different red and blue ratios were used as the light sources (B: blue light; 5R5B: red light: blue light =5:5; 7R3B: red light: blue light =7:3; 8R2B: red light: blue light =8:2, R: red light). The cool white fluorescent lamp served as the CK. Each group was treated with 10 plantlets and repeated three times. The root growth parameters were measured using a root scanner, and the shoot lengths were measured with a scale.

Determination of transplant survival rate

Following growth enhancement, the plantlets were transplanted into a culture room for domestication. The transplanting substrate was mixture of pine forest soil, perlite, and vermiculite at a ratio of 2:1:1. Each SRP was transferred into a dedicated flask. Each group was treated with 10 plants and repeated three times. The plantlets were cultured in culture room under cool white fluorescent light (16/8h) at 23, and watering once a week. The survival rate of the plantlets was calculated after six months.

Statistical analysis

The transplant survival rate was evaluated using an analysis of variance (ANOVA and Principal component analysis (PCA)). Means were compared by Duncan's honestly significant difference test at $p < 0.05$. All analyses were done using SPSS version 19 (IBM, Armonk, NY, USA) and R (R Core Team 2019).

Results

Enhancement of SRPs by culture conditions: sucrose, liquid medium, and transplantation stroma

The needles of the SRPs were sparse and yellow-green under a 10 g/L sucrose treatment, showing an obvious nutrient deficiency phenomenon. When the sucrose concentration was increased to 20 and 30 g/L, the needles were darker in color and the plant height was significantly higher than under the 10 g/L treatment. However, when the sucrose concentration was raised to 40 g/L, the growth of the SRP was inhibited. Furthermore, the plant height of the SRP was higher in the 1/2 WPM and GD than the DCR and LP liquid medium.

Similarly, the plant height of the SRP in perlite and vermiculite = 1:1 medium treatment was higher than that of only the perlite and vermiculite. In general, the 20 and 30 g/L sucrose, liquid medium GD and 1/2 WPM and culture stroma (perlite: vermiculite = 1:1) were observed to significantly enhance the SRP growth (Fig. 2 A).

With further study, the sucrose concentration (shoot, $p< 0.001$; root, $p = 0.002$), liquid medium (shoot,$p < 0.001$ and root, $p < 0.001$) and culture stroma (shoot, $p = 0.03$; roots, $p = 0.09$) significantly affected the SRPs growth. Under the sucrose treatments, when the concentration was doubled from 10 to 20 g/L, the shoot lengths of the SRPs were increased from 1.00 to 2.05 cm, and the root lengths were increased from 2.04 to 3.23 cm. Simultaneously, the lengths of both the shoots and roots reached their peak. However, when the sucrose concentration was raised to 30 g/L, the shoots and root lengths of the SRPs began to decrease. When the sucrose concentration was further increased to 40 g/L, the growth of the SRPs was significantly inhibited (Fig. 2 B). Under the liquid medium treatments, the longest shoots (1.44 cm) and roots (4.47 cm) of SRPs were obtained with the 1/2 WPM medium, followed by the GD liquid medium treatment (shoots = 1.24, roots = 3.89 cm). However, among all the tested liquid media, the shortest shoots (0.54 cm) and roots (0.75 cm) of the SRPs were obtained with the DCR medium (Fig. 2 C), which was obviously not suitable for the growth of*P. thunbergii* SRPs.

For the transplantation stroma treatments, the minimum shoot (0.72 cm) and root (1.48 cm) values were obtained under the perlite treatment, followed by the vermiculite treatment (shoot = 0.77 cm, root = 1.90 cm). However, the lengths of the SRP shoots and roots cultured in a stroma mixture of perlite and vermiculite (v/v = 1:1) were significantly higher than when they were cultured in perlite and vermiculite alone. The results revealed that the shoot and root lengths were significantly ($p < 0.05$) increased to 1.26 cm and 3.89 cm, when the vermiculite and perlite were mixed at a 1:1 ratio as the transplantation stroma (Fig. 2 D). This suggested that the 20 g/L sucrose, 1/2 WPM liquid medium, and transplantation stroma of vermiculite and perlite mixed at a 1:1 ratio were the most suitable for the growth of post-germination nematode-resistant *P. thunbergii*plantlets.

Effects of BR on the growth of SRPs

The BR content significantly ($p < 0.001$) affected the growth of roots and shoots of SRPs without rooting. Statistical results showed that the length of roots and shoots of SRPs under the treatment of 1 μg/L BR was significantly higher ($p < 0.05$) than that of the 10 μg/L BR and control group (Fig. 3 A). In addition, the needles were light green and the root system consisted of only one primary root, with no lateral roots in the control group. When the rooting medium was supplemented with 1 μg/L BR, the needles were dark green and a well-developed root system emerged (more lateral roots, greater root volume, and more root tips). However, when the BR concentration was increased to 10 μg/L, the SRPs showed a similar differentiation to the control group (Figs. 3 B, C, D). These results clearly indicated that 1 μg/L BR promoted the growth of *P. thunbergii* SRPs via root system differentiation. Further, they implied that the BR promoting the growth of *P. thunbergii* SRPs might related to photosynthetic regulation.

Effects of red and blue LED light sources on the growth of SRPs

The light treatment had significant ($p < 0.001$) effects on the shoot length and root differentiation of the SRPs. The shoot length of SRPs was significantly ($p < 0.05$) increased under the blue light treatment, while the needles were light green in contrast to the CK treatment. Additionally, the root differentiation was significantly ($p < 0.05$) promoted under the R, 7R3B, and 8R2B treatments (Fig. 4). With further statistical analysis, the greatest shoot length (3.56 cm) was obtained under the blue light treatment (Fig. 4 A). In terms of root differentiation, the total length of roots, their volume, and the number of root tips all reached the peak under the 8R2B treatment (Figs. 4 B, D, E). However, the root surface area and average diameter reached their peak under the R treatment (Fig. 4 C, F). In general, the blue light promoted the longitudinal growth of the shoots, whereas the red light was beneficial for root development. In addition, the optimal combination for the enhancement of the SRP was 8R2B.

SRP survival rate

The treatments that effectively promoted the growth of SRPs also contributed to the survival rate of the transplanted plants. For example, the SRPs treated with 20 g/L sucrose obtained the highest survival rate (85.2 %). Similarly, following the liquid medium and transplantation stroma treatments, the highest survival rate was obtained in the 1/2 WPM and mixture of vermiculite and perlite (1:1 (v/v)). Thus, the optimal enhancement for *P. thunbergii* SRPs was 20 g/L sucrose, 1/2 WPM liquid medium, and a transplantation stroma of vermiculite and perlite (1:1 (v/v)). Among the spectral treatments, the highest survival rate occurred under the 8R2B, while the lowest survival rate was under the B treatment. Furthermore, the survival rate of SRPs was significantly promoted in the rooting medium with 1 μg/L BR (Fig. 5). As for the ratio of roots to shoots (R/S), the results revealed that when the ratio of roots to shoots < 4cm, there was no relationship with survival rate. When the ratio of roots to shoots > 4cm, it was positively related to survival rate (Fig. 6). Among all treatments, those under the light spectrum had the highest survival rate. Thus, based on the light spectrum treatments, principal component analysis showed that the factor that had the greatest influence on the survival rate was the tips (Fig. S1). Moreover, the correlation analysis also showed that the correlation coefficient of the root tips on survival was the greatest ($p< 0.001$; r=0.53), while the highest correlation with the root tips was the root volume ($p < 0.001$; r=0.38). The root length had the lowest correlation coefficient ($p < 0.05$; r = 0.18) for the survival rate compared to the root tips, surface area, volume, and diameter, (Fig. 7).

Discussion

The results indicated that the liquid medium (shoot $p < 0.001$, root $p < 0.001$) conveyed the greatest effect on the growth of shoots and roots in *P. thunbergii* SRPs compared to sucrose (shoot $p < 0.001$, roots $p = 0.002$) and the transplantation stroma (shoot $p = 0.03$, root $p = 0.09$). For nematode-resistant*P. thunbergii* SRPs, the optimal liquid medium was 1/2 WPM followed by the GD medium. However, the DCR medium was obviously not suitable for the growth of *P. thunbergii* SRPs. Similarly, the slash pine plantlet showed a better growth performance in the GD medium than DCR medium (Zhu and Wu 2005). Further analysis revealed that the NO_3^-, NH_4^+, PO_4^{3-}, Mg^{2+}, and Ca^{2+} contents in the DCR medium were not significantly different compared to the 1/2 WPM medium; however, the K^+ content was only half that of the 1/2 WPM medium (Table S2). It is well known that plant growth is modulated by K^+ (Claussen et al. 1997). Tode and Luthen (2001) reported that the enhancement of plant growth via the fungal toxin fusicoccin and auxin both required K^+ uptake in maize coleoptiles. However, the K^+ content of the DCR medium was lowest of all the media. Thus, we speculated that the poor growth performance of SRPs under the DCR medium might have been related to a lower K^+ content. Additionally, the 1/2 WPM medium contained sufficient minor elements (e.g., Mn, Zn, and Ni) in contrast to the other media. The Mn and Zn ions improved chlorophyll synthesis and photosynthesis, which resulted in the modulation of plant growth, such as tomato (Shenker et al., 2004; Hansch and Mendel, 2009). As a component of urease, Ni promoted plant growth of *Caryophyllaceae* and Cruciferae, by modulating the transport of nitrogen from the roots to the leaves (Aller et al. 2010). This indicated that the improved performance of SRPs in the 1/2 WPM medium might be correlated with the concentration of trace elements.

In this experiment, the mixture of perlite and vermiculite at a 1:1 ratio as transplantation stroma was superior to the perlite and vermiculite only, for improving the growth of *P. thunbergii* SRPs. The pH of the transplantation stroma affected the root differentiation, whereas its porosity was positively correlated with the plant height (Gao et al. 1992; Wang et al. 2014). Thus, the transplantation stroma mixture improved plant growth through the regulation of these elements. Similarly, the *P. elliottii* root length was improved by the mixture of perlite and vermiculite compared to perlite only (An et al., 2011). As for carbohydrates, the quality of *P. thunbergii* SRPs were improved under sucrose concentrations of from 20 to 30 g/L. Moreover, the color of the SRPs needles was significantly darkened. Bhattacharyya et al. (2006) reported that 20 g/L sucrose efficiently enhanced the vertical growth and rooting of *Dendrobium nobile*plantlets. Further, Zhu et al. (2018) reported that photoassimilates were continuously increased when the *Hevea* leaf color changed from light to dark green, which was consistent with our research. Although sugar is essential for plant growth, in excess it can be detrimental. We found that the shoot and root lengths of *P. thunbergii* SRPs were inhibited when the sucrose concentration reached 40 g/L, as high concentrations of sucrose hindered

photosynthesis (Hdider and Desjardins, 1994). This was evidenced by the restricted growth of rice and maize when they accumulated higher concentrations of sugars in their leaves (Eom et al. 2011). Appropriate sucrose concentrations, which promoted the growth of *P. thunbergii* SRPs, might have been related to the regulation of photosynthesis. Akin to the sucrose treatment, the 1 μg/L BR root treatment showed the similarly enhancement for SRPs (Fig. 3B). BR mutants of *Arabidopsis* display a stronger dwarf phenotype and de-etiolated growth in the dark (Tanaka et al., 2003), and the application of BR increased the plant height of papaya (de Assis-Gomes et al. 2018). Further, Gao et al. (2017) reported the enhancement of photosynthesis in maize by foliar spraying with BR. The *P. thunbergii* SRPs enhancement by the BR might relate to the regulation of photosynthesis through the photosynthesis (the needles green to darker in 1 μg/L BR). However, the growth of *P. thunbergii* SRPs was inhibited when the BR concentration reached 10 μg/L; thus, the promotion of somatic plantlets using exogenous BR was optimized at lower concentrations. The application of BR for roots at lower concentrations was more effective for plantlet growth (Arteca et al., 2001; Pandey et al., 2020).

Moreover, in our study, monochromatic red light promoted the elongation of taproots, while monochromatic blue light promoted shoot elongation, the optimal combination of spectra for *P. thunbergii* SRPs growth was the red and blue at 8R:2B ratio. Kvaalen (1999) reported that red light wavelengths inhibited the elongation of Norway spruce (*Picea abies*) shoots compared to blue light and cool white fluorescent light. However, for lettuce, shoot growth resulted from red light exposure rather than blue light (Zhao et al., 2007), which indicated that light spectrum treatments on plant growth was variable between species. As is known, the photoreceptors for red light are the phytochromes (PhyA and PhyB), while the blue light photoreceptors are phototropins (Briggs et al., 2001; Rockwell et al., 2007). In terms of light regulators, phytochrome interacting factors (PIFs) played a key role in the modulation of plant growth. In green seedlings, elongation is primarily mediated by PIF4 and PIF5 (Lorrain et al., 2008; Hornitschek et al., 2012). In an *Arabidopsis thaliana* study, blue light stimulated the expression of phytochrome interacting factor4 (PIF4) and PIF5 in seedlings, whereas the PIF4 and PIF5 negatively modulated auxin signaling. For example, PIF4 and PIF5 repressed the expression of auxin-responsive marker genes IAA5 and GH3-LIKE (Sun et al., 2013). This suggested that blue light promoted stem elongation of *Arabidopsis thaliana* by suppressing the expression of auxin genes. The results of our light spectrum treatments were consistent with this study, which indicated that the blue light promotion of shoot elongation in conifers might be associated with low concentrations of auxin. Further, our results indicated that although the growth of the aboveground portions of the plantlets was suppressed, root development was promoted by red light. Adjusting the red to blue light ratio combined the advantages of monochromatic light; for example, the higher number of root tips, root volume, root length, and survival rate of nematode-resistant *P. thunbergii* were obtained under the 8R2B treatment. The promotion of root development by red light has been reported (Casal, 2000, 2013; Li et al., 2021). Ranade et al. (2016) suggested that combined red and blue light treatments enhanced the biomass as well as fiber size, resulting in stable tree structures. In general, light spectrum treatments have been recognized as an important factor for improving plant production and quality, which is extensively used in horticulture (Li et al., 2010). Conifer seedlings ware also known to respond to light spectrum treatment (Ranade SS and Gil MRG., 2016). A novel study was conducted regarding the effects of red and blue LED combinations, as well as white light treatments during post-germination and root development in nematode-resistant *P. thunbergii* plantlets. In this report, we discussed the effects of red and blue light, and white light treatments during root and shoot development of nematode-resistant *P. thunbergii* plantlets following germination, which influenced root development, shoot elongation, and the survival rate of plantlets after transplantation. Our studies on post-gemination plantlets revealed that the light quality could be manipulated to obtain high quality plantlets (improved shoot and root growth).

All treatments to enhance the growth of *P. thunbergii* SRPs contributed to its survival rate, which indicated that it is necessary to promote plant growth and improve the quality of plantlets to enhance the survival rate. Luis et al. (2009) considered that larger seedlings may augment transplantation performance in contrast to smaller seedlings. Further, although no statistically significant correlation was identified between the rootstock ratio and survival rate in our study, the high survival rate was focused on a rootstock ratio of

about 2 and 10. Interestingly, at a rootstock ratio of ˜10, and a root length of from between 10 and 30 cm, the survival rate was >60 %. However, at a rootstock ratio of 2 and root length less than 5 cm, the survival rate was also >60 % in half of the plantlets, which may have been related to their high quality. The correlation between root development and the survival rate showed that root tips had the greatest impact on the survival rate, followed by root surface area, root volume, and root diameter, with the root length having the lowest. Thus, for evaluating plantlet quality, the root tips, root surface area, and volume were the main criteria. In short, this study showed that enhancing SRPs quality, especially, promoting the plant root development is an effective strategy for improving the SRPs survival rate. This study makes a big step forward for pines afforestation strategy using SRPs. This study had made pines breeding progress in afforestation strategy using somatic embryo seedlings closer to success.

Conclusion

For this study, we initially articulated the acclimation protocols for the somatic plantlets (SRPs) of nematode-resistant *P. thunbergii* , and monitored their survival rates following transplantation. The results indicated that a growth medium containing 20 g/L sucrose, 1/2 WPM liquid medium, and a transplantation stroma (vermiculite and perlite = 1:1) was optimal for improving the *P. thunbergii* plantlet quality. Moreover, blue light promoted the shoot length, whereas red light promoted the main root length. The survival rates of regenerated plants were improved by all treatments that enhanced the SRP growth. This study investigated the factors involved in the transition from the heterotrophic (organic nutrient growth) to autotrophic (inorganic nutrient growth) stage of SRPs. The culture conditions were identified to improve the quality and the survival rates of the SRPs that were transplanted into the field. This research provides the foundation for future research toward increasing the survival rates of plantlets, and promotes the application of elite clones (nematode-resistant *P. thunbergii* plantlets)

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Competing interests

The authors declare that they have no competing interests.

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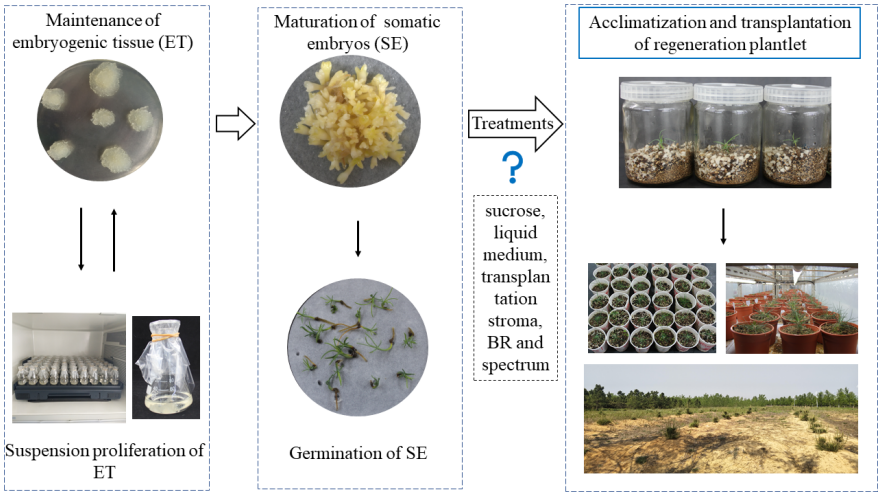

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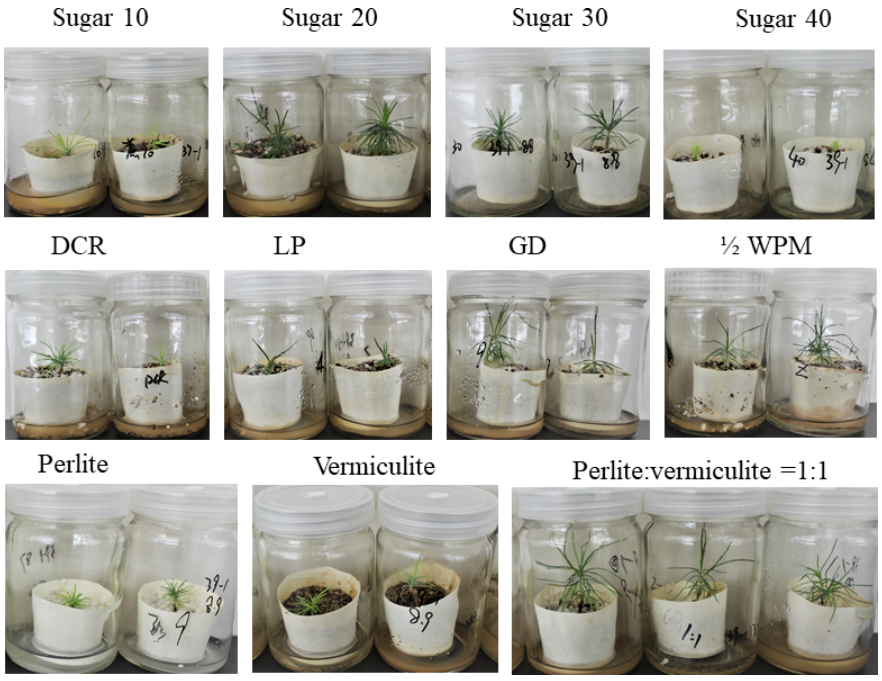
Fig. 1 Plant regeneration via somatic embryogenesis in nematode-resistant *Pinus thunbergii.*



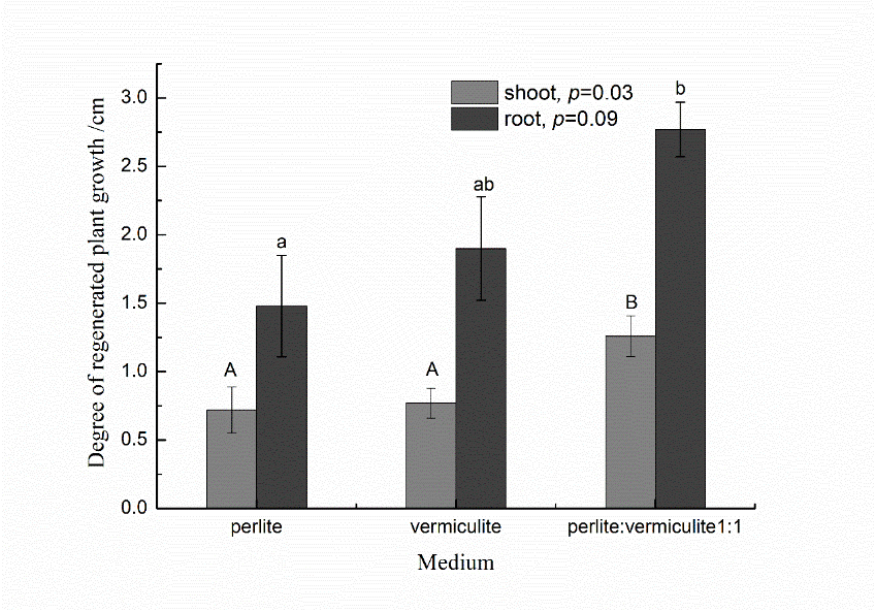


Fig. 2 Effects of (A) sugar content, (B) liquid medium, and (C) transplantation stroma on growth of nematode-resistant *Pinus thunbergii* plantlets. D: Performance of plantlet growth in treatments. Data represent mean ± standard error (SE). Different letters indicate significant difference ($p < 0.05$ by Duncan's test).

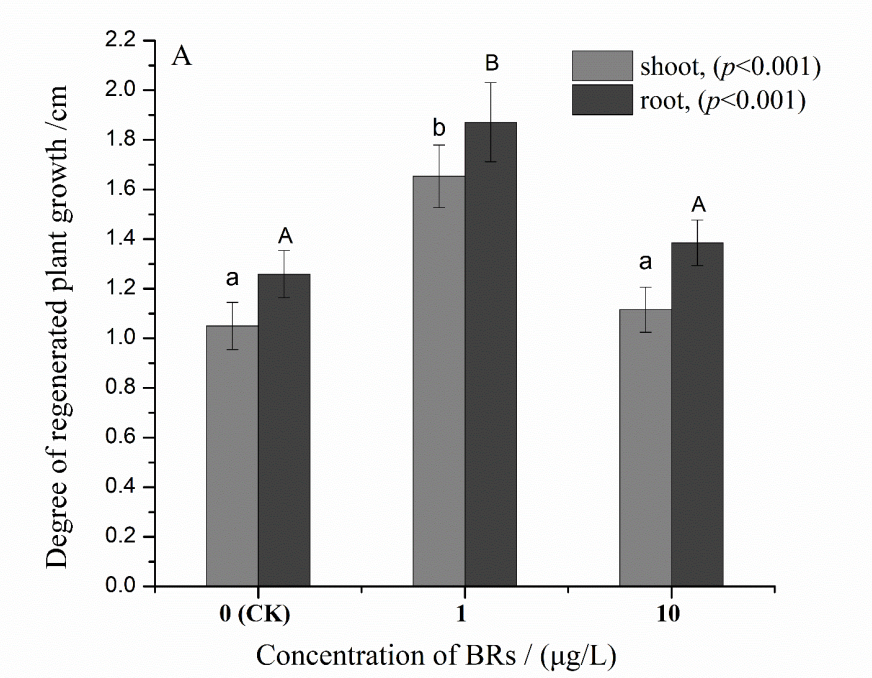


Fig. 3 Effects of BR on growth of nematode-resistant *Pinus thunbergii* plantlets.

A, B, and C are the performance of plantlet growth under CK, 1 μg/L, and 10 μg/L BR treatments D: Statistical analysis of root and shoot length of plantlets. Data represent mean ± standard error (SE). Significant differences between variances were calculated by two-way ANOVA. Different letters indicate significant differences between roots and shoots ($p < 0.05$, by Duncan's test).

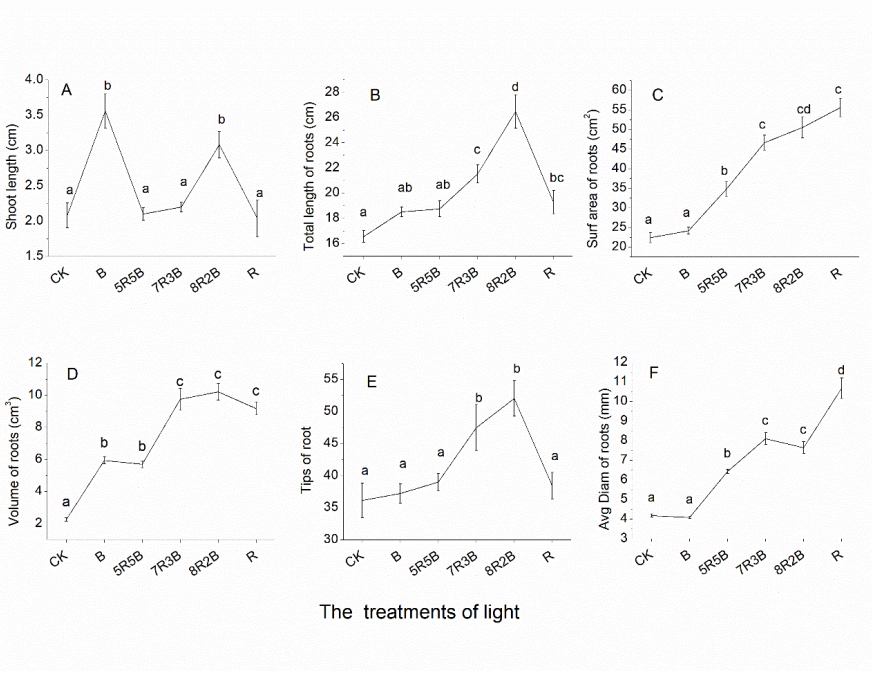


Fig. 4 Effects of spectrum treatments on growth of (A) shoot length, (B) total length of roots, (C) surface area of roots, (D) volume of roots, (E) root tips, (F) average diameter of roots in nematode-resistant *Pinus thunbergii* plantlets. Data represent mean ± standard error (SE). Different lowercase letters indicate significant differences between spectrum treatments ($p < 0.05$, by Duncan's test). G: Performance of plantlet growth under different light spectrum treatments.

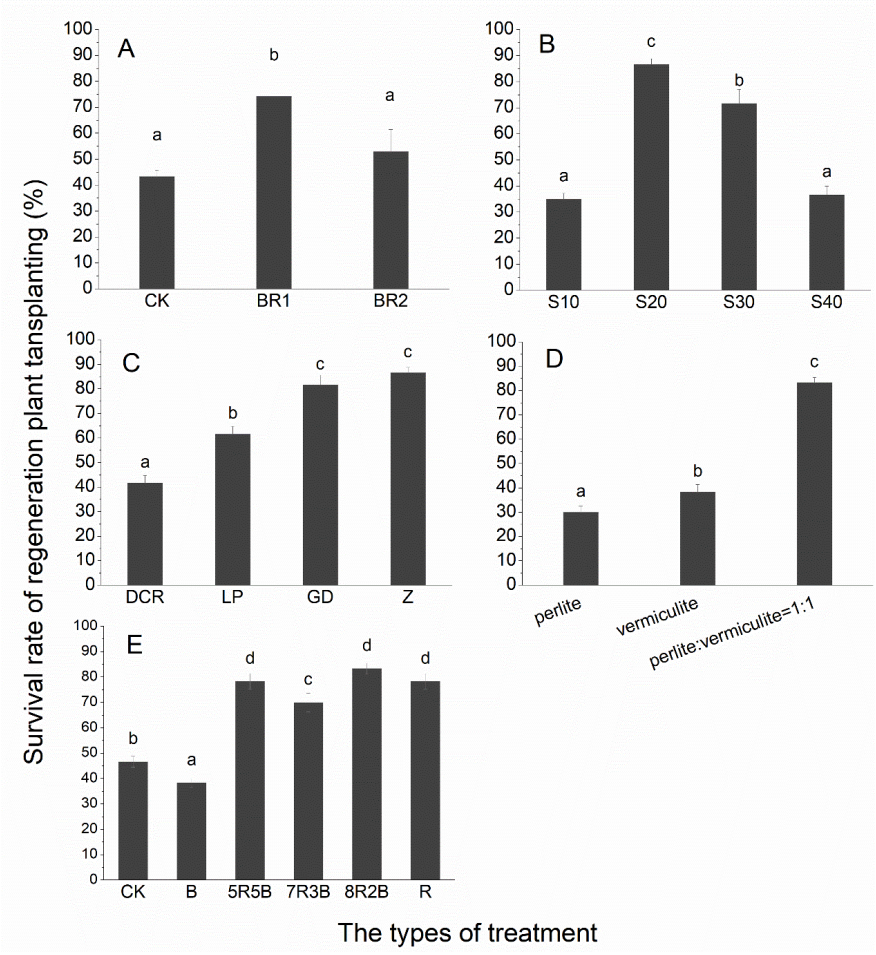


Fig. 5 Survival rates of (A) BR, (B) sugar, (C) liquid medium, (D) transplantation stroma, (E) spectrum treatments of nematode-resistant *Pinus thunbergii* plantlets. (F) Performance of transplantation plantlets after spectrum treatments for one month. Data represent mean ± standard error (SE). Different lowercase letters indicate significant differences between spectrum treatments ($p < 0.05$, by Duncan's test).

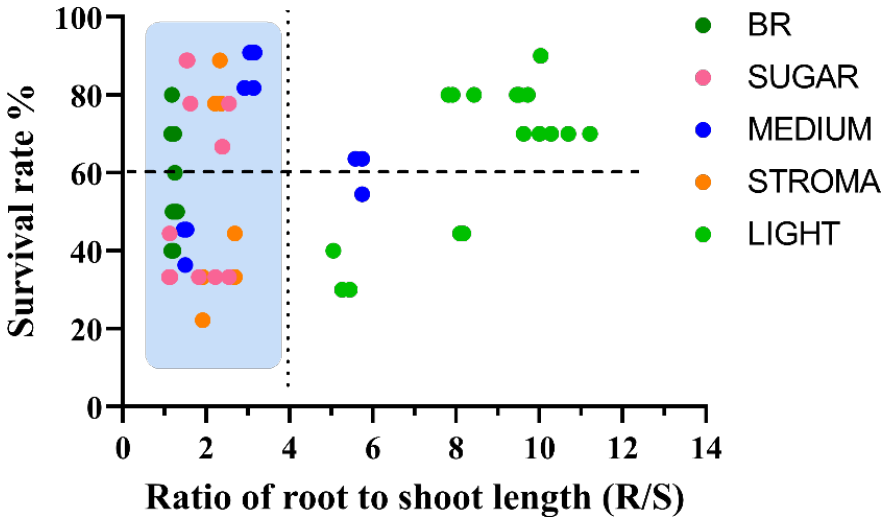


Fig. 6 Effects of root to shoot length ratios on survival rate. BR: BR treatments, SUGAR; sugar content treatment, MEDIUM: liquid medium treatment, STROMA: transplantation stroma treatment, LIGHT: spectrum treatment. Light blue shading indicates root length < 5cm. Dots represent the mean of each treatment.

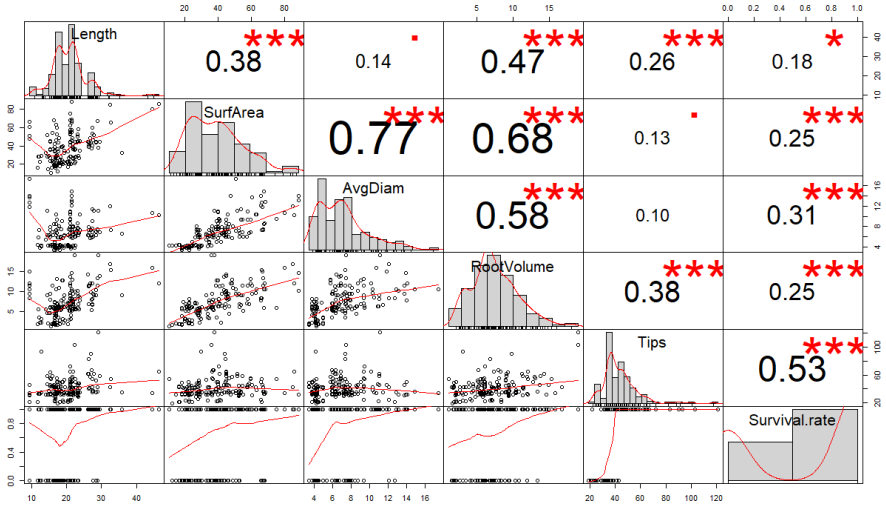


Fig.7 Correlation analysis of various factors affecting the survival rate. * and ** indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively.

Table S1. Basic composition of liquid medium.

Components	Components	Basic medium 1/2WPM mg/L	Basic medium GD mg/L	Basic medium LP mg/L	Basic medium DCR mg/L
Major elements	NH4NO3	400	400	200	400
	KNO3	0	1515	909.9	340
	KH2PO4	170	90	136.1	170
	CaNO3	556	30	236.2	556
	Cacl2	96	150	0	85

	MgSO4	370	250	246.5	370
	K2SO4	1000	300	0	0
	MgNO3	0	0	256.5	0
	MgCL2	0	0	101.5	0
Minor elements	KI	0	0	4.1	0.83
	H3BO3	0	3	15.5	6.2
	MnSO4	44.6	10	10.5	22.3
	ZnSO4	17.2	3	14.7	8.6
	Na2MoO4	0.5	0.25	0.125	0.25
	CuSO4	0	0.25	0.173	0.25
	COCL2	0	0.25	0.125	0.3
	NICL2	12.4	0	0	0.3
Iron salt	FeSO4	22.24	13.9	13.9	27.8
	EDTA	29.84	18.65	18.7	37.3
Organic additives	Nicotinic acid	0.5	0.1	0.5	0.5
	VB6	0.5	0.1	0.5	0.5
	VB1	1	1	1	1
	Glycine	2	0	2	2

Supplemental Information:

Table S2. Basic contents of main elements in liquid medium.

Parameters	Basic medium 1/2WPM mg/L	Basic medium GD mg/L	Basic medium LP mg/L	Basic medium DCR mg/L
NH_4^+	400	400	200	400
NO_3^-	956	1945	1602.6	1296
Total nitrogen	1356	2345	1802.6	1696
PO_4^{3-}	170	90	136.1	170
K^+	1170	1905	1050.1	510.83
Ca^{2+}	652	180	236.2	641
Mg^{2+}	370	250	604.5	370
B^{3+}	0	3	15.5	6.2
Mn^{2+}	44.6	10	10.5	22.3
Zn^{2+}	17.2	3	14.7	8.6
Na^+	0.5	0.25	0.125	0.25
Cu^{2+}	0	0.25	0.173	0.25
NI^+	12.4	0	0	0.3
Fe^{2+}	22.24	13.9	13.9	27.8

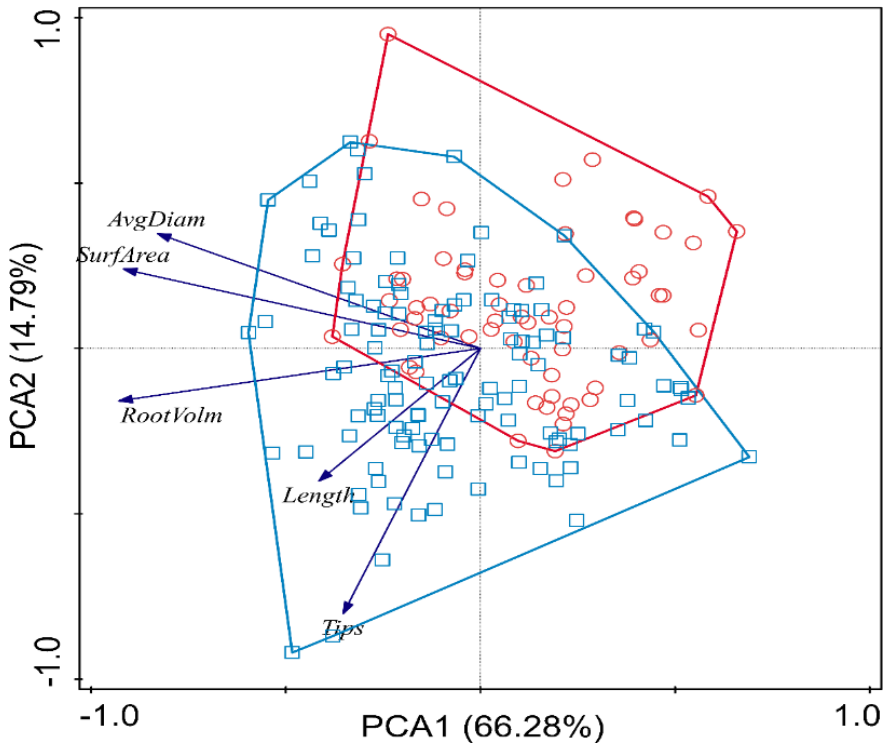


Fig. S1 Principal component analysis of plantlet growth for nematode-resistant *Pinus thunbergii*. Red circles represent dead plantlets and blue rectangles indicate living plantlets.