Somatic embryogenesis of slash pine (Pinus elliottii Engelm.): initiation, maturation, germination and mycorrhization of regenerated plantlets

QI FEI

feiqi1999@njfu.edu.cn

Nanjing Forestry University

You-Mei Chen

Nanjing Forestry University

Xin Ke

Nanjing Forestry University

Jian-Ren Ye

Nanjing Forestry University

Lihua Zhu

Nanjing Forestry University https://orcid.org/0000-0003-2740-4980

Research Article

Keywords: Pinus elliottii, Somatic embryo, Initiation, Maturation, Germination, Ectomycorrhizal fungi

Posted Date: March 13th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4017225/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on June 1st, 2024. See the published version at https://doi.org/10.1007/s11240-024-02789-3.

Somatic embryogenesis of slash pine (*Pinus elliottii* Engelm.): initiation, maturation, germination and mycorrhization of regenerated plantlets

Qi Fei[1,2], You-Mei Chen[1,2], Xin Ke[1,2], Jian-Ren Ye [1,2], Li-hua Zhu[1,2*]

1 Collaborative Innovation Center of Sustainable Forestry in Southern China, Nanjing-210037, People's Republic of China, **2** Institute of Forest Protection, College of Forestry and Grassland, Nanjing Forestry University, Nanjing- 210037, People's Republic of China.

*Corresponding author. E-mail: lhzhu@njfu.com.cn

Abstract

Slash pine (*Pinus elliottii* Engelm.), an important economic tree species with a high resin yield, is extensively cultivated in southern China. Somatic embryogenesis (SE) technology could hasten the breeding of *P. elliottii* through improving seedling quality and number, but mass production of somatic embryos remains constrained by various factors. We described an efficient SE system through indirect route and investigate the effects of genotype, phytohormones and culture condition on SE. Immature zygotic embryos of three open-pollinated mother trees collected during 2020–2023 were utilized for initiation of embryogenic callus (EC). The initiation of EC was significantly influenced by seed sources (families) ($p < 0.05$). The addition of 2,4-dichlorophenoxyacetic acid (6 mg/L), 6-benzylaminopurine (1 mg/L) and kinetin (2 mg/L) effectively improved initiation rates by up to 15.67 ± 1.89%. Furthermore, glutamine addition to the maturation medium enhanced the yield of SE during culture. The optimum concentration range of activated carbon within the germination medium spanned from 3–4.5 g/L. Inoculation with ectomycorrhizal fungus *Laccaria amethystea* enhanced the root length of regenerated plantlets, reaching 7.92 ± 4.06 cm. The regenerated plantlets inoculated with mycorrhizae *Pisolithus orientalis* and *Xerocomus chrysenteron* showed improved survival during the acclimatization phase, with 100% survival of mycorrhizae-treated plantlets after 3 months of acclimatization in the

greenhouse. We clarified the SE conditions suitable for *P. elliottii*, and the SE system and mycorrhizal method we used can be applied to mass production of plantlets.

Keywords *Pinus elliottii* · Somatic embryo · Initiation · Maturation · Germination · Ectomycorrhizal fungi

Declarations

Funding

This research was financially supported by the National Natural Science Foundation of China (No. 31971659), the Graduate Student Scientific Research Innovation Projects in Jiangsu Province (No. KYCX23_1252) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Conflicts of interest/Competing interests

The authors declare that they have no competing interests.

Ethics approval

Compliance with ethical standards

Consent to participate

The authors have seen and agree with the contents of the manuscript

Consent for publication

The authors that the submission is original work and is not under review at any other publication.

Availability of data and material

The data sets supporting the results of this article are included within the article and its additional files.

Code availability

Not applicable

Authors' contributions

QF and LHZ developed the idea of the study, participated in its design and coordination and helped to draft the manuscript. YMC and XK contributed to the acquisition and interpretation of data. LHZ and JRY revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This research was financially supported by the National Natural Science Foundation of China (No. 31971659), the Graduate Student Scientific Research Innovation Projects in Jiangsu Province (No. KYCX23_1252) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). Thanks are due to Xi'po State-owned Forest Farm for supplying pine cones.

Abbreviations

SE, Somatic embryogenesis
EC, Embryogenic callus
ECL, Embryogenic cell line
MG, Megagametophyte
PEM, Pro-embryogenic masses
2,4-D, 2,4-Dichlorophenoxyacetic acid
6-BA, 6-Benzylaminopurine
VC, Vitamin C
KT, Kinetin
NAA, 1-naphthylacetic acid
AC, Activated carbon

Introduction

Slash pine (*Pinus elliottii* Engelm.), a significant species of economic trees with characteristics of rapid growth, straight dry form and high resin content, and originates from the southern United States (Li et al. 1987). In the late 1940s, slash pine was brought to China and started being extensively cultivated in the southern regions by the late 1970s (Wen et al. 2004). Since 1978, pine needle brown spot has occurred in southern China, severely hindering *P. elliottii* development (Li et al. 1987). It is economically and ecologically significance to study *P. elliottii* resistance breeding. Since 1982, healthy trees have been selected from areas affected by the pine needle brown spot began in Fujian, China (Ye et al.

1991), and a disease-resistant seed orchard was established in 1986 (Ye and Li 1996). Nonetheless, the conventional approaches of breeding do not suffice to fulfill the demands of extensive mass production swiftly.

Plant somatic embryogenesis (SE) serves as a potent instrument in the field of plant biotechnology, offering an efficient method for the swift spread of superior genotypes (Pullman et al. 2005), and maximizing the advantages of conventional breeding practices by cultivating trees with advantageous traits for plantation forestry (Pullman and Bucalo 2014). The entire SE process beginning with initiation of an initial early-stage somatic embryos and continuing until germination is conducted in sterile conditions (in vitro) (Egertsdotter 2019). Beginning in 1985, with the report of successful SE in immature zygotic embryos of *Picea abies* (Chalupa 1985; Hakman et al. 1985) and *Larix decidua* (Nagmani and Bonga 1985), there has been significant advancement in the in vitro development of conifers. Successful SE system has been reported for at least 36 species of *Pinus* spp., such as *Pinus densiflora* (Kim and Moon 2014), *Pinus taeda* (Pullman et al. 2015), *Pinus pinaster* (Arrillaga et al. 2019), *Pinus radiata* (Montalban and Moncalean 2019) and *Pinus thunbergia* (Sun et al. 2019). Jain et al. (1989) were the first to report the embryogenic callus (EC) initiation and production of Stage-1 somatic embryos on culture medium in *P. elliottii*, with the successful regeneration of plantlets through SE being the initial report by Liao and Amerson (1995b). Newton et al. (2005) outlined an intricate procedure for regenerating plants using SE in *P. elliottii*. In recent years, enhancements have been made to each process in SE of *P. elliottii*, including initiation, proliferation, maturation and germination. Yand et al. (2020) reported a SE system in *P. elliottii* with an initiation rate of 36%, and found that 88.9% of plants were phenotypically normal and free of genetic variation when the genetic stability of 14 regenerated plantlets at SSR loci was tested.

However, current findings indicate that the SE of *P. elliottii* remains limited, including infrequent initiation of EC, subpar yield and quality of somatic embryos, low regeneration rates of plantlet and inefficiency in field transference (Lelu-Walter et al. 2016; Yang et al. 2020). As stated above, SE has been successful in many *Pinus* spp., but the currently available SE laboratory methods vary widely in their effectiveness across species and genotypes (Egertsdotter

92 2019). Numerous research efforts have been directed towards promoting the initiation or maturation through altering
93 media formulations containing plant growth regulators, polyamines and hormone inhibitors (Pullman and Bucalo 2014;
94 Peng et al. 2021; Xia et al. 2021; Chen et al. 2023). The acclimatization stage is also viewed as a significant hurdle in
95 the effective micropropagation of conifers. (Mohammed et al. 1988).
96 Plantlets that have been regenerated in sterile tissue cultures do not interact with microbes, making it difficult to
97 successful cultivate and acclimatize them to the environment under non-sterile conditions, resulting in low survival
98 rates post-transplant. Inoculation with mycorrhizal fungi can enhance the development of plantlets grown in vitro, and
99 boost the adaptation of these plantlets to a non-sterile environment (Zhu et al. 2010). Mycorrhiza denotes to the
100 symbiosis between plants and the fungi that colonize their roots (Niemi et al. 2004). Researches have shown that root
101 inoculation with ectomycorrhizal fungi enhances the absorption of nutrients and water (Chen et al. 2016), increase
102 resilience against to diseases and pests (Wang et al. 2022) and enhance ability withstand stress from drought (Gehring et
103 al. 2017), salinity (Guo et al. 2022) and microplastics stress (Zhang and Gao 2023). Consequently, inoculation of
104 ectomycorrhizal fungi onto regeneration plantlets grown in isolation serves as a crucial technique to develop plantlets
105 that can withstand diverse stressors. Ectomycorrhizal fungi vary in their ability to obtain nutrients and water from the
106 medium and distribute them to the hosts (Abuzinadah and Read 1989a; Abuzinadah and Read 1989b; Dighton and
107 Harrison 1990; Dighton and Coleman 1992; Dighton et al. 1993). Earlier researches have shown how ectomycorrhizal
108 fungi might influence specific traits of host plants prior to and throughout the transplantation phase (Niemi and
109 Häggman 2002; Niemi et al. 2004; Zhu et al. 2010; Chen et al. 2022). However, there have been no reports on how
110 different ectomycorrhizal fungi impact plantlets that have regenerated from *P. elliottii* somatic embryos.
111 This study aimed to develop a proficient SE protocol for *P. elliottii*. Immature zygotic embryos from three
112 open-pollinated mother trees (families) of the disease-resistant *P. elliottii* were investigated to determine their ability for
113 SE. The effects of seed family, developmental stage of zygotic embryos and formulation of culture medium were

evaluated, along with the influence of glutamine on maturation capacity of somatic embryos. Additionally, the study examined the impact of activated carbon (AC) on the germination of somatic embryos and the effort of ectomycorrhizal fungi on growth of regenerated plantlets.

Materials and methods

Plant material

Green cones from three open-pollinated mother trees (families) (7#, 27# and 30#) in disease-resistant *P. elliottii* from Xi'po State-owned Forest Farm, Hua'an, Fujian (25°06′34.58″N, 117°33′8.47″E) were gathered between 2020 and 2023 (29 June to 20 July 2020, 28 June to 15 July 2021, 1 to 15 July 2022 and 27 June to 15 July 2023), with cones collected every 5–7 days. The cones underwent surface disinfection with alcohol cotton and were preserved in darkness at 4°C for a duration of one week. Subsequently, immature seeds were removed from the cones, sterilized using 75% (v/v) ethanol for 30 s followed by 30% (w/w) H_2O_2 for 10 min and washed thrice with sterile distilled water. Intact megagametophytes were removed sterilely from seeds and cultured horizontally on the initiation medium (Zhang et al. 2016). Only proliferating ECs were considered for quantification of the initiation of embryogenic cell line (ECL).

Initiation and proliferation of EC

Effect of seed family on EC initiation

For assessing the effect of seed family on EC initiation, approximately 30 megagametophytes (mostly at stages 2–5) were selected from each of the three mother trees (7#, 27#, and 30#) each year (2020-2023) and cultured on the initiation medium. The EC initiation medium contained LP basic medium (Arnold and Eriksson 1977) supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/L 6-benzylaminopurine (6-BA), 1 mg/L Vitamin C (VC), 3% (w/v) maltose, 1 g/L myoinositol, 500 mg/L casein acid hydrolysate, 250 mg/L 2-(4-morpholino) ethane-sulfonic acid (MES) and 500 mg/L glutamine, as described by Zhang et al. (2016). Prior to autoclaving, the initiation medium

was adjusted to pH 5.8 and followed by the addition of 6.5 g/L agar. After 7-8 weeks, the initiation rate of EC was determined. Each treatment was repeated at least three times.

Effect of embryo development stages on EC initiation

For this experiment seeds from the family 7# were utilized. Female cones which differ in maturity were collected at four different dates in 2020 (29 June and 6, 13 and 20 July). On various collection dates, Zygotic embryos were removed from megagametophytes and examined under a stereomicroscope (Leica MZ16, Wetzlar, Germany) for rapid assessment of their developmental stages. The developmental stages of the embryo were categorized from 1-8 according to the loblolly pine zygotic embryo development stage system established by Pullman and Webb (1994), based on embryonic morphology. Every treatment involved 30 megagametophytes and was conducted a minimum of three times. The initiation rate of EC was determined after 7-8 weeks.

Effect of 2,4-D, 6-BA and kinetin (KT) concentration on EC initiation

The seeds of family 7# collected on 8 July 2021 were tested to determine the influence of 2,4-D, 6-BA and KT concentrations on EC initiation. The various levels of 2,4-D, 6-BA and KT treatments were arranged using a fully randomized design method (Table 1). To determine the optimal combination, orthogonal arrays comprising three factors, each with three concentrations. Experiments consisted of three replicates with 30 megagametophytes per treatment. Initiation rate of EC was recorded after 7-8 weeks.

Cytological observation of EC

The EC was observed by staining with acetocarmine and Evans blue. Small pieces of EC were positioned on a microscopic slide, stained with 2% (w/v) acetocarmine for 1 min and treated with 0.05% (w/v) Evans blue for 30 s and the stain was washed off prior to being mounted with a cover slip (Gupta and Holmstrom 2005). The ECs were examined under a stereomicroscope.

Maintenance and proliferation of EC

157 After 8 weeks on initiation medium in darkness, ECs were isolated from the micropylar end of megagametophytes and
158 transferred to maintenance medium to enhance their proliferation. The maintenance medium was made up of LP basic
159 medium added with 1 mg/L 1-naphthylacetic acid (NAA), 1 mg/L VC, 0.3 mg/L 6-BA, 0.3 mg/L KT, 1.5% (w/v)
160 maltose, 1 g/L myoinositol, 500 mg/L casein acid hydrolysate, 250 mg/L MES, 500 mg/L glutamine and 6.5 g/L agar.
161 The EC was cultured in darkness at 23 ± 2°C and sub-cultured onto fresh medium every 15 days.

162 **Maturation and germination of somatic embryos**

163 *Establishment of suspension culture*

164 At 12–13 days after culture, the embryonic cell suspension culture was started by suspending 1 g of fresh EC in 30 mL
165 of liquid proliferation medium. Liquid medium contained 0.5 mg/L NAA, 0.5 mg/L VC, 0.15 mg/L 6-BA and
166 0.15 mg/L KT. Erlenmeyer flasks were placed on a shaker at 90 rpm and cultured at 25°C in darkness. Liquid cultures
167 were sub-cultured once a week. The EC was sub-cultured in fresh medium maintaining a 1:5 (v/v) ratio, which
168 transferring 5 mL of cell suspension to a flask containing 25 mL of fresh liquid medium. After one week remove the
169 supernatant, 5 mL of cell suspension was spread evenly on filter paper (Whatman No. 2, 55 mm). The Filter papers
170 were laid on the maturation medium (Zhang et al. 2016). The maturation medium was made up of LP basic medium
171 added with 2 mg/L abscisic acid, 130 g/L polyethylene glycol 8000, 1.5 mg/L gibberellin, 3% (w/v) maltose, 1 g/L
172 myoinositol, 500 mg/L casein acid hydrolysate, 250 mg/L MES and 500 mg/L glutamine. Each treatment was
173 replicated three times and incubated at 23 ± 2°C for 10 weeks under dark conditions.

174 *Effect of glutamine concentration on somatic embryo maturation*

175 Four ECLs (2107-1, 2107-8, 2107-12 and 2107-16) showing the most vigorous maturity were tested to determine the
176 effect of glutamine concentration (0, 0.45, 0.90, 1.35 and 1.80 g/L) on somatic embryo maturation. Other additives
177 maintained identical concentrations as in the maturation medium. The yield of mature somatic embryos was counted
178 after 10 weeks.

179 *Effect of AC concentration on somatic embryo germination*

180 Somatic embryos from four ECLs (2107-1, 2107-8, 2107-12 and 2107-16) were transferred onto medium without plant
181 growth regulators for germination. The germination medium contained DCR basic medium (Gupta and Durzan 1985)
182 added with 1.0, 1.5, 3.0, 4.5 and 6.0 g/L AC, 20 g/L maltose, 0.1 g/L myoinositol and 8 g/L agar. In total, 150
183 well-developed embryos (10 somatic embryos × 3 replicates × 5 AC treatments) were tested. Culture plates were
184 incubated for an initial period of 3 days in darkness and then transferred to light incubation with an intensity of
185 ~36 μmol/m^2·s from cool white fluorescent lamps. Germination rate was recorded after 5 weeks.

186 **Mycorrhization of regenerated plantlets**

187 The 8-month-old regenerated plantlets from ECL 2127-1 were carried out inoculation treatments, plantlets of similar
188 size were selected to minimize within-sample variation. The fungal isolates used were *Pisolithus orientalis*, *Lactarius*
189 *insulsus, Laccaria amethystea*, *Xerocomus chrysenteron* and *Rhizopogon luteolus*. The *Pis. orientalis* was purchased
190 from the Agricultural Culture Collection of China, Beijing, China. The other fungi were obtained from the Forest
191 Pathology Laboratory, Nanjing Forestry University (Nanjing, China). The fungi were cultured on potato dextrose agar
192 medium before use. The plantlets were transferred into sterilized containers, containing sterilized pearlite as substrate.
193 Rooted plantlets were inoculated with ectomycorrhizal fungal mycelia, and plantlets without ectomycorrhizal fungi
194 served as control. Each treatment was replicated three times with 15 regenerated plants per replication. Plantlets were
195 watered regularly with DCR solution, with 17.5 g/L sucrose and 0.1 g/L myoinositol added. After 4 months, root length
196 was recorded, and the regenerated plantlets were transferred to plastic pots (10 cm in diameter, 8.5 cm in height) filled
197 with sterile soil, vermiculite and perlite. The regenerated plantlets were acclimatized in greenhouse according to Sun et
198 al. (2019). The survival rate was recorded 3 months after transfer.

199 **Statistical analysis**

Data were evaluated using analysis of variance (ANOVA). All tests were carried out in SPSS 26.0 and GraphPad Prism 8.0. Data represent mean ± standard deviation (SD).

Results

Initiation and proliferation of EC

Effect of seed family on EC initiation

There were significant differences ($p < 0.05$) between mean initiation rates among the three seed families collected during 2020–2023(Fig. 1). The initiation rate for family 7# (8.89–27.78%) was significantly higher than for the other two families. The second highest rate was for family 27# (4.45–25.56%). The initiation rate of seed family 30# was the lowest (1.11–21.11%). Family 7# collected in 2020 showed the highest initiation response with a mean value of 27.78 ± 3.85%, followed by family 7# collected in 2023 with 26.67 ± 3.34%.

The initiation rate of the same family collected in different years also showed significant differences ($p < 0.05$). In 2020, all seed families showed the highest initiation rate (21.11–27.78%), which significantly differed from those of the other years. All families showed the lowest induction rate in 2022 (1.11–8.89%).

Effect of embryo development stages on EC initiation

The developmental stage of zygotic embryos of disease-resistant *P. elliottii* according to Pullman's criteria was divided into eight stages (Fig. 2). As the zygotic embryos evolved, the embryo transitioned from translucent to white and opaque, and the cotyledons gradually elongate but remain open as the suspensor increases radially, the shoot apical meristem can be seen when viewed from a right angle. In this study, 66.67% of immature zygotes collected on 29 June 2020 were at 2–3 stage, while the proportion of immature zygotes collected on 6 July 2020 increased to 93.33%. The maturity of the zygotes gradually increased as the time of seed collection increased.

The EC initiation was related to the embryo development stage (Table 2). The immature zygotes collected on 6 July 2020 had the highest rate of initiation (21.83 ± 4.95%), and predominantly clustered in stage 2–3, follow by the

initiation rate was for immature zygotes collected on 13 July 2020 (12.97 ± 0.52%). The initiation rate gradually decreased as the immature zygotes continued to develop. On 20 July 2020, the initiation rates were only 1.27 ± 0.34%.

Effect of 2,4-D, 6-BA and KT concentration on EC initiation

The initiation of EC was significantly affected by the 2,4-D, 6-BA and KT concentrations ($p < 0.05$), with rate of initiation peaking at number 3 treatment (initiation medium added with 6 mg/L 2,4-D, 1 mg/L 6-BA and 2 mg/L KT), with 15.67 ± 1.89%, followed by number 1 treatment (initiation medium added with 6 mg/L 2,4-D and 3 mg/L 6-BA), with 9.00 ± 1.41% (Table 3). Among the initiation medium tested, the lowest initiation rate was for number 8 treatment (initiation medium added with 4 mg/L 2,4-D, 1 mg/L 6-BA and 1 mg/L KT), with an average of 1.00 ± 1.41%.

Maintenance and proliferation of EC

Following an 8-week period of positioning the megagametophytes on the initiation medium, white or translucent EC were observed extruding from the micropylar end of megagametophyte in several explants (Fig. 3A and B), and the EC begins to proliferate continuously on medium (Fig. 1C). Double staining revealed two typical embryogenic structures: embryogenic cells reacted redly to acetocarmine, which were rounded; and elongated, vacuolated suspensor cells, which reacted bluely to Evans blue. According to Filonova (2000), an individual pro-embryogenic mass (PEM) undergoes a sequence of distinct stages (I, II and III), characterized by distinct cell aggregates. The three stages of PEM and early somatic embryo exist at the same time (Fig. 3D–F).

Maturation and germination of somatic embryos

Effect of glutamine concentration on somatic embryo maturation

Addition of glutamine to the maturation medium enhanced the yield of somatic embryos during culture (Fig. 4A). Glutamine concentrations of 0–1.8 g/L had significant effects on yield of somatic embryos ($p < 0.05$). The callus surface color changed from light yellow white to dark brown in response to increasing glutamine concentration (Fig. 4B). The ECL 2107-8 had the highest yield of somatic embryos with 0.9 g/L glutamine (223.00 ± 18.24 per dish). The

244 ECL 2107-12 produced the most somatic embryos with 0.9 g/L glutamine (98.67 ± 8.38 per dish), the ECL 2107-1 and
245 2107-16 had optimal production with 0 and 1.8 g/L glutamine, respectively (15.67 ± 3.09 and 12.00 ± 2.16 per dish,
246 respectively) (Fig. 4C). Overall, yield of somatic embryos differed according to the glutamine concentration in the
247 maturation medium.

248 *Effects of AC concentration on somatic embryo germination*

249 The germination rate of somatic embryos varied among medium with different AC concentration (Fig. 5A). The
250 optimal growth performance and the highest germination rate of somatic embryos were obtained when 3-4.5 g/L AC
251 was added to culture medium. Applying a substantial concentration of AC (6 g/L) in the culture medium resulted in a
252 slower germination rate and diminished growth in germinating somatic embryos, in contrast to the 3-4.5 g/L AC
253 treatment. As AC concentrations of 3-4.5 g/L, The ECL 2107-1, 2107-12, and 2107-16 showed the highest germination
254 rates of 90.00 ± 8.16%, 90.00 ± 8.16%, and 93.33 ± 2.89%, respectively. However, when 4.5 g/L AC was added, the
255 ECL 2107-8 showed the lowest germination rate (26.67 ± 4.71%). Different ECLs needed different AC content for
256 germination.

257 **Mycorrhization of regenerated plantlets**

258 The perlite substrate of inoculated mycorrhiza fungi plantlets was rapidly colonized (Fig. 6A). The inoculated plantlet
259 roots were enveloped in either a yellow or white layer of hyphae, with no visible root hairs on their surface. Conversely,
260 the control plantlet without inoculation exhibited root hairs (Fig. 6B). The root length of regenerated plantlets
261 inoculated with different ectomycorrhizal fungi and non-inoculated varied significantly ($p < 0.05$) (Fig. 6D). After 4
262 months inoculated with *Pis. orientalis*, *L. amethystea*, *L. insulsus* and *X. chrysenteron*, a variety of dichotomously
263 branched ectomycorrhiza-like structures were observed on the roots. Mass epitaxial hyphae were observed at the
264 bifurcation of mycorrhizae, which replace some root hairs in function and expand the absorption area of the root system.
265 When regenerated plantlets were inoculated with *L. amethystea*, the average root length was 7.92 ± 4.06 cm,

266 significantly longer than for other treatments ($p < 0.05$). When inoculated with *R. luteolus*, regenerated plantlets had the
267 shortest root lengths (3.58 ± 1.73 cm).
268 The regenerated plantlets were moved from sterile tissue-cultured containers to plastic pots containing substrates for
269 acclimatization. After 3 months, the needles of regenerated plantlets inoculated with *R. luteolus* appeared yellow-green,
270 whereas other plantlets remained green (Fig. 6C). Among them, 100% of regenerated plantlets survived after
271 transferring when inoculated with *Pis. orientalis* and *X. chrysenteron*, which significantly higher than for the other
272 treatments ($p < 0.05$).

273 Discussion

274 In this study, three seed families of mother trees in Xi'po State-owned Forest Farm (Huaan, Fujian) were studied, and
275 an effective plant regeneration program for SE of *P. elliottii* was established. It was possible to obtain a high EC
276 initiation rate and improve the maturation and germination capacity of somatic embryos with the described method.
277 Furthermore, this mycorrhization method was helpful to improve the survival rate of plantlets after transplantation.
278 The key factors of EC initiation of *P. elliottii* include seed family, zygotic embryo development stage, hormone
279 concentration and so on (Pinto et al. 2008).The genetic factors are recognized as crucial drivers in initiation phase of SE
280 (Park 2002). Previous researches have pointed out that the heritability of SE is higher than other quantitative traits,
281 especially in *P. halepensis*, *P. taeda* and *P. radiata* (Pullman and Johnson 2002; Hargreaves et al. 2009; Montalban et al.
282 2013). This study showed that various seed families exhibited significantly different capacities in initiating EC, with
283 range 1.11–27.78%, and family 7# was more responsive than families 27# and 30#. Similar significant differences in
284 EC initiation capacity among different pine families were also observed in *P. elliottii* (Liao and Amerson 1995a; Liao
285 and Amerson 1995b; Yang et al. 2020), *P. taeda* (Tang et al. 2001; Pullman and Johnson 2002), *P. pinaster* (Miguel et al.
286 2004; Park et al. 2006), *P. pinea* (Carneros et al. 2009), *P. radiata* (Hargreaves et al. 2009), *P. halepensis* (Montalban et
287 al. 2013), hybrid *P. elliottii* × *P. caribaea* (Nunes et al. 2018) and *P. massoniana* (Xia et al. 2021; Chen et al. 2023). The

288 results described the influence of genotype on EC initiation rates, and future studies could explore a wider range of
289 seed family at the initiation stage. Thus, further screening of culture conditions could have been carried out for those
290 genotypes that perform well in terms of initiation capacity, which is of high reference value for future SE study. In
291 addition, our experimental results show that the same seed family collected in different years showed different initiation
292 rates, possibly due to different stages of seed development.

293 The initiation rate was also affected by embryo development stages and collection date in this study. The general
294 correlation between the maturity of explant and the rate of initiation suggested that it is important to collect seed at
295 appropriate development stages. Studies in *P. densiflora* (Wu et al. 2015) and *P. sylvestris* (Wang et al. 2023) also
296 confirmed the significance of embryo developmental stage on the rate of initiation. Maturity of the explant increased
297 with the duration of seed collection. In this study, the highest initiation rate (21.83 ± 4.95 %) was observed when the
298 immature zygotes were predominantly concentrated in stage 2–3, consistent with the findings of Hu et al. (2019) and
299 Cheng et al. (2023). Exogenous hormones play crucial roles in regulating EC initiation and proliferation (Teixeira da
300 Silva and Malabadi 2012). Appropriate application of plant exogenous hormones in culture medium is not only the
301 decisive factor for the successful initiation of EC, but also the main factor affecting the initiation rate. The main
302 exogenous hormones affecting EC formation of pine immature embryos are 2,4-D, 6-BA and KT (Tang et al. 1996;
303 Chen and Lai 2001). High 2,4-D concentrations have been found to promote production of EC in the initial stage of
304 initiation; as cytokinin, 6-BA and KT generally promote the initiation rate at low concentration (Gifford and Tolley
305 1989; Go et al. 1993). We found that medium containing 6 mg/L 2,4-D, 1 mg/L 6-BA and 2 mg/L KT increased the
306 average EC initiation rate in *P. elliottii*. Numerous studies have shown that the optimal hormone concentration in
307 initiation medium varies among conifer species. For example, Zhou et al. (2010) found that the addition of 8 mg/L
308 2,4-D and 2 mg/L 6-BA consistently increases EC initiation rate in *P. bungeanaas*. Sun et al. (2015) reported increased
309 EC initiation in *P. thunbergii* using 4 mg/L 2,4-D and 2 mg/L 6-BA or 4 mg/L 2,4-D and 1 mg/L KT. In our study, some

ECs were unable to proliferate in subsequent experiments. Not all callus could survive on maintenance medium, similar to previous reports by Hosoi and Maruyama (2012) and Sun et al. (2019).

There are many factors affecting maturation capability of somatic embryos have been reported, including medium composition (Miguel et al. 2004; Zhang et al. 2016; Gao et al. 2020). The addition of glutamine (0.9 g/L) significantly improved the regeneration ability of somatic embryos, thereby promoting somatic embryo maturation, and yield of somatic embryos was significantly increased compared to culture medium without glutamine. Studies have showed that exogenous glutamine stimulates de novo protein synthesis and cause rapid embryogenic cells division (Stasolla et al. 2001), and that an increase in glutamine concentration also slightly increased the osmotic pressure of the culture medium, which in turn affects maturation response (Garin et al. 2000). Khlifi and Tremblay (1995) noted that in the absence of any form of inorganic nitrogen in the culture medium, glutamine alone was able to maintain maturation of *P. mariana* (Mill) somatic embryos.

Supplementation of suitable concentrations of AC promotes somatic embryo germination. Therefore, the addition of AC to germination medium is a common method for SE in many conifers, including *P. nigra* (Salajová and Salaj 2005), *P. densiflora* (Maruyama et al. 2005) and *P. armandii* (Maruyama et al. 2007). In this experiment, AC at 3–4.5 g/L promoted somatic embryo germination rates in *P. elliottii*. Analogously, the role of AC has been widely reported in other conifers (Kim and Moon 2007; Ahn and Choi 2017; Jiang et al. 2021). However, the underlying mechanisms by which AC promotes germination of somatic embryos are still poorly understood. AC is known to regulate the concentration of various plant growth regulators, plant metabolic wastes and toxic metabolites in the culture medium (Pan and Staden 1998; Von Aderkas et al. 2002; Thomas 2008). In addition to this, the AC may alter the pH of culture medium as well as absorb components essential for plant growth, such as plant growth regulators, vitamins and ions (Ebert and Taylor 1990; Van Winkle et al. 2003). Jiang et al. (2021) theorized that AC may simultaneously assimilate multiple components of the culture medium, including both favorable and unfavorable components for germination

of somatic embryos, that an overabundance of hormones during maturation of somatic embryos is a key impediment to germination and that the absorption of corresponding components can in theory greatly enhance germination rate of somatic embryos. Consequently, it is important to elucidate the role played by AC and to determine the optimal concentration of AC in the culture medium in order to substantially increase the germination rate of somatic embryos. Inoculation with different mycorrhizal fungi caused different efforts on the growth of seedlings, and inoculating appropriate mycorrhizal fungi could improve the adaptability of isolated regenerated plantlets to the transplant environment (Niemi and Häggman 2002). The survival rate of transplanted cultured plantlets of *P. densiflora* were effectively improved when inoculated the mycorrhizal fungi *R. luteolus*, *Pis. tinctorius* and *Boletus edulis* (Wang et al. 2015). And Zheng et al. (2003), Zhu et al. (2010) and Chen et al. (2023) showed that using ectomycorrhizal fungi *Pis. orientalis* can promote plant development in *P. massoniana*. In our study, regenerated plantlets inoculated with *L. amethystea* had an average length of root of 7.92 cm before acclimatization, which was higher than for other treatments. However, the root length of plantlets inoculated with the other four mycorrhizal fungi was lower than that of the control group. It has also been shown that use of specific ectomycorrhizal fungi can promote the growth of the aboveground portion of the plantlet, but not root formation (Niemi et al. 2007). When there is a physiological imbalance between the host and the mycorrhizal fungi in inoculation it may hinder infection, the prevailing in vitro conditions favoring the fungi result in fungi growing more vigorously than plantlets at this time (Sasa and Krogstrup 1991). Acclimatization of the regeneration plantlets to ex vitro conditions was improved by *Pis. orientalis* and *X. chrysenteron*. After 3 months of acclimatization, the survival rate of plantlets inoculated with *Pis. orientalis* and *X. chrysenteron* reached 100%, which was significantly higher than for the control group. Only one of the five mycorrhizal fungi inhibited the growth and development of plants in our experiment. Thus, inoculation with an excellent and suitable ectomycorrhizal fungus is a potential tool to improve later stages of SE.

This study described the factors playing crucial roles in initiation, maturation, germination and acclimatization in SE of disease-resistant *P. elliottii*. We confirmed that stages 2–3 are the optimal development stage suitable for EC initiation and identified the seed family and hormone concentration most favorable for embryogenesis initiation. The addition of glutamine (0.9 g/L) enhanced the maturation of somatic embryos. The impact of AC concentration on the germination of somatic embryos was investigated. Subsequently, the regenerated plantlets underwent inoculation with ectomycorrhizal fungi and were adapted for transplantation. Inoculation with *Pis. orientalis* and *X. chrysenteron* improved the *P. elliottii* survival rate at 3 months after transfer. We believe that the current protocol will be available in the future for large-scale production of plantlets and labor savings, as well as accelerating breeding programs to combat pine needle brown spot disease.

References

Abuzinadah R, Read D (1989a) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants: V. Nitrogen transfer in birch (*Betula pendula*) grown in association with mycorrhizal and non-mycorrhizal fungi. New Phytol 112(1):61-68.

Abuzinadah R, Read D (1989b) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants: IV. The utilization of peptides by birch (*Betula pendula* L.) infected with different mycorrhizal fungi. New Phytol 112(1):55-60.

Ahn CH, Choi YE (2017) In vitro clonal propagation and stable cryopreservation system for *Platycladus orientalis* via somatic embryogenesis. Plant Cell Tissue Organ Cult 131(3):525-525. https://doi.org/10.1007/s11240-017-1307-3

Arnold SV, Eriksson T (1977) A revised medium for growth of pea mesophyll protoplasts. Physiol Plan 39(4):257-260. https://doi.org/10.1111/j.1399-3054.1977.tb01879.x

Arrillaga I, Morcillo M, Zanón I, Lario F, Segura J, Sales E (2019) New approaches to optimize somatic embryogenesis in maritime pine. Front Plant Sci 10:138. https://doi.org/10.3389/fpls.2019.00138

Carneros E, Celestino C, Klimaszewska K, Park YS, Toribio M, Bonga JM (2009) Plant regeneration in Stone pine (*Pinus pinea* L.) by somatic embryogenesis. Plant Cell Tissue Organ Cult 98(2):165-178. https://doi.org/10.1007/s11240-009-9549-3

Chalupa V (1985) Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.) Kaerst. Commun Inst For Cech 14:57-63.

Chen BL, Yang KT, Huang S, Gong JY, LI QL, Wang XY, Su LH (2022) Effects of mycorrhizal fungi interaction on the growth and physiology of Paphiopedilum hirsutissimum seedlings in vitro. J Southwest Forestry University (Natural Sciences) 42(02):19-25. https://doi.org/10.11929/j.swfu.202105008

Chen W, Koide RT, Adams TS, DeForest JL, Cheng L, Eissenstat DM (2016) Root morphology and mycorrhizal symbioses together shape nutrient foraging strategies of temperate trees. Proc Natl Acad Sci USA 113(31):8741-8746. https://doi.org/10.1073/pnas.1601006113

Chen YJ, Lai ZX (2001) Researches and utilization of somatic embryogenesis in fruits and trees. J Fujian Agric and For Univ(03):420-426.

Chen YM, Fei Q, Xia XR, Ke X, Ye JR, Zhu LH (2023) *Pinus massoniana* somatic embryo maturation, mycorrhization of regenerated plantlets and its resistance to *Bursaphelenchus xylophilus*. Frontiers Plant Science 14 https://doi.org/10.3389/fpls.2023.1130471

Cheng F, Sun TY, Ye JR (2023) Induction of embryogenic callus from immature zygotic embryos of slash pine (*Pinus elliottii*) resistant to brown spot needle blight (*Lecanosticta acicola*) J Nanjing For Univ:1-11. https://doi.org/10.12302/j.issn.1000-2006.202203002

Dighton J, Coleman DC (1992) Phosphorus relations of roots and mycorrhizas of *Rhododendron maximum* L. in the southern Appalachians, North Carolina. Mycorrhiza 1:175-184.

Dighton J, Harrison A (1990) Changes in phosphate status of Sitka-spruce plantations of increasing age, as determined by root bioassay. Forest Ecol Manag 31(1-2):35-44.

Dighton J, Poskitt JM, Brown TK (1993) Phosphate influx into ectomycorrhizal and saprotrophic fungal hyphae in relation to phosphate supply; a potential method for selection of efficient mycorrhizal species. Mycol Res 97(3):355-358.

Ebert A, Taylor H (1990) Assessment of the changes of 2, 4-dichlorophenoxyacetic acid concentrations in plant tissue culture media in the presence of activated charcoal. Plant Cell Tissue Organ Cult 20:165-172.

Egertsdotter U (2019) Plant physiological and genetical aspects of the somatic embryogenesis process in conifers. Scand J Forest Res 34(5):360-369. https://doi.org/10.1080/02827581.2018.1441433

Gao F, Peng CX, Wang H, Tretyakova IN, Nosov AM, Shen HL, Yang L (2020) Key Techniques for Somatic Embryogenesis and Plant Regeneration of *Pinus koraiensis*. Forests 11(9) https://doi.org/10.3390/f11090912

Garin E, Bernier-Cardou M, Isabel N, Klimaszewska K, Plourde A (2000) Effect of sugars, amino acids, and culture technique on maturation of somatic embryos of *Pinus strobus* on medium with two gellan gum concentrations. Plant Cell Tissue Organ Cult 62:27-37.

Gehring CA, Sthultz CM, Flores-Renteria L, Whipple AV, Whitham TG (2017) Tree genetics defines fungal partner communities that may confer drought tolerance. Proc Natl Acad Sci USA 114(42):11169-11174. https://doi.org/10.1073/pnas.1704022114

Gifford DJ, Tolley MC (1989) The seed proteins of white spruce and their mobilization following germination. Physiol Plant 77(2):254-261.

Go NE, Perez-Orozeo GD, Halos SC (1993) In vitro response of embryos from different provenances of *Pinus caribaea* var. *hondurensis* Morelet. Plant Cell Tissue Organ Cult 32:1-7.

Guo W, Hao H, Zhang W-H, Hu Z-H, Leng P-S (2022) Ectomycorrhizal fungi enhance salt tolerance of *Quercus mongolica* by regulating ion balance. Chin J Appl Ecol 33(12):3303-3311. https://doi.org/10.13287/j.1001-9332.202212.003

Gupta PK, Durzan DJ (1985) Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Rep 4:177-179.

Gupta PK, Holmstrom D (2005) Double staining technology for distinguishing embryogenic cultures. In: Jain SM & Gupta PK (eds) Protocol for somatic embryogenesis in woody plants. Springer, Netherlands, pp 573-575.

Hakman I, Fowke LC, Arnold SV, Eriksson T (1985) The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). Plant Sci 38(1):53-59.

Hargreaves CL, Reeves CB, Find JI, Gough K, Josekutty P, Skudder DB, van der Maas SA, Sigley MR, Menzies MI, Low CB, Mullin TJ (2009) Improving initiation, genotype capture, and family representation in somatic embryogenesis of *Pinus radiata* by a combination of zygotic embryo maturity, media, and explant preparation. Can J For Res 39(8):1566-1574. https://doi.org/10.1139/X09-082

Hosoi Y, Maruyama TE (2012) Plant regeneration from embryogenic tissue of *Pinus luchuensis* Mayr, an endemic species in Ryukyu Island, Japan. Plant Biotechnology 29(4):401-406. https://doi.org/10.5511/plantbiotechnology.12.0530a

Hu JW, Guo WB, Deng LP, Zhong SY, Wang WM, Zhao FC, Huang T, Wu HS, Li YL, Liao FY (2019) Somatic embryogenesis and plantlet regeneration in *Pinus elliottii* and its hybrids. J South China Agric Univ 40(01):107-115. https://doi.org/10.7671/j.issn.1001-411X.201804001

Jain SM, Dong N, Newton R (1989) Somatic embryogenesis in slash pine (*Pinus elliottii*) from immature embryos cultured in vitro. Plant Sci 65(2):233-241.

Jiang SF, Chen XY, Gao Y, Cui Y, Kong LS, Zhao J, Zhang JF (2021) Plant regeneration via somatic embryogenesis in *Larix principis-rupprechtii* Mayr. Forests 12(10):1335. https://doi.org/10.3390/f12101335

Khlin S, Tremblay FM (1995) Maturation of black spruce somatic embryos. Part I. Effect of L-glutamine on the number and germinability of somatic embryos. Plant Cell Tissue Organ Cult 41:23-32.

Kim YW, Moon HK (2007) Enhancement of somatic embryogenesis and plant regeneration in Japanese larch (*Larix leptolepis*). Plant Cell Tissue Organ Cult 88(3):241-245. https://doi.org/10.1007/s11240-007-9202-y

Kim YW, Moon HK (2014) Enhancement of somatic embryogenesis and plant regeneration in Japanese red pine (*Pinus densiflora*). Plant Biotechnol Rep 8:259-266. https://doi.org/10.1007/s11816-014-0319-2

Lelu-Walter M-A, Klimaszewska K, Miguel C, Aronen T, Hargreaves C, Teyssier C, Trontin J-F (2016) Somatic embryogenesis for more effective breeding and deployment of improved varieties in Pinus spp.: bottlenecks and recent advances. In: Loyola-Vargas VM & Ochoa-Alejo N (eds) Somatic embryogenesis: fundamental aspects and applications. pp 319-365.

Li CD, Han ZM, Ye JR, Zhen WP, Zhang ZH, Zheng RH, Zhang HT (1987) Devepment of brown-spot needle blight in slash pine plantations. J Nanjing For Univ(01):1-7.

Liao Y, Amerson H (1995a) Slash pine (*Pinus elliottii* Engelm.) somatic embryogenesis I. Initiation of embryogenic cultures from immature zygotic embryos. J For 10:145-163.

Liao Y, Amerson H (1995b) Slash pine (*Pinus elliottii* Engelm.) somatic embryogenesis II. Maturation of somatic embryos and plant regeneration. N For 10:165-182.

Maruyama E, Hosoi Y, Ishii K (2005) Propagation of Japanese red pine (*Pinus densiflora* Zieb. et Zucc.) via somatic embryogenesis. Propag Ornam Plant 5:199-204.

Maruyama E, Hosoi Y, Ishii K (2007) Somatic embryogenesis and plant regeneration in yakutanegoyou, *Pinus armandii* Franch. var. *amamiana* (Koidz.) Hatusima, an endemic and endangered species in Japan. Vitr Cell Dev Biol Plant 43(1):28-34. https://doi.org/10.1007/s11627-006-9003-8

Miguel C, Gonçalves S, Tereso S, Marum L, Maroco J, Oliveira MM (2004) Somatic embryogenesis from 20 open-pollinated families of Portuguese plus trees of maritime pine. Plant Cell Tissue Organ Cult 76(2):121-130. https://doi.org/10.1023/B:TICU.0000007253.91771.e3

Mohammed GH, Vidaver WE, Culture O (1988) Root production and plantlet development in tissue-cultured conifers. Plant Cell Tissue Organ Culture 14:137-160. https://doi.org/10.1007/BF00043405

Montalban IA, Moncalean P (2019) Rooting of *Pinus radiata* somatic embryos: factors involved in the success of the process. J Forestry Res 30(1):65-71. https://doi.org/10.1007/s11676-018-0618-5

Montalban IA, Setien-Olarra A, Hargreaves CL, Moncalean P (2013) Somatic embryogenesis in *Pinus halepensis* Mill.: an important ecological species from the Mediterranean forest. Trees 27(5):1339-1351. https://doi.org/10.1007/s00468-013-0882-0

Nagmani R, Bonga J (1985) Embryogenesis in subcultured callus of *Larix decidua*. Can J For Res 15(6):1088-1091.

Newton RJ, Tang W, Jain SM (2005) Slash Pine (*Pinus elliottii* Engelm.). In: Jain SM & Gupta PK (eds) Protocol for somatic embryo genesis in woody plants. Springer, Dordrecht, pp 1-10.

Niemi K, Häggman H (2002) *Pisolithus tinctorius* promotes germination and forms mycorrhizal structures in Scots pine somatic embryos in vitro. Mycorrhiza 12:263-267. https://doi.org/10.1007/s00572-002-0181-x

Niemi K, Sarjala T, Chen X, Häggman H (2007) Spermidine and the ectomycorrhizal fungus *Pisolithus tinctorius* synergistically induce maturation of Scots pine embryogenic cultures. J Plant Physiol 164(5):629-635. https://doi.org/10.1016/j.jplph.2006.03.019

Niemi K, Scagel C, Häggman H (2004) Application of ectomycorrhizal fungi in vegetative propagation of conifers. Plant Cell Tissue Organ Culture 78(1):83-91. https://doi.org/10.1023/B:TICU.0000020379.52514.72

Nunes S, Marum L, Farinha N, Pereira VT, Almeida T, Sousa D, Mano N, Figueiredo J, Dias MC, Santos C (2018) Somatic embryogenesis of hybrid *Pinus elliotti* var. *elliottii* x *P.caribaea* var. *hondurensis* and ploidy assessment of somatic plants. plant Cell Tissue Organ Cult 132(1):71-84. https://doi.org/10.1007/s11240-017-1311-7

Pan M, Staden Jv (1998) The use of charcoal in in vitro culture–A review. Plant Growth Reg 26:155-163.

Park YS (2002) Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Ann For Sci 59(5-6):651-656. https://doi.org/10.1051/forest:2002051

Park YS, Lelu-Walter MA, Harvengt L, Trontin JF, MacEacheron I, Klimaszewska K, Bonga JM (2006) Initiation of somatic embryogenesis in *Pinus banksiana*, *P.strobus*, *P.pinaster*, and *P.sylvestris* at three laboratories in Canada and France. Plant Cell Tissue Organ Cult 86(1):87-101. https://doi.org/10.1007/s11240-006-9101-7

Peng C, Gao F, Wang H, Shen H, Yang L (2021) Optimization of maturation process for somatic embryo production and cryopreservation of embryogenic tissue in *Pinus koraiensis*. Plant Cell Tissue Organ Culture 144(1):185-194. https://doi.org/10.1007/s11240-020-01918-y

Pinto G, Silva S, Park Y-S, Neves L, Araujo C, Santos C (2008) Factors influencing somatic embryogenesis induction in *Eucalyptus globulus* Labill.:basal medium and anti-browning agents. Plant Cell Tissue Organ Cult 95(1):79-88. https://doi.org/10.1007/s11240-008-9418-5

Pullman GS, Bucalo K (2014) Pine somatic embryogenesis: analyses of seed tissue and medium to improve protocol development. N For 45(3):353-377. https://doi.org/10.1007/s11056-014-9407-y

Pullman GS, Gupta PK, Timmis R, Carpenter C, Kreitinger M, Welty E (2005) Improved Norway spruce somatic embryo development through the use of abscisic acid combined with activated carbon. Plant Cell Rep 24(5):271-279. https://doi.org/10.1007/s00299-005-0933-0

Pullman GS, Johnson S (2002) Somatic embryogenesis in loblolly pine (*Pinus taeda* L.):: improving culture initiation rates. Ann For Sci 59(5-6):663-668. https://doi.org/10.1051/forest:2002053

Pullman GS, Webb DT An embryo staging system for comparison of zygotic and somatic embryo development. In: TAPPI R&D Division biological sciences symposium, 1994. pp 31-34. https://hdl.handle.net/1853/67549

Pullman GS, Zeng X, Copeland-Kamp B, Crockett J, Lucrezi J, May SW, Bucalo K (2015) Conifer somatic embryogenesis: improvements by supplementation of medium with oxidation-reduction agents. Tree Physiol 35(2):209-224. https://doi.org/10.1093/treephys/tpu117

Salajová T, Salaj J (2005) Somatic embryogenesis in *Pinus nigra*:: maturation and regeneration ability of embryogenic tissue initiation, established cell lines. Biol Plant 49(3):333-339. https://doi.org/10.1007/s10535-005-0003-z

Sasa M, Krogstrup P (1991) Ectomycorrhizal formation in plantiets derived from somatic embryos of Sitka spruce. Scand J Forest Res 6(1-4):129-136. https://doi.org/10.1080/02827589109382655

Stasolla C, Loukanina N, Ashihara H, Yeung EC, Thorpe TA (2001) Ascorbic acid changes the pattern of purine metabolism during germination of white spruce somatic embryos. Tree Physiol 21(6):359-367. https://doi.org/10.1093/treephys/21.6.359

Sun TY, Wang YL, Zhu LH, Wu XQ, Ye JR (2019) Plant regeneration by somatic embryogenesis in *Pinus thunbergii* resistant to the pine wood nematode. Can J For Res 49(12):1604-1612. https://doi.org/10.1139/cjfr-2018-0522

Sun TY, Ye JR, Wu XQ, Zhu LH, Wu J, Liang F (2015) Induction of Embryonic Callus from *Pinus thunbergii* for Resistance to Pine Wilt Disease. J Northeast For Univ 43(09):96-99. https://doi.org/10.13759/j.cnki.dlxb.20150721.016

Tang W, Guo ZC, Ouyang F (2001) Plant regeneration from embryogenic cultures initiated from mature loblolly pine zygotic embryos. In Vitro Cell Dev Biol Plant 37(5):558-563. https://doi.org/10.1007/s11627-001-0097-8

Tang W, Yang YG, Gui YL, Guo ZC, Wu MB (1996) Study and application on somaticembry ogenesis in conifers. Chin Bull Bot(01):26-32.

Teixeira da Silva JA, Malabadi RB (2012) Factors affecting somatic embryogenesis in conifers. J For Res 23:503-515. https://doi.org/10.1007/s11676-012-0266-0

Thomas TD (2008) The role of activated charcoal in plant tissue culture. Biotechnol Adv 26(6):618-631. https://doi.org/10.1016/j.biotechadv.2008.08.003

Van Winkle SC, Johnson S, Pullman GS (2003) The impact of Gelrite and activated carbon on the elemental composition of two conifer embryogenic tissue initiation media. Plant Cell Rep 21(12):1175-1182. https://doi.org/10.1007/s00299-003-0637-2

Von Aderkas P, Label P, Lelu M-A (2002) Charcoal affects early development and hormonal concentrations of somatic embryos of hybrid larch. Tree Physiol 22(6):431-434.

Wang ST, Zhang PX, Wang M, Wang H, Meng P (2023) Preliminary Study of Embryogenic Callus Induction of *Pinus sylvestris* var. mongolica Clone (GS1). Molecular Plant Breeding 21(18):6088-6095. https://doi.org/10.13271/j.mpb.021.006088

Wang YH, Dai Y, Kong WL, Zhu ML, Wu XQ (2022) Improvement of Sphaeropsis Shoot Blight Disease Resistance by Applying the Ectomycorrhizal Fungus *Hymenochaete* sp. Rl and Mycorrhizal Helper Bacterium *Bacillus pumilus* HR10 to *Pinus thunbergii*. Phytopathology 112(6):1226-1234. https://doi.org/10.1094/PHYTO-09-21-0392-R

Wang ZL, Ye JR, Zhu LH, Wu XQ, Sun TY (2015) Mycorrhization of tissue-cultured plantlets of nematode-resistant *pinus densiflora*. J Nanjing For Univ 39(01):27-32. https://doi.org/10.3969/j.issn.1000-2006.2015.01.006

Wen XS, Kuang YY, Shi MQ, Li HZ, Luo LS, Deng RL (2004) Biology of Hylobitelus xiaoi (Coleoptera: Curculionidae), a new pest of slash pine, *Pinus elliottii*. J Econ Entomol 97(6):1958-1964. https://doi.org/10.1603/0022-0493-97.6.1958

Wu J, Zhu LH, Xu JX, Wu XQ, Ye JR (2015) Induction and proliferation of embryogenic callus in nematode-resistant *pinus densiflora*. J Nanjing For Univ 39(01):17-21. https://doi.org/10.3969/j.issn.1000-2006.2015.01.00

Xia XR, Yang F, Ke X, Chen YM, Ye JR, Zhu LH (2021) Somatic embryogenesis of masson pine (*Pinus massoniana*): initiation, maturation and genetic stability analysis at SSR loci. Plant Cell Tissue Organ Culture 145(3):667-677. https://doi.org/10.1007/s11240-021-02036-z

Yang F, Xia X-R, Ke X, Ye J, Zhu L-h (2020) Somatic embryogenesis in slash pine (*Pinus elliottii* Engelm): improving initiation of embryogenic tissues and maturation of somatic embryos. Plant Cell Tissue Organ Culture 143(1):159-171. https://doi.org/10.1007/s11240-020-01905-3

Ye JR, Han ZM, Li CD, Zhou CG, Chen G, Hu CY, Xu GH, Zhou GH, Min SB, Gan SP (1991) Establishment technique of resistance clones seed orchard of slash pine to brown spot disease. J Nanjing For Univ (02):23-29.

Ye JR, Li CD (1996) Research progress on resistance of *Pinus elliottii* to pine needle brown spot in China. Forest Res(02):84-90.

Zhang CY, Zhu LH, Tan JJ, Chen TT, Pan J, Liang F, Ye JR (2016) Somatic embryogenesis and plantlet regeneration of diseaseresistant Slash Pine (*Pinus elliottii* Engelm.) to Brown Spot Needle Blight. J Northeast For Univ 44(6):17-22. https://doi.org/10.3969/j.issn.1000-5382.2016.06.006

Zhang L, Gao B (2023) Physiological response of ectomycorrhizal fungi (*Lactarius delicious*) to microplastics stress. Global Nest J 25(3):115-124. https://doi.org/10.30955/gnj.004575

Zheng YL, Li WD, Cheng XF, Hua XM (2003) Study on Ecto-endomycorrohizae Associated by *Pisolithus tinctorius* with Pine. Forest Res(03):262-268.

Zhou T, Li H, Xiao N, Li BL, Zhang JF (2010) Research on induction of embryogenic callus from mature zygotic embryo of *pinus bungeanaas*. J Anhui Agric Sci 38(21):11063-11066. https://doi.org/10.13989/j.cnki.0517-6611.2010.21.157

Zhu LH, Wu XQ, Qu HY, Ji J, Ye JR (2010) Micropropagation of *Pinus massoniana* and mycorrhiza formation in vitro. Plant Cell Tissue Organ Culture 102(1):121-128. https://doi.org/10.1007/s11240-010-9711-y

573

574 **Table 1** Orthogonal array of nine initiation medium consisting of three concentrations of each of 2,4-D, 6-BA and KT

575

Levels	Factors		
	2,4-D（mg/L）	6-BA（mg/L）	KT（mg/L）
1	2	1	0
2	4	2	1
9	6	3	2

576

577 **Table 2** Embryo developmental stage and initiation rate for four collection dates in *Pinus elliottii*

Collection date	Embryo developmental stage (%)					Initiation rate (%)
	Stage1	Stage2-3	Stage4-5	Stage6-7	Stage8	
6/29	33.33	66.67	0	0	0	2.53±0.47c
7/06	6.67	93.33	0	0	0	21.83±4.95a
7/13	0	20.00	66.67	13.33	0	12.97±0.52b
7/20	0	0	13.33	46.67	40.00	1.27±0.34c

578 Data represent the mean ± SD of replicates. Different lowercase letters above the bars indicate significant differences according to
579 ANOVA and Duncan's test ($p < 0.05$)

580

581

582 **Table 3** Results of the influence of 2,4-D, 6-BA and KT on EC initiation in *Pinus elliottii*

Number	Factors			Initiation rate (%)
	2,4-D（mg/L）	6-BA（mg/L）	KT（mg/L）	
1	6	3	0	9.00 ±1.41b
2	2	2	2	4.33 ±1.89cd
3	6	1	2	15.67 ±1.89a
4	2	3	1	6.67 ±2.87c
5	4	3	2	2.00 ±1.41d
6	6	2	1	3.00cd
7	4	2	0	2.00 ±1.41cd
8	4	1	1	1.00 ±1.41d
9	2	1	0	2.00 ±1.41d

583 Data represent the mean ± SD of replicates. Different lowercase letters above the bars indicate significant differences according to
584 ANOVA and Duncan's test ($p < 0.05$)

585

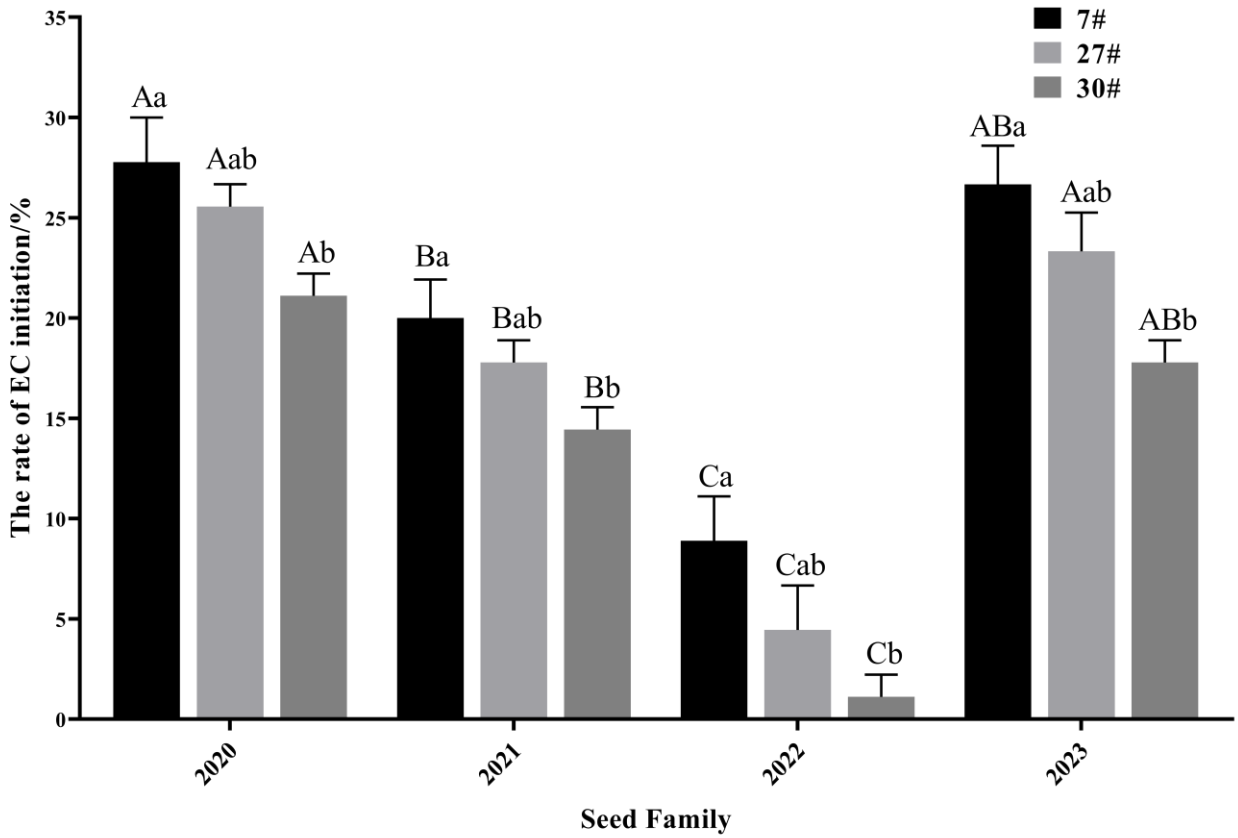
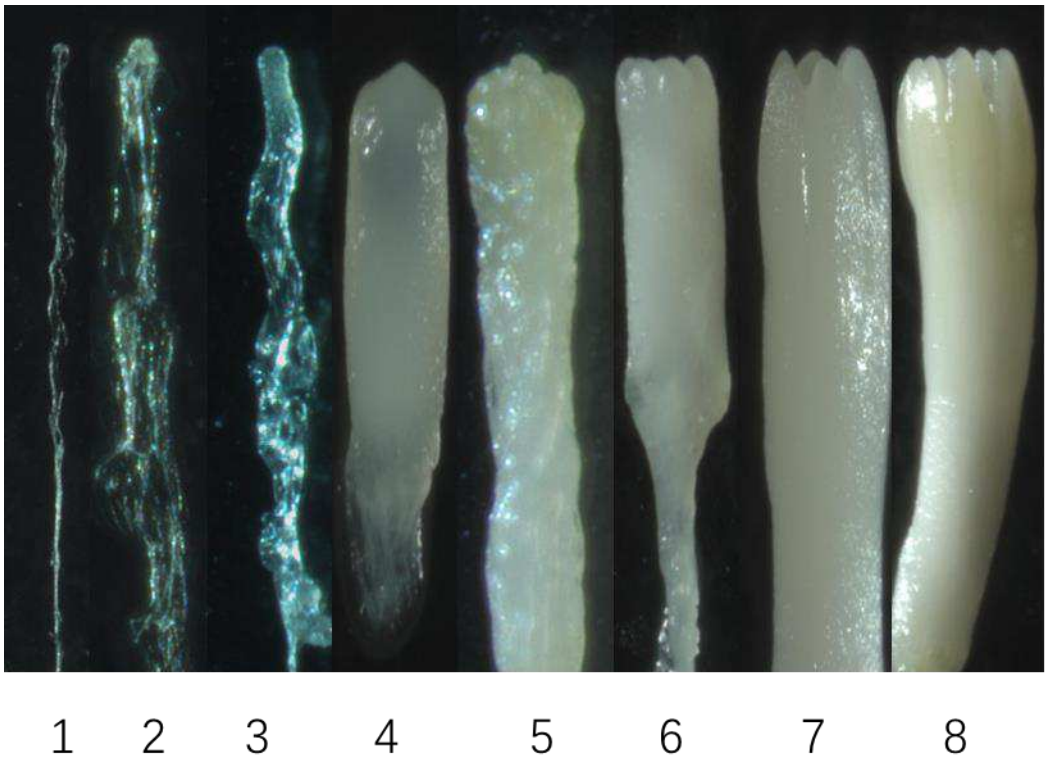


Fig. 1 Effect of seed family on embryogenic callus initiation rates in *Pinus elliottii.* Data represent the mean ± SD of replicates. According to ANOVA and Duncan's test, different capital letters above the bar indicate significant differences between years in the same seed family and different lowercase letters indicate significant differences in seed families in the same year ($p < 0.05$)



592
593 **Fig. 2** Photographs of *Pinus elliottii* zygotic embryos stripped from megagametophytes. Embryo developmental stages of *P. elliottii*
594 zygotic embryos were sorted according to the *P. taeda* zygotic embryo development stage system of Pullman and Webb (1994).
595 Sequence of *P. elliottii* somatic embryo development showing similar developmental stages 1–8.

596

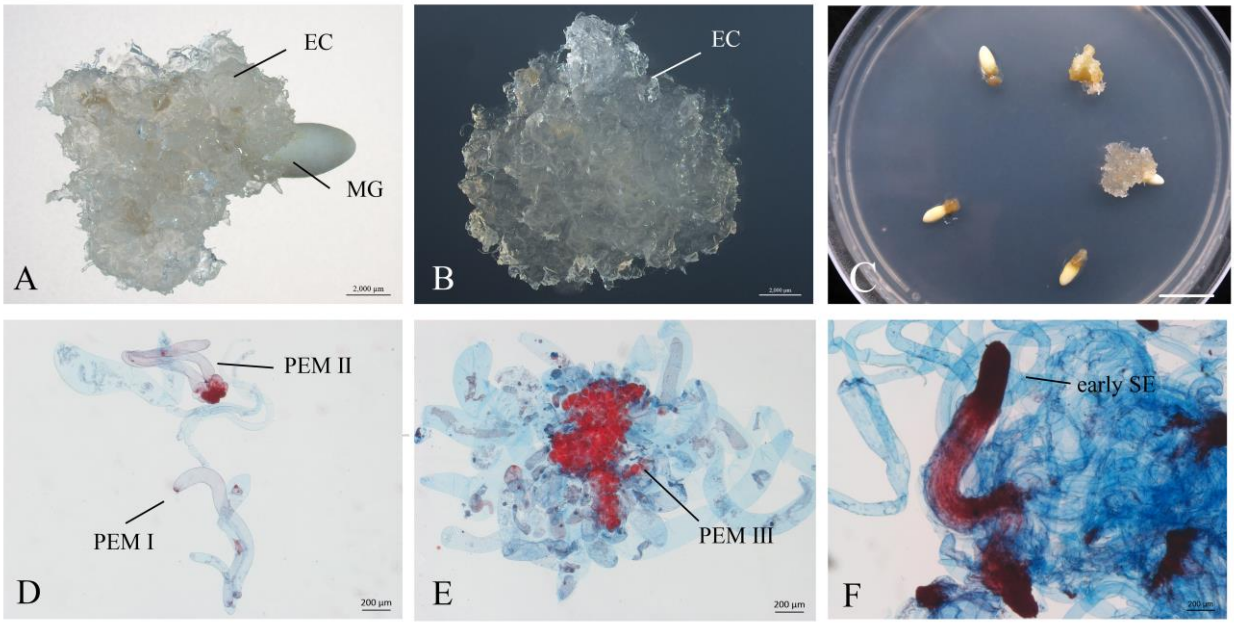


Fig. 3 Initiation, proliferation and cytological observation of embryogenic callus (EC) in *Pinus elliottii.* (A) The EC protruded from micropylar end of the megagametophyte (MG) explant, bar = 0.2 cm. (B) The EC stably proliferating on medium, bar = 0.2 cm. (C) Immature seeds start EC after being cultured for 8 weeks in the induction culture period, bar = 1 cm. (D–F) The EC developmental stages: PEM I-III and early somatic embryo, bar = 0.2 cm

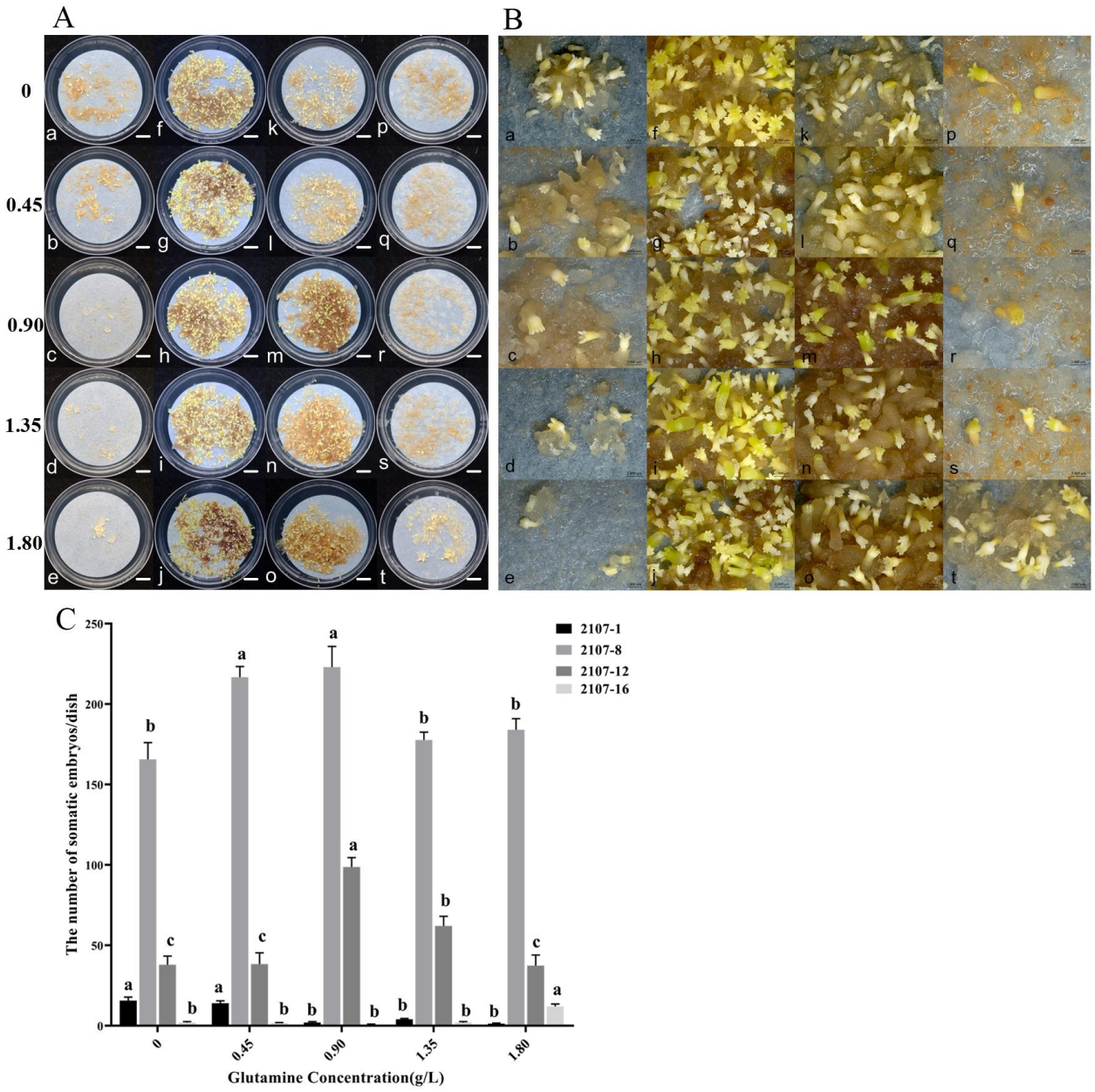


Fig. 4 Effect of glutamine concentration on somatic embryo maturation. (A) Concentrations of glutamine from top to bottom of picture are 0, 0.45, 0.90, 1.35 and 1.80 g/L in solid culture, and somatic embryos were obtained after 10 weeks, bar = 1.0 cm. (B) Further close-up of (A) under the stereomicroscope. Scale bar = 0.2 cm. (C) Number of somatic embryos in different treatments after 10 weeks of maturation. Different letters above the bars of the same color indicate significant differences according to ANOVA and Duncan's test ($p < 0.05$). Letters represent maturation medium composition supplemented with (a–e) ECL 2107-1, (f–j) ECL 2107-8, (k–o) ECL 2107-12 and (p–t) ECL 2107-16

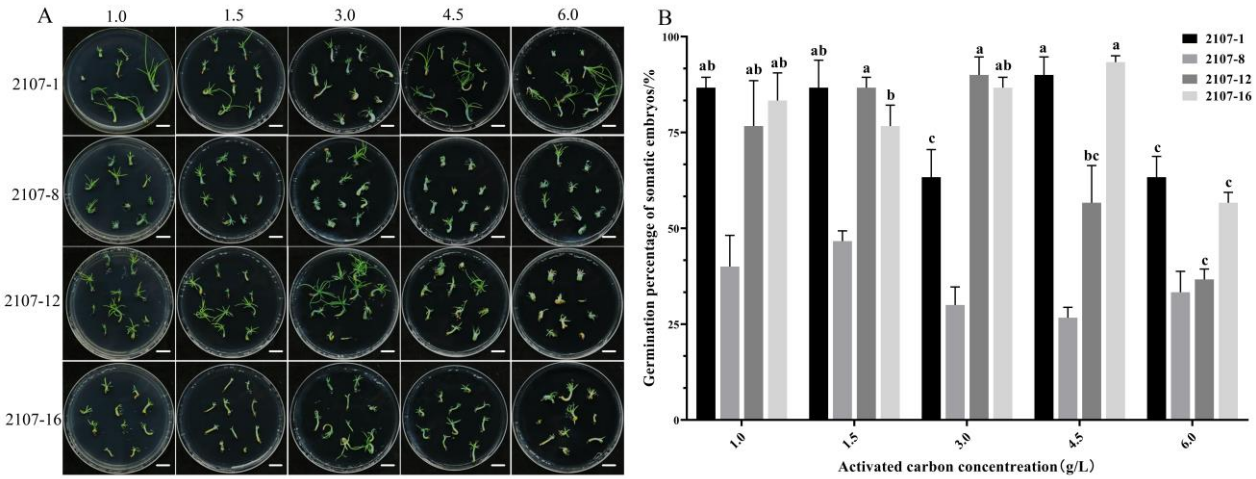
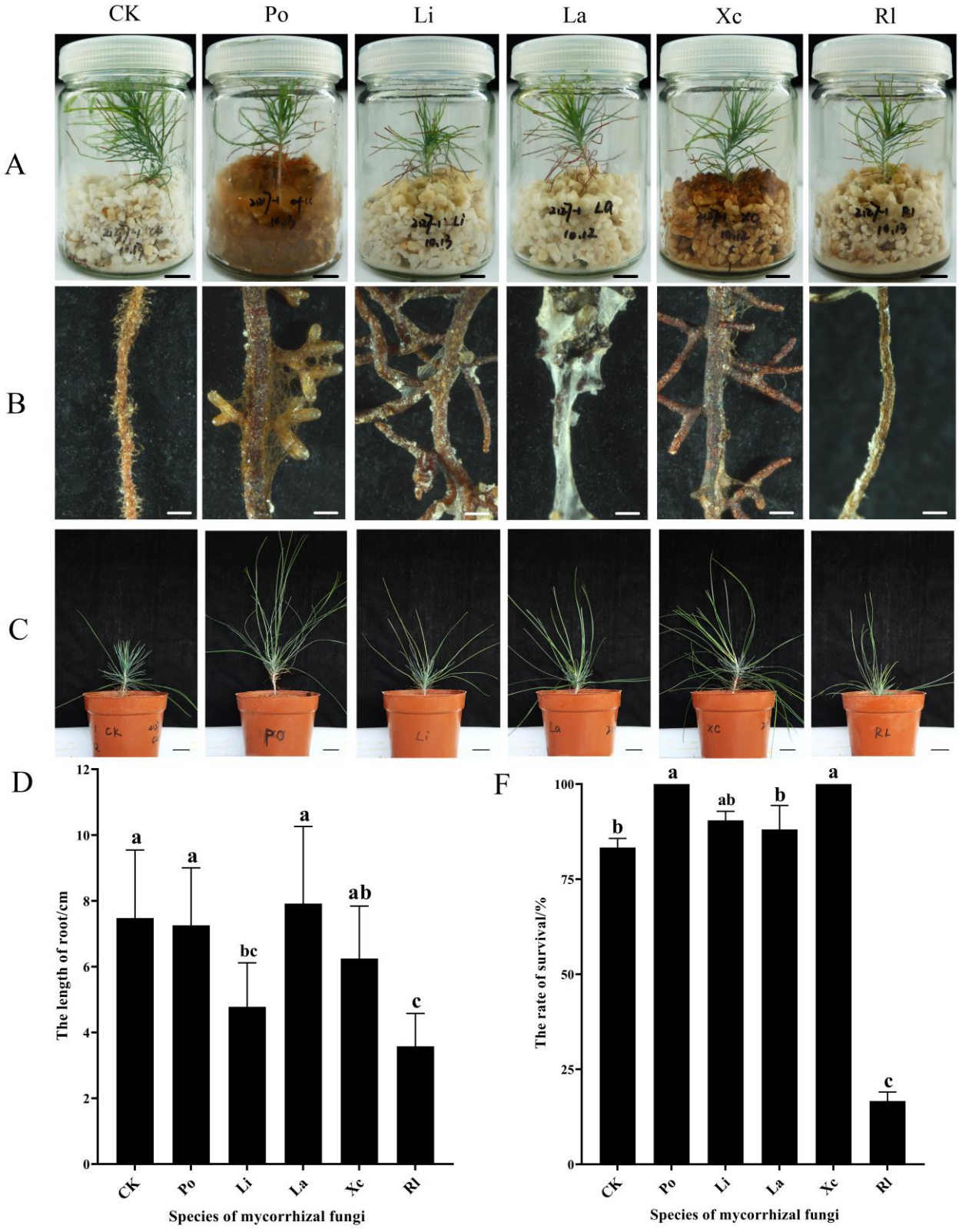


Fig. 5 Effect of activated carbon concentration on somatic embryo germination. (A) Germination of somatic embryos on germination medium after 5 weeks, bar = 1.0 cm. (B) Germination rate of somatic embryos. Data represent the mean ± SD of the sample. Different letters above the bars of the same color indicate significant differences according to ANOVA and Duncan's test ($p < 0.05$)


CK
Po
Li
La
Xc
Rl
A
B
C
D
F
The length of root/cm
Species of mycorrhizal fungi
The rate of survival/%
Species of mycorrhizal fungi

622 **Fig. 6** Effect of ectomycorrhizal fungi on regenerated plantlets. (A) Regenerated plantlets inoculated with mycorrhizal fungi under
623 sterile conditions for 4 months and uninoculated plantlets. (B) Details of a root structures viewed under microscope, bar = 0.05 cm.
624 (C) Regenerated plants at 3 months after transfer, bar=1 cm (D) Rate of survival in regenerated plantlet at 3 months after transfer.
625 Data represent the mean ± SD of replicates. Different lowercase letters above the bars indicate significant differences according to
626 ANOVA and Duncan's test ($p < 0.05$)

627