# Sterile seedlings cultivation and transplanting of *Idesia polycarpa* seeds

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## Abstract

**Background.** *Idesia polycarpa* Maxim is not only a high-quality and high-yield woody edible oil tree species, but also an industrial oil raw material tree species in China, which plays a crucial role in ensuring national food and oil security, promoting ecological environment construction, and facilitating rural revitalization. It was included in the catalog of national strategic reserve forests in April 2020. Currently, *I. polycarpa* is mainly propagated by seeding, but its seeds exhibit dormancy, and their natural germination rate is low, which seriously restricts the development of the *I. polycarpa* industry. Tissue culture technology can reproduce quickly and has a large reproduction coefficient, with no seasonal restrictions, and can provide good-quality seedlings in a short time.

**Methods.** This study used *I. polycarpa* seeds as the experimental material to establish a seed aseptic and efficient germination system by investigating the effects of different disinfection times, basic medium variations, activated carbon concentrations, as well as hormone types and concentrations on seed germination. Subsequently, bacteria-free seedlings were used as explants to explore the impact of various factors on rooting and determine the optimal conditions for transplanting *I. polycarpa* tissue culture seedlings.

**Results.**The results showed that a medium consisting of 1/2MS+1.0 mg·$L^{-1}$ $GA_3$+1.0 g·$L^{-1}$ AC exhibited maximum efficiency in promoting seed germination with a rate of 96.0%. Additionally, MS+0.3 mg·$L^{-1}$ IBA + 0.5 mg·$L^{-1}$ NAA proved to be the most suitable medium for rooting, resulting in a rooting rate of 100% and an average rooting numbers of 22.17. The regenerated plantlets were transplanted into a mixture comprising organic soil, perlite, vermiculite (2:1:1, V/V/V), the survival rate reached 96.67%. These experimental outcomes can provide valuable technical support for factory breeding of *I. polycarpa* seedlings.

**Key words:** *Idesia polycarpa*; tissue culture; sterile germination; acclimatization and transplanting

## Introduction

*Idesia polycarpa* Maxim (genus *Idesia*, family Salicaceae)is a tall deciduous, perennial trees, the species is widely distributed in low-altitude hillsides and valleys across China(*Wang et al., 2023*). It is distributed both domestically and internationally. *I. polycarpa* fruit yield, with a high oil content and a high percentage of unsaturated fatty acids (88.67%) and more than 80% linoleic acid. Its oil also contains a lot of vitamin E, squalene, sterol, and other nutrients, which can help lower blood lipid levels and prevent cardiovascular and cerebrovascular diseases(*Li et al., 2024*). The oil can be converted into biodiesel, industrial oils that satisfies standards using a process called alkali-catalyzed transesterification, which is a new, pollution-free energy source known as "Tree Oil Depot"(*Xiang et al., 2023*). In addition, *I. polycarpa* has a well-developed root system, remarkable adaptability, and resilience to drought and barren conditions, making it an excellent ecological barrier tree. It is a significant timber tree species due to its quick growth, straight trunk, and ability to be utilized as raw material for furniture and construction. It also has strong ornamental qualities and yellow-green blooms. It is also a great choice for roadside plantings and gardens(*Sui, 2011*). *I. polycarpa* is a multipurpose, multi-functional, important woody oil tree that has social, economic, and ecological value. It is crucial for preserving the nation's grain and oil security, fostering the creation of ecological environments, and assisting in the restoration of rural areas. *I. polycarpa* was included to the national strategic Reserve Forest list in April 2020 (*Wu et al., 2023*).

At present, *I. polycarpa* is mostly sown and propagated. However, its seeds have a low germination rate in natural conditions, the propagation cycle is long, and an incomplete tissue culture and quick propagation system, severely limiting the development of industry(*Liu et al., 2009*). Cutting and grafting can help with problematic reproduction and the preservation of good traits, however cuttings demand high natural conditions, are prone to rot, and are difficult to take root. Grafting is expensive and technically demanding, and grafting duration, temperature, and scion lignification degree can all have an impact. Plant tissue culture technology's high reproduction coefficient, quick rate of reproduction, and lack of seasonal constraints allow it to produce a large number of seedlings quickly. The foundation and starting point for the ensuing tissue culture and transgenic research is the successful cultivation of a vaccination devoid of microorganisms. Tissue culture inoculatory vaccines with long cycles and large-scale manufacturing are hard to come by at the moment, as tissue culture rapid propagation system of *I. polycarpa* seed

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The introduction contains precious information about the species. However, it could be more effectively organized and clarified. Attention to grammatical detail and readability should be taken into account to improve the overall quality of the manuscript. The introduction provides a solid background on the species and its significance, but limited detail on the previous studies that have attempted similar tissue culture techniques. The main purpose of this research is somewhat buried, so it might be more effective to state the objectives of the research work clearly for the reader

**Commented [TK2]:** This sentence is vague. It has to be more informative to specify the regions or countries where it is found outside China.

**Commented [TK3]:** lacks clarity in describing the yield and composition. Please revise it

technology is still lacking(*Li, 2023a*). This experiment investigated tissue culture and rapid propagation technology using mature *I. polycarpa* seeds as the raw material, screened the most favorable culture conditions by adding plant growth regulators at different culture stages, and conducted extensive research on the cultivation and transplanting technology of cultured bacteria-free seedlings, which could obtain a large number of high-quality seedlings in a short amount of time. These steps were taken in order to establish a high quality and high efficiency seedling technology system of excellent single plant of *I. polycarpa*. It offers the practical foundation and technical assistance needed for large-scale, industrial seedling cultivation in the future.

## Materials & Methods

### Materials

In this study, we used the *I. polycarpa* seeds obtained from Qishe Town, Xingyi City, Guizhou Province, China (104°47'E ,24°56'N) at the end of November 2022. After harvest, the pericarp was removed, the seeds were washed, broken grains, shrunken grains and impurities were discarded, then the seeds were dried indoors and stored at 0 to 10 ℃ for reserve.

### Establishment of aseptic germination system of *I. polycarpa* seed

Took the reserved *I. polycarpa* seed, first carried on the pretreatment: put the *I. polycarpa* seed into the clear water, screened out the floating impurities, shrunken grains and empty grains, and repeated this operation for 2 or 3 times, then added appropriate amount of washing powder to soak for 2 hours, wrapped with gauze, kneaded repeatedly to remove the waxy layer, and rinsed with running water for 30 min. Then the seeds were soaked in pure water at 30 to 40℃ for 30 min and moved into the ultra-clean worktable.

### Effects of different disinfection time on *I. polycarpa* seed germination

Selected full *I. polycarpa* seed (1000-seed weight: 4.007 g), and put it in the culture bottle. Directly added 0.1% $HgCl_2$ solution to disinfect (time is 6, 8, 10 and 12 min), and rinsed with sterile water for 3 to 5 times, then poured the seed on sterile filter paper to absorb surface water, and inoculated it on the seed germination medium. 10 seeds were inoculated in each bottle, 10 bottles in each treatment, and 3 groups were repeated. After 30 days, the germination rate and pollution rate of *I. polycarpa* seed were calculated. Germination rate (%) = (30 d germinated seeds / inoculated seeds) × 100, pollution rate (%) = (30 d polluted seeds / inoculated seeds) × 100.

### Effects of different basic medium on *I. polycarpa* seed germination

Selected full *I. polycarpa* seed after disinfection treatment, and inoculated into three basic medium: MS, 1/2MS and WPM, respectively. All the medium were supplemented with 1.0 $mg \cdot L^{-1} GA_3$ + 30 $g \cdot L^{-1}$ sucrose + 8.0 $g \cdot L^{-1}$ Agar + 1.0 $g \cdot L^{-1}$ AC. 10 seeds were inoculated in each bottle, 10 bottles in each treatment, and 3 groups were repeated. After 30 days, the germination rate and pollution rate of *I. polycarpa* seed were counted, and the seedling height was measured with a ruler.

### Effects of different concentrations of activated carbon on *I. polycarpa* seed germination

Selected full *I. polycarpa* seed after disinfection treatment. Different concentrations of activated carbon (0, 1.0, 2.0, 3.0 $g \cdot L^{-1}$) were added to the medium with 1/2MS+1.0 $mg \cdot L^{-1}$ $GA_3$ + 30 $g \cdot L^{-1}$

**Commented [TK4]:** This sentence is too long and complex. Revise it for better clarity and flow of the Idea

**Commented [TK5]:** The existing bits of knowledge are not well articulated in this area. There are different works related to propagating *I. polycarpa* via tissue culture. So, it is important to clearly state what have so far done, the gap of those previous works, and indicate the contribution of your work to fill the gap.

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The methodology is comprehensive and detailed which is important for reproducibility. However, additional clarification on procedures, consistency in units, terminology, and formatting are needed to enhance the readability and simplicity.
The experiment is well structured, but the rationale for selecting specific treatment for example the concentration of each treatment should be explained more explicitly to strengthen the work..
Even though the methodology provides many details, some areas lack detailed information for complete reproducibility such as the exact formulation of the rooting powder solution and the conditions of the greenhouse environment during the transplanting phase.

**Commented [TK7]:** Mention for how long the seeds were stored before use.

**Commented [TK8]:** Could you specify the amount of water used during rinsing

**Commented [TK9]:** Is it a total sample for all treatment or for this specific treatment?. Make it clear

**Commented [TK10]:** Why you selected these time duration? Is there any standard? Please briefly explain why you select these time duration.

**Commented [TK11]:** Use an appropriate term, because pollution rate is not a scientific term in seed studies

**Commented [TK12]:** You should describe the methods of adding activated carbon. For instance, was it mixed thoroughly with the medium?

sucrose + 8.0 g·L$^{-1}$ Agar, aiming to screen the most effective concentration of activated carbon for *I. polycarpa* seed germination. Inoculated 10 seeds per bottle, 10 bottles per treatment, and repeated 3 groups. After 30 days, the germination rate and pollution rate of *I. polycarpa* seed were counted, and the seedling height was measured with a ruler.

**Effects of different hormone types and concentrations on *I. polycarpa* seed germination**

The sterilized seeds were inoculated in the medium containing different types and concentrations of plant growth regulators ($GA_3$: 0.5, 1.0, 1.5, 2.0 mg·L$^{-1}$; 6-BA: 0.5, 1.0, 1.5, 2.0 mg·L$^{-1}$; NAA: 0.5, 1.0, 1.5, 2.0 mg·L$^{-1}$). 10 seeds were inoculated in each bottle, 10 bottles in each treatment，and 3 groups were repeated. No plant growth regulator was added as the control group. After 30 days, the germination rate and pollution rate of *I. polycarpa* seed were counted, and the seedling height was measured with a ruler. The best conditions for *I. polycarpa* seed germination were screened.

**Commented [TK13]:** Describe the method of hormone application. Were they dissolved in a solvent before being added?

**Rooting culture of *I. polycarpa* aseptic seedlings**

**Effects of different sucrose concentrations on rooting of aseptic seedlings of *I. polycarpa* seed**

Selected the robust *I. polycarpa* aseptic seedlings with a height of 4 to 7 cm and trimmed the base of the hypocotyl. Different concentrations of sucrose (10, 20, 30 g·L$^{-1}$) were added to the MS medium. Each treatment was inoculated with 20 bottles. The rooting and growth of aseptic seedlings were observed 30 days after inoculation, and the related indexes such as rooting rate, average root length, and average rooting number were counted. Rooting rate (%) = (number of rooting explants / total number of explants) × 100, average root number = total number of roots / number of rooting plants, average root length = sum of root length / number of roots.

**Effects of different plant growth regulators on rooting of *I. polycarpa* aseptic seedlings**

Selected the robust *I. polycarpa* aseptic seedlings with a height of 4 to 7 cm and trimmed the base of the hypocotyl. Different concentrations of IBA (0.1, 0.3, 0.5, 0.7, 0.9 mg·L$^{-1}$) or IAA (0.05, 0.1, 0.5, 1.0, 1.5 mg·L$^{-1}$) or NAA (0.01, 0.05, 0.1, 0.5, 1.0 mg·L$^{-1}$) were added to MS medium. Different plant growth regulators and concentrations suitable for rooting of *I. polycarpa* aseptic seedlings were preliminarily screened. 30 bottles of each concentration were inoculated with 1 or 2 strains per bottle. The effects of different combinations of IBA (0.1, 0.3, 0.5 mg·L$^{-1}$) and NAA (0.1, 0.3, 0.5 mg·L$^{-1}$) on rooting were further studied, with no plant growth regulators as the control group. 30 bottles of each concentration were inoculated with 1 or 2 strains per bottle. The rooting and growth of aseptic seedlings were observed 30 days after inoculation, and the related indexes such as rooting rate, average root length, and average rooting number were counted.

**Study on the transplanting technique of *I. polycarpa* tissue culture seedlings**

**Effect of different substrate ~~ratio~~ ratios on transplanting survival rate of *I. polycarpa* aseptic seedlings**

Suitable transplanting substrate can significantly improve the transplanting survival rate and growth of tissue culture seedlings. Selected the tissue culture seedlings with good growth after rooting for 30 days and transplanted them into transparent plastic cups filled with different transplanting substrates. When transplanting, washed off the residual culture medium with clean

water, and then dipped seedlings in the rooting powder solution with the concentration of 10 $g \cdot L^{-1}$~15 $g \cdot L^{-1}$ for 5 to 15 s. Paid attention to gentle manipulation in the process of transplanting to avoid root damage. The specifications of transparent plastic cup is: bottom diameter is 8 cm, height is 14.5 cm, and bottle mouth diameter is 11.5 cm. Covered the cup with a lid, and opened an air vent with a diameter of 3 to 6 mm on the side of the lid and the side at a height of 0.5 mm from the bottom. keeping the water content of the substrate above 70%. Cultured in a light culture room for 15 days, and then transferred to the greenhouse for 10 to 15 days, the temperature was 25-35℃. Calculated the survival rate and observed seeding growth after 25 days of culture of a transparent plastic cup. ~~And then~~Then the seedlings were transplanted into seedling bags and cultured in the greenhouse for 7 days. Finally, ~~placed~~ the seedlings in the nursery covered with a sunshade net. Sprayed water timely according to the humidity of the ~~substrate, and~~substrate and sprayed 0.1% urea once a month.

Organic nutrient soil (BALTIC PEAT), perlite, and vermiculite were selected as substrates. Three treatments were designed, that was, the volume ratios were 1:1:1, 2:1:1, and 1:2:1. 30 rooting seedlings were transplanted in each treatment. After transplanting, the growth was observed and the related indexes were counted after 25 days of culture. Transplanting survival rate (%) = (transplanting survival / total transplanting number) × 100.

**Effects of different transplanting containers on the transplanting survival rate of *I. polycarpa* tissue culture seedlings**

The tissue culture seedlings with good growth after rooting for 30 days were transplanted into transparent plastic cups and seedling trays filled with organic nutrient soil (BALTIC PEAT), perlite and vermiculite at a 2:1:1 volume ratio, respectively. The culture method of seedlings transplanted to the transparent plastic cup was the same as

**Effect of different substrate ratio on transplanting survival rate of *I. polycarpa* aseptic seedlings**. The tissue culture seedlings in the seedling trays were cultured in closed ~~bottle~~ bottles and opened ~~bottle~~ bottles for 7 days in the greenhouse. The culture medium was washed off when transplanting~~,~~ and then dipped seedlings in the rooting powder solution with ~~the~~ a concentration of 10 $g \cdot L^{-1}$~15 $g \cdot L^{-1}$ for 5 to 15 s, and directly ~~transpla nted~~transplanted into the seedling tray. The humidity of the two culture methods were basically the same and were cultured in the greenhouse. After 25 days, the survival rate, average seedling height and seedling growth were calculated.

**Culture conditions**

MS or 1/2MS was mainly used as the basic medium, with sucrose content of 3% (rooting of 1%), Agar content of 0.8% (rooting of 0.7%), pH 5.7~5.9, and autoclaving conditions of 116℃ 30 min or 121℃ 15 min. Different plant growth regulators were added to the culture medium of different culture stage. The temperature of the culture room was 25 ±2℃, the light intensity was 50~60 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and the light time was 12 $h \cdot d^{-1}$.

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**Data processing**

The results were processed and analyzed by SPSS 26.0 and Excel 2016 software, Image ~~manipul ation~~manipulation using Photoshop 2022.

## Results

**Primary culture of *I. polycarpa* seed**

**Effects of different disinfection time on *I. polycarpa* seed germination**

As the 0.1% $HgCl_2$ disinfection time was extended, the rate of seed contamination dropped (*Table 1*). The seeds started to sprout and reveal the radicle after 8 days of inoculation; by 15 days, the majority of the cotyledon had fully unfurled; after 30 days, the seed shell had fallen off; and no vaccine had grown rapidly. The germination rate increased to 77% after a 6-minute disinfection period. The maximum germination rate was 96.0% when the disinfection period was increased to 10 minutes. Following that, the germination rate dropped to 79.67% as the disinfection period was extended. The findings showed that the germination rate decreased with increasing disinfection duration and increased seed damage. Combining germination rate and contamination rate, 10 min was the best disinfection time for this experiment.

**Commented [TK15]:** It is not clear

**Commented [TK16]:** You mentioned about germination rate, but lack detail on reporting about contamination rate. As I stated before use appropriate term instead of contamination rate

**Effects of different basic medium on *I. polycarpa* seed germination**

*I. polycarpa* seeds were screened using 1/2MS, MS, and WPM as the basic media, in that order, for sterile germination. *Table 2* illustrates how slowly and erratically the seeds inoculated on WPM and MS media grew. Even though a large number of seeds germinated after 30 days, some of them had abnormalities that prevented normal growth. After 30 days, the germination rate of the seeds inoculated on 1/2MS medium was 94.67%, which differed from the other two basic media ($P<0.05$). The seedlings grew quickly and reached a height of 4.2 cm, with green leaves. Thus, 1/2MS basic medium was appropriate for *I. polycarpa* seed aseptic germination culture.

**Effects of different concentrations of activated carbon on *I. polycarpa* seed germination**

It was evident how varying concentrations of activated carbon affected seed germination, and the rate of germination with activated carbon was higher than the rate without it (*Table 3*). The maximum germination rate was observed at 1.0 $g \cdot L^{-1}$, with the plant growing to 4.13 cm in 30 days. Plant growth slowed ~~down~~down, and the rate of seed germination declined as the amount of activated carbon increased. Later on, the inoculum without activated carbon developed slowly, and the leaves somewhat yellowed.

**Effects of different plant growth regulators and concentrations on *I. polycarpa* seed germination**

To varying degrees, three distinct plant growth regulators increased seed germination (*Table 4*). The seeds could also germinate in the absence of an external plant growth regulator, with a 73.33% germination rate. The seed germination rate rose initially upon the addition of $GA_3$, NAA, and 6-BA to the medium, but as the concentration increased, it subsequently declined. Plant growth was much higher than that of the control group, with minimal variation in germination rates of 96.0% and 88.0%, respectively, at the optimal doses of $GA_3$ and NAA, which were 1.0 $mg \cdot L^{-1}$ and 0.5 $mg \cdot L^{-1}$, respectively. 6-BA had the poorest overall germination impact; the seed coat ruptured and the radicle expanded ten days later, and the initial germination time was delayed. The seedlings grow slowly. The seed bark cracked on day six, the radicle began to grow on day eight, and the cotyledon unfurled on day fourteen when the concentration of $GA_3$ was at its best. In conclusion, $GA_3$ is the most effective plant growth regulator for

inducing a bacterial-free vaccine of *I. polycarpa*, and as a result, 1/2MS+1.0 mg·$L^{-1}$ $GA_3$+1.0 g·$L^{-1}$ AC is the ideal medium for seed germination (*Fig. 1A*).

**Rooting culture of *I. polycarpa* aseptic seedlings**

**Effects of different sucrose concentrations on rooting of *Idesia polycarpa* aseptic seedlings**

For the purpose of inducing roots, sterile seedlings measuring 4 to 7 cm and exhibiting strong growth were chosen and injected into the root medium. After 6 days, the roots formed root primordia at the location where the stem segment's base touched the medium. The roots were strong and lengthy after 30 days of nonstop culture (*Fig. 1B*). *Table 5* shows that treatment 2 had the greatest rooting effect on *I. polycarpa* aseptic seedlings, with an average root number of 16.85 and a length of 2.66 cm. With an average root number of 12.3 and an average root length of 2.38 cm, treatment 3 had the lowest results. Increases in sugar concentration resulted in weaker plant development, a progressive drop in the number of roots, an inconspicuous change in average root length, and sporadic leaf yellowing. According to this perspective, the ideal rooting concentration is 10 g·$L^{-1}$ of sucrose.

**Effects of different auxins on rooting of *I. polycarpa* aseptic seedlings**

IBA, IAA, and NAA were introduced to MS medium in varying quantities to aid in the rooting of tissue culture seedlings. After 30 days, tissue culture seedlings were inspected and counted for rooting rate, average root length, and average root number, as indicated in *Table 6*. Auxin types varied in their ability to accelerate the rooting rate of *I. polycarpa* tissue culture seedlings. The inoculant's average root length was 0.98 cm, and its rooting rate was just 33.33% in the absence of any plant growth regulator. The tissue culture seedlings' rooting rate exhibited an increasing and then declining pattern when the IBA concentration was gradually increased. Callus also formed at the base of the stem segments, and the roots thickened and extended. The maximum rooting rate of 96.77%, the longest root length of 3.05 cm, and the largest average number of roots of 13.6 were observed at an IBA concentration of 0.7 mg·$L^{-1}$. During the IAA treatment, the maximum ~~rootin~~ grooting rate of 1.0 mg·$L^{-1}$ was achieved at 92.0%. The average number of roots was 10.37, and the length of the roots was 2.38 cm. The average number of roots was 12.8, the root length was 2.22 cm, and the rooting rate of 0.05 mg·$L^{-1}$ treated with NAA was 94.00%. Direct skin rooting was the primary rooting mechanism of IAA from a growth standpoint. A tiny amount of callus would form at the base of the stem segment as concentration increased, and there were fewer overall roots and shorter roots overall. Usually, NAA first causes a pale yellowish green callus at the stem segment's base before it begins to produce roots. The callus forms more quickly as the concentration rises, which has an impact on root growth. The clumping of roots and the yellowing or falling of leaves have an impact on the subsequent nurturing and transplantation of seedlings. The rooting rate of tissue culture seedlings exhibited an increasing and then declining trend under the three distinct ~~auxin~~ auxins and its concentrations, with the rooting rate and concentration steadily increasing from low concentration to high concentration. As can be observed, auxin at low concentrations will effectively encourage rooting, however, auxin at high concentrations can prevent *I. polycarpa* from rooting. It is evident from comparing the three treatments that IBA is most suited for assisting *I. polycarpa* aseptic seedlings in rooting (*Fig. 1C*).

Using varying IBA and NAA concentrations, inoculate the non-bacterial vaccine, which is robust, into MS media at a distance of 4-7 cm (*Table 7*). After 8 days or so, adventitious roots began to sprout. The roots flourished and grew, and after thirty days, the plants became green (*Fig. 1D*). Different amounts of root formation can be induced by each medium formula: more roots could be produced when IBA and NAA were combined than when auxin was used alone. The T6 variety exhibited the highest rooting effect, with a 100% rooting rate, average root length of 3.4 cm, and average root number of 22.17 cm. Additionally, the roots were robust and had a few lateral roots, making them excellent for transplanting. In comparison to other treatments, the T1 therapy had the shortest average root length (1.96 cm) and the lowest average root number (9.93). Considering the rooting conditions from the previous experiment, the root growth in the T6 treatment group and the 0.7 $mg \cdot L^{-1}$ IBA treatment group was nearly identical. T6 exhibited more roots and a greater rate of rooting, but a little lower survival rate. As a result, the T6 treatment group ~~provi ded~~provided a superior rooting media.

**Transplanting of *I. polycarpa* sterile seedlings**

**Effect of different substrate ~~ratio~~ ratios on transplanting survival rate of *I. polycarpa* aseptic seedlings**

Selected tissue culture seedlings with a developed root system were screened and injected with MS+0.7 $mg \cdot L^{-1}$ IBA+10 $g \cdot L^{-1}$ sucrose + 7.0 $g \cdot L^{-1}$ Agar media. The rooted seedlings that grew well were moved into clear plastic cups filled with a volume ratio of 1:1:1, 2:1:1, and 1:2:1 of organic nutrition soil (BALTIC PEAT), perlite, and vermiculite, respectively. Over ~~a 25 day period~~25 days, the average height of the seedlings, the transplant survival rate, and the material growth were recorded (*Table 8*) (*Fig. 1E*). After that, they were moved into seedling bags containing organic nutrient soil, perlite, and vermiculite in a volume ratio of 2:1:1. They were then raised in a greenhouse for a further 7 days, and on rainy days or in the morning and evening, they were put in a nursery covered by a sunshade net (*Fig. 1F*). Tissue culture seedlings treated with A1 and A2 had transplanting survival rates of 90.0% and 96.67%, respectively. In addition to its great water retention, the organic nutrient soil (BALTIC PEAT) has good air permeability and water ~~retenti on~~retention. It can also have its content increased by adding perlite and vermiculite. The group treated with A3 had the lowest survival rate (86.67%). The elevated perlite content resulted in poor root development, decreased root-matrix contact surface, and increased matrix porosity. To ~~summary ze~~summarize, the initial two treatments exhibit strong transplant viability and can be employed for the transplantation of tissue culture seedlings of *I. polycarpa*. In comparison to A1 and A3, A2 treatment is marginally superior, and the matrix combination ratio makes sense. Thus, organic nutrient soil: perlite: vermiculite (2:1:1) was determined to be the ideal culture medium formulation for *I. polycarpa*.

**Effects of different transplanting containers on the transplanting of *I. polycarpa* tissue culture seedlings**

*I. polycarpa* tissue culture seedlings that were essentially the same height and growth were chosen and then transferred into growing hole trays with the best substrate selection and transparent plastic cups, respectively. For 25 days, the growing hole trays' seedlings were cultivated in a greenhouse. During that time, measurements were made of the materials' growth,

average seedling height, and transplanted survival rate (*Table 9*). The height of the seedlings, the diameter of the ground, and the quantity of leaves did not significantly change after 10 days of transplanting. Although many of the seedlings in the growing hole trays were cultivated prior to transplanting, many of them perished within the first three days of their new home due to a lack of environmental adaptation. As the number of transplanting days rose, the transparent plastic cup's survival rate reached 96.67%, the height of the seedlings reached 26 cm, the quantity of leaves also much increased, and the growth was outstanding. In the growing hole trays, the seedlings develop slowly, with thin stems and leaves that eventually turn yellow and fall off. As a result, the transparent plastic cup is better for the development of *I. polycarpa* tissue culture seedlings.

## Discussion

In plant tissue culture, genetic transformation and other experiments need numerous bacteria-free seedlings, seed disinfection is particularly critical. I~~i~~nvestigated ~~In~~ the sterile germination of *I. polycarpa* seeds, initially disinfecting them with 70% ethanol for 2 minutes, followed by 0.1% $HgCl_2$ for 5 minutes, controlling contamination within 2%. ~~Combining~~ Combined with a 30% hydrogen peroxide solution achieved a viability rate of 90%. However, this study found that only using 0.1% $HgCl_2$ for 10 minutes resulted in a better outcome, with a viability rate of 96%, effectively addressing difficulties and complexities in seed sterilization for *I. polycarpa* seeds(*Shen~~,~~,2015*). Compared to 6-BA, 1.0 $mg \cdot L^{-1}$ $GA_3$ was most conducive to the germination of *I. polycarpa* seeds, followed by NAA, which was inconsistent with the result that 6-BA was suitable for the induction of aseptic vaccine of *Streptocaulon griffithii* (*Xiao et al.,2023*). Activated carbon can provide a dark environment for seed germination, improve gas conditions in the culture medium, and adsorb toxic substances such as quinones and phenols produced during plant growth(*Zhao et al.,2017*). In this experiment, 1.0 $g \cdot L^{-1}$ activated carbon was most suitable for the *I. polycarpa* seeds germination and growth of bacteria-free seedlings. Excessive content of activated carbon may adsorb plant growth regulators in the culture medium, resulting in poor seedling growth. Similar results have been reported in the seed germination experiment of *Syzygium album* (*Chen ,2021)*. Moreover, suitable basic culture media can facilitate the rapid germination of explants and regulate growth status. WPM medium has a lower concentration of inorganic salts, while MS is a rich ~~salt-balanced~~salt-balanced medium with higher concentrations of inorganic salts and ions. 1/2 MS medium reduces the trace elements in MS medium by half. From the perspective of seed ~~germin-ation~~germination and growth, 1/2 MS medium is better than MS and WPM medium for *I. polycarpa* seeds, It can be seen that reducing the concentration of inorganic salt in MS medium was beneficial to seed germination. Increasing sucrose concentration in the culture medium led to increases in foliar starch and ~~sugars~~ sugar content and decreases in water potential in ~~in~~ vitro plantlets. At the same time, the root length, shoot length, root number, and leaf number of the plant were higher(*Badr A, Angers P &Desjardins Y,2015*). In addition, *Gaspar et al. (2002)* reported that~~,~~ the sucrose added to the medium ~~hinderedchlorophyll~~ hindered chlorophyll synthesis, Calvin cycle, and photosynthesis, ~~anddisturbed~~ and disturbed the overall carbon metabolism in in vitro cultured plantlets.

**Commented [TK17]: General comments on the discussion**
The discussion effectively summarizes the study's key findings, comparing the results with the previous studies. It also delineates the effects of different treatments on the sterilization and growth of *I. polycarpa* seeds. Moreover, it translates the experimental findings into practical recommendations for improving the efficiency of tissue culture and seedling production, which is valuable for applied research.
However, improvement is required in the flow of information and coherence. A language edition is also required. Some sentences are too long, so try to make them short and clear for the reader

Cultivation under different sucrose concentrations revealed that 10 g·$L^{-1}$>20 g • $L^{-1}$>30 g·$L^{-1}$, indicating that lower concentrations of sucrose are more suitable for the rooting culture of *I. polycarpa* tissue culture seedlings. Some studies have reported that 20 g • $L^{-1}$ sucrose is suitable for rooting ~~of~~ *I. polycarpa*, which is inconsistent with the results of this study, which may be due to the different types of basic medium used, and variations in the amount of sucrose added may also be due to the different test materials(*Hong et al., 2023)*.

The growth and differentiation of plant roots are closely related to the types and concentrations of plant growth regulators. Some studies suggested that NAA has an excellent inducing effect on rooting in coniferous tree materials (*Browne, Davidson & Enns 2000*; *Goldfar et al., 2010*). *Costa Junior et al. (2018)* believed IBA to be more stable and the most effective auxin for inducing rooting in the plant tissue culture. This study found that IBA has the best rooting effect on *I. polycarpa* seeds, which is ~~basically~~ consistent with previous research results. Additionally, the effect of different auxin concentrations on the rooting efficiency of tissue-cultured seedlings varies. *I. polycarpa* seeds can be induced to root on MS medium without adding any plant growth regulators, but the root quantity is ~~low~~low, and the roots are short, which is not conducive to transplanting. Yet adding an appropriate concentration of a single auxin promotes rooting in *I. polycarpa* seeds. 0.7 mg·$L^{-1}$ IBA has the best relative effect on inducing the rooting of *I. polycarpa* seeds, which can rapidly induce the differentiation and growth of roots, and the induced roots are numerous and robust. Increasing the IBA concentration will inhibit *I. polycarpa* seeds rooting, resulting in increased callus tissue at the plant base and yellowing of the leaves. The roots become shorter and thicker, hindering root growth, consistent with the research results of *Yang, Liu & Xie (2013)* on *I. polycarpa* seeds rapid propagation rooting experiments, albeit with slight differences in concentration. In the culture medium ~~with1.0~~with 1.0 mg·$L^{-1}$ IBA, the root regeneration rate of *H. lucidum* subsp. *lucidum* is the highest, and the number and length of roots are also the largest (*Gianguzzi V et al.,2024*). IAA induces fewer roots that are slender and longer, generally without lateral roots. In the rooting research of *Asarum sieboldii*, it was found that 0.6~0.8 mg·$L^{-1}$ NAA can rapidly promote root differentiation and growth, forming thick roots (*Huang et al.,2016*). When the NAA concentration exceeds 0.05 mg·$L^{-1}$, shallow yellow-green callus tissue appears at the base, and the roots ~~would~~ become shorter, thicker, and enlarged in a spongy shape, which is easy to break off from the stem base during seedling washing, and easy to die when transplanted into the substrate in later stage (*Li, 2023b*). This is inconsistent with the finding of 0.05 mg·$L^{-1}$ NAA being the optimal concentration in this study, possibly due to different basal culture media or sources of experimental materials (germplasm). Both IBA and NAA can induce adventitious root formation, and their synergistic effect is better than when used alone. At the same time, some experiments showed that the rooting effect of mixed use of multiple auxins was better than that of single auxins(*Pandey N et al.,2019*). Moreover, during rooting culture, rooting rate, root length, and root quantity are influenced not only by plant growth regulators but also by factors such as seedling size~~-~~, leaf quantity, and operational approach. If the cost is considered cost, 0.7 mg·$L^{-1}$ IBA can be used alone.

The quality of the substrate is a key factor in determining the quality of seedlings, so choosing a suitable substrate and container for the growth of seedlings is crucial for the development of the forestry and seedling industry. *Jiang et al. (2006)* found in their study on the transplantation of tissue culture seedlings of *I. polycarpa* that vermiculite(V): perlite(V): peat(V)=6:3:1, requires 25 days to ~~survive,and~~survive, and the survival rate reaches 85%, it is the optimal mixed substrate ratio. Through comparative analysis of three formulations, the optimal substrate formulation was selected as follows: organic nutrient soil (BALTIC PEAT):~~perlite:vermiculite~~perlite: vermiculite=2:1:1, with a survival rate of 96%. This formula has a long-lasting fertilizer effect, strong water and fertilizer retention ability, good permeability and drainage, robust seedling growth, and high quality. This experiment was ~~cultivated~~ conducted in Changsha City, Hunan Province from June to July. The temperature was high, and although the *I. polycarpa* is a sunny plant, the seedlings have poor heat resistance. During the cultivation process, it is best to choose cloudy and rainy days or in the morning and evening. The temperature is more suitable, and the moisture content of the substrate is also the key to survival. High temperature and low humidity lead to severe leaf ~~curling,yellowing~~curling, yellowing of leaf edges, and shrinkage of stems and stems due to water loss in the seedlings. In high temperature and humidity environments, the substrate is prone to mold, causing seedlings to be infected with pathogens and prone to root rot and stem rot. This experiment used the conventional seedling tray and ~~transpar- ent~~ transparent plastic cup, and the results showed that the seedling tray is prone to root pit and crooked, which limits the growth of the root system of *I. polycarpa*. However, the use of transparent ~~plast- ic~~plastic cups can significantly improve the survival rate, seedling height, diameter, and root elongation of *I. polycarpa* tissue culture seedlings. The tray is placed in a constant temperature, humidity, and ~~nutrient rich~~nutrient-rich environment provided by the light culture room, close to the tissue culture bottle, dipped in a suitable concentration of rooting powder solution~~,~~ and stimulated by appropriate light to put the plants into a ~~self nourishing~~self-nourishing state, enhancing the resistance of the seedlings. Moving to a greenhouse avoids the burning of leaves by strong light, gradually stabilizing and further domesticating the tissue culture seedlings. At the same time, the transitional stage of the light culture room allows the growth of seedlings and root development to quickly adapt to the new environment, thus improving the survival rate of seedlings. The use of transparent plastic cups eliminates the traditional time for tissue culture bottle cultivation and greatly shortens the seedling cultivation cycle. In summary, this study used *I. polycarpa* seed as an explant to establish a sterile tissue culture rapid propagation system for *I. polycarpa* seedlings~~,~~ and explored ways to significantly improve the survival rate and growth of *I. polycarpa* tissue culture seedlings. This can obtain a large number of high-quality seedlings in a short ~~period of time~~period, reduce production management costs, improve seedling efficiency, and provide technical support and a Apractical basis for future large-scale and factory seedling cultivation.

## Conclusions

Use 0.1%$HgCl_2$ to disinfect the seeds of *I. polycarpa*, with an optimal time of 10 minutes. The optimal culture medium for promoting seed germination of *I. polycarpa* is 1/2MS+1.0 mg $L^{-1}$

**Commented [TK18]:** In summary of discussion it is better to addresses how the results contribute to the field of plant tissue culture, specifically for *I. polycarpa*, and suggests potential directions for future research.

**Commented [TK19]:** The conclusion summarizes the key findings but does not clearly emphasize the novelty or uniqueness of the research
Potential challenges or limitation of the work is not clearly stated

$GA_3$+1.0 g $L^{-1}$ AC, with a germination rate of 96.0%. The optimal culture medium for rooting is MS+0.3 mg $L^{-1}$ IBA+0.5 mg $L^{-1}$ NAA, resulting in a rooting rate of 100% and an average rooting numbers of 22.17. The tissue cultured seedlings were transplanted into a mixed substrate of organic nutrient soil (BALTIC PEAT): perlite: vermiculite at a ratio of 2:1:1 (V/V/V), with a survival rate of up to 96.67%. The use of transparent plastic cups can significantly improve the survival rate, seedling height, ground diameter, and root elongation of ~~tissue cultured~~tissue-cultured seedlings of *I. polycarpa*, and shorten the seedling cultivation cycle. Future research should focus on the anatomical study of rooting and the mechanical study of tissue culture of *I. polycarpa* in combination with new techniques such as cell engineering and transgene.

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