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ESTABLISHMENT OF A REGENERATION SYSTEM FROM SEED EXPLANTS OF *SAURURUS CHINENSIS*

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Abstract

Saururus chinensis is as a relic plant and its seed germination is notably slow and inefficient under natural conditions. To find a fast propagation method, a regeneration system of *S. chinensis* seedlings is established utilizing seeds as explant, employing plant tissue culture technique. The results showed that the optimal disinfection protocol was determined to be 75% ethanol treatment for 30 s followed by 0.1% (w/v) $HgCl_2$ treatment for 10 min. This treatment resulted in a sterile germination rate of 37.41% and a contamination rate of only 5.56%. Furthermore, soaking seeds in 1.00 mg/L GA_3 for 2 hours and supplementing the culture medium with 0.20 mg/L IBA significantly improved germination, reaching a rate of 52.22%. During the seedling growth stage, the optimal medium for seedling development was identified as MS supplemented with 0.20 mg/L NAA+0.50 mg/L GA_3+1.00 mg/L IBA, under these conditions, seedlings reached an average height of 3.43 cm, developed dense root systems, and exhibited an average root length of 1.17 cm. To promote growth vigor, the subculture medium was further optimized to 0.50 mg/L NAA, 2.00 mg/L GA_3 and 0.20 mg/L IBA; this optimization produced seedlings with broad, dark green leaves, a plant height of 4.13 cm, dense root systems, and root lengths of 1.32 cm. Finally, regenerated seedlings were acclimatized in sterile soil and successfully transplanted to natural soil conditions, achieving a survival rate of 88%. Our work effectively supports the aseptic propagation and large-scale production, germplasm conservation, artificial breeding, and biological research of *S. chinensis*.

Key words: *Saururus chinensis;* Seed explant; Rapid propagation system; Aseptic seedling

Introduction

Saururus chinensis, as a relict species of the Paleotropical flora of the Tertiary period (Peng 2011; Zhuang *et al*., 2014), is a perennial hydrophytic herb belonging to the family *Saururaceae* and the genus *Saururus*, closely related to *Houttuynia cordata, Gymnotheca chinensis* and *Gymnotheca involucrata*. According to the *Flora of China*, the genus *Saururus* comprises approximately 3 species, distributed across East Asia and North America. In China, only one species, *S. chinensis*, is found, mainly in the Yellow River basin and southern provinces such as Hebei, Shandong, and Henan. The plant features a well-developed rhizome system and emits a distinct aromatic scent. Morphologically, it is characterized by bisexual flowers lacking a perianth, borne with conspicuous white bracts and typically 1–3 terminal spike-like inflorescences. The fruit is a capsule. It grows in moist habitats such as riverbanks, swamps, and wetlands at altitudes ranging from 200 to 1800 m. Phytochemical studies have revealed that *S. chinensis* is rich in secondary metabolites, including essential oils, lignans, alkaloids, flavonoids, and terpenoids (Zhai & Zhao, 2021). The entire plant is used medicinally and exhibits a wide range of pharmacological activities, such as anti-inflammatory (Mengy *et al*., 2015; Zhang *et al*., 2021; Liu *et al*., 2024), anticancer (Jeong *et al*., 2015), hypoglycemic (Patel *et al*., 2024), antioxidant (Basavegowda *et al.*, 2016; Gao *et al*., 2022), hepatoprotective, and neuroprotective effects (Zhai & Zhao, 2021). Specifically, bioactive compounds such as quercetin and afzelin isolated from *S. chinensis* have been reported to alleviate diabetic complications, including cataracts, neuropathy, and vasculopathy (Duan, 2019). Total flavonoids, polysaccharides, and amino acids from the plant have demonstrated significant hypoglycemic effects in diabetic models (Dong & Zhen, 2015). Additionally, *S. chinensis* effectively reduced alanine alanine aminotransferase (ALT) levels in hepatocytes, with this activity contributing to its hepatoprotective effects (Patel *et al*., 2024). It also suppresses inflammatory responses and mitigates acute lung injury through modulation of neutrophil activity (Han *et al*., 2013; Li *et al*., 2023) and hypoglycemic effect (Hayashi *et al*., 2022). Traditionally, *S. chinensis* has been used in traditional medicine for treating dysuria, leucorrhea, nephritic edema, beriberi, and urinary tract infections (Zhuang *et al*., 2014). Beyond its medicinal value, the plant's unique variegated foliage enhances its ornamental value.

Currently, the propagation of *S. chinensis* primarily relies on vegetative reproduction via rhizomes; however, this method limits large-scale cultivation. Experimental observations have demonstrated that seed germination of *S. chinensis* under natural conditions is notably slow and inefficient, typically requiring more than one month in germination. Furthermore, the collection of *S. chinensis* still depends largely on wild harvesting. Due to its seasonally restricted growth and the rapid development of the traditional Chinese medicine (TCM) industry in recent years, *S. chinensis*, as a valuable medicinal herb (Patel *et*

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al., 2024; Sun-Waterhouse. *et al*., 2024), has experienced steadily increasing market demand. In addition, with the growing public interest in health and wellness, *S. chinensis* has gradually expanded into the nutraceutical and functional food markets, further broadening its commercial applications. Plant tissue culture offers an efficient approach to address rising market demand while providing sterile seedlings for experimental studies. This approach can overcome barriers in natural reproduction and ensure the sustainable utilization and large-scale production of *S. chinensis* (Barthawal *et al*., 2024).

Studies have demonstrated that tissue culture and micropropagation can significantly enhance the propagation coefficient and reduce the production cycle. Tissue culture is typically conducted under sterile and controlled environmental conditions, where essential nutrients required for plant growth are supplied and combined with specific plant growth regulators (PGRs) to promote cell differentiation and organogenesis (Nimavat & Parikh, 2024; Pasternak & Steinmacher, 2024). PGRs include auxins, cytokinins (Song *et al*., 2023), and gibberellins, each of which plays a critical role in various stages of plant development, such as cell division, callus formation, adventitious bud induction, and root development (Long *et al*., 2022).For instance, NAA (naphthaleneacetic acid), a synthetic auxin, primarily facilitates root development and, at appropriate concentrations, also promotes stem elongation and leaf growth (Wang *et al*., 2025). IBA (indole-3-butyric acid), another widely used auxin in plant tissue culture, effectively induces adventitious root formation and enhances rooting efficiency (Adsul *et al*., 2019; Gharari *et al*., 2021). GA_3 (gibberellic acid) is known to break seed dormancy and significantly improve seed germination, as well as stimulate shoot elongation and leaf expansion when applied at optimal concentrations (Luo *et al*., 2025; Sulaiman & Toma, 2023; Zhang *et al*., 2024). The synergistic interaction among different PGRs, when used in suitable concentrations and ratios, can further accelerate plantlet development and reduce the time to seedling establishment (Ardhani *et al*., 2024; Nimavat & Parikh, 2024). For example, the combination of NAA and GA_3 has been shown to significantly influence seed germination and root development (Bramhanapalli *et al*., 2016), while the joint application of NAA and IBA can promote shoot elongation and enhance root system formation (Soni *et al*., 2023). Additionally, 6-BA (6-benzylaminopurine), a commonly used synthetic cytokinin, effectively induces adventitious bud formation and enhances shoot multiplication rates (Adsul *et al*., 2019; Pang *et al*., 2023).

In recent years, research reports on the tissue culture of *S. chinensis* are rather limited. A study published in 2001 investigated the tissue culture system of *S. chinensis*, utilizing rhizomes, stem segments, and leaves as explants. The researchers employed a suspension culture technique and identified MS medium supplemented with 0.2 mg/L NAA and 1.0 mg/L BA as the optimal medium for stem explant growth. For suspension culture, the best medium formulation was MS supplemented with 2.0 mg/L BA and 1.5 mg/L 2,4-D (Jiang *et al*., 2001). Notably, no aseptic regeneration system using seed explants has been reported for S. chinensis thus far. This study aimed to develop a rapid propagation system using *S. chinensis* seeds as explants. Seeds present several advantages as explant sources: (1) Seeds are derived from mature plants and share identical genetic characteristics with the maternal line, ensuring genetic stability and uniformity; (2) As vertically transmitted propagules, seeds typically harbor fewer pathogens and contaminants, making them ideal for in vitro culture initiation (Lei *et al*., 2021); (3) The presence of a protective seed coat facilitates effective surface sterilization and contributes to higher disinfection success rates (Pepe *et al*., 2021; Rezaei *et al*., 2023). In this study, seeds of *S. chinensis* were selected as explants, and various disinfection protocols were evaluated to determine the most effective sterilization method, thereby establishing a solid foundation for seed-based tissue culture. In combination with optimized plant growth regulator (PGR) treatments, the study systematically investigated the best culture media for seed germination, seedling development, and subculture proliferation. Furthermore, an efficient regeneration system for *S. chinensis* was developed through an acclimatization-transplanting pipeline. The outcomes of this research provide key technical support for the large-scale propagation of *S. chinensis*, while also contributing valuable experimental foundations for artificial breeding, germplasm conservation, and the production of seedlings for biological research.

Material and Methods

Plant materials: Whole plants of *S. chinensis* were collected from Jima Village, Liping County, Qiandongnan Miao and Dong Autonomous Prefecture, Guizhou Province, China (E109°19’23.700”, N25°52’23.592”), and transplanted into the greenhouse of the Key Laboratory of Mountain Environment at the West Campus of Guizhou Normal University. The fruiting spikes of *S. chinensis* typically reach maturity between August and September and were harvested from the same greenhouse facility. Mature, disease-free plants with robust stems, intact leaves, and well-developed fruits were selected for seed collection. Seeds were naturally air-dried in the laboratory, sealed in plastic bags, and stored at 5°C in a Haier refrigerator for subsequent use as explants.

Methods

Sterilization of explants: As explants may harbor a variety of microorganisms both on their surface and internally, effective surface sterilization is essential to obtain aseptic plantlets. Therefore, the use of appropriate sterilizing agents is critical for establishing an efficient and contamination-free rapid propagation system. Intact and undamaged seeds of *S. chinensis* were selected as explants. Due to their small size and hard seed coats, the outer seed coats were manually removed prior to sterilization to enhance disinfection efficiency. The de-coated seeds were then enclosed in 3 × 3 cm non-woven fabric bags to facilitate subsequent sterilization procedures. Before transferring to the sterile laminar flow hood, seeds were soaked in a detergent solution for 20 min, followed by rinsing under low-flow tap water for 3 hours to remove surface contaminants. For disinfection, the seeds were immersed and gently agitated in 75% ethanol for 30 s, followed by a 1-minute rinse with sterile distilled water. Subsequently, seeds were treated with 0.1% mercuric chloride ($HgCl_2$) for 8, 10, or 12 min to assess the effect of different exposure durations on sterilization efficacy.

Throughout the $HgCl_2$ treatment, seeds were continuously agitated to ensure full contact with the disinfectant. After sterilization, seeds were rinsed five times with sterile water (1 minute per rinse) to eliminate residual disinfectant. Sterile filter paper was used to blot dry the seeds, which were then evenly inoculated onto the surface of Murashige and Skoog (MS) basal medium without any PGRs (plant growth regulators). Due to the limited availability of seeds, three treatments were established. Each treatment consisted of three culture bottles, with 10 seeds per bottle, and three biological replicates (Table 1). Contamination rates were recorded after 15-20 d of culture, and germination rates were assessed between 15-50 d.

Sterilized seeds were transferred into 240 mL glass culture bottles, each containing 30 mL of autoclaved MS basal medium. The culture medium was autoclaved at 121°C for 20 min. All reagents and instruments used in the experiment were sterilized prior to use and exposed to UV light in the laminar flow hood for 30 min to minimize contamination. The culture conditions were maintained at 23-25°C with a light intensity of 1500–2000 lux under a photoperiod of 12 h light/12 h dark.

Basal medium: All experiments were conducted using Murashige and Skoog (MS) medium as the basal formulation, supplemented with 15 g/L sucrose, 10 g/L agar, and 0.1g/L activated charcoal. The pH of the medium was adjusted to 6.0 ± 0.1 using 1 mol/L NaOH or HCl. Subsequently, 30 mL of the medium was dispensed into 240 mL glass culture bottles and autoclaved at 121°C for 20 min. After sterilization, the medium was allowed to cool and solidify under sterile conditions for later use. The MS basal medium was employed for the induction of the tissue culture regeneration system, including the formation of adventitious shoots and callus. Depending on the developmental stage of the explants, appropriate concentrations of PGRs were added to the medium, such as naphthaleneacetic acid (NAA), GA_3, IBA, and 6-BA.

Effects of pre-treatment and plant growth regulators on seed germination and seedling development: To investigate the effects of different pre-treatment methods on seed germination and seedling growth, the experiment was divided into two parts: (1) Seeds were subjected to different gibberellic GA_3 soaking treatments and subsequently cultured on MS medium supplemented with 0.50 mg/L GA_3 to assess their influence on germination. The treatments included: a control group (no GA_3), 0.5 mg/L GA_3 soaking for 0.5 h, 0.5 mg/L GA_3 soaking for 2 h, 1.0 mg/L GA_3 soaking for 0.5 h, and 1.0 mg/L GA_3 soaking for 2 h. (2) Based on the treatment of soaking seeds in 1.0 mg/L GA_3 for 2 h, different PGRs were added to the culture medium to assess their effects on seed germination and seedling growth. The medium was supplemented with various concentrations of GA_3 (0.50 and 1.00 mg/L), 6-BA (0.50 and 1.00 mg/L), and IBA (0.20 and 1.00 mg/L). Due to the limited availability of seeds, each treatment consisted of 6 culture bottles, with 5 seeds per bottle, and 3 replicates per treatment (Table 2). Seed germination rates were recorded over a period of 15-50 d, and seedling growth characteristics were monitored to identify the optimal germination medium.

Preliminary screening of seedling elongation and strengthening medium: Seedlings grown for 42 days on the optimal germination medium, with 3-4 true leaves and exhibiting vigorous growth, were selected for subculture onto seedling-strengthening media. Murashige and Skoog (MS) basal medium was used as the foundation, with different combinations of PGRs designed as follows: NAA (0.20, 0.50 mg/L)+GA_3 (0.10, 0.30, 0.50 mg/L), 6-BA (0.20, 0.50, 1.00 mg/L), IBA (0.20, 0.60, 1.00 mg/L). A total of 9 treatment combinations were designed (Table 3). For each treatment, five culture bottles were established, with one seedling per bottle, in three biological replicates. After 40 days of culture, the average number of shoots, plant height, root number, and root length of regenerated seedlings were recorded and analyzed to determine the optimal medium for seedling growth and development.

Screening of Subculture Medium: Based on the preliminary screening of seedling-strengthening media and identification of the optimal PGRs combination, a refined subculture medium was developed to further enhance the growth of *S. chinensis* seedlings. The medium formulations included combinations of NAA (0.50, 1.00 mg/L), GA_3 (0.20, 2.00 mg/L), and IBA (0.20, 0.50, 1.00 mg/L), resulting in four treatments (Table 4). Seedlings that had been cultured for 40 days and exhibited healthy growth on the primary medium were transferred to the new subculture media. MS basal medium was used as the foundation. Each treatment consisted of 5 bottles, each containing a single seedling, with three biological replicates per treatment. After 40 days of cultivation number of leaves, plant height, root number, and root length were recorded and analyzed to determine the optimal subculture medium for seedling development.

Acclimatization and transplantation: To facilitate successful adaptation and further propagation of plantlets in natural conditions following in vitro culture, a two-phase acclimatization and transplantation protocol was established. First, plantlets were transferred to sterile soil for 10 days of acclimatization. A substrate mixture composed of decomposed leaf litter and natural soil at a 1:2 ratio was prepared. Each 450 mL glass culture bottle was filled with 100 g of the soil mixture and supplemented with 35 mL of tap water. The substrate was autoclaved at 121°C for 100 min and cooled to room temperature before use. Healthy subcultured plantlets with heights ranging from 3 to 5 cm were transplanted into sterile soil and placed in a controlled greenhouse environment (25°C, 1500–2000 lx light intensity, 12 h light/12 h dark photoperiod). Fifteen days later, the culture bottle caps were gradually opened for acclimatization. During days 1-5, caps were opened by 1 cm daily to prevent abrupt moisture loss. From days 6-9, caps were completely removed to allow adaptation to the ambient environment, with misting applied three times daily (morning, noon, and evening). Once seedlings adapted to ambient temperature, they were transplanted into natural soil. For the first 5 days post-transplantation, direct sunlight exposure was avoided, and seedlings were misted twice daily (morning and evening). Subsequently, watering was performed once every 2 days. Seedling survival rate and growth status were recorded at 40 days post-transplantation. The experiment consisted of three parallel groups, with 10 bottles per group.

Table 1. Seed sterilization results.

Experimental group	Sterilization duration of 75% ethanol (s)	Sterilization duration of 0.1% $HgCl_2$ (min)	Contamination rate (%)	Germination rate (%)
A1	30	8	17.04 ± 7.40a	16.29 ± 5.29b
A2	30	10	5.56 ± 3.34b	37.41 ± 5.79a
A3	30	12	0.74 ± 1.28b	32.34 ± 9.52a

Note: Different lowercase letters indicate statistically significant differences among groups at $p<0.05$

Table 2. Effects of pretreatment and GA_3 on seed germination and growth.

Experimental group	Seed pretreatment	GA_3 (mg/L)	6-BA (mg/L)	IBA (mg/L)	Germination rate (%)	Growth vigor
CK	-	-	-	-	12.22 ± 5.09f	Two leaves, slender roots
B1	0.50 mg/L GA_3 soaking for 0.5 h	0.50	-	-	16.30 ± 0.64ef	Four leaves, slender roots
B2	0.50 mg/L GA_3 soaking for 2.0 h	0.50	-	-	17.78 ± 1.92def	Two to three leaves, slender roots
B3	1.00 mg/L GA_3 soaking for 0.5 h	0.50	-	-	38.89 ± 3.85c	Two leaves, slender roots
B4	1.00 mg/L GA_3 soaking for 2.0 h	0.50	-	-	40.00 ± 3.33c	Two to three leaves, slender roots
B5	1.00 mg/L GA_3 soaking for 2.0 h	1.00	-	-	22.22 ± 3.85de	ale-green leaves with slender roots
B6	1.00 mg/L GA_3 soaking for 2.0 h	-	0.50	-	22.22 ± 1.92de	Thickened leaves with absent root system
B7	1.00 mg/L GA_3 soaking for 2.0 h	-	1.00	-	23.33 ± 3.34d	Thickened, chlorotic leaves lacking roots
B8	1.00 mg/L GA_3 soaking for 2.0 h	-	-	0.20	52.22 ± 1.92a	Hypertrophied leaves with elongated slender roots
B9	1.00 mg/L GA_3 soaking for 2.0 h	-	-	1.00	45.55 ± 3.85b	Hypertrophic leaves with slender, elongated roots

Note: Lowercase letters have the same meanings as those shown in Table 1. ($p<0.05$). CK (control), B1- B9: Effects of pretreatment methods and plant growth regulators (PGRs) on seed germination

Table 3. Screening of seedling-strengthening culture media.

Experimental group	Hormone combination	Leaf number (leaves)	Bud umber (buds)	Plant eight (cm)	Root number (piece)	Root length (cm)
C1	0.50 mg/L NAA+0.10 mg/L GA_3	10.33 ± 1.53a	1.00 ± 0.00c	2.4 ± 0.17b	10.00 ± 1.00e	1.52 ± 0.13a
C2	0.50 mg/L NAA+0.30 mg/L GA_3	5.33 ± 1.53d	1.00 ± 0.00c	1.83 ± 0.15c	18.33 ± 1.15d	0.52 ± 0.08d
C3	0.50 mg/L NAA+0.20 mg/L 6-BA	5.33 ± 0.58d	2.33 ± 0.58c	1.97 ± 0.06c	13.33 ± 3.06e	0.47 ± 0.06d
C4	0.50 mg/L NAA+1.00 mg/L 6-BA	4.67 ± 0.58de	1.67 ± 0.58c	1.33 ± 0.15d	0.00 ± 0.00f	0.00 ± 0.00e
C5	0.20 mg/L NAA+0.50 mg/L GA_3+0.50 mg/L 6-BA	2.67 ± 0.58e	7.67 ± 3.06b	1.87 ± 0.15c	0.00 ± 0.00f	0.00 ± 0.00e
C6	0.20 mg/L NAA+0.50 mg/L GA_3+1.00 mg/L 6-BA	4.67 ± 1.15de	34.00 ± 2.65a	1.00 ± 0.00e	0.00 ± 0.00f	0.00 ± 0.00e
C7	0.20 mg/L NAA+0.50 mg/L GA_3+0.20 mg/L IBA	5.67 ± 0.58cd	1.00 ± 0.00c	2.37 ± 0.12b	30.33 ± 2.52c	1.00 ± 0.05c
C8	0.2 mg/L NAA+0.50 mg/L GA_3+0.60 mg/L IBA	7.67 ± 2.08bc	1.00 ± 0.00c	2.27 ± 0.15b	35.67 ± 2.52b	1.40 ± 0.13a
C9	0.2 mg/L NAA+0.50 mg/L GA_3+1.00 mg/L IBA	8.67 ± 1.53ab	1.00 ± 0.00c	3.43 ± 0.32a	42.00 ± 7.21a	1.17 ± 0.03b

Note: Lowercase letters have the same meanings as those shown in Table 1. ($p<0.05$)

Table 4. Screening of subculture media.

Experimental group	NAA mg/L	GA_3 mg/L	IBA mg/L	Leaf number (leaves)	Plant height (cm)	Root number (piece)	Root length(cm)
D1	0.50	2.00	0.20	7.33±0.58	4.13±0.12a	59.00±3.61	1.32±0.16a
D2	0.50	2.00	0.50	5.67±0.58	3.17±0.15b	51.33±6.11	1.05±0.05ab
D3	0.50	0.20	1.00	7.33±1.53	2.67±0.29c	59.67±6.03	0.72±0.26c
D4	1.00	0.20	1.00	7.67±1.15	2.23±0.21d	54.00±14.93	0.85±0.09bc

Note: Lowercase letters have the same meanings as those shown in Table 1. ($p<0.05$)

Data collection and statistical analysis: Experimental data were recorded and organized using Microsoft Excel, and statistical analyses were performed using SPSS version 26.0. All results were expressed as mean ± standard error (SE). Differences among treatment groups were assessed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test to determine significant differences between means at a confidence level of $p<0.05$. The contamination rate and germination rate were calculated as follows:

$$\text{Contamination rate (\%)} = \frac{\text{Number of contaminated explants}}{\text{Total number of explants per treatment}} \times 100$$

$$\text{Germination rate (\%)} = \frac{\text{Number of germinated explants}}{\text{Total number of explants per treatment}} \times 100$$

Results and Discussion

Effects of different disinfection treatments on explants: In plant tissue culture, surface sterilization of explants is one of the most critical steps for preventing microbial contamination and ensuring successful in vitro propagation. According to the experimental data, *S. chinensis* seeds demonstrated highly effective sterilization with relatively simple disinfection procedures. The most effective method involved soaking the seeds in 75% ethanol with shaking for 30 s, followed by shaking in 0.1% mercuric chloride solution for 10 min. This treatment resulted in a low contamination rate of 5.56% and a germination rate of 37.41%.

The disinfection efficiency varied with treatment time, as shown in Table 1. With increasing exposure time to 0.1% $HgCl_2$, the contamination rate of seeds was decreased significantly. A1 (8 min) had a contamination rate of 17.04%, while A3 (12 min) achieved the lowest contamination rate of 0.74%. However, seed germination was also affected by sterilization duration. A1 had a germination rate of 16.29%, whereas A2 (10 min) and A3 (12 min) showed increased germination rates of 37.41% and 32.34%, respectively. The highest germination rate was observed in A2. These results suggest that an optimal disinfection duration (10 min) is crucial to effectively control microbial contamination while maintaining seed viability. Excessive sterilization time, although reducing contamination, may damage seed tissue and impair

germination. Therefore, the most suitable seed sterilization protocol for *S. chinensis* was determined to be immersion in 75% ethanol with shaking for 30 s, followed by shaking in 0.1% $HgCl_2$ for 10 min.

Effects of GA_3 pretreatment and plant growth regulators on seed germination: In plant tissue culture, seed germination rate and seedling vigor are critical indicators of successful culture establishment. In this study, the effects of different soaking durations and concentrations of GA_3, as well as various PGRs, on seed germination were evaluated (Table 2; Fig. 1). The results showed that seeds without any pretreatment (CK) began to germinate after 30 days, whereas seeds subjected to different treatments initiated germination within 15-20 d. By day 50, significant differences were observed in seedling performance among the treatments. In the control group, the germination rate was only 12.22%, although the morphology appeared normal, the seedlings were short with narrow leaves. In contrast, the GA_3-treated groups (Table 2- B1 & B4) exhibited earlier germination (15-20 d), and germination rates were increased with both higher GA_3 concentrations and longer soaking times. The B4 (1.00 mg/L GA_3 for 2 h) achieved the highest germination rate of 40.00%, producing healthy, though relatively short, seedlings. The B2 (0.50 mg/L GA_3 immersion for 2 h) exhibited a germination rate of 17.78%, with resultant seedlings developed 4-6 dark green true leaves and slender, pale green roots. Overall, B4 exhibited the most effective germination promotion, indicating that GA_3 pretreatment significantly enhanced seed germination and shortened germination time in *S. chinensis*. Based on the optimal GA_3 pretreatment (1.00 mg/L for 2 h), additional experiments were conducted by supplementing the medium with various PGRs (GA_3, 6-BA, and IBA). It was found that further increasing GA_3 concentration in the medium to 1.00 mg/L (Table 2 B5) reduced germination rate to 22.22%, and seedlings displayed abnormal chlorotic leaves with signs of vitrification near the medium surface (Fig. 1 a5).When 6-BA was added alone (Table 2-B6 & B7), germination rates decreased to 22.22% and 23.33%, respectively. These seedlings exhibited thickened leaves but failed to develop roots, suggesting that while 6-BA supports leaf development, it may inhibit root formation, leading to rootless seedlings. In contrast, treatment with IBA alone (Table 2- B8 & B9) significantly improved germination rates to 52.22% and 45.55%, respectively. These seedlings exhibited robust growth, with expanded leaves and well-developed root systems (Fig. 1- a8 & a9). Therefore, IBA plays a key role in promoting both seed germination and root development, resulting in vigorous and healthy seedlings. Taken together, these results indicate that the optimal condition for *S. chinensis* seed germination is soaking seeds in 1.00 mg/L GA_3 for 2 hours, followed by culture on a medium supplemented with 0.20 mg/L IBA, as this combination maximizes both germination rate and seedling quality.

Screening of optimal seedling growth medium: The application and ratio of PGRs had a significant influence on the growth of *S. chinensis* seedlings (Table 3; Fig. 2). Experimental results showed that supplementing the basal MS medium with a low concentration of GA_3 (0.10 mg/L) promoted both root and shoot growth. However, higher concentrations of GA_3 (0.30 mg/L) inhibited leaf and root development and resulted in stunted, thickened roots (Fig. 2-b1 & b2).When the basal medium was supplemented with 0.50 mg/L NAA combined with different concentrations of 6-BA (0.20 and 1.00 mg/L), it was observed that increasing 6-BA concentrations enhanced shoot proliferation, while overall seedling vigor declined. At the lower 6-BA concentration (0.20 mg/L), seedlings exhibited better overall growth with a greater number of leaves and an average plant height of 1.97 cm, though significant root browning was noted. In contrast, at the higher 6-BA concentration (1.00 mg/L), seedling growth was suppressed and root development was completely inhibited (Fig. 2- b3 & b4).When the basal medium was supplemented with 0.20 mg/L NAA + 0.50 mg/L GA_3 and different concentrations of 6-BA (0.50 and 1.00 mg/L), shoot proliferation capacity increased with increasing 6-BA concentration, reaching 7.67% and 34.00%, respectively. However, root number, leaf number, and leaf quality declined correspondingly (Fig. 2- b5 & b6). These results suggest that a medium containing 0.50 mg/L NAA + 0.20 mg/L 6-BA is suitable for shoot proliferation. Furthermore, when 0.20 mg/L NAA + 0.50 mg/L GA_3 was combined with varying concentrations of IBA (0.20, 0.60, and 1.00 mg/L), seedling growth improved with increasing IBA concentration. At 1.00 mg/L IBA (Fig. 2 b9), seedlings exhibited optimal performance with an average of 8.67 leaves, a plant height of 3.43 cm, dense root formation (42 roots), and a root length of 1.17 cm. This indicates that IBA effectively promotes root development, and that higher IBA concentrations enhance root number, leaf number, and plant height.

Based on these findings, the optimal shoot proliferation medium is 0.50 mg/L NAA + 0.20 mg/L 6-BA, while the optimal seedling strengthening medium is 0.20 mg/L NAA + 0.50 mg/L GA_3 + 1.00 mg/L IBA.

Screening of Subculture Medium: The experimental results demonstrated that different subculture media had significant effects on the growth performance of *S. chinensis* seedlings (Table 4; Fig. 3). After 55 days of culture, notable differences in seedling development were observed across the treatments.

Under low GA_3 concentration (0.20 mg/L), as the NAA concentration was increased (treatments D3 and D4), plant height was decreased progressively (2.67 cm and 2.23 cm, respectively), and root length was also reduced (0.72 cm and 0.85 cm). This suggests that higher concentrations of NAA (1.00 mg/L) may inhibit root elongation and result in thickened and shortened roots. Although the number of leaves was relatively higher (7.33 and 7.67 leaves), the overall growth was stunted, and plant morphology was compact (Fig. 3-c3 & c4). These findings indicate that excessive NAA may lead to root thickening, browning, and suppressed shoot elongation, which is detrimental to healthy seedling development. In contrast, under high GA_3 concentration (2.00 mg/L), increasing the IBA concentration (Table 4-D1 & D2) resulted in elongated but sparse roots, which changed color from pale yellow to light green. However, the number of leaves was decreased slightly, along with slightly reduced plant height. Among all treatments, D1 exhibited the most favorable results, with broad, dark green leaves, a plant height of approximately 4.13 cm, densely developed roots, of 1.32 cm length. These results suggest that a moderately elevated GA_3 concentration promotes overall seedling growth. Furthermore, a lower concentration of IBA is beneficial for shoot elongation and root development, while excessive IBA may reduce plant height and leaf number, thus affecting plant vigor. Therefore, the optimal subculture medium was determined to be: 0.50 mg/L NAA + 2.00 mg/L GA_3 + 0.20 mg/L IBA.

Acclimatization and transplantation: After 45 days of in vitro culture, the *S. chinensis* plantlets were transferred to sterile soil and maintained in a greenhouse for one week. During this period, the leaf color gradually changed from light green to dark green, and leaf expansion was observed, indicating the plantlets' adaptation to external environmental conditions. Following one week in sterile soil, gradual acclimatization was carried out by slowly opening the culture vessel lids. Plants were misted three times a day (morning, noon, and evening) to minimize transpiration and prevent excessive water loss. After 5 days of acclimatization, the plantlets showed healthy growth, with over 90% leaf expansion, darker leaf coloration, well-developed root systems, and emergence of new lateral shoots in some individuals. The survival rate reached 83.33%, demonstrating strong adaptability. Subsequently, the acclimatized plantlets were transplanted into natural soil. After 10 days of cultivation under ambient conditions, the seedlings continued to grow normally, and the survival rate increased to 88% (Fig. 4).

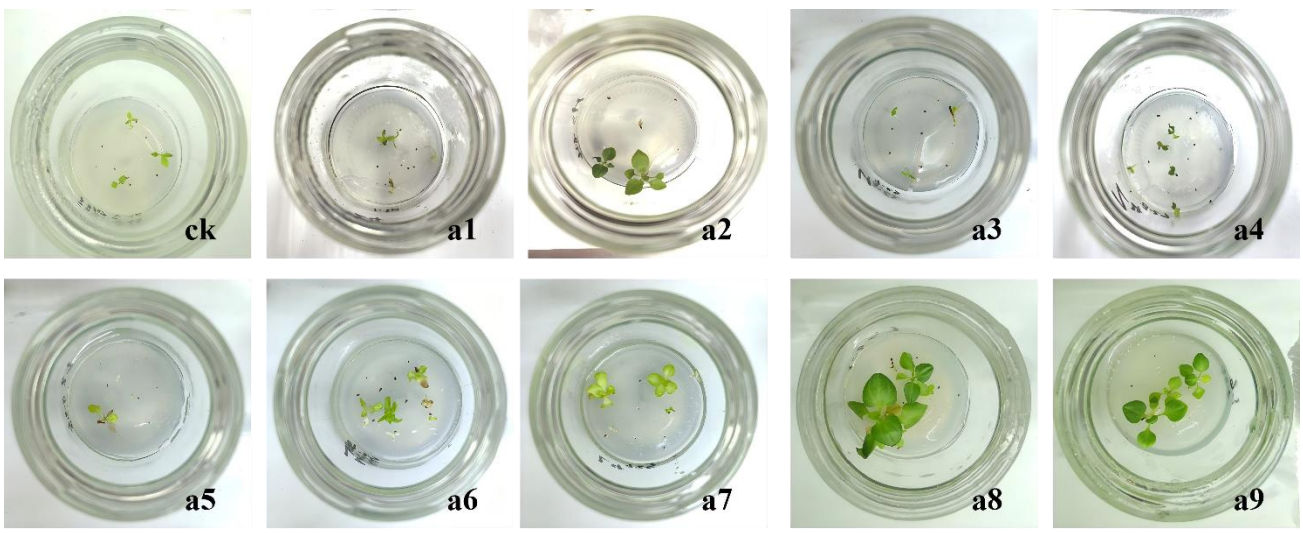

Fig. 1. Effects of different pretreatments and plant growth regulators on seed germination.
CK: Untreated control group; a1-a4: Germination status of seeds soaked in 0.50 or 1.00 mg/L GA_3 for varying durations; a4-a9: Under the precondition of 1.00 mg/L GA_3 soaking treatment, germination rates and seedling growth performance on media supplemented with varying concentrations of GA_3 (0.50, 1.00 mg/L), 6-BA (0.50, 1.00 mg/L), and IBA (0.20, 1.00 mg/L) PGRs.

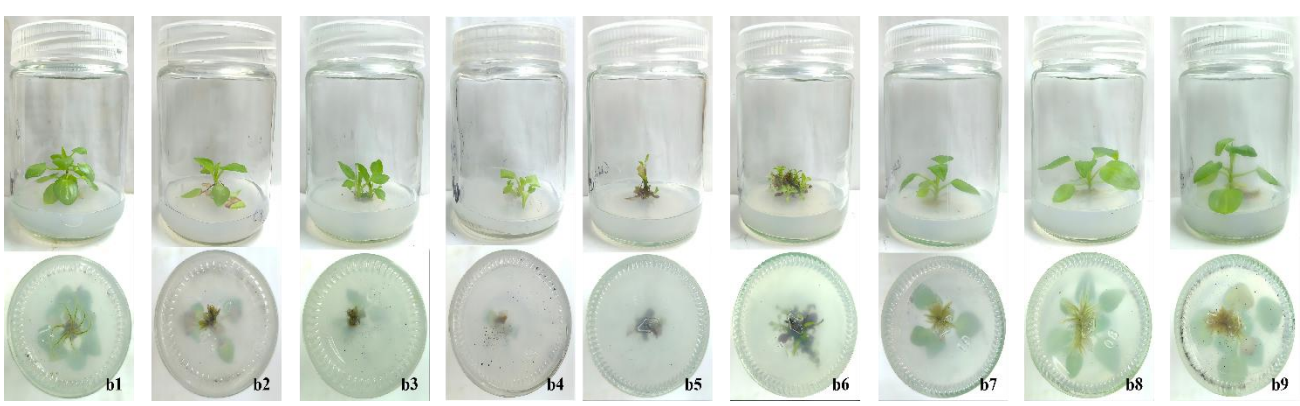

Fig. 2. Screening of seedling-strengthening culture media.
b1-b9 represent the effects of different hormone combinations on seedling growth. b1-b2: 0.50 mg/L NAA + GA_3 (0.10, 0.30 mg/L), b3-b4: 0.50 mg/ L NAA + 6-BA (0.20, 1.00 mg/L), b5-b6: 0.20 mg/L NAA + 0.50 mg/L GA_3 + 6-BA (0.50, 1.00 mg/L), b7-b9: 0.20 mg/L NAA + 0.50 mg/L GA_3 + IBA (0.20, 0.60, 1.00 mg/L.)

Discussion

Surface sterilization prior to plant tissue culture is a critical initial step that significantly affects explant survival and subsequent in vitro propagation. Residual disinfectants on explants can cause tissue damage, leading to an increased rate of browning after sterilization (Rezaei *et al*., 2023). Therefore, identifying the optimal sterilization protocol is essential for the success of tissue culture experiments. Seeds are generally the plant part with the lowest endophytic microbial load (Aline *et al*., 2010; Li *et al*., 2024), which greatly reduces microbial contamination during tissue culture (Brieuc *et al*., 2025; Kandel *et al*., 2017). In this study, *S. chinensis* seeds, protected by a hard seed coat similar to that of *Houttuynia cordata* (Ye *et al*., 2022), were shown to be well-suited for sterilization, with minimal detrimental effects from disinfectants. Previous studies have demonstrated that GA_3, as a plant growth regulator, is effective in breaking seed dormancy and promoting germination (Zhang *et al*., 2024). In this study, soaking *S. chinensis* seeds in GA_3 significantly broke dormancy and accelerated the onset of germination. Furthermore, the addition of IBA to the culture medium further enhanced germination rates and enhanced seedling establishment rate. These results are consistent with findings in *Sophora moorcroftiana* tissue culture systems (Bramhanapalli *et al*., 2016).

During root induction and seedling strengthening stages of *S. chinensis*, the combination of GA_3 and NAA exhibited excellent performance in promoting root development and seedling growth. The addition of IBA further enhanced seedling vigor, resulting in taller plants with more developed root systems. However, when the concentration of NAA was too high, root thickening and shortening occurred, and root browning was observed, which also inhibited seedling height-adversely affecting overall plant health. These observations aligned with the findings reported by Wang *et al.* (Wang *et al*., 2025). The study also indicated that the differentiation of adventitious buds was primarily regulated by 6-BA, a synthetic cytokinin. At optimal concentrations, 6-BA effectively induced bud proliferation. However, excessive concentrations led to the over-proliferation of shoots and inhibited root development, which is detrimental to seedling establishment. These results corroborate the findings reported by Nayan Nimavat *et al* (Nimavat & Parikh, 2024).

It has been reported that the addition of IBA and NAA to the regeneration medium of *Artemisia annua* effectively induces adventitious root formation (Soni *et al*., 2023). Consistently, the present study demonstrates that a specific ratio of NAA and IBA in the subculture medium significantly promotes seed germination in *S. chinensis*. Furthermore, the incorporation of GA_3 into the medium enhances root elongation and increases plant height, ultimately resulting in more vigorous seedling growth.

S. chinensis is a valuable medicinal plant and possesses ornamental potential due to its distinctive foliage and floral morphology. Establishing an efficient tissue culture and rapid propagation system is greatly significant with increasing market demand for *S. chinensis* quality and yield. In this study, selecting seed as explants, a high-efficiency rapid propagation protocol was successfully developed under aseptic conditions through the optimization of PGRs combinations. Optimal culture conditions of seed germination, seedling development, and subculture propagation were determined. The system establishment effectively shortens the cultivation cycle, overcomes seasonal growth constraints, and enables large-scale propagation to meet industrial demand. it provides a scientific basis and technical support for genetic breeding, germplasm conservation, and the generation of sterile plantlets for experimental purposes.

Future research will focus on optimizing the tissue culture conditions for *S. chinensis*, shortening the seedling development period, enhancing seedling robustness, and elucidating the symbiotic mechanisms between *S. chinensis* and its endophytic microbiota.

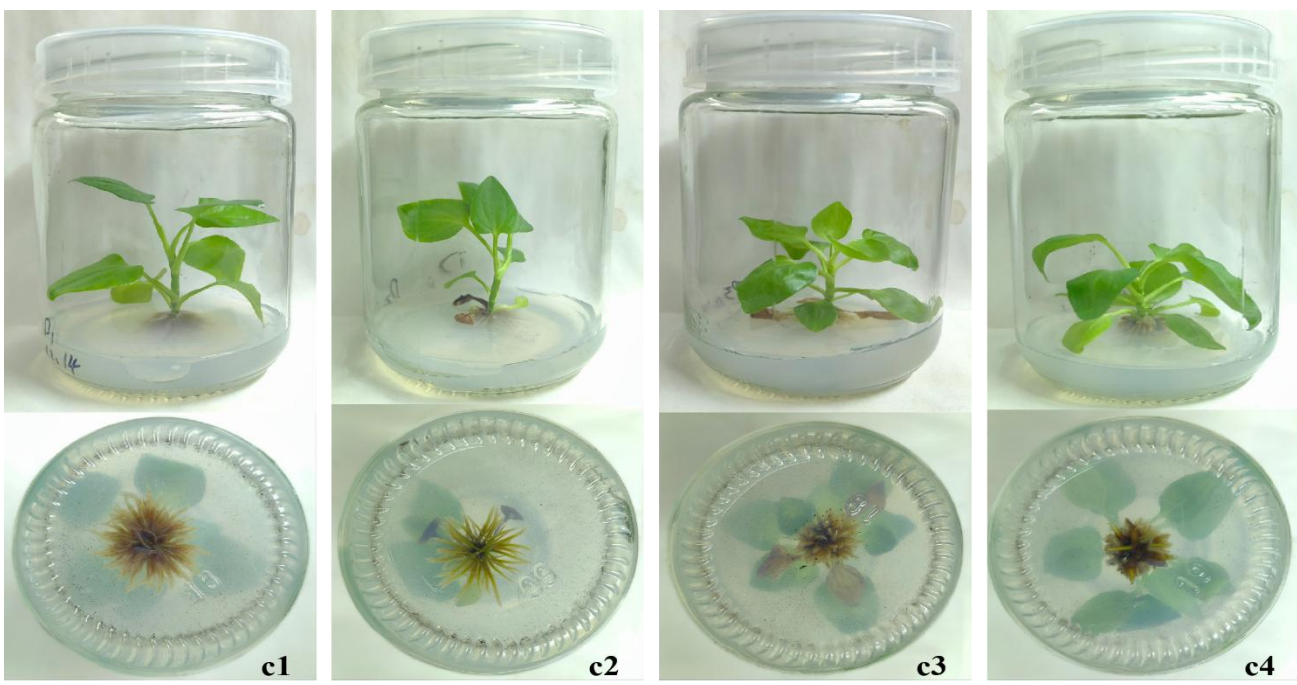

Fig. 3. Effects of different hormone combinations in subculture media on seedling growth.
c1: 0.50 mg/L NAA + 2.00 mg/L GA_3 + 0.20 mg/L IBA; c2: 0.50 mg/L NAA + 2.00 mg/L GA_3 + 0.50 mg/L IBA; c3: 0.50 mg/L NAA + 0.20 mg/L GA_3 + 1.00 mg/L IBA; c4: 1.00 mg/L NAA + 0.20 mg/L GA_3 + 1.00 mg/L IBA.

Fig. 4. Results of acclimatization in natural soil.

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