

Synergistic regulation of plant growth regulators on somatic embryogenesis and optimization of rejuvenation culture in *Sinocrassula indica* (Decne.) Berger

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Abstract

To address the industrial bottlenecks of *Sinocrassula indica* (Decne.) Berger, including prolonged propagation cycles, poor genetic stability, and low acclimatization survival rates, this study established an efficient *in vitro* rapid propagation system. An L_9 (3^4) orthogonal design was employed to optimize the hormonal combinations of 6-BA (0.1–1.0 mg/L), NAA (0.05–0.5 mg/L), and KT (0.5–2.0 mg/L), integrated with paclobutrazol (PP333)-mediated rejuvenation culture to enhance plantlet quality. Results demonstrated that the optimal hormonal combination (0.1 mg/L 6-BA + 0.3 mg/L NAA + 1.0 mg/L KT) achieved a proliferation coefficient of 93.5 ($P < 0.05$). Furthermore, 0.01 mg/L PP333 treatment significantly improved acclimatization survival rates from 54.83–100%, increased stem diameter by 48% ($P < 0.01$), and preserved the characteristic rosette morphology. Notably, this system maintained genetic fidelity during long-term cultivation. The established protocol provides a robust solution for germplasm conservation and industrial-scale propagation of Crassulaceae plants, offering dual benefits for ecological preservation and commercial horticultural applications.

Key message

A "hormone-induced embryogenesis-paclobutrazol rejuvenation-acclimatization" system resolves *Sinocrassula indica* mass propagation and establishes a Crassulaceae micropropagation paradigm.

Introduction

The concept of succulent plants was first proposed by Swiss botanist Jean Bauhin in 1619, referring to plants with highly succulent vegetative organs (roots, stems, or leaves) and specialized water-storage functions (Mao 2014). In horticultural science, these plants are commonly termed "succulents" or "fleshy plants," characterized by thickened, moisture-rich tissues and diverse life cycles ranging from annual to perennial species (Hu and Shen 2013; Xie and Xu 1999). Succulents hold significant value in landscaping, ecological restoration (e.g., green roofs), and environmental engineering (Wang et al. 2009; Zhou et al. 2008). Beyond ornamental applications, certain species exhibit edible and medicinal properties, such as the culinary cactus *Opuntia ficus-indica* (L.) Mill. 'Milpa Alta' and *Aloe vera* (L.) Burm. f., renowned for antitumor and burn-healing effects (Zhao et al. 2018; Song et al. 1999; Wan et al. 2013). Recently, their morphological diversity, vibrant coloration, and low maintenance have propelled succulents to dominate the miniature horticulture market (Yasin et al. 2012; Zheng 2019). The global succulent industry has surpassed RMB 20.9 billion since 2019, with sustained growth (Feng and Xie 2022). Rising consumer demand has intensified efforts in introducing premium varieties and advancing industrial-scale propagation technologies, marking a new trend in sector development (Ke and Tang 2016; Zhang et al. 2019).

Sinocrassula indica (Decne.) Berger, a perennial herbaceous species in the Crassulaceae family, is naturally distributed across the Mexican valley region (Li 2019), Himalayan foothills (Nepal, India), and continuous habitats spanning China's Hengduan Mountains to Qinling Mountains, covering nine

provinces including Tibet, Yunnan, and Gansu (Flora of China Editorial Committee 1984; Kunming Institute of Botany 1997; Flora of China Editorial Committee 2001). Its typical habitats include gravel-rich riparian zones, shallow weathered rock crevices, and arid valley slopes, with elevational ranges of 1,300–3,300 m in Yunnan's distribution core (e.g., Kunming and Lijiang plateau basins) (Kunming Institute of Botany 1997). As a model succulent, *S. indica* exhibits morphological adaptations characterized by: 1) rosette phyllotaxis, 2) thickened cuticular layers, and 3) Crassulacean Acid Metabolism (CAM), conferring extreme thermotolerance (Ha Tran et al. 2024). Beyond ornamental merits (compact rosettes and glaucous wax-coated leaves), its phytochemical profile—rich in flavonoid glycosides and triterpenoids—imparts notable anti-inflammatory and tissue-repair activities. Ethnobotanical records document its use in Tibetan medicine for treating dysentery, hematochezia, and metrorrhagia, and in Tujia ethnomedicine for trauma, hemorrhage, and venomous bites (Yoshikawa et al. 2007; Editorial Committee of Chinese Ethnic Medicine 2005).

The Crassulaceae family, a highly representative group within succulent plants, occupies a pivotal position in plant taxonomy. This family encompasses 35 natural genera, 23 artificial hybrid genera, and over 1,600 species. Crassulaceae plants distinguish themselves through diverse morphological forms, remarkable phenotypic plasticity, and broad ecological adaptability. Their morphological spectrum ranges from compact and charming varieties to peculiar and distinctive forms, demonstrating extraordinary ornamental appeal. These exceptional characteristics have established Crassulaceae as an indispensable core commodity group in modern horticulture, garnering widespread enthusiasm among gardening enthusiasts (Huang et al. 2016; He and He 2019; Eggli 2012; Hassan et al. 2021). Furthermore, their unique miniaturization characteristics and superior storage tolerance have facilitated extensive applications in urban vertical greening, achieving a utilization rate of 37.6% (Huang et al. 2016; Sun et al. 2016). However, current commercial cultivars predominantly consist of artificial hybrids and somaclonal variants (87.7%), with native species constituting merely 12.3%. The accelerated cultivar replacement cycle (9–14 months) imposes stringent requirements on propagation technologies (Yan et al. 2020). Conventional vegetative propagation methods, including leaf cuttings and division, exhibit significant limitations: prolonged propagation cycles (8–12 weeks), low multiplication coefficients (< 5.0), and elevated genetic variation rates (18.7%), substantially hindering industrial-scale production (Liu et al. 2016). Since Haberlandt's pioneering work in 1902, plant tissue culture technology has achieved substantial progress. Through precise regulation of explant dedifferentiation, optimization of phytohormone ratios, and controlled environmental parameters, year-round mass propagation of succulents has been successfully realized (Li et al. 2022; Wang et al. 2017; Tao et al. 2018; Min 2016). Research demonstrates that tissue culture systems achieve 7.3-fold higher multiplication coefficients than conventional methods ($p < 0.01$), while SSR marker analysis confirms 98.2% genetic stability, significantly improving seedling export compliance rates (Hu 2014; Dui 2016; Wang et al. 2022).

This study focuses on *S. indica* to address critical challenges in its industrial-scale production, including low natural propagation efficiency, genetic instability in traditional vegetative propagation, and prolonged production cycles. By systematically investigating somatic embryogenesis mechanisms and regulatory patterns of plant growth regulators, we aim to establish a highly efficient and stable artificial propagation

system. This approach not only overcomes the proliferation limitations of conventional leaf-cutting and division methods but also enables standardized, large-scale seedling production to meet the urgent demand for high-quality plantlets in the miniature horticulture market. Simultaneously, it provides a consistent raw material source for sustainable exploitation of medicinal bioactive compounds (flavonoid glycosides and terpenoids) in *S. indica*, facilitating its transition from an ornamental plant to a medicinal resource. Furthermore, optimized *in vitro* conservation techniques will alleviate pressure on wild germplasm collection and supply stress-resistant germplasm reserves for vegetation restoration in ecologically fragile regions. The findings also offer technical references for artificial propagation of other Crassulaceae succulents, promoting the healthy and sustainable development of the entire succulent plant industry.

Materials and methods

Plant materials

Five potted *S. indica* plants (10 individuals) were provided by Ms. Meirong Wang from Yunnan Suifeng Jiayuan Agricultural Technology Co., Ltd. These plants were transplanted with pots to the experimental greenhouse of the Research Center for Medicinal Plant Germplasm Improvement and Engineering Propagation, Yunnan University of Chinese Medicine (25°24'36"N, 103°22'10"E; Altitude: 1878.3 m). The species identification was authenticated by Professor Yu Hong from Yunnan University.

Culture media and chemical reagents

The basal medium used was Murashige and Skoog (MS) medium. Plant growth regulators (PGRs), including 6-benzylaminopurine (6-BA), kinetin (KT), 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and paclobutrazol (PP333), as well as sucrose (analytical grade), agar, mercuric chloride (HgCl_2), and sodium hydroxide (NaOH), were purchased from Beijing Dingguo Biotechnology Co., Ltd. (Beijing, China). Unless otherwise specified, all concentrations refer to mass concentration. In the MS medium, sucrose was added at 3% (w/v), agar at 0.47% (w/v), and the pH was adjusted to 5.6–5.8. The medium was sterilized in an autoclave at 122°C for 22 min and stored for subsequent use.

Establishment of aseptic system and initiation culture

Leaf explants (approximately 2.0 cm in length) were rinsed under running water for 20 min to remove surface debris, followed by surface sterilization in a laminar flow hood: sequential immersion in 75% (v/v) ethanol for 30 s, 0.1% HgCl_2 solution for 10 min, and three rinses with sterile water (≥ 3 min per rinse). Sterilized materials were blotted dry with sterile filter paper, and approximately 0.3 cm of damaged tissue at the leaf base was excised using a sterile scalpel. Explants were vertically oriented according to their physiological polarity and inoculated onto MS medium supplemented with 1.0 mg/L 6-

BA (three explants per jar, with five jars inoculated). After obtaining the first batch of aseptic seedlings through initiation culture, their leaves were repeatedly transferred to fresh medium for multiple subcultures to generate sufficient homogenized material.

Anatomical and histological observations

Morphological structures of leaf explants at different developmental stages were examined under a stereomicroscope. Subsequently, paraffin sections were prepared using the method described by Gnasekaran et al. Gnasekaran (2016), stained with 0.05% toluidine blue solution, and observed under an optical microscope to analyze early-stage tissue organization.

Single-Factor PGRs experiment

Five plant growth regulators (PGRs) were tested at varying concentration gradients: 6-BA (0.1, 0.5, 1.0, 2.0, 3.0 mg/L), NAA (0.1, 0.5, 1.0, 2.0, 3.0 mg/L), KT (0.5, 1.0, 2.0, 3.0 mg/L), IBA (0.1, 0.5, 1.0, 2.0 mg/L), and 2,4-D (0.1, 0.5, 1.0, 2.0 mg/L). Somatic clumps obtained from initiation culture were cut into 1.0–1.5 cm² fragments and transferred to the respective media, with 10 explants per jar and five jars per treatment group. After 50 d, leaf regeneration was observed and recorded, and the proliferation coefficient was calculated.

L₉(3⁴) Orthogonal experiment

Table 1
Orthogonal experimental design of proliferation culture L₉(3⁴).

Levels	Factors(mg/L)		
	6-BA (A)	NAA (B)	KT (C)
1	0.1	0.1	0.5
2	0.3	0.3	0.8
3	0.5	0.5	1.0

Based on the single-factor experiment results, three factors—6-BA (A), NAA (B), and KT (C)—were selected for an L₉(3⁴) orthogonal design (Table 1). Seedling-derived leaf explants from the single-factor experiment were used as materials, with five jars per group (10 explants per jar) and three replicates. After 60 d, growth performance was observed, the proliferation coefficient was calculated, and the optimal treatment was identified through statistical analysis.

Rejuvenation culture

In vitro seedlings obtained from the orthogonal experiment exhibited dense growth due to limited space, resulting in slender stems and weak plants. At this stage, acclimatization yielded a survival rate of only 54.83%. To address this, a rejuvenation culture experiment was conducted. Stem tips (approximately 1.0 cm in length with 5–8 leaves) were cultured on unmodified MS medium supplemented with PP333 at varying concentrations (0.01, 0.1, 0.5, 1.0, 2.0, 3.0 mg/L), with five jars per group (10 explants per jar). Growth parameters were observed and recorded after 40 d. To validate reliability and stability, the optimal PP333 concentration identified was further tested in 10 independent replicates to assess its consistent effects on seedling growth.

Acclimatization and transplantation

After 40 d of rejuvenation culture, *in-vitro* seedlings reaching 2–3 cm in height with robust roots and fully expanded leaves were deemed suitable for transplantation. Seedling-containing jars were exposed to natural light for 7 d for pre-acclimatization. Lids were then removed, and plants were gently extracted, with residual medium carefully rinsed from roots under running water to avoid root damage. Seedlings were transplanted into a sterilized substrate (peat moss : perlite = 3:1, v/v), pre-treated with 0.2% carbendazim, and placed in foam boxes (inner dimensions: 27 × 18 × 17 cm) with a substrate depth of 3–5 cm. After transplantation, boxes were covered with plastic film to maintain stable conditions: temperature at $25 \pm 2^{\circ}\text{C}$ and humidity at 50–70%. Survival rates were assessed after 70 d, and surviving seedlings were transferred to round plastic pots (10 cm diameter × 8 cm height) filled with standard humus soil.

To evaluate potential residual effects of PP333, control seedlings (untreated with PP333) underwent identical acclimatization and transplantation procedures. Comparative growth analysis between PP333-treated and untreated groups was conducted after the 70-d period to preliminarily assess PP333's long-term impact on seedling development.

Culture conditions

The culture room was maintained at a temperature of $(22 \pm 1)^{\circ}\text{C}$, with a light intensity of 1500–2000 lx and a photoperiod of 10 h/d. Contaminated cultures were promptly discarded, and experimental jars were replenished with fresh explants to maintain the required sample size.

Data analysis

Experimental data were collated and organized using Excel 2021. Results for each treatment group were expressed as mean $\pm$ standard error. Statistical analysis, including analysis of variance (ANOVA) and Duncan's test, was performed using SPSS 27.0 software to determine significant differences ($p \leq 0.05$) among treatments. Figures were formatted and edited using Photoshop 2024 and GraphPad Prism 2025.

Proliferation coefficient = Number of viable subcultures / Original number of explants

Survival rate (%) = (Number of surviving seedlings / Total transplanted seedlings) × 100

Results and analysis

Initiation culture

After being sterilized, the leaves were placed in the initiation medium for culture. After 20 days, swollen bases with pale yellow granular protrusions were observed across all leaf specimens (Fig. 1A). By day 30 of cultivation, these protrusions exhibited accelerated proliferation, changed to a light green color, and showed visible volumetric expansion (Fig. 1B). Forty days after cultivation, the protrusions maintained proliferative activity without visible bud differentiation, displaying characteristic bipolar growth patterns of somatic embryos and forming clustered structures (Fig. 1C). By day 50, the proliferation rate of clustered cultures decelerated, exhibiting green crystalline structures at the base while developing shoot primordia with white adventitious roots at the apex (Fig. 1D).

growth status after 25 days of culture(**A**); growth status after 30 days of culture(**B**); growth status after 40 days of culture(**C**); growth status after 50 days of culture(**D**). Scale = 3.0 cm.

It should be noted that not all leaf explants successfully developed into clustered cultures during the entire initiation phase. Statistical analysis revealed that approximately 50–65% of leaves ceased further development after forming basal protrusions. Although these leaves exhibited somatic embryo-like proliferation in subsequent stages, they demonstrated markedly suboptimal developmental status, with some displaying yellowing and others undergoing gradual withering. However, such anomalies occurred exclusively during the initiation culture phase and were not observed in subcultures, indicating a strong correlation with residual disinfectants.

Morphological and anatomical observations

After 5 days of cultivation in the initiation medium, leaf explants developed tightly arranged pale green granular protrusions at their bases (Fig. 2A). By day 7, these protrusions exhibited significant volumetric enlargement, intensified green coloration, and a glossy surface texture (Fig. 2B). At day 10, the number of protrusions increased with continued expansion (Fig. 2C). After 15 days, the clustered basal structures differentiated distinct morphological features, displaying vivid surface coloration and characteristics typical of cotyledonary embryo morphology (Fig. 2D). By day 20, leaf primordia emerged at the apex of the clustered structures, tightly connected to the original epidermal tissues (Fig. 2E). Following 30 days of cultivation, selected protrusions achieved complete morphogenesis of juvenile shoots (Fig. 2F).

Paraffin sections revealed 1–3 layers of tightly arranged parenchyma cells beneath the epidermis. Localized epidermal regions contained small, densely stained embryonic structures (Fig. 3A). As cultivation progressed, these structures gradually protruded to form conical protrusions, consistent with the anatomical features of somatic embryos (Fig. 3B).

Single-Factor experiment

Plant growth regulators (PGRs) exerted significantly differential regulatory effects on somatic embryo differentiation and proliferation in *S. indica* leaves (Fig. 4). Cytokinin-type regulators (6-BA, KT) induced elevated proliferation coefficients and adventitious root formation but consistently produced morphologically abnormal regenerants, including underdeveloped leaf expansion and excessive internode elongation. Specifically, 6-BA (optimal concentration: 0.1 mg/L) restricted leaf expansion in clustered shoots (Fig. 5A, B), while KT (optimal concentration: 1.0 mg/L) triggered chlorosis in regenerated tissues (Fig. 5C). In contrast, the auxin-type regulator NAA at 0.5 mg/L maintained moderate proliferation efficiency while producing regenerants with complete morphogenesis (fully expanded leaves) and stable adventitious root differentiation (Fig. 5D). Both IBA and 2,4-D treatments exhibited low proliferation efficiency, inducing plant dwarfing and severe chlorosis, respectively (Fig. 5E, F). Based on these results, 6-BA, NAA, and KT were selected as optimal PGR candidates for *S. indica* micropropagation. Effective concentration ranges were determined as 0.1–0.5 mg/L for 6-BA and NAA, and 0.5–1.0 mg/L for KT.

Somatic embryo induction and proliferation

In the orthogonal experiment, somatic embryos were induced in all leaf explants with varying efficiencies, accompanied by distinct proliferation (Table 2). Range analysis showed that the primary effects of the factors were 6-BA ($R = 36.00$) > NAA ($R = 10.09$) > KT ($R = 5.69$). Since all R -values were higher than the blank error term ($R = 3.27$), the regulatory reliability of these factors was confirmed. Variance analysis (Table 3) further demonstrated that only 6-BA significantly influenced proliferation coefficients ($P < 0.05$), while NAA and KT showed no statistical significance ($P > 0.05$). Duncan's multiple range test (Table 4) revealed superior proliferation efficacy at 0.1 mg/L 6-BA (Level 1) compared to 0.3 mg/L (Level 2) and 0.5 mg/L (Level 3). Comprehensive mean analysis and interaction effects identified the optimal hormone combination for *S. indica* somatic embryo induction and proliferation as A1B2C3 (0.1 mg/L 6-BA + 0.3 mg/L NAA + 1.0 mg/L KT).

Table 2
Results of $L_9(3^4)$ orthogonal experiment on proliferation of somatic embryos in *S. indica*.

Number	PGRs (mg/L)				Proliferation coefficient
	6-BA (A)	NAA (B)	KT (C)	Error (D)	
1	0.1	0.1	0.5	1	75.38 ± 0.51
2	0.1	0.3	1.0	2	93.5 ± 0.32
3	0.1	0.5	0.8	3	88.72 ± 0.51
4	0.3	0.1	1.0	3	63.62 ± 0.65
5	0.3	0.3	0.8	1	68.39 ± 0.48
6	0.3	0.5	0.5	2	62.42 ± 0.90
7	0.5	0.1	0.8	2	46.38 ± 0.46
8	0.5	0.3	0.5	3	52.74 ± 0.92
9	0.5	0.5	1.0	1	50.49 ± 0.98
K_1	85.87	61.79	63.51	65.09	
K_2	65.14	71.88	68.16	67.43	
K_3	49.87	67.21	69.20	68.36	
R	36.00	10.09	5.69	3.27	
Note K : mean; R : range ($R = K_{\max} - K_{\min}$); Mean $\pm$ Standard error					

Table 3
Results of analysis of variance of somatic embryonic proliferation coefficient in *S. indica*.

Factor	Source	Type III sum of square	df	Mean square	F value	Sig. (P)
Proliferation coefficient	6-BA	1958.491	2	979.246	26.119	$P < 0.05$
	NAA	152.792	2	76.396	0.226	$P > 0.05$
	KT	55.080	2	27.540	0.078	$P > 0.05$
	Error	17.080	2	8.540		

Table 4
Duncan's test at three levels of 6-BA

Level	Mean	Significance
1	85.87	a
2	65.14	b
3	49.87	c
Note: Different lowercase letters in the same column indicate significant differences ($P < 0.05$).		

Repeating the optimal hormone combination, somatic embryos were uniformly induced at leaf bases after 20 days of cultivation, accompanied by the formation of primary leaf primordia (Fig. 6A). By day 40, somatic embryos entered a pronounced proliferation phase, with early-stage embryos differentiating into morphologically intact, jade-green regenerants, while abundant white adventitious roots emerged at the base (Fig. 6B). At 60 days, regenerants exhibited sustained biomass accumulation, marked increases in plant height and leaf number, and dense adventitious root system development (Fig. 6C). After 80 days of cultivation, regenerants occupied the effective space within culture vessels, displaying elongated morphology due to physical constraints, yet achieving an average of over 20 leaves per plant (Fig. 6D). At this stage, plants retained vigorous proliferation potential, with leaves serving as secondary explants for subsequent subcultures, yielding a proliferation coefficient exceeding 95.0.

Rejuvenation culture

Results demonstrated significant regulatory effects of PP333 concentrations on *in vitro* plantlet morphogenesis. At 0.01 mg/L, plants exhibited typical healthy morphology with compact, turgid leaves and robust root systems densely covered with white adventitious roots, devoid of physiological abnormalities (Fig. 7A). Under 0.1 mg/L treatment, plants maintained normal leaf expansion with axial elongation tendencies but displayed reduced root system density (Fig. 7B). At 0.5 mg/L, leaves thickened with reduced surface area, concomitant with localized root browning (Fig. 7C). The 1.0 mg/L treatment induced shortened internodes, partial leaf chlorosis, and delayed root development accompanied by gradual browning (Fig. 7D). At 2.0 mg/L concentration triggered pronounced dwarfing phenotypes, intensified basal leaf chlorosis, and inhibited adventitious root formation (Fig. 7E). The highest concentration (3.0 mg/L) severely suppressed morphogenesis, resulting in overall plant malformation, marked chlorosis, loss of normal organ differentiation capacity, and only sporadic adventitious root primordia (Fig. 7F).

Replicate verification using MS medium supplemented with 0.01 mg/L PP333 demonstrated that *in vitro* plantlets initiated axial expansion growth patterns after 10 days of cultivation, with yellow-green swollen meristematic structures at the base and adventitious root primordia formation (Fig. 8A). By day 20, plants exhibited significant biomass accumulation, enhanced chlorophyll content intensifying leaf coloration, differentiation of swollen tissues into new leaf primordia, and a dense initiation phase of basal adventitious roots (Fig. 8B). After 30 days, plant morphology approached structural maturity, with

substantial photosynthetic pigment accumulation imparting vivid organ coloration and roots entering rapid developmental stages (Fig. 8C). Upon completion of 40-day cultivation, *in vitro* plantlets achieved full morphogenesis, forming regenerants with fully differentiated organs, characterized by well-differentiated leaf cuticle structures, compact plant architecture, and highly branched root systems (Fig. 8D).

Acclimatization and Transplantation

Untreated *in vitro* plantlets demonstrated temporal gradient adaptability during acclimatization. After 10 days of cultivation, partial plants exhibited phenotypic variations, including abnormal leaf morphology and swollen apical meristems (Fig. 9A). By day 70, significant growth heterogeneity emerged within the population, with chlorosis or mortality observed in some individuals, resulting in a survival rate of only 54.83% (Fig. 9B). In contrast, PP333-rejuvenated plantlets displayed superior adaptability during early acclimatization (10 days), characterized by dark-green leaves and fully developed organs (Fig. 9C). After completing the 70-day acclimatization cycle, these plants maintained stable physiological status, with only sporadic senescent chlorosis in leaves, achieving a 100% survival rate (Fig. 9D).

Plants from different treatment groups exhibited temporal morphological developmental characteristics post-transplantation. Non-rejuvenated plants entered an active vegetative growth phase 2 months after transplantation, displaying anthocyanin deposition features at leaf bases (Fig. 10A). By month 5 of cultivation, leaves developed axially elongated morphologies with expanded anthocyanin accumulation zones (Fig. 10B). At 8 months, increased leaf arrangement hierarchy and significant photosynthetic pigment accumulation were observed (Fig. 10C). Rosette architecture was fully established with stable phenotypes after 10 months (Fig. 10D). In contrast, rejuvenated plants initially showed relatively slower biomass accumulation rates during early transplantation (2 months), but featured more developed leaf cuticle structures (Fig. 10E). By month 5, leaves exhibited compact spatial arrangements (Fig. 10F). Leaf arrangement expansion patterns initiated after 8 months of cultivation (Fig. 10F), culminating in complete reconstruction of typical rosette morphology at 10 months (Fig. 10G). Morphological observations confirmed no statistically significant differences in final phenotypic characteristics between the two treatment groups.

4. Discussion

In vitro propagation modes of *S. indica*

Somatic embryogenesis, as a critical strategy for plant micropropagation, exhibits a unique direct formation pattern in *S. indica*. Unlike species such as *Lilium brownii* var. *viridulum* Baker. (Zhai et al. 2011) and *Portulaca oleracea* L. (Sedaghati et al. 2019), *S. indica* somatic embryogenesis completely bypasses the callus phase, with embryogenic primordia directly originating from the leaf epidermis or subepidermal 1–2 layers of parenchyma cells. Histological evidence reveals localized epidermal rupture during early cultivation, followed by polar division of subjacent parenchyma cells to form bipolar conical embryogenic structures. This direct mechanism aligns with patterns reported in succulent species such

as *Haworthia retusa* (L.) Duval. (Kim et al. 2019) and *Crassula ovata* (Mill.) Druce 'Gollum' (Muhsen Almasoody et al. 2024), but markedly differs from indirect pathways reliant on callus intermediation, as observed in *Salicornia brachiata* Roxb. (Rathore et al. 2015) and *Caralluma bhupenderiana* Sarkaria. (Pachipala et al. 2023). Direct somatic embryogenesis offers distinct advantages: shortened developmental cycles, significantly enhanced embryo yield per explant, reduced microbial contamination risks by avoiding callus formation, and ensured phenotypic uniformity of regenerants, thereby providing an ideal technical pathway for large-scale production.

However, the high proliferation efficiency observed in this study resulted in morphological anomalies such as slender stems and elongated internodes *in vitro* plantlets, a phenomenon analogous to physiological stress effects reported in *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (Chu and Sun 2009). Notably, traditional optimization of hormone ratios failed to ameliorate these issues in the *S. indica* system, suggesting complex synergistic-antagonistic interactions between endogenous hormones and exogenous plant growth regulators. This study innovatively introduced a PP333-based rejuvenation strategy, which suppressed endogenous gibberellin biosynthesis and activated cell wall lignin deposition pathways, significantly enhancing stem thickness and leaf cuticle development in regenerated plantlets, ultimately achieving a breakthrough in transplantation survival rates from 54.83–100%. Rejuvenated plants maintained typical rosette architecture during long-term cultivation without genetic phenotypic shifts, confirming that this technology enables "quality-efficiency co-optimization" through metabolic reprogramming while preserving proliferation efficiency. This finding establishes a replicable technical framework for the industrial-scale micropropagation of Crassulaceae succulents (Fig. 11).

Synergistic regulation of medium and PGRs

In plant tissue culture systems, the culture medium serves as the material foundation for explant morphogenesis, with its composition requiring precise alignment with the nutritional demands of distinct developmental stages (Ren et al. 2019; Lu et al. 2023). Similar to other succulent species (Tuo et al. 2019; Zhao et al. 2022; Wang et al. 2017), this study employed MS basal medium, whose high nitrate and ammonium concentrations effectively supported somatic embryo induction and proliferation in *S. indica*. Spatiotemporal regulation of plant growth regulators (PGRs) constitutes the core driving force of somatic embryogenesis, where the type, concentration, and combinatorial patterns directly govern explant dedifferentiation and redifferentiation processes (Singh 2019; Nowakowska et al. 2019).

Experimental results demonstrated that the synergistic effects of cytokinins (6-BA, KT) and auxin (NAA) exerted decisive influences on *S. indica* somatic embryogenesis. Low-concentration 6-BA (0.1 mg/L) efficiently induced epidermal cells to initiate embryogenic programs, forming bipolar conical embryogenic primordia. However, proliferation efficiency markedly declined when concentrations exceeded 1.0 mg/L. This phenomenon aligns with mechanisms reported in *Aloe vera* (L.) Burm. f., where high 6-BA concentrations presumably disrupt proliferation by perturbing endogenous hormone

homeostasis, interfering with cell cycle regulation networks, and suppressing embryogenesis-related gene expression (Das and Bora 2018). The auxin NAA exhibited dose-dependent bidirectional regulation: 0.3 mg/L NAA promoted adventitious root differentiation via activation of auxin response factors, whereas elevation to 1.0 mg/L likely induced metabolic imbalance of endogenous auxins, inhibiting root development. This "low-promotion and high-inhibition" effect closely parallels findings in *Sedum sediforme* (Jacq.) Pau and *Sedum engleri* Hamet (Xing et al. 2010). Notably, variance analysis in the orthogonal experiment revealed nonsignificant primary effects of NAA ($P > 0.05$), suggesting its role in the *S. indica* system may depend on synergistic interactions with 6-BA rather than independent regulation.

Compared with the previously mentioned PGRs, 2,4-D showed strong callus-inducing ability, which resulted in chlorosis and necrosis of the explants. This phenomenon aligns with studies on succulent species such as *S. brachiata* (Rathore et al. 2015) and *bhupenderiana* (Pachipala et al. 2023), where 2,4-D likely suppresses the expression of genes critical for somatic embryo induction and development—including those governing embryogenic cell differentiation and organogenesis—thereby obstructing somatic embryogenesis. Consequently, 2,4-D must be entirely excluded from the *S. indica* rapid propagation system to prevent callus-mediated interference with the direct somatic embryogenesis pathway.

Dose-Dependent rejuvenation effects and stress resistance enhancement of pp333, and its dual-edged nature in morphological regulation and residual risks

As a triazole-type plant growth retardant, PP333 demonstrated significant rejuvenation effects in *S. indica* micropropagation by suppressing endogenous gibberellin biosynthesis. Low-concentration treatments (0.01–0.1 mg/L) effectively enhanced stem robustness and leaf compactness *in vitro* plantlets, achieving 100% transplantation survival rates. However, this study identified clear dosage-associated risks: concentrations exceeding 0.5 mg/L induced irreversible phenotypes including malformed leaf reduction, root browning, and distorted plant architecture, consistent with PP333-triggered organ deformities reported in *Sempervivum tectorum* L. (Chen et al. 2011). Notably, research shows that even at the optimal concentration, residual PP333 can cause delayed growth inhibition after the acclimatization process. This is manifested as hidden abnormalities like abnormal shortening of internodes and disrupted leaf arrangement patterns. Moreover, the risks are increased when the tissue culture and acclimatization protocols are not ideal (Zhang et al. 2019; Luo et al. 2011).

During the acclimatization phase of *S. indica*, PP333 underwent progressive metabolic degradation, leading to gradual attenuation of endogenous concentrations. This dynamic process resulted in nonsignificant differences in morphological parameters (plant height, internode spacing) between treated and untreated groups, while maintaining enhanced leaf cuticle thickness and root lignification. These observations confirm that PP333-mediated rejuvenation effects are time-limited and non-inductive of genetic phenotypic shifts, aligning with reversible chemical regulation patterns reported in *Echeveria* 'Moon Gad varnish', *Sedum pachyphyllum* Rose, and *Echeveria runyonii* 'Topsy Turvy' (Song et

al. 2019). Notably, PP333 continued to improve drought resistance during post-transplantation stages by modulating stomatal aperture frequency and osmolyte accumulation, demonstrating dual functional value in both morphological optimization and stress resilience enhancement for potted cultivation management.

Additionally, characteristic red pigmentation observed at leaf apices of transplanted plants may correlate with light-induced anthocyanin accumulation. The intensity of coloration changes could result from prolonged cultivation periods and plant regulatory responses to seasonal temperature fluctuations and UV radiation variations. Potential mechanisms include low-temperature-induced pigmentation shifts or adaptations to diurnal temperature variation in the Kunming region, a hypothesis consistent with Zhang et al.'s (Zhang et al. 2016) findings on chromatic variation factors in three Crassulaceae succulent species. However, the species-specificity of this phenomenon in *S. indica* and the interaction mechanisms between UV/thermal signaling pathways require systematic elucidation through controlled environmental experiments.

Conclusion

This study successfully established an efficient *in vitro* propagation system for *S. indica* by integrating optimized hormonal regulation with PP333-mediated rejuvenation culture. The orthogonal experiment identified 0.1 mg/L 6-BA + 0.3 mg/L NAA + 1.0 mg/L KT as the optimal combination, achieving a proliferation coefficient of 93.5 while preserving genetic fidelity. Crucially, 0.01 mg/L PP333 supplementation enhanced acclimatization survival rates to 100% through metabolic reprogramming, significantly improving stem robustness and leaf cuticle development without compromising proliferation efficiency. The direct somatic embryogenesis pathway, bypassing callus formation, ensured rapid propagation cycles and phenotypic uniformity, overcoming critical bottlenecks in traditional methods. Furthermore, PP333 degradation during acclimatization eliminated residual growth inhibition risks while maintaining enhanced drought resistance via stomatal regulation and osmolyte accumulation. This dual optimization of proliferation efficiency and plantlet quality establishes a scalable technical framework for industrial production of *S. indica* and related Crassulaceae species. Future research should focus on elucidating UV/thermal signaling interactions underlying anthocyanin dynamics and refining PP333 metabolic clearance protocols to further enhance commercial viability. The protocol demonstrates significant potential for ecological conservation and sustainable exploitation of medicinal compounds in succulent plants.

Abbreviations

MS

Murashige and Skoog

PGRs

Plant growth regulators

6-BA

6-benzylaminopurine
KT
kinetin
NAA
1-naphthaleneacetic acid
IBA
indole-3-butyric acid
2,4-D
2,4-dichlorophenoxyacetic acid
PP333
paclobutrazol
 HgCl_2
mercuric chloride
NaOH
sodium hydroxide.

Declarations

Conflicts of Interest:

The authors declare no conflicts of interest.

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Author Contributions:

Conceptualization: Hengyu Huang and Aili Zhang; Experimental design: Hengyu Huang and Aili Zhang; Investigation and validation: Liqing Cheng and Shuang Liu; Data analysis: Liqing Cheng and Shuang Liu; Writing—original draft: Liqing Cheng and Shuang Liu; Writing—review and editing: Hengyu Huang and Aili Zhang; Funding acquisition: Hengyu Huang and Aili Zhang. All authors have read and agreed to the published version of the manuscript.

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Data will be made available upon reasonable request.

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Figures

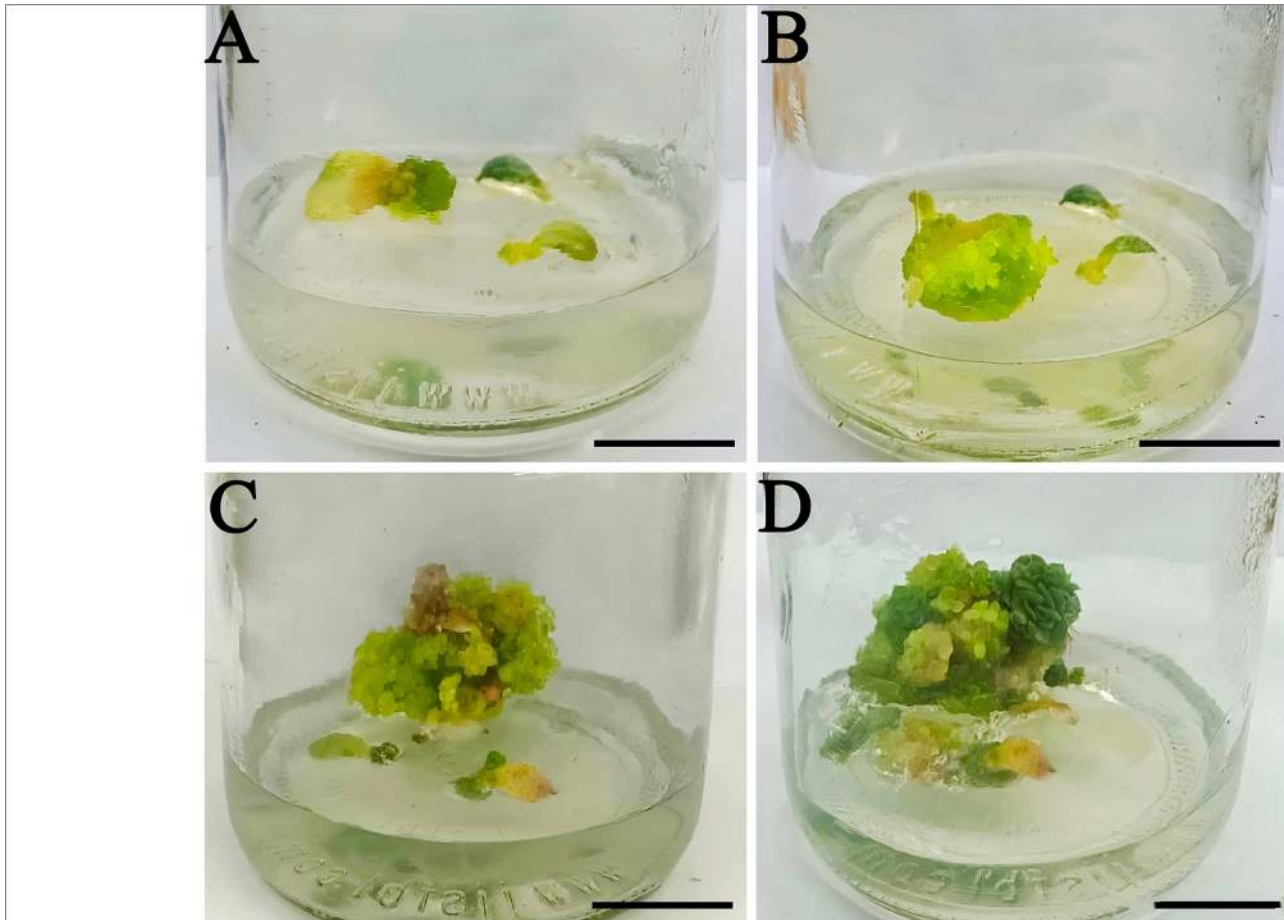


Figure 1

Initiation culture of the leaves of *S. indica*.

growth status after 25 days of culture(A); growth status after 30 days of culture(B); growth status after 40 days of culture(C); growth status after 50 days of culture(D). Scale = 3.0 cm.

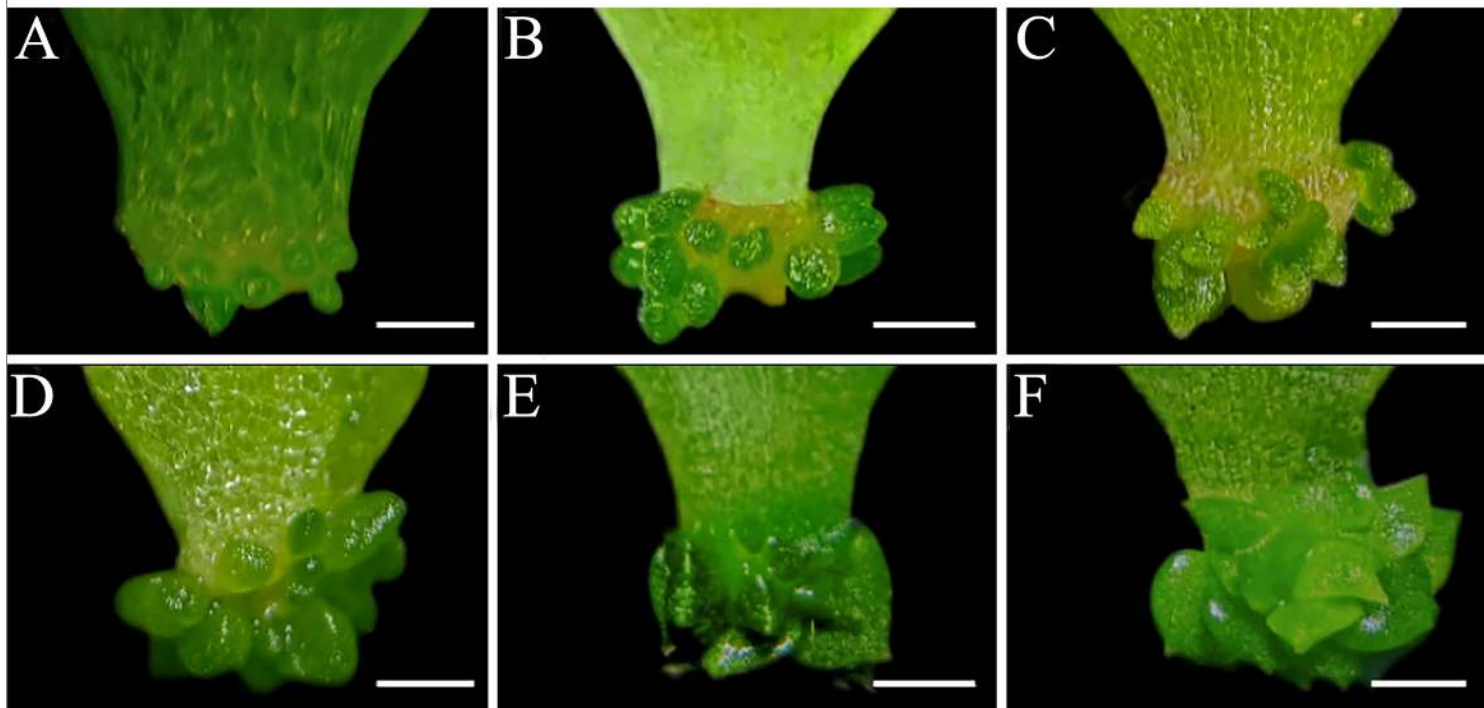


Figure 2

Morphological observation of the leaf explants of *S. indica* under the dissecting microscope.

growth after 5 days of culture (A); growth after 7 days of culture(B); growth after 10 days of culture(C); growth after 15 days of culture(D); growth after 20 days of culture(E); growth after 30 days of culture(F). Scale = 0.2 cm

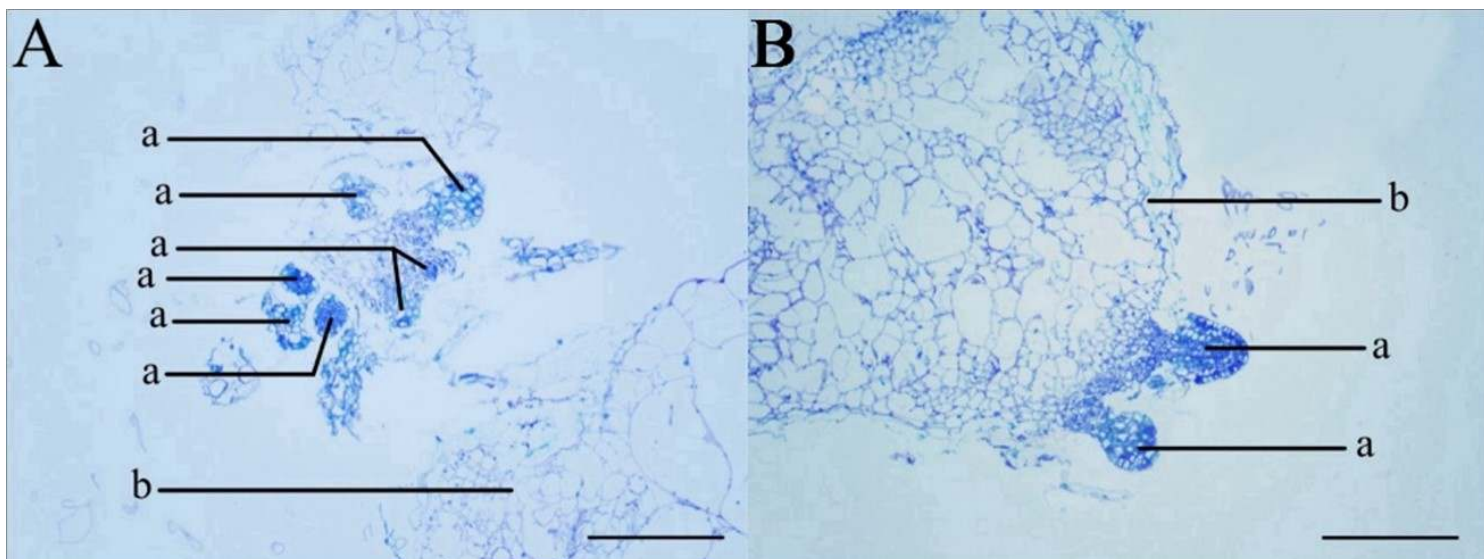


Figure 3

Histological characterization of somatic embryogenesis in *S. indica* by paraffin section micrographs.

cross-section of explants cultured for 5 days (A): (a) somatic embryo; (b) parenchyma cells.
morphogenesis of somatic embryos after 10 days of culture(B): (a) somatic embryo; (b) parenchyma cells. Scale bar = 200 μ m.

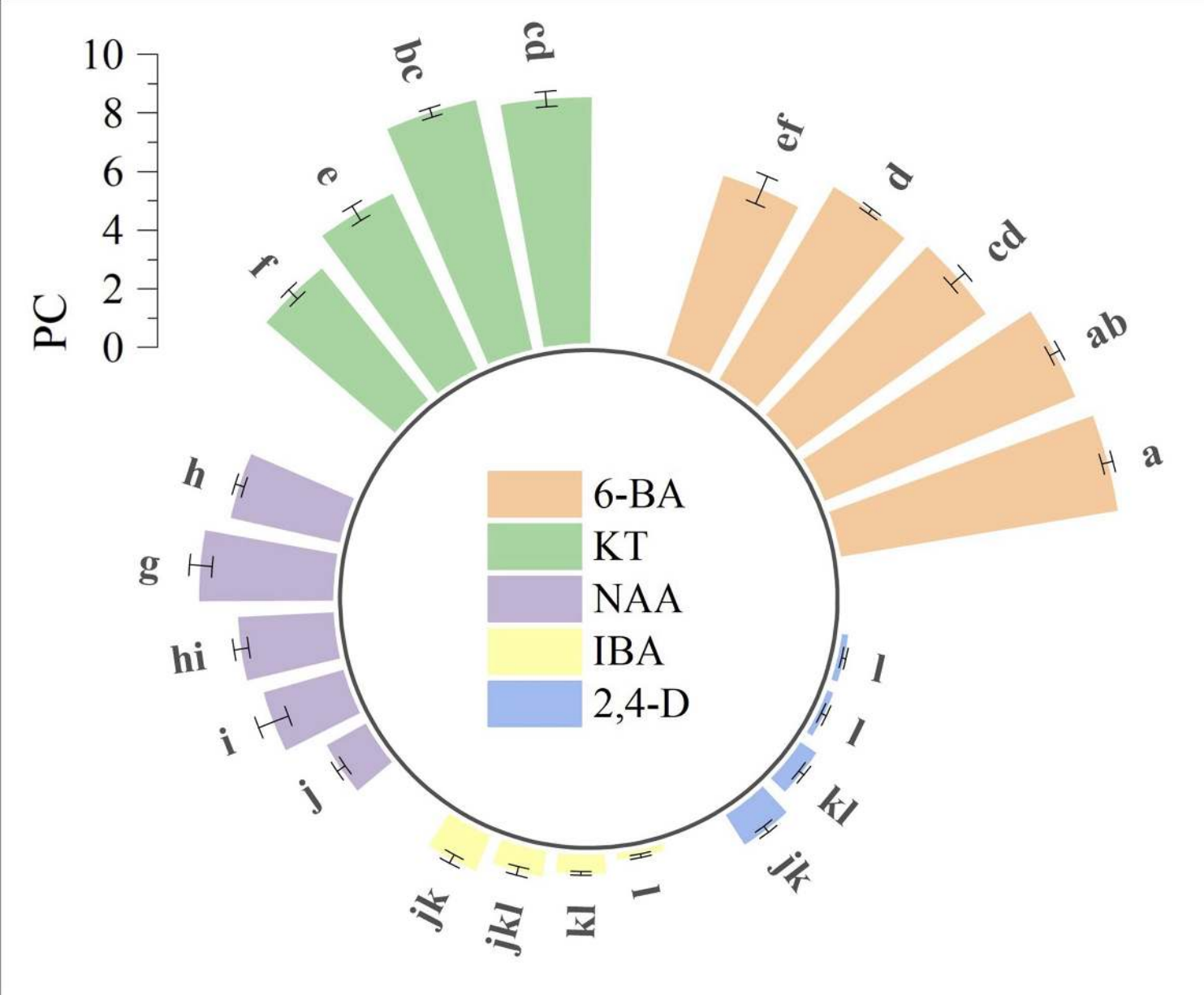


Figure 4

Radial column chart of the single-factor proliferation coefficient.

PC: proliferation coefficient. Different lowercase letters indicate significant differences ($P < 0.05$)

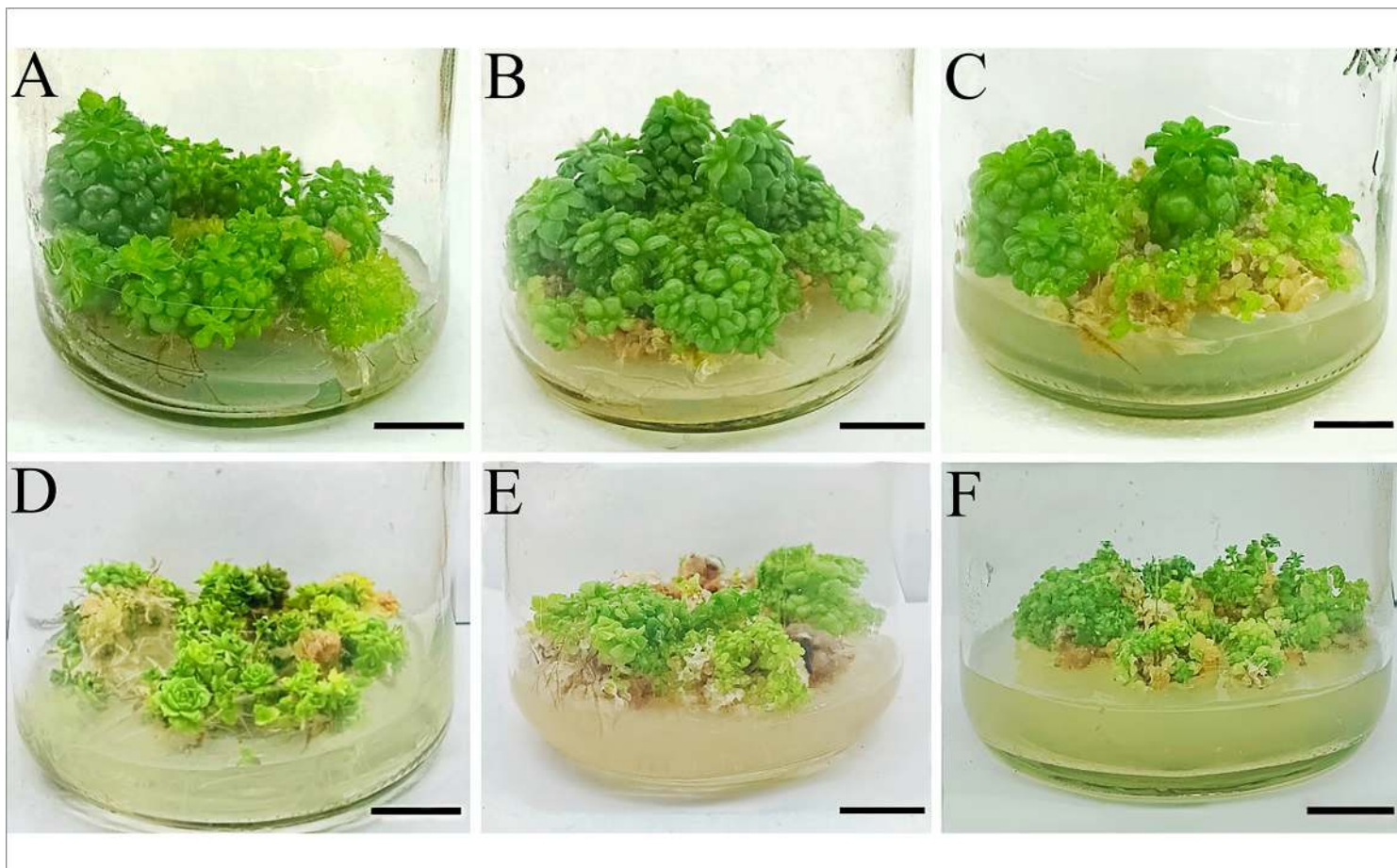


Figure 5

Results of the single-factor experiment in *S. indica*.

growth in MS with 0.1 mg/L 6-BA (**A, B**); growth in MS with 1.0 mg/L KT (**C**); growth in MS with 0.5 mg/L NAA (**D**); growth in MS with 0.1 mg/L IBA (**E**); growth in MS with 0.1 mg/L 2,4-D (**F**). Scale bar = 2.0 cm.

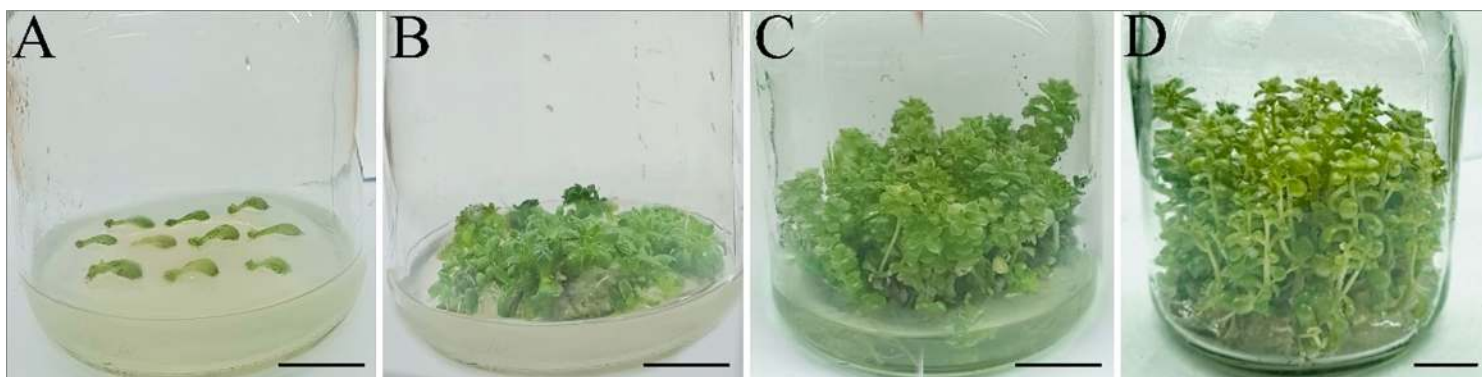


Figure 6

Results of repeated experiments with the optimal orthogonal combination in *S. indica*.

growth after 20 days (A); growth after 40 days (B); growth after 60 days (C); growth after 80 days (D). Scale bar = 2.5 cm.

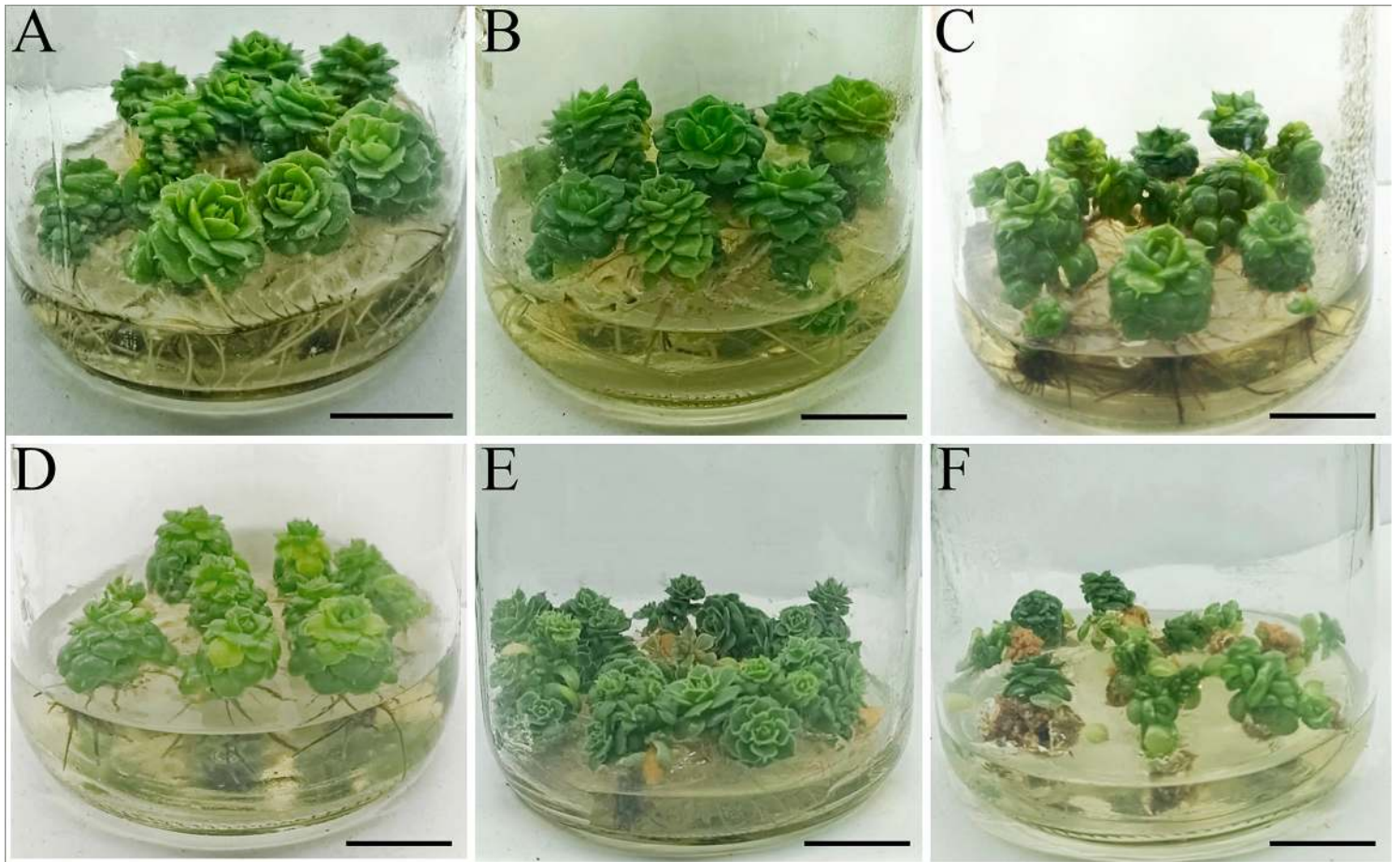


Figure 7

Rejuvenation cultivation in *S. indica*.

growth in MS with 0.01 mg/L PP333 (A); growth in MS with 0.1 mg/L PP333 (B); growth in MS with 0.5 mg/L PP333 (C); growth in MS with 1.0 mg/L PP333 (D); growth in MS with 2.0 mg/L PP333 (E); growth in MS with 3.0 mg/L PP333 (F). Scale bar = 2.5 cm.

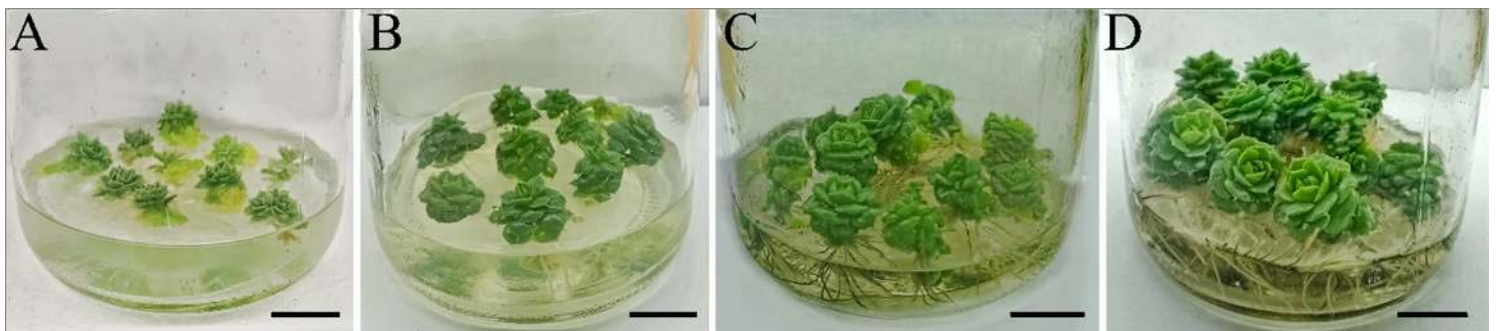


Figure 8

Repeated verification of rejuvenation culture in *S. indica*.

growth after 10 days (A); growth after 20 days (B); growth after 30 days (C); growth after 40 days (D).
Scale bar = 2.0 cm.

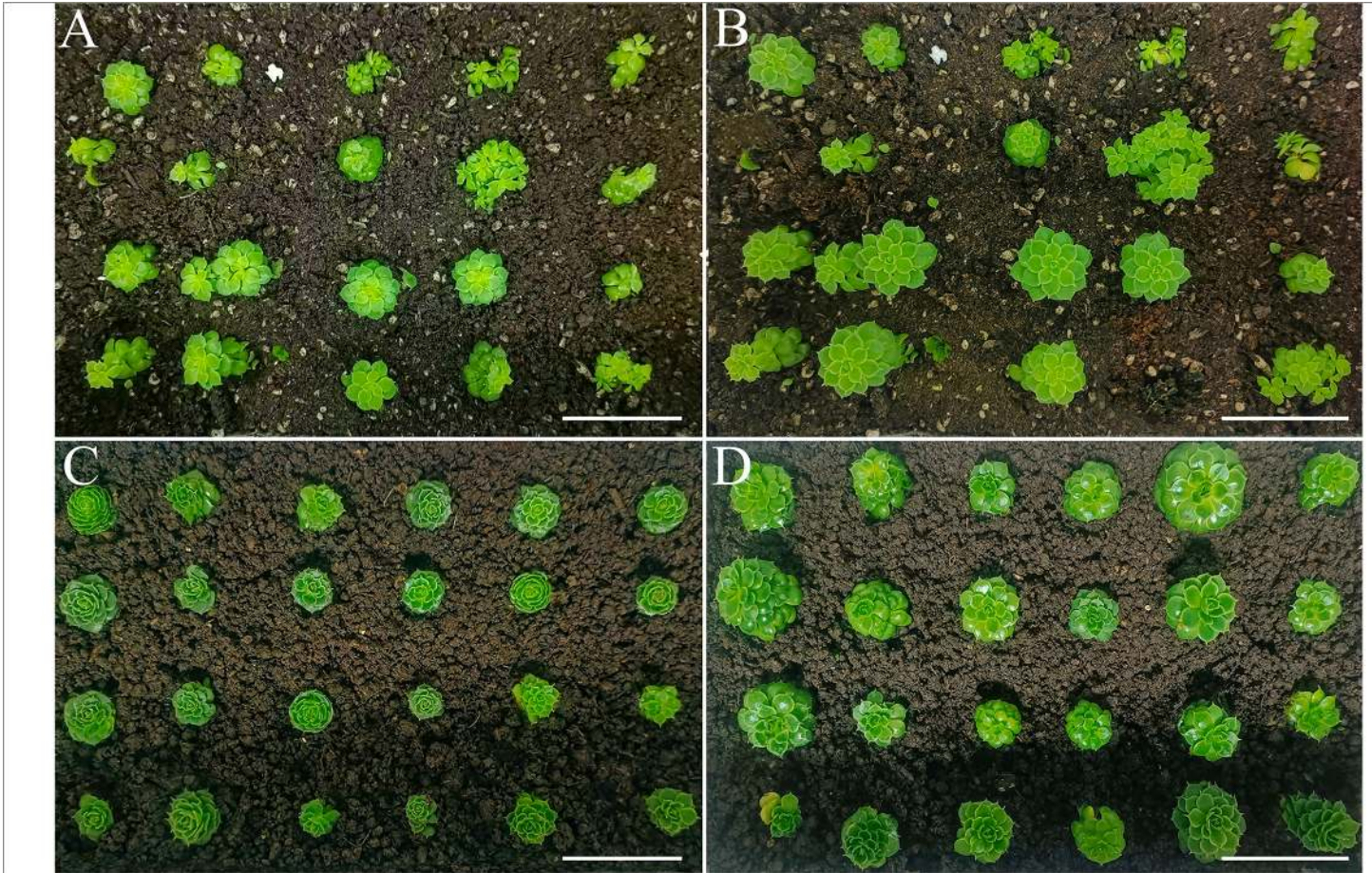


Figure 9

Comparative observation of acclimatization in *S. indica* *in vitro* plantlets with or without paclobutrazol-mediated rejuvenation.

growth of un-rejuvenated test-tube seedlings 10 days after acclimatization (A); growth of un-rejuvenated test-tube seedlings 70 days after acclimatization (B); growth of rejuvenated test-tube seedlings 10 days after acclimatization (C); growth of rejuvenated test-tube seedlings 70 days after acclimatization (D).
Scale bar = 5.0 cm.

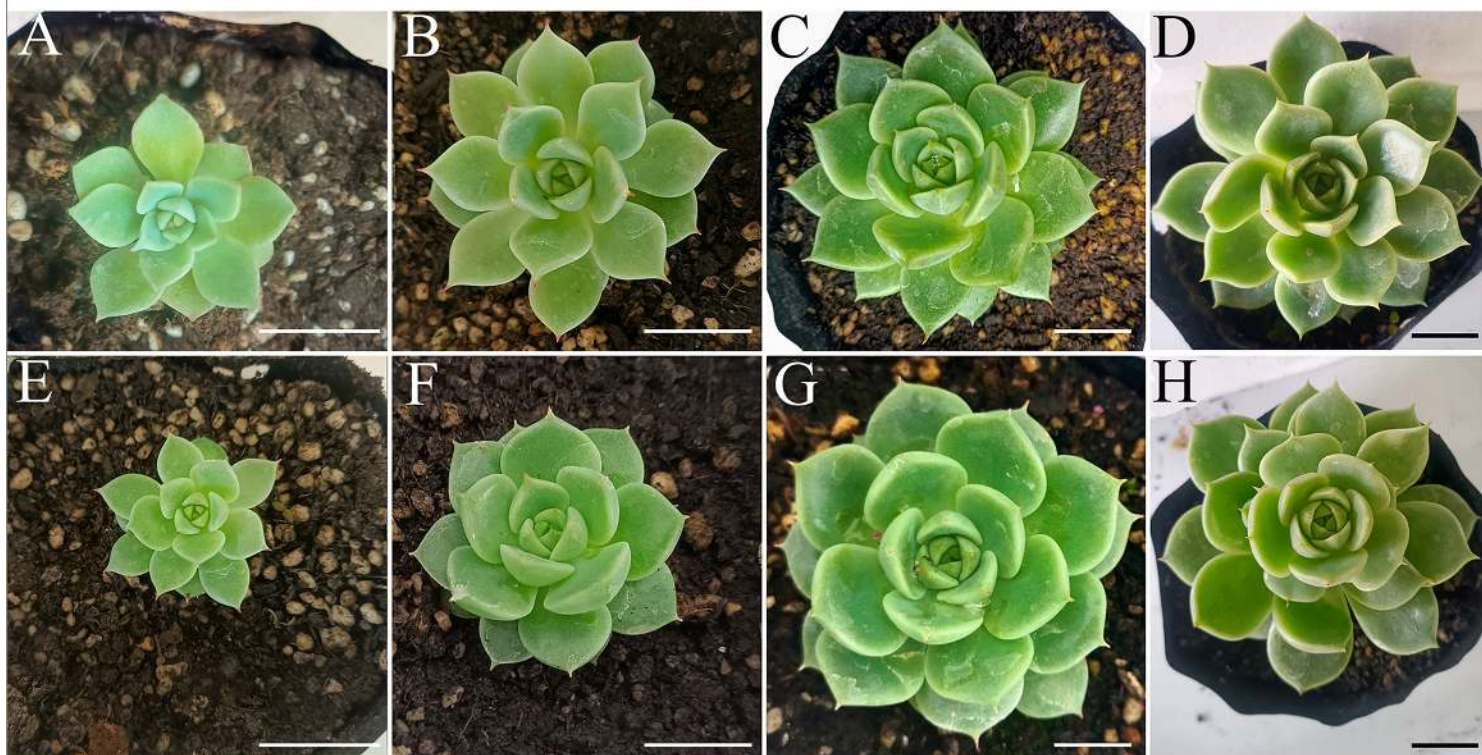


Figure 10

Growth performance of acclimatized *S. indica* plantlets (with or without paclobutrazol-mediated rejuvenation) after transplantation into round pots.

growth process of normal plants ten months after being transplanted into flowerpots (**A-D**); growth process of the plants rejuvenated with paclobutrazol ten months after being transplanted into flowerpots (**E-H**). Scale bar = 2.0 cm.

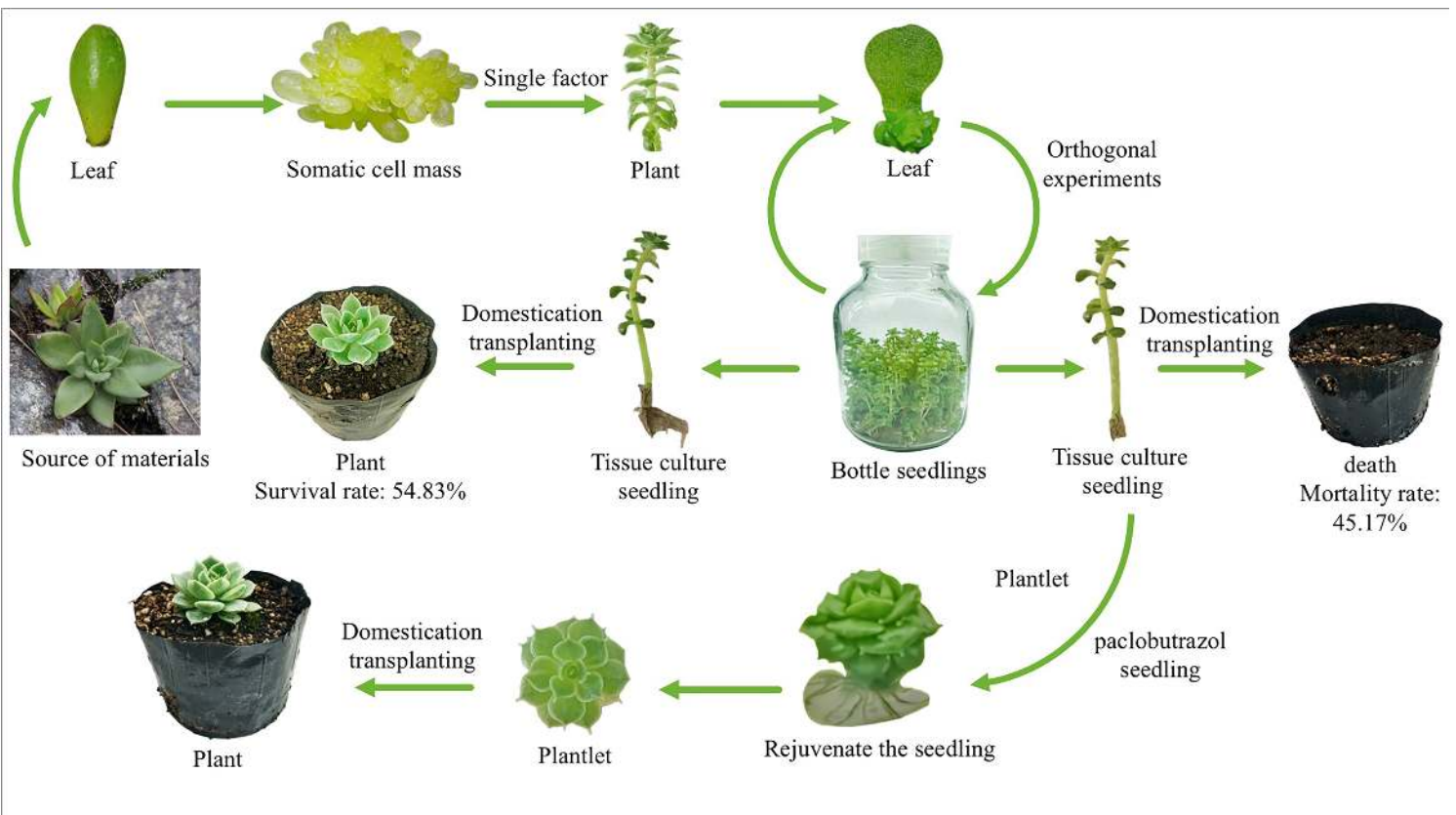


Figure 11