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Establishment of a High-Efficiency Direct Regeneration System from 'Guichang' Kiwifruit Leaves: Postprint

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Abstract

Using 'Guichang' kiwifruit leaves as explants, adventitious buds were induced directly through dedifferentiation, and the adventitious bud proliferation and rooting systems were optimized to establish an efficient direct regeneration system. The results showed that when leaves were cultured on MS medium supplemented with 4.0 mg/L 6-BA and 0.4 mg/L NAA, the adventitious bud induction rate reached 95.8%, with an average bud number of 15.7 per leaf. Adventitious buds cultured on MS medium containing 3.0 mg/L 6-BA, 0.3 mg/L NAA, and 0.2 mg/L GA3 achieved a proliferation rate of 100%, with an average multiplication rate of 8.15 across generations 1–6. For rooting, adventitious buds were first induced on 1/2 MS solid medium supplemented with 1.0 mg/L IBA for 7 days, then sequentially cultured for 14 days each on 1/2 MS solid medium and perlite fully saturated with 1/2 MS liquid medium, resulting in a rooting rate of 98.61% with well-developed root systems. When 50 plantlets were transplanted into nursery pots containing a substrate of perlite and field soil at a volume ratio of 1:4, 49 survived after 2 weeks, giving a survival rate of 98%. This study successfully established an efficient leaf regeneration system for 'Guichang' kiwifruit. This method features a short adventitious bud induction period, high bud induction rate with numerous buds, high proliferation coefficient, high rooting rate, and well-developed root systems in plantlets, laying a foundation for in vitro rapid propagation and genetic transformation of 'Guichang' kiwifruit.

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Full Text

Establishment of a High-Efficiency Direct Regeneration System from Leaf Explants of 'Guichang' Kiwifruit

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Abstract

A highly efficient direct regeneration system was established for 'Guichang' kiwifruit using leaf explants that undergo dedifferentiation to produce adventitious buds directly, with subsequent optimization of bud proliferation and rooting protocols. Results demonstrated that on MS medium supplemented with 4.0 mg/L 6-BA and 0.4 mg/L NAA, leaf explants achieved an adventitious bud induction rate of 95.8% with an average of 15.7 buds per leaf. For bud proliferation, MS medium containing 3.0 mg/L 6-BA, 0.3 mg/L NAA, and 0.2 mg/L GA yielded a proliferation rate of 100% and an average multiplication coefficient of 8.15 across generations 1–6. For rooting, adventitious buds were first induced for 7 days on 1/2 MS solid medium containing 1.0 mg/L IBA, then sequentially cultured for 14 days each on 1/2 MS solid medium and perlite saturated with 1/2 MS liquid medium, achieving a rooting rate of 98.61% with well-developed root systems. Fifty plantlets were transplanted to pots containing a substrate of perlite and field soil (1:4 v/v), and 49 survived after two weeks, giving a survival rate of 98%. This study successfully established a high-efficiency regeneration system for 'Guichang' kiwifruit leaves, characterized by short induction cycles, high bud induction rates and numbers, large proliferation coefficients, high rooting rates, and robust root systems, providing a foundation for rapid in vitro propagation and genetic transformation of this cultivar.

Keywords: 'Guichang' kiwifruit, leaves, adventitious buds, plant regeneration

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Introduction

Kiwifruit belongs to the genus *Actinidia* Lindl. of the family Actinidiaceae Hutch and is a perennial vine fruit tree. China is recognized as the native origin of kiwifruit, possessing the world' s largest population and distribution range, earning it the reputation as the "home of kiwifruit" [1]. Kiwifruit is celebrated as the "king of fruits" due to its high vitamin C content, abundant mineral elements, and dietary fiber [2]. 'Guichang' kiwifruit (*Actinidia chinensis* cv. 'Guichang') is an excellent cultivar of Chinese kiwifruit developed in Guizhou Province and introduced to northern Guizhou in 1990 [3]. Its individual fruits can weigh up to 130 g with sugar content reaching 15%, and are rich in vitamin C, carotenoids, and trace elements such as iron and zinc. The cultivar is favored by consumers for its refreshing aroma, balanced sweet-sour taste, tender flesh,

strong fragrance, and excellent storage properties. Moreover, it demonstrates outstanding yield performance in production with promising economic benefits and application prospects [3]. In September 2011, 'Guichang' kiwifruit was designated as a National Geographical Indication Product by the General Administration of Quality Supervision, Inspection and Quarantine [4]. However, as kiwifruit is dioecious, sexual reproduction through seeds readily leads to cultivar degeneration [5], thereby limiting the promotion of improved varieties. Additionally, kiwifruit cultivation is susceptible to diseases such as bacterial canker [7], which can cause yield losses of up to 20% annually [8].

Plant tissue culture technology can overcome the cultivar degeneration associated with sexual reproduction and enable large-scale seedling propagation [9-12]. Furthermore, an efficient and stable regeneration system is fundamental for plant genetic engineering breeding [13-14], which provides a new pathway for disease control in plants [15]. In plant tissue culture, explants can regenerate through callus induction followed by adventitious bud differentiation [16], or directly from explants without callus formation [17]. The former approach involves longer differentiation cycles and higher mutation rates, whereas the latter features shorter cycles and lower variation, making it an excellent system for plant regeneration and genetic transformation [18]. While several reports on kiwifruit in vitro propagation exist [19-20], studies on direct regeneration are limited and show low regeneration frequencies [21], and no reports have been published on the in vitro regeneration of 'Guichang' kiwifruit. This study establishes a high-efficiency direct regeneration system using 'Guichang' kiwifruit leaf explants, laying the foundation for rapid in vitro propagation and disease-resistant genetic engineering breeding of this cultivar.

Materials and Methods

Plant Materials

Young leaves of 'Guichang' kiwifruit (*Actinidia chinensis* cv. 'Guichang') were collected from the kiwifruit base in Xiuwen Gubao Township, Guizhou Province.

1.2.1 Leaf Explant Preparation

Leaves were washed clean, surface-sterilized with 75% ethanol for 40 seconds, then with 0.1% HgCl for 9 minutes, and rinsed 3–4 times with sterile water before being trimmed to approximately 1 cm × 1 cm for use.

1.2.2 Adventitious Bud Induction

Explants obtained from Section 1.2.1 were inoculated onto media containing different combinations of plant growth regulators, pre-cultured in darkness for 28 days, then transferred to light conditions. After 14 days of light culture, the number and induction rate of adventitious buds were recorded.

1.2.3 Adventitious Bud Proliferation

Adventitious buds measuring 0.5–0.8 cm were inoculated onto proliferation media. After 42 days, bud numbers were counted and the proliferation coefficient was calculated as the ratio of proliferated buds to inoculated buds. Proliferation across generations 1–6 was also investigated.

1.2.4 Adventitious Bud Rooting

New shoots 2–3 cm in length were inoculated onto 1/2 MS solid medium containing IBA (Table 3) for 7 days of pre-culture. The shoots were then transferred to 1/2 MS solid medium for rooting culture for 14 days, after which rooting rate, average root number (total roots/inoculated shoots), and average root length (total root length/total root number) were recorded. Plantlets with roots were subsequently transferred to 1/2 MS medium with perlite as substrate to promote root development. After 14 days, root development was assessed for transplantation.

1.2.5 Plantlet Transplantation

Well-rooted plantlets were acclimatized by gradually opening the culture vessel caps for one week. Plantlets were then removed, their roots washed, and transplanted to pots containing a mixture of perlite and field soil (1:4 v/v). Appropriate watering was applied daily, and survival rates were recorded after 14 days.

All experiments were conducted under sterile conditions using MS or 1/2 MS medium (supplemented with 8 g/L agar, 30 g/L sucrose, pH 5.85). Culture conditions were 4,500 lx light intensity (16 h/d photoperiod) at (25±2)°C. Adventitious bud induction media are listed in Table 1 , proliferation media in Table 2 , and rooting media in Table 3. Each medium treatment had three replicates. The first 28 days of bud induction involved dark culture, while all subsequent stages were under light conditions.

Results

2.1 Effects of Plant Growth Regulators on Adventitious Bud Regeneration from Leaves

After leaf inoculation, veins began to swell around day 14, with incision sites showing meristematic activity, particularly at vein cuts, accompanied by minimal callus formation (Figure 1 [Figure 1: see original paper]). Bud primordia emerged by day 21, and distinct leaf primordia appeared by day 28, displaying a yellow coloration (Figure 2 [Figure 2: see original paper]), likely due to insufficient chlorophyll synthesis under dark conditions. Following 28 days of dark culture, explants were transferred to light, where adventitious buds continued

to differentiate and elongate rapidly. After 14 days of light culture, statistical analysis showed that yellow bud points developed into light green shoots (Figure 3 [Figure 3: see original paper]). The highest differentiation rates of 95.8% were observed on media A5 and A8, though these were not significantly different from A6 (83.3%) and A9 (87.5%) at P 0.05 (Table 1). However, analysis of average bud numbers revealed that A5, A6, A8, and A9 produced 15.7, 10.3, 11.3, and 10.0 buds per leaf, respectively. Notably, A5 medium yielded the highest average bud number, with significant differences from other media according to variance analysis. Therefore, the optimal medium for adventitious bud regeneration from 'Guichang' kiwifruit leaves in this study was A5: MS + 4.0 mg/L 6-BA + 0.4 mg/L NAA.

2.2 Effects of Plant Growth Regulators on Adventitious Bud Proliferation

Newly differentiated adventitious buds were inoculated onto various proliferation media. New bud primordia began emerging after approximately 14 days, with the best proliferation observed on B2, B3, and B6 media, achieving 100% proliferation rates. The poorest performance was on B4 medium (61.1% proliferation), though variance analysis showed no significant differences among B2, B3, B5, and B6 (P 0.05), indicating that 'Guichang' kiwifruit adventitious buds readily proliferate under appropriate 6-BA and NAA ratios with good efficacy. To promote rapid shoot growth, GA was added to proliferation media, resulting in vigorous growth and clustered shoot formation (Figure 4 [Figure 4: see original paper]) (Table 2). Adventitious buds exceeded 2 cm in length with distinct stem and leaf differentiation, facilitating individual plantlet separation and subsequent root induction. The study also investigated subculture proliferation across generations, with statistical analysis revealing that B2 medium produced the highest average multiplication coefficient of 8.15 across generations 1–6, significantly different from other formulations (P 0.05), with vigorous bud growth (Figure 4 [Figure 4: see original paper]). Thus, B2 medium (MS + 3.0 mg/L 6-BA + 0.3 mg/L NAA + 0.2 mg/L GA) was identified as the optimal proliferation medium for 'Guichang' kiwifruit adventitious buds.

2.3 Adventitious Bud Rooting and Plantlet Transplantation

Adventitious buds were treated with various IBA concentrations in 1/2 MS solid medium (Table 3), then transferred to 1/2 MS solid medium for root induction, followed by root proliferation in a root-strengthening medium. IBA-treated buds rooted rapidly, with rooting rate, average root number, and root length increasing as IBA concentration increased. At 1.0 mg/L IBA, maximum values were achieved: 98.61% rooting rate, 5.31 roots per shoot, and 0.81 cm average root length (C5 root length was not significantly different from C3, C4, and C6). However, IBA concentrations exceeding 1.0 mg/L decreased these parameters, likely due to inhibitory effects of high IBA concentrations on root initiation. Plantlets rooted on C5 medium were transferred to perlite substrate contain-

ing 1/2 MS liquid medium for root proliferation, showing vigorous growth with significantly increased root length and number, accompanied by lateral root proliferation. Within approximately 14 days, extensive root systems developed (Figure 5 [Figure 5: see original paper]), which enhanced transplant survival rates. Therefore, the optimal rooting protocol for 'Guichang' kiwifruit adventitious buds involved induction on 1/2 MS + 1.0 mg/L IBA solid medium for 7 days, followed by sequential culture on 1/2 MS solid medium and then perlite saturated with 1/2 MS liquid medium for 14 days each, producing high-quality root systems.

Fifty plantlets with well-developed root systems were selected for transplantation experiments. After one week of acclimatization by gradually opening culture vessel caps, plantlets were removed, roots washed, and transplanted to pots containing perlite and field soil (1:4 v/v). With daily watering, 49 out of 50 plantlets survived after 14 days, achieving a 98% survival rate with good growth (Figure 6 [Figure 6: see original paper]).

Discussion

Kiwifruit represents a plant of significant research interest with broad application prospects. This study investigated tissue culture of 'Guichang' kiwifruit, a National Geographical Indication Product [4], and established a high-efficiency direct regeneration system from leaf explants. Previous studies have used stem segments, leaves, and petioles for kiwifruit tissue culture [22-23], but most suffered from long induction periods, low regeneration rates, and few buds per explant. For example, *Actinidia chinensis* showed regeneration frequencies of 90% with 4–8 buds per callus [5]; *A. deliciosa* leaf callus induction was only 80% [23]; red-fleshed kiwifruit had 85.7% bud differentiation [12] with merely 3.7 buds per explant [12]; *A. kolomikta* required 98 days to achieve only 68.7% regeneration [24]; and *A. chinensis* 'Funiu 95-2' produced 87.5% bud formation with 4.2 buds after 40 days [21]. Although some studies reported higher induction rates—such as *A. polygama* petiole and stem segments achieving 100% regeneration with 8–10 buds per callus [22]—these used expensive zeatin, inevitably increasing tissue culture costs. Furthermore, most previous research employed a two-step process of callus induction followed by adventitious bud differentiation, which inevitably extends the differentiation cycle. Additionally, the growth regulators and ratios used for callus induction and bud differentiation were not identical [1, 23], requiring explants to adapt to different media during subculture, which further increases the time from explant to adventitious bud and may affect induction rates and bud numbers.

In this study, leaf explants directly differentiated adventitious buds after 28 days on MS + 4.0 mg/L 6-BA + 0.4 mg/L NAA (Figure 2 [Figure 2: see original paper]), achieving a differentiation rate of 95.8% and an average of 15.7 buds per leaf—substantially reducing the time from explant to bud while increasing

both differentiation rate and bud number per explant. By eliminating the callus culture stage, this direct regeneration system reduces the risk of cultivar variation and preserves elite traits. Using 6-BA instead of zeatin, consistent with Zhao et al. [17], also reduces tissue culture costs for 'Guichang' kiwifruit.

Proliferation culture enables mass propagation of adventitious buds. Previous studies reported multiplication coefficients of 3.7 for red-fleshed kiwifruit [25], 6.5 for 'Jinfu' kiwifruit [6], and 4.8 for *A. deliciosa* [26]. However, most research focused only on the first generation multiplication coefficient [5, 10, 26], with few investigating continuous proliferation across multiple generations [1]. This study examined proliferation across generations 1–6 for 'Guichang' kiwifruit, demonstrating stable proliferation on MS + 3.0 mg/L 6-BA + 0.3 mg/L NAA + 0.2 mg/L GA with an average multiplication coefficient of 8.15 (Figure 4 [Figure 4: see original paper]).

Rooting culture is essential for plantlet regeneration in vitro, with root quantity and quality directly affecting acclimatization and transplant survival [17]. Previous kiwifruit studies reported relatively low rooting rates: 62.5% for *A. kolomikta* after 20 days [24], 86.67% for *A. chinensis* 'Funiu 95-2' [15], and 81.8% for red-fleshed *A. chinensis* [25]. While some achieved 100% rooting for *A. deliciosa* [23] and *A. kolomikta* [24], published images showed underdeveloped root systems, such as unilateral root formation with few roots in *A. kolomikta* [24]. Few reports address root proliferation methods for kiwifruit, and underdeveloped root systems may compromise transplant survival. This study achieved excellent rooting by first inducing with 1.0 mg/L IBA, then transferring to 1/2 MS solid medium for rooting, and finally to a root-strengthening medium for proliferation. This approach significantly increased root length and number with lateral root proliferation, forming extensive root systems within approximately 14 days (Figure 5 [Figure 5: see original paper]). Fifty plantlets with robust root systems were acclimatized and transplanted to perlite and field soil (1:4 v/v), achieving 98% survival (Figure 6 [Figure 6: see original paper]).

In summary, the optimal medium for direct adventitious bud differentiation from leaves was MS + 4.0 mg/L 6-BA + 0.4 mg/L NAA, achieving 95.7% differentiation with 15.7 buds per leaf. For proliferation, MS + 3.0 mg/L 6-BA + 0.3 mg/L NAA + 0.2 mg/L GA produced 100% proliferation with an average coefficient of 8.15 across generations 1–6. For rooting, induction on 1/2 MS + 1.0 mg/L IBA for 7 days, followed by sequential culture on 1/2 MS solid medium and perlite with 1/2 MS liquid medium for 14 days each, yielded 98.61% rooting with well-developed root systems. Fifty plantlets transplanted to perlite and field soil (1:4 v/v) showed 98% survival after two weeks. This study successfully established a high-efficiency direct regeneration system for 'Guichang' kiwifruit leaves, addressing previous limitations in kiwifruit tissue culture and providing a foundation for rapid propagation and disease-resistant genetic engineering breeding.

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Figure 1-6. Plant regeneration from leaf of 'Guichang' kiwifruit

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