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Somatic Embryogenesis Induction and Genetic Stability Assessment of Plants Regenerated from Immature Seeds of *Akebia trifoliate* (Thunb.) Koidz

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Abstract: *Akebia trifoliata* is a perennial woody plant with considerable potential in nutrition, food, and health, and the production of seedlings with high quality is critical for its economic utilization. Plant regeneration through somatic embryogenesis is a powerful alternative for propagating many plant species. In this study, a simple and practicable protocol was developed for plant regeneration from immature seeds of *A. trifoliata* via somatic embryogenesis, and the genetic stability of regenerated plants was also assessed. In the somatic embryo (SE) induction stage, the highest frequency of somatic embryogenesis (35.2%) was observed on the WPM medium containing 1.0 mg L^{-1} of thidiazuron (TDZ) and 1.0 mg L^{-1} of 6-benzyladenine (6-BA). The concentration of 6-BA was optimized at 1.0 mg L^{-1} for the proliferation and maturation of the induced SEs, and the combination of 2.0 mg L^{-1} of indole-3-butyric acid (IBA) and 0.5 mg L^{-1} of TDZ was the most responsive for root development and plant growth. The leaf morphological characteristics greatly varied among the established plants, and they could be grouped into three plant types, namely the normal type, Type I, and Type II. Remarkable differences in the number, size, shape, and color of the leaflets were observed among the three types, while their ploidy level was the same via flow cytometry analysis. The Type II and the Type I plants had the highest and the lowest net photosynthesis rate, transpiration rate, and stomatal conductance among the three groups, respectively, and both had a smaller size of stomatal guard cells than the normal type. Simple sequence repeat (SSR) analysis detected that 41 bands (43.62%) were different from those observed in the wild, indicating a high degree of polymorphism between the regenerants and their donor parent. The obtained plants might hold potential for future genetic improvement and breeding in *A. trifoliata*, and the established regeneration protocol might serve as a foundation for in vitro propagation and germplasm preservation of this crop.

Keywords: flow cytometry; morphological characteristics; photosynthesis; somatic embryo; SSR

Citation: Zhang, Y.; Cao, Y.; Wang, Y.; Cai, X. Somatic Embryogenesis Induction and Genetic Stability Assessment of Plants Regenerated from Immature Seeds of *Akebia trifoliate* (Thunb.) Koidz. *Forests* **2023**, *14*, 473. https://doi.org/10.3390/f14030473

Academic Editors: Marcos Edel Martinez Montero and Judit Dobránszki

Received: 6 February 2023
Revised: 22 February 2023
Accepted: 23 February 2023
Published: 26 February 2023

1. Introduction

Akebia trifoliata, a perennial woody vine belonging to the Lardizabalaceae family, mainly grows along forest edges, in scrub forests, or by streams and rivers in eastern Asia, such as China, Korea, and Japan [1,2]. Its stems, leaves, and seeds have been traditionally used as ingredients in Chinese herbal medicine for over 2000 years owing to its diverse pharmacological properties, such as anti-inflammatory, reducing diuresis, relieving nervousness, and activating blood circulation [2,3]. Previous phytochemical studies have revealed that the extracts from the stems, pericarps, and leaves of *A. trifoliate* contain many bioactive compounds and have high levels of antimicrobial and antioxidant properties [3,4]. In China, *A. trifoliata* is commonly consumed as not only an edible fruit crop [2,5] but also a healthy tree oil [6].

With the expansion of growing areas of *A. trifoliata*, the production of seedlings with high quality is critical for economic utilization. Well-developed plants can be produced through seed-based propagation in this crop [5,7]. However, this propagation method is

undesirable due to the high heterozygosis of the seed offspring of *A. trifoliata* since this species is monoecious and self-incompatible [1]. Vegetative propagation via cutting has also been reported in *A. trifoliate* [5], while this approach is labor-intensive and low in reproductive efficiency. Thus, there is a strong need for developing other propagation methods for this crop species.

The in vitro culture technique provides a powerful and reproducible alternative for the rapid propagation of elite, rare, and endangered plants, and has been widely used in the large-scale multiplication of true-to-type plants in numerous plant species [8,9]. In *A. trifoliate*, several attempts have been made to develop an efficient in vitro regeneration protocol for plant production. Calli have been induced from the leaves, petioles, and hypocotyl of *A. trifoliate* [10], whereas callus differentiation and plant regeneration were not obtained in this study. Plants have been successfully regenerated in vitro from the stems with axillary buds of *A. trifoliate* [11], while this system has several issues to be improved, such as a relatively low multiplication rate, a high contamination rate, and serious browning in the primary culture. Somatic embryogenesis is considered a powerful alternative used for in vitro propagation of plantlets in a number of plant species, especially in woody plants [12–15]. Generally, the somatic embryo (SE) is believed to frequently originate from a single cell and have a typical bipolar structure [16,17], and thus plant regeneration via somatic embryogenesis has several advantages over organogenesis, such as a higher number of regenerants, a lower level of chimeras, and a reduced frequency of somaclonal variations [8,18]. Recently, plants were also successfully reproduced from immature and mature zygotic embryos of *A. trifoliate* via direct [19] and indirect somatic embryogenesis [20], respectively. These studies greatly contribute to mass propagation and future genetic modification of this crop. However, young and very small embryos needed to be separated from the immature seeds of *A. trifoliata* in these studies [19,20], which is a difficult and laborious process and may result in a high level of explant contamination. Therefore, an optimized protocol for SEs induction is needed for this species.

Moreover, it is of great necessity to evaluate the genetic stability of in vitro-regenerated plants when producing plants for commercial applications [8,9]. However, the genetic uniformity of the tissue culture-derived plants of *A. trifoliate* remains unexplored in the previous reports.

Furthermore, the collection and long-term preservation of germplasm resources are important for plant biodiversity and food safety [12,21]. Wild *A. trifoliata* has a low fruit set and seed germination rate in its natural state, and the natural populations have gradually declined in China over the past decades due to destructive harvesting practices and habitat degradation [22]. Therefore, immediate efforts are required to maintain the genetic diversity of *A. trifoliata* for their domestication and commercial utilization. Conventionally, orthodox seeds with low water content are the most convenient and widely used materials for the long-term conservation of plant biodiversity [21]. However, Xiong et al. [7] found that the germination rate was only 14.7% when the moisture content of *A. trifoliata* seeds was reduced to 7.8%, indicating that the seeds of this crop are recalcitrant and very sensitive to desiccation. This means that *A. trifoliata* seeds are unsuitable for biodiversity conservation by establishing seed banks. In addition to seed banks, germplasm conservation can also be realized by in vitro culture approaches [21]. As compared with other methods, in vitro conservation was a more inexpensive and reliable way for germplasm preservation of recalcitrant seeds and asexually propagated plant species. Thus, it is very necessary to develop an efficient in vitro culture protocol not only for rapid propagation but also for germplasm conservation in *A. trifoliata*.

It is a routine procedure to induce somatic embryogenesis in many plant species, as plant SEs are the optimum explants for plant reproduction, germplasm preservation, and production of artificial seeds [12,17]. In the present study, plants were regenerated in vitro from the immature seeds of wild *A. trifoliata* via direct somatic embryogenesis, and the genetic homogeneity of the regenerants was evaluated based on morphological, stomatal, flow cytometric, gas exchange, and molecular analysis. This study aimed to establish a

simple and reproducible protocol for somatic embryogenesis of wild *A. trifoliata*, which will contribute to commercial reproduction and germplasm preservation of this valuable plant species.

2. Materials and Methods

2.1. Plant Materials and Explant Disinfection

Immature fruits were harvested from a wild *A. trifoliate* tree growing in a natural forest in the western Hubei of China in early July 2020 (about ten weeks after open pollination) and were stored at 4 °C until further processing. For disinfection, the fruits were firstly thoroughly washed with running water for 30 min and then immersed in 75% (*v*/*v*) ethyl alcohol for 15 min, and next burned for 15 s using an alcohol lamp to remove contamination in a laminar flow cabinet. The fruits were then cut into two halves using a sterilized surgical blade, and the undeveloped seeds were aseptically isolated from the fruits and used for the subsequent experiment.

2.2. Explant Inoculation and SE Initiation

The basic medium consisted of Murashige and Skoog (MS) [23] or woody plant medium (WPM) [24] supplemented with 500 mg L^{-1} of malt extract, 40 g L^{-1} of sucrose, and 7.0 mg L^{-1} of agar, and the pH was adjusted to 5.8. For somatic embryo induction (SEI), each basic medium was added with any two of the three plant growth regulators (PGRs), including 1.0 mg L^{-1} of thidiazuron (TDZ), 1.0 mg L^{-1} of 6-benzyladenine (6-BA), and 1.0 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D), respectively. Five 100 mL Erlenmeyer flasks containing 40 mL of fresh medium were used for each treatment and nine seeds were cultured per flask, and each medium treatment was designed with three replicates. After inoculation, the cultures were maintained in a growth room at a temperature of 25 ± 2 °C, photoperiod of 14 h, and photon flux density of 45 μmol m^{-2} s^{-1}, provided by cool white fluorescent lamps. Two months later, somatic embryogenesis was frequency calculated as a percentage of the number of explants forming SEs divided by the total explant number, and the browning rate was determined by the following equation:

$$\text{browning rate (\%)} = (\text{number of browning explants}/\text{total number of explants}) \times 100.$$

2.3. Proliferation and Maturation of SEs

For proliferation, the SEs obtained from the immature seeds were excised and cultured in somatic embryo maturation (SEM) medium, i.e., WPM medium containing different concentrations of 6-BA (0.5, 1.0, or 2.0 mg L^{-1}), 40 g L^{-1} of sucrose, and 7.0 g L^{-1} of agar (pH 5.8), under the same conditions described above. The developed cotyledonary SEs were transferred onto a similar medium for embryo maturation. Eight SEs were cultured on each Erlenmeyer flask and each treatment contained three flasks with three replications, and cultures were sub-cultured once a month and browning parts of the SEs were excised at each inoculation. Two months later, the number of secondary SEs per explant was counted. Four months later, the number of SEs with adventitious shoots, number of adventitious shoots per explant, and shoot height were recorded. The germination frequency of SEs was calculated using the following formula:

$$\text{germination frequency of SEs (\%)} = (\text{number of SEs with shoots}/\text{total number of explants}) \times 100.$$

2.4. Whole Plant Regeneration

For root induction (RI), two types of explants, namely the SE-derived shoots and the germinated SEs, were separated and transferred to 1/2 WPM medium supplemented with 1.0 or 2.0 mg L^{-1} of indole-3-butyric acid (IBA) and 0.5 or 1.0 mg L^{-1} of TDZ. All media contained 20 g L^{-1} of sucrose, 7.0 g L^{-1} of agar, and 0.5 g L^{-1} of activated charcoal at pH 5.8. Four explants were cultured on each flask under the culture conditions as described in the SE initiation, and twelve explants were used for each treatment with three replications. Two

months later, the number of shoots with roots, adventitious root number per plant, total root length per plant, and total leaf number per plant were counted. The root induction rate was calculated as follows:

root induction rate (%) = (number of explants producing roots/total number of explants) × 100.

2.5. Acclimatization and Transplantation

For acclimatization, plants with well-developed roots were removed from the culture flasks and carefully washed with running water, and then planted in transparent plastic cups filled with a sterilized commercial mix from Jiangsu Peilei Biotechnology Co., Ltd., Zhenjiang, China. Each transplanted plant was covered with another cup on the top to prevent dehydration and then incubated in a growth chamber at 28/20 °C (day/night) with a photoperiod of 14 h light/10 h dark and a light intensity of about 110 μmol m^{-2} s^{-1}. The covers were removed 15 days later when new leaves emerged. Next, the plants were maintained for another 15 days, and irrigation was applied every 5 days. Then, the surviving plants were individually transferred into plastic pots (top diameter: 24.0 cm, bottom diameter: 9.0 cm, and height: 26.5 cm) containing the commercial mix mentioned above and incubated in a greenhouse at 30/20 °C (day/night) with natural light. Five months after transfer to the pots, the genetic stability of the plants was analyzed.

2.6. Evaluation of Leaf Morphological Characteristics

Seven morphological characteristics of the mature leaves were evaluated, including terminal leaflet length and width, lateral leaflet length and width, leaf rachis length and diameter, and leaf SPAD value. The length and width of the terminal and lateral leaflets were measured with a ruler, and the leaf rachis length was measured from the end of the rachis to the end of the apical leaflet. The rachis diameter was taken at the middle part of the leaf rachis using an electronic digital caliper. The leaf chlorophyll content was determined by using a portable chlorophyll meter (SPAD-502 plus, Konica Minolta, Tokyo, Japan). Nineteen plants were randomly selected, and measurements of each parameter were replicated ten times for each plant after five months of growth in the greenhouse. The coefficient of variation (CV) was calculated for each parameter to evaluate the genetic stability of leaf morphological characteristics.

2.7. Nuclei Isolation and Relative Nuclear DNA Content Analysis

Intact nuclei were prepared following Zhang et al. [25] with some modifications. Briefly, a piece of young leaf was excised and rinsed with water to remove adherent dirt, and then approximately 1.0 cm^2 of fresh leaf segments were chopped using a sharp single-sided blade in 500 μL of cold lysis buffer at pH 7.5, containing 10 mmol/L of $MgSO_4 \cdot 7H_2O$, 50 mmol/L of KCl, 5 mmol/L of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1% (m/v) polyvinylpyrrolidone (PVP), and 0.5% (m/v) Triton X-100. For staining the released nuclei, the chopped samples were filtered through a nylon mesh (40 μm), and 50 μg of propidium iodide (PI, 100 μg mL^{-1}, Shanghai Yuanye Bio-Technology Co., Ltd., Shanghai, China) and 100 μL of RNase A (100 μg/mL) were then added into the filtrate. The samples were maintained in the dark for 20 min before sample analysis on a Beckman Coulter CytoFLEX flow cytometer (Suzhou, China). A minimum of 3000 nuclei were analyzed in each sampling with 3 replications.

2.8. Measurements of Stomatal and Gas Exchange Parameters

After growth in the pots for five months, stomatal and gas exchange parameters of the established plants were analyzed. Stomatal characteristics including guard cell length, guard cell width, and stomatal density were measured according to Zhao et al. [26]. A portable photosynthesis instrument (LI-6400, Li-Cor, Lincoln, NE, USA) was used to measure leaf gas exchange parameters such as the net photosynthetic rate, stomatal conductance,

and transpiration rate, according to the suggestions of the manufacturer. At least five functional leaves per plant were used for the analysis of stomatal and gas exchange parameters.

2.9. Genomic DNA Isolation and SSR Analysis

Genomic DNA was isolated from approximately 100 mg of young leaf tissue of the 19 randomly selected plants and their wild parent by using the modified cetyltrimethylammonium bromide (CTAB) method [27]. The concentration and quality of the extracted DNA were verified using a NanoDrop^{TM} One C spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) and a 1.0% agarose gel electrophoresis in 1 × Tris-Borate-EDTA (TBE) running buffer at a constant voltage of 110 V for 30 min. To evaluate the genetic stability of the regenerants, five pairs of simple sequence repeat (SSR) primers, including MT15, MT19, MT28, MT31, and MT45, developed by Li et al. [28], were used in this study. SSR-PCR was performed in a total reaction volume of 20 µL consisting of 2.0 µL of template DNA (25 ng/µL), 10.0 µL of 2 × Taq PCR StarMix with loading dye (GenStar, Beijing, China), 1.0 µL of each primer (10 µM), and 6.0 µL of ddH_2O. The PCR amplification was carried out on a T100 Thermo Cycler (BIO-RAD, Singapore) as follows: initial denaturalization of the DNA at 94 °C for 4 min, followed by 32 cycles of amplification consisting of denaturation at 94 °C for 60 s, annealing at a temperature depending on the Tm of the primer pairs used for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. The microsatellite alleles were separated on non-denaturing 8% polyacrylamide gels in 1 × TBE buffer using a vertical electrophoresis instrument (DYCZ-24G, Beijing Liuyi Biotechnology Co., Ltd., Beijing, China) and visualized by silver staining, as described previously [29]. A 1000 bp DNA marker was used to evaluate the molecular weight of the detected bands.

2.10. Statistical Analysis

The experiment was carried out as a completely randomized design. Data were analyzed using SPSS 23.0 software to generate descriptive statistics, and one-way ANOVA was conducted to assess statistically significant differences. Prior to analysis, data of the somatic embryogenesis frequency, browning rate, and germination frequency of SEs were arcsine-square root-transformed. Mean values were expressed with standard deviation, and the significant differences were determined at $p < 0.05$ using Duncan's multiple range test.

3. Results

3.1. Initiation of SEs from Immature Seeds

In this study, the disinfected seeds from the immature fruits of *A. trifoliate* (Figure 1A) were inoculated on six SEI media for SE induction. After two months of culture, somatic embryogenesis was observed on the surface of the seeds cultured on the somatic embryo induction (SEI) media, namely the SEI2 and SEI5, without an intermediate callus phase (Figure 1B), and no embryo formation was found on the other four SEI media. Explant browning was observed on all these medium treatments, especially on the SEI3 and SEI6 media (Figure 1B). As shown in Table 1, different medium compositions have profound effects on the somatic embryogenesis frequency and the browning rate of the immature seeds. The highest frequency of somatic embryogenesis (35.2%) and the lowest browning rate (64.1%) were both recorded on the SEI5 medium, and a significant difference was found between the SEI5 and the other medium treatments for the two indexes. A relatively high frequency of somatic embryogenesis was also observed on the SEI2 medium (20.4%), which contained a similar PGRs composition, but a different basic medium (MS) compared with the SEI5 medium. These data suggested that in *A. trifoliate*, the addition of 1.0 mg L^{-1} of TDZ and 1.0 mg L^{-1} of 6-BA in the medium might have an active effect on SEs initiation, and WPM was more suitable for somatic embryogenesis than MS. On the other media, no SE was induced from the immature seeds, as might result from an almost 100% browning rate of the explants.

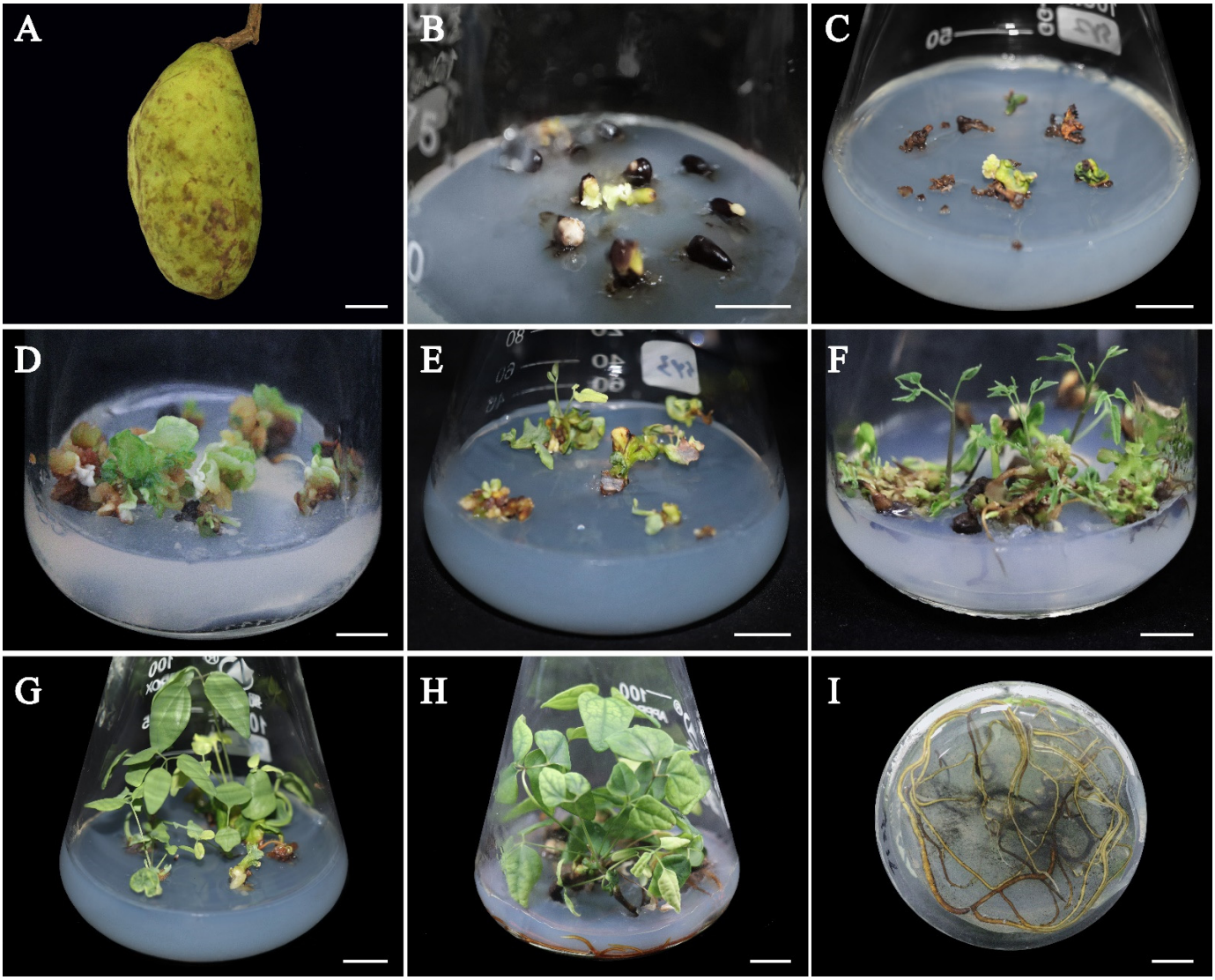

Figure 1. SEs induction and plant regeneration from the immature seeds of wild *A. trifoliate*. (**A**) Immature fruits harvested from a wild *A. trifoliate* tree 10 weeks after open pollination. (**B**) Induced SEs after 2 months of culture on the WPM medium containing 1.0 mg L^{-1} of TDZ and 1.0 mg L^{-1} of 2,4-D (the SEI5 medium). (**C**) Browning and even dead SEs observed on the WPM medium containing 2.0 mg L^{-1} of 6-BA (the SEM3 medium) after culture for one month. (**D**) Secondary SEs occurred on the WPM medium containing 1.0 mg L^{-1} of 6-BA (the SEM2 medium) after 2 months of culture. (**E**) Adventitious shoots initiated after 3 months of culture on the SEM2 medium. (**F**) Numerous adventitious shoots regenerated on the SEM2 medium after culture for 4 months. (**G**) Roots initiated from the SEs after about 20 days of culture on the WPM medium containing 1.0 mg L^{-1} of IBA and 1.0 mg L^{-1} of TDZ (the RI2 medium). (**H**,**I**) Plants regenerated (**H**) and well-developed roots formed (**I**) after 2 months of culture on the RI2 medium. Bars = 1 cm.

Table 1. Effects of different somatic embryo induction (SEI) media on the somatic embryogenesis frequency and the browning rate of the immature seeds after 2 months of culture.

Basic Medium		PGRs (g L^{-1})			Somatic Embryogenesis Frequency (%)	Browning Rate (%)
		TDZ	6-BA	2,4-D		
SEI1	MS	1.0	–	1.0	0.0 ± 0.0 c	92.6 ± 5.7 b
SEI2		1.0	1.0	–	20.4 ± 8.4 b	77.8 ± 9.9 c
SEI3		–	1.0	1.0	0.0 ± 0.0 c	100.0 ± 0.0 a
SEI4	WPM	1.0	–	1.0	0.0 ± 0.0 c	96.3 ± 9.1 ab
SEI5		1.0	1.0	–	35.2 ± 14.8 a	61.1 ± 9.3 d
SEI6		–	1.0	1.0	0.0 ± 0.0 c	100.0 ± 0.0 a

Means (±standard deviation) within columns followed by a similar letter are not significantly different ($p < 0.05$) according to Duncan's range test.

3.2. Proliferation and Maturation of SEs

The number of induced primary SEs was small, and thus these embryos were transferred into the somatic embryo maturation (SEM) medium containing different concentrations of 6-BA for proliferation and maturation. After one month of culture, browning was observed, and some SEs even died on all the culture media, especially on the SEM3 medium containing 2 mg L^{-1} of 6-BA (Figure 1C). Two months later, browning was reduced due to more vigorous growth and development of the cultured explants with the increase of subculture times, and some light-green globular secondary SEs were found to emerge from most of the primary SEs (Figure 1D). These globular embryos could develop further into the cotyledonary stage, while most of them were malformed with deformed, multiple, or fused cotyledons (Figure 1E). After three months of culture, several short and thin adventitious shoots were formed from a part of the malformed embryos (Figure 1E). At the end of the fourth subculture, numerous shoots with several small green leaves were regenerated from the matured SEs in all the culture media, especially the SEM2 medium, and some of them generated a few short but thick roots (Figure 1F). These results indicated that it took as long as four months for the conversion of SEs, and the germinated SEs had a poor shoot performance, which might be due to the malformations of the cultured SEs.

All four evaluated variables, including the number of secondary SEs per explant, the germination frequency of SEs, the number of adventitious shoots per explant, and shoot height, were significantly higher when the SEs were cultured on the SEM2 medium than those cultured on the SEM1 and SEM3 media (Figure 2A–D), which reached mean values of 14.92, 81.25%, 1.90, and 2.63 cm, respectively. The SEs cultured on the SEM3 medium produced a significantly increased shoot number (0.65 per explant) compared to those cultured on the SEM1 medium (Figure 2C), while no significant difference was observed between the two treatments for the other three variables (Figure 2A,B,D). Conclusively, among the three concentrations of 6-BA examined, 1.0 mg L^{-1} of 6-BA was proven to be the most suitable for the proliferation and germination of the induced SEs in *A. trifoliate*.

3.3. In Vitro Rooting

In this experiment, about 2 cm shoots regenerated from the SEs were excised and cultured on the root induction (RI) medium, as presented in Table 2. However, these shoots failed to root even after three months of culture (data not shown), indicating difficulties in rooting from the regenerated shoots in *A. trifoliate*. In another rooting experiment, roots were initiated from the SEs with a single shoot after about 20 days of culture on all 4 media treatments (Figure 1G). After another month of culture, plants with well-developed roots and shoots were obtained on all the culture media, especially on the RI2 and RI3 media (Figure 1H,I).

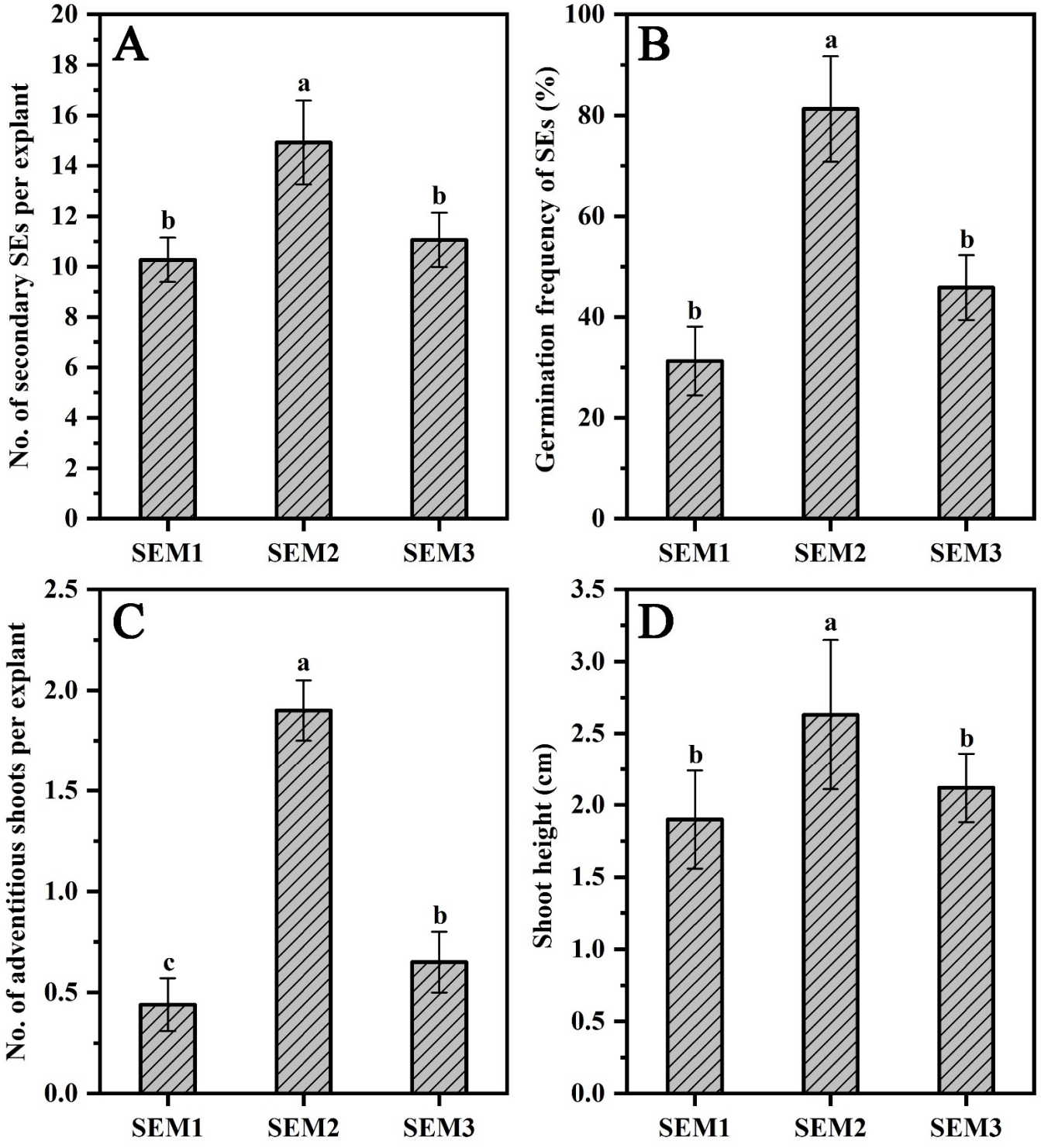

Figure 2. Effects of different concentrations of 6-BA, including 0.5 (SEM1), 1.0 (SEM2), and 2.0 mg L^{-1} of 6-BA (SEM3), on the number of secondary SEs per explant (**A**), the germination frequency of SEs (**B**), the number of adventitious shoots per explant (**C**), and the shoot height (**D**). Different letters indicate significant differences at the 5% level via Duncan's range test (n = 9).

Table 2. Effects of different concentrations of IBA and TDZ on in vitro rooting and plant performance of the matured SEs after 2 months of culture.

Medium	PGRs (mg L^{-1})		Root Induction Rate (%)	Adventitious Root Number Per Plant	Total Root Length Per Plant (cm)	Plant Height (cm)	Total Leaf Number Per Plant
	IBA	TDZ					
RI1	1.0	0.5	33.3 ± 12.9 b	6.5 ± 1.0 b	21.6 ± 3.7 a	4.1 ± 0.5 ab	12.7 ± 2.1 b
RI2	1.0	1.0	100.0 ± 0.0 a	4.8 ± 1.3 b	9.2 ± 1.3 b	3.5 ± 0.3 b	12.3 ± 1.5 b
RI3	2.0	0.5	95.8 ± 11.2 a	13.7 ± 2.8 a	19.2 ± 1.9 a	5.2 ± 0.9 a	20.3 ± 4.2 a
RI4	2.0	1.0	41.7 ± 12.9 b	6.2 ± 1.9 b	17.5 ±1.8 a	4.0 ± 0.5 ab	19.7 ± 2.3 a

The mean values within columns followed by a similar letter are not significantly different ($p < 0.05$) according to Duncan's range test.

Effects of different concentrations of IBA and TDZ on the root induction and plant performance after two months of culture are shown in Table 2. Root formation from the matured SEs was observed in the entire RI medium with a frequency that varied from 33.3% to 100.0%. The highest root induction rate (100%) was observed on the RI2 medium, while they had the lowest value of the adventitious root number per plant, the total length of roots per plant, the plant height, and the leaf number per plant among the four medium treatments. Although the regenerated plants on the RI3 medium showed a lower root induction rate (95.8%) and total root length (19.2 cm) than those cultured on the RI2 and RI1 media, respectively, they had the largest number of adventitious roots (13.7) and leaves (20.3) and the highest plant height (5.2 cm). Moreover, no significant difference was observed between the RI3 and RI2 treatments for the root induction rate, and between the RI3 and RI1 treatments for the total root length. These findings indicated that the SEs with one single shoot cultured on the RI3 medium produced the best-developed root system, which might result in the most excellent plant performance.

3.4. Plant Establishment and Genetic Stability Analysis Based on Leaf Morphological Characteristics

Finally, 116 well-developed plants (Figure 3A) regenerated from the immature seeds were acclimated ex vitro. After one month of acclimatization, approximately 82.8% of plants survived, and most plants produced many new leaves (Figure 3B). After two months of growth in the plastic pots, these plants grew normally and vigorously (Figure 3C). After growth for five months, nineteen plants were randomly selected, and descriptive statistics of leaf characteristics were carried out, as shown in Table 3. The terminal leaflet length and width ranged from 3.63 to 6.93 cm and 2.73 to 4.90 cm, with an average of 5.53 and 3.55 cm, respectively, and the lateral leaflet length and width varied from 3.42 to 6.12 cm and 2.35 to 3.88 cm, respectively. The minimum value of the leaf rachis length was only 1.67 cm, while the maximum reached 8.00 cm, which resulted in the highest coefficient of variation (CV, 48.83%) among all the analyzed parameters. The leaf rachis diameter varied from 0.69 to 1.09 mm with an average of 0.93 mm, and the leaf SPAD value reached 58.82, with a mean of 51.47. These results indicated that the in vitro-regenerated plants had a wide genetic variation in leaf morphology.

3.5. Main Morphological Variation Types and Flow Cytometry, Stomatal, and Gas Exchange Analysis

Based on close and continuous visual observation, the 19 plants could be classified into 3 types, namely normal type and two variation types (Type I and Type II), after 5 months of growth. Fourteen normal-type plants showed similar leaf morphology to the wild parent (Figure 4A), and the remaining exhibited remarkably different leaf morphology from the wild parent, including two plants of the Type I (Figure 4B) and three plants of the Type II (Figure 4C). Each compound leaf of the normal type and the Type II had three leaflets, while the common number of leaflets of the Type I was four (Figure 4A–C). In addition, the leaflets of the Type II had a dark green color with large white blotches (Figure 4C).

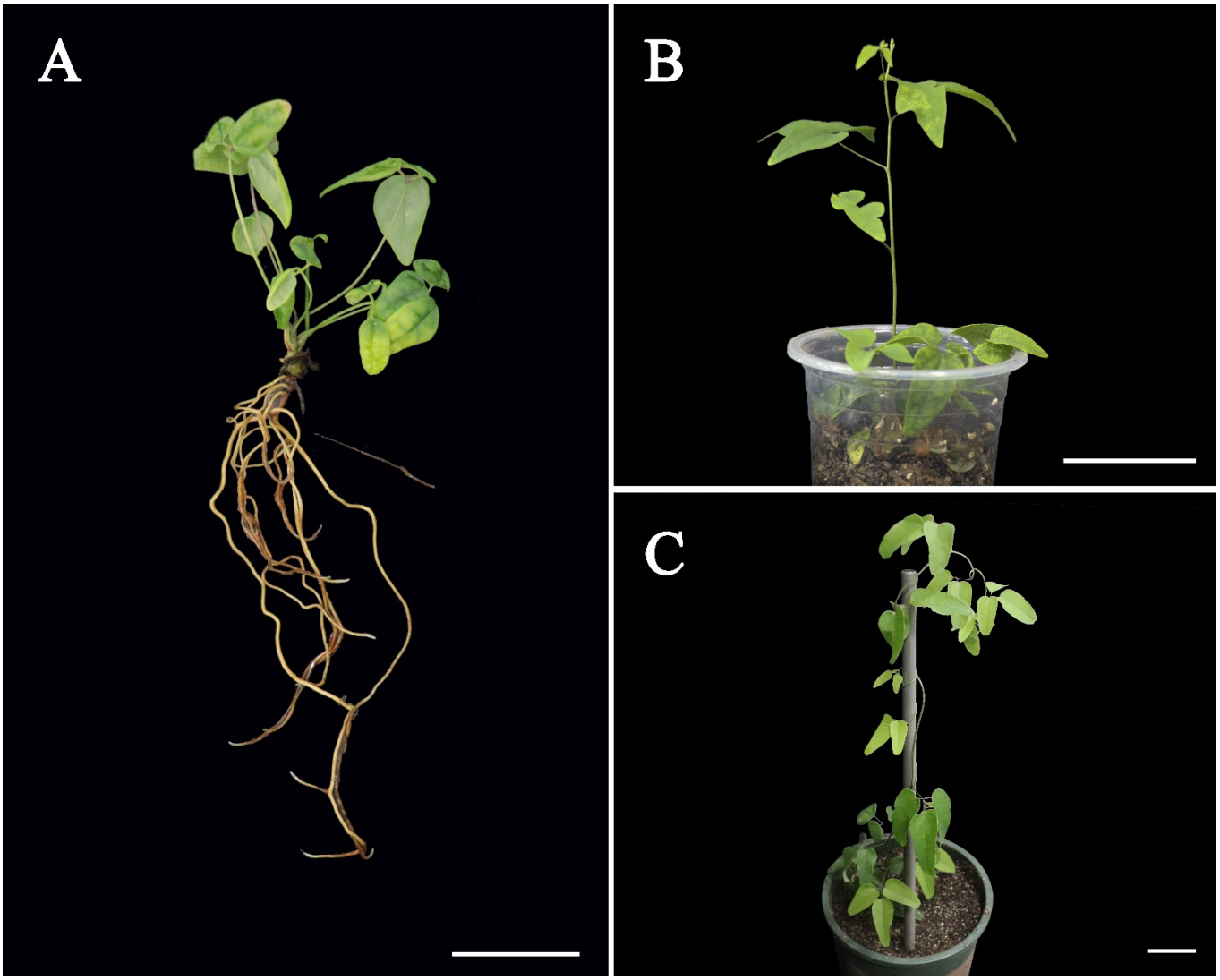

Figure 3. Plant acclimatization ex vitro and growth in plastic pots. (**A**) In vitro-regenerated plants with well-developed roots. (**B**) Surviving plants with new leaves after one month of acclimatization. (**C**) Vigorously growing plants after 2 months of culture in the plastic pots. Bars = 5 cm.

Table 3. Descriptive statistics for the leaf morphological characteristics of the plants regenerated in vitro from immature seeds of wild *A. trifoliate* after 5 months of growth in plastic pots.

Characters	Min	Max	Mean	SD	CV (%)
Terminal leaflet length (cm)	3.63	6.93	5.53	0.69	12.51
Terminal leaflet width (cm)	2.73	4.90	3.55	0.60	16.94
Lateral leaflet length (cm)	3.42	6.12	4.67	0.62	13.24
Lateral leaflet width (cm)	2.35	3.88	2.82	0.42	14.84
Leaf rachis length (cm)	1.67	8.00	3.88	1.89	48.83
Leaf rachis diameter (mm)	0.69	1.09	0.93	0.11	11.83
Leaf SPAD value	37.03	58.82	51.47	5.19	10.08

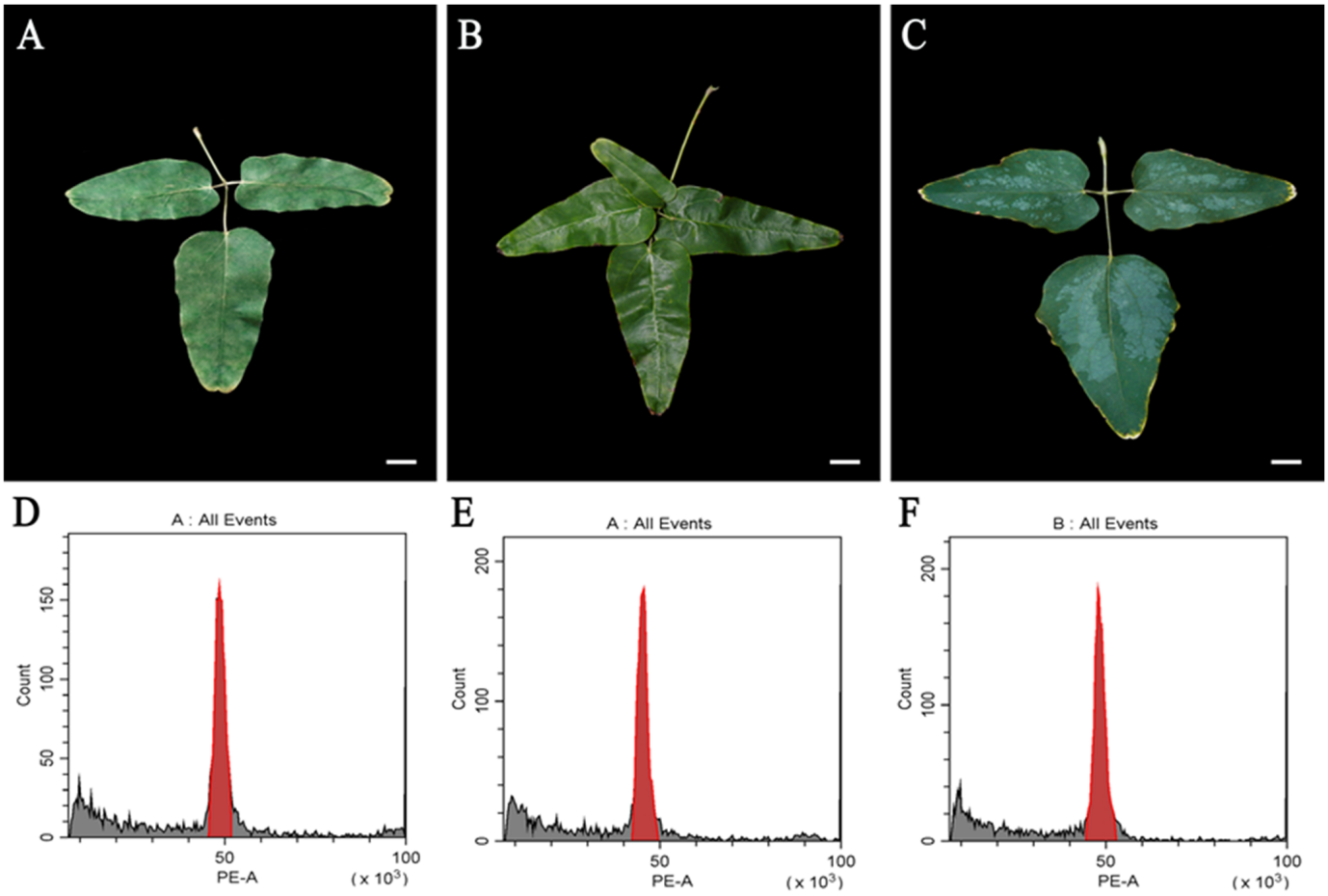

Figure 4. Leaf morphology and flow cytometry analysis of the three types of plants regenerated from the immature seeds of wild *A. trifoliate*. (**A**,**D**) Normal type: the plants showing a similar leaf phenotype to the wild plant. (**B**,**E**) Type I: each compound leaf having 4 leaflets. (**C**,**F**) Type II: the leaves exhibiting large white blotches. Bars = 5 cm.

Remarkable differences in leaf morphological characteristics were observed among the three plant types (Table 4). As compared with the normal type and Type I, the terminal leaflets of the Type II plants had a significantly higher length and width and a significantly lower length/width ratio, suggesting that the Type II had larger and rounder terminal leaflets. The Type II also had significantly longer and wider lateral leaflets, while the leaf length/width ratio was not significantly different among the three groups. A significantly longer leaf rachis (5.46 cm) was observed in the Type I, while the Type II plants shared the largest leaf rachis diameter (1.07 cm). As for the leaf SPAD value, the Type II reached the highest (55.40), followed by the normal type (51.05), which was consistent with the observed leaf color of the three types of plants.

Flow cytometry, stomatal, and gas exchange analysis were used to further compare the differences among the three types. All three types had only one main peak of relative fluorescence intensity, and the 2C peaks were all situated at a value of about 50 in the histogram (Figure 4D–F), suggesting that the ploidy level of these plants was identical. Stomatal analysis showed that no significant difference was observed among the three types in terms of the guard cell length the stomatal density (Table 4). Both the Type I and the Type II had a significantly lower guard cell width than the normal type, while no significant difference was detected between them (Table 4). The highest net photosynthetic rate, transpiration rate, and stomatal conductance were all recorded in the Type II, which was significantly higher than that of the Type I plants, respectively (Table 4). No significant difference was detected between the normal type and the Type II for the three gas exchange

parameters, while the normal type had a significantly higher transpiration rate and stomatal conductance than the Type I (Table 4).

Table 4. Morphological, stomatal, and gas exchange analysis of the three types of plants regenerated in vitro from the immature seeds of wild *A. trifoliate*.

Characteristics	Normal Type	Type I	Type II
Terminal leaflet length (cm)	5.36 ± 0.51 b	5.92 ± 0.41 b	6.86 ± 0.71 a
Terminal leaflet width (cm)	3.28 ± 0.44 b	3.10 ± 0.33 b	5.04 ± 0.65 a
Leaf length/width ratio of terminal leaflets	1.64 ± 0.09 b	1.93 ± 0.25 a	1.37 ± 0.06 c
Lateral leaflet length (cm)	4.23 ± 0.87 b	4.43 ± 0.48 b	5.81 ± 0.83 a
Lateral leaflet width (cm)	2.46 ± 0.25 b	2.71 ± 0.29 b	3.36 ± 0.75 a
Leaf length/width ratio of lateral leaflets	1.73 ± 0.24 a	1.76 ± 0.31 a	1.69 ± 0.09 a
Leaf rachis length (cm)	2.96 ± 0.43 b	5.46 ± 0.25 a	2.44 ± 0.54 b
Leaf rachis diameter (mm)	0.77 ± 0.16 b	0.91 ± 0.07 ab	1.07 ± 0.06 a
Leaf SPAD value	51.05 ± 7.55 ab	48.13 ± 3.97 b	55.40 ± 4.68 a
Stomatal guard cell length (μm)	23.80 ± 2.24 a	21.36 ± 2.08 a	21.67 ± 2.80 a
Stomatal guard cell width (μm)	17.83 ± 2.59 a	14.55 ± 1.46 b	13.28 ± 1.74 b
Stomatal density (no./mm^2)	210.94 ± 20.23 a	218.24 ± 2.54 a	203.04 ± 20.44 a
Net photosynthesis rate (μmol CO_2 m^{-2} s^{-1})	7.32 ± 0.86 ab	6.74 ± 0.20 b	7.93 ± 0.54 a
Transpiration rate (mmol H_2O m^{-2} s^{-1})	1.86 ± 0.01 a	1.59 ± 0.09 b	1.95 ± 0.32 a
Stomatal conductance (mol H_2O m^{-2} s^{-1})	0.18 ± 0.01 a	0.13 ± 0.01 b	0.19 ± 0.02 a

Means (±standard deviation) within rows followed by different letters are significantly different at the 5% level via Duncan's range test.

3.6. SSR Analysis of the Wild Parent and the in Vitro Regenerants

Genetic characterization of the 19 plants and the wild parent was performed by SSR analysis. Of the 5 pairs of SSR primers used, 4 primer combinations produced a total of 164 observable DNA bands with the desired size, and only MT15 (Figure 5A) and MT28 (Figure 5B) could clearly detect polymorphisms between the regenerants and their donor parent. The MT15 and the MT28 primers amplified a total of 94 bands, of which 41 (43.62%) were polymorphic compared with the wild parent (Table 5). The maximum percentage of polymorphic bands (59.57%) was amplified by the MT15 primer, of which 28 of 47 amplified bands were polymorphic (Table 5). The three plant types also exhibited various degrees of polymorphism within their groups. For the two Type I plants, both exhibited two new alleles of 200–300 bp with the primer MT15 (Figure 5A, lanes 6–7), and lost one small DNA band of about 200 bp amplified by the primer MT28 (Figure 5B, lanes 6–7). As for the three Type II plants, two plants yielded one new allele of 200–300 bp and one plant had two new alleles of 200–300 bp amplified by the primer MT15 (Figure 5A, lanes 18–20), whereas two plants had similar DNA banding profiles to the wild plant and the remaining lost one DNA band of about 200 bp with the primer MT28 (Figure 5B, lanes 18–20). Furthermore, some polymorphism DNA bands were also detected among the 14 plants of the normal-type group, especially when amplified by the MT15 primer. These results suggested that the regenerants from the immature seeds of *A. trifoliata* exhibited a high degree of polymorphism via SSR analysis.

Table 5. Number of amplified bands, number of polymorphic bands, and percentage of polymorphic bands between the wild parent and the regenerants.

Primers	No. of Amplified Bands	No. of Polymorphic Bands	Percentage of Polymorphic Bands (%)
MT15	47	28	59.57
MT28	47	13	14.89

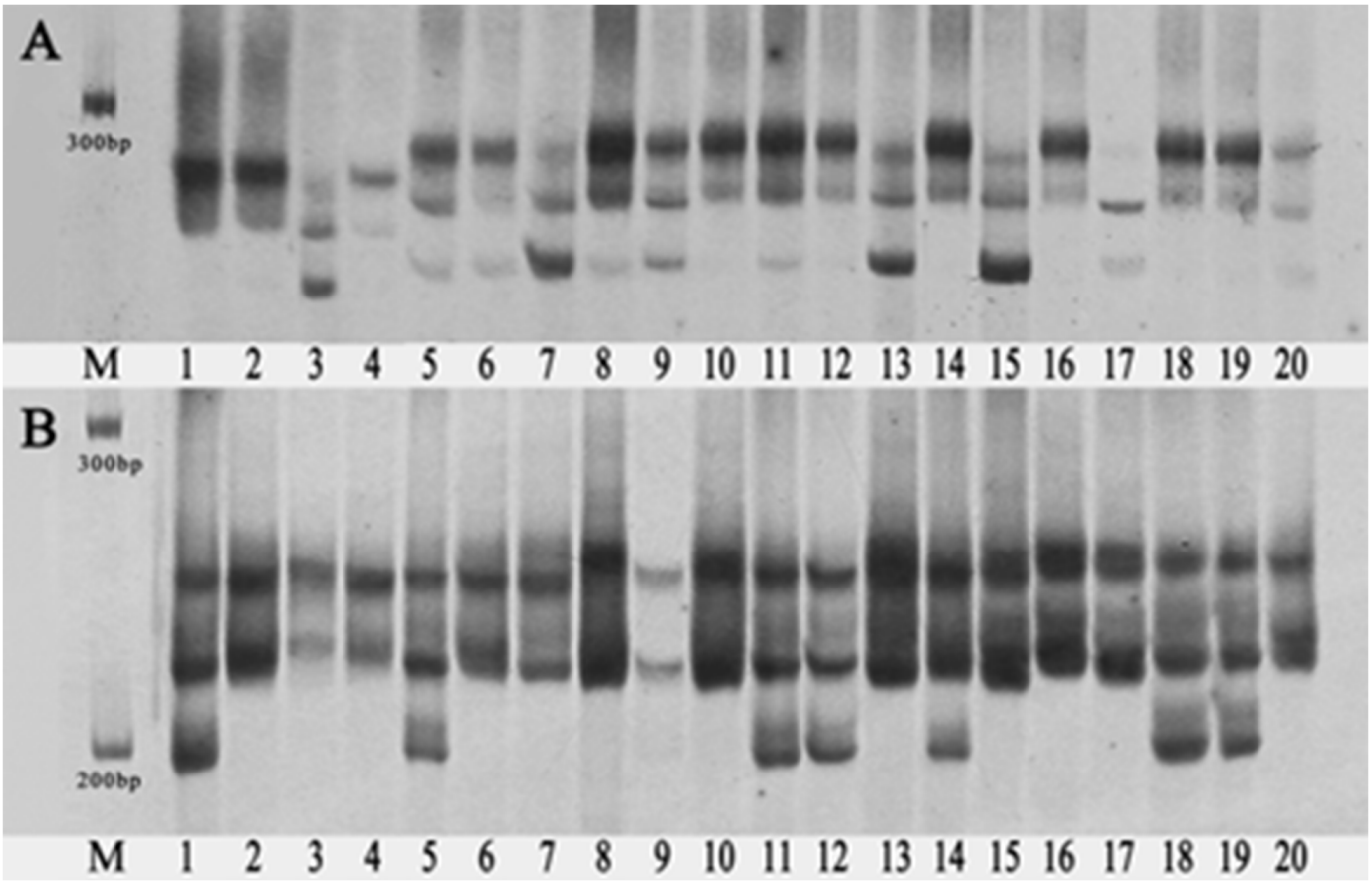

Figure 5. SSR profiles of the wild plant and 19 randomly selected plants regenerated from immature seeds of wild *A. trifoliate*. (**A**) Banding patterns of SSR marker MT15. (**B**) Banding patterns of SSR marker MT28. M: 1000 bp DNA marker; 1: the wild plant; 2–20: the 19 randomly selected plants, including two Type I plants (lanes 6–7) and three Type II plants (lanes 18–20).

4. Discussion

4.1. Factors Affecting SE Induction

SEs can be in vitro-induced from a range of plant explant types, such as embryogenic callus tissues, cotyledons, and zygotic embryos, under appropriate culture conditions [9,16,30]. In general, immature tissues and organs are easier to produce SEs compared with old or adult plant explants [31], and immature embryos were the most frequently used explants in somatic embryogenesis [16,19]. As far as we know, SEs have been successfully induced only from the embryos from immature or mature seeds in *A. trifoliata* [19,20]. However, the process of embryo isolation was difficult due to their small size and hard seed coats, which may result in a high frequency of explant contamination [20]. In this study, the intact seeds of a wild *A. trifoliata* were used as explants and SEs were successfully induced from the seed surfaces, which might avoid the difficulties in isolating embryos from the seeds compared with the previous reports [19,20]. It has also been reported that immature seeds were directly used as the initial materials for successful somatic embryos' induction in other woody crops, such as *Pinus attenuate* × *P. radiata* [13] and *Citrus limon* [32].

It has been documented that nutrient medium and PGRs have a great influence on SEs' induction [30,33]. To support the growth and development of the explants in tissue culture, WPM medium is frequently used for woody plants, whereas MS medium is often applied in herbaceous plants [34]. In this study, we also found that WPM medium was more suitable for somatic embryogenesis in *A. trifoliata* (a perennial woody vine) than MS medium. In addition, TDZ is believed to have stronger effects on plant growth and development in vitro and has been reported to be effective in the induction of various

morphogenic responses, including somatic embryogenesis [30,33,35]. In this study, the SEs were induced with the best results on the medium containing 1.0 mg L^{-1} of TDZ and 1.0 mg L^{-1} of 6-BA. Studies on somatic embryogenesis of *Hippeastrum hybridum* [30] and *Cajanus cajan* [35] have also demonstrated that the presence of TDZ in the medium increased the induction frequency of SEs.

Besides, several other factors, such as plant genotypes and the developmental stage of the explants, can strongly affect the induction frequency of SEs [16,18]. The highest frequency of SE induction was only 35.2% in the present study, which was much lower compared with the report on the SE induction from immature embryos of *A. trifoliata* by Zou et al. [19]. This might be attributed to the differences in the genetic background and fruit harvesting time. Faisal et al. [18] also found that the harvest days of the immature seeds of *Brassica juncea* had a significant impact on in vitro embryogenesis. In addition, although it is more convenient when using the intact immature seeds as explants compared with the isolated embryos, the seed coats may limit in vitro development of the immature embryos in this study.

4.2. Pathways of Plant Regeneration via Somatic Embryogenesis

A critical step in somatic embryogenesis is the dedifferentiation of somatic cells into totipotent embryonic cells [12,36]. In general, two different regeneration methods are commonly involved in somatic embryogenesis, including direct and indirect somatic embryogenesis [18,30,32]. Direct somatic embryogenesis is often observed when microspores, ovules, and zygotic embryos are used as explant materials [36]. In *A. trifoliate*, Zou et al. [19] reported that SEs arose directly from the root zone of the immature embryos, whereas calli were firstly induced from the excising embryos from the mature seeds and then plants were regenerated via somatic embryogenesis [20]. In the present study, the SEs were also directly induced from the surface of the immature seeds after two months of culture on the SEI2 and SEI5 media. These results indicated that seed maturity has a determining effect on the subsequent regeneration pathway in *A. trifoliate* using the seeds or embryos as initial explants. In addition, previous studies suggested that the types and concentrations of PRGs are crucial factors in determining the developmental pathways of somatic embryogenesis [12,31,36]. For example, Zhang et al. [31] observed direct and indirect somatic embryogenesis in *Camellia oleifera* when the immature seeds were cultured in the medium containing 2,4-D alone and the combination of 2,4-D and TDZ, respectively. In the present study, no callus was observed on any culture medium with or without 2,4-D or TDZ during the SEs' induction. These results suggested that whether there are determinative effects of PGRs on callus formation in somatic embryogenesis may vary from species to species.

4.3. SE Maturation and Plant Regeneration

Somatic embryogenesis involves several complex developmental processes, including pro-embryogenic mass formation and the formation, maturation, and conversion of SEs [14,36]. Generally, exogenous auxin is vital for SEs' proliferation and their early development, but maturation and germination can be carried out on a PGR-free medium once SEs are formed [9,16,36]. From the current investigation, we found that a medium concentration of 6-BA (1.0 mg L^{-1}) was the most responsive for the development and germination of the SEs, and the regenerated plants had a high survival rate and vigorously grew in plastic pots. A certain concentration of 6-BA had a positive influence on embryo maturation, which was also reported in *Ormosia henryi* [17] and *Citrus limon* [32]. In addition, several stress treatments, such as treating with abscisic acid (ABA), increasing sucrose concentration, and cold storage [12,37,38], are considered effective for stimulating the maturation of SEs. In this study, it took too long (four months) for SE maturation and conversion, and hence some stress treatments, as mentioned above, should be attempted in the future to shorten the period of SE maturation.

Furthermore, a well-developed root system is crucial to the late growth of SE-derived plants. However, a low rooting percentage and poor performance of the regenerated

plants remain constraints in somatic embryogenesis in some plant species [37]. In this study, the matured SEs also had a low rooting percentage and a poor shoot performance when cultured on the SEM media. In somatic embryogenesis, abnormal SEs may lead to a poor germination rate and lose the ability to convert into normal plants [39]. The occurrence of malformed somatic embryos is one of the common phenomena during plant somatic embryogenesis, which might be related to many factors, such as plant genetic background, medium composition, and culture condition [12,16]. In this study, most of the induced cotyledonary embryos were malformed, which may explain their low rooting frequency and poor shoot development. Jiang et al. [20] also observed that the matured SEs of *A. trifoliate* had a very low rooting rate, and it was necessary to transfer the germinated embryos onto a fresh medium to produce vigorously growing plants. In this study, a high rooting capacity and a well-developed root system were also observed when the germinated SEs were cultured on the RI3 medium for two months.

4.4. Genetic Stability Analysis of the Regenerated Plants from the Immature Seeds via Somatic Embryogenesis

It is essential to assess the genetic uniformity of in vitro-regenerated plants before large-scale production for commercial application [8,9], as somaclonal variation has been frequently observed during tissue culture [40–42]. In general, the genetic stability of in vitro-produced plants can be assessed via analysis of phenotypic traits, the ploidy level, and genetic markers [26,40,43]. However, many reports have shown a limited level of somaclonal variations among the recovered plants in somatic embryogenesis, especially in direct somatic embryogenesis [8,18]. Contrary to these studies, our results showed that the 19 randomly selected regenerants had a wide genetic variation via morphological analysis and SSR characterization. Wild *A. trifoliata* is self-incompatible, and significant differences in morphological traits have been recorded among their sexual progenies [1], indicating a high genetic heterozygosity of the seeds. Therefore, the high genetic differences of the regenerants obtained in this study could be largely attributed to the genetic heterogeneity of the initial explants used (the immature seeds harvested from a wild *A. trifoliate* tree ten weeks after open pollination) and the potential epigenetic variation generated during in vitro culture. Although the obtained plants had a high degree of genetic variation, they might be valuable germplasm materials for genetic improvement and breeding of this crop. In addition, true-to-type plants might be produced via somatic embryogenesis when the initial explants of the same genetic composition, such as the same cell line, or a single seed are used. Therefore, the established protocol would contribute to mass propagation of *A. trifoliate* via somatic embryogenesis.

Additionally, wild resources of *A. trifoliata* have gradually declined in China due to overharvesting and habitat deterioration over the past decades [22], and thus germplasm conservation is very necessary for biodiversity and genetic breeding. In vitro conservation is a widely used and efficient method to maintain plant materials, especially in asexual propagated species or the plants producing recalcitrant seeds [12,21]. The seeds of *A. trifoliata* are very sensitive to desiccation [7], which makes them unsuitable for establishing seed banks. In this study, we established a simple protocol for SE induction and plant regeneration in wild *A. trifoliata*. Although a relatively high degree of genetic variation was observed among the regenerants, the established method could be helpful not only for in vitro propagation but also for germplasm conservation in *A. trifoliate*.

5. Conclusions

In this study, we established a simple and practicable method of producing whole plants from immature seeds of *A. trifoliata* via direct somatic embryogenesis, and the genetic uniformity of the regenerated plants was also evaluated. A significantly higher frequency of SE induction was obtained on the WPM medium containing 1.0 mg L^{-1} of TDZ and 1.0 mg L^{-1} of 6-BA. The presence of 1.0 mg L^{-1} of 6-BA in the WPM medium significantly improved the proliferation and maturation of the induced SEs, and the addition of

2.0 mg L^{-1} of IBA and 0.5 mg L^{-1} of TDZ was the most responsive for root system development and plant growth. A wide genetic variation was found among the regenerants based on morphological, stomatal, flow cytometric, gas exchange, and SSR analysis, which could provide useful plant materials for future genetic improvement and breeding in *A. trifoliata*. Moreover, the established in vitro regeneration protocol might serve as a foundation for in vitro propagation and germplasm conservation of this crop.

Author Contributions: Conceptualization and data curation, Y.Z. and Y.C.; formal analysis, Y.C. and Y.W.; funding acquisition, X.C.; investigation, Y.Z. and Y.W.; methodology, Y.C. and X.C.; writing—review and editing, X.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Key Research and Development Project of Hubei Province (No. 2021BBA096).

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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