Efficient plant regeneration system for New Guinea *Impatiens* (*Impatiens hawkeri* W. Bull) CV. ‘Violet’ and ‘Scarlet Bronze Leaf’

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Abstract

New Guinea *Impatiens* (*Impatiens hawkeri* W. Bull) is an eye-popping landscaping plant of bright and colorful blooms. A highly efficient in vitro plant regeneration system via direct shoot organogenesis was established for the first time from hypocotyl with partial cotyledons of New Guinea *Impatiens*. Our results showed that the explant sterilization method, basic medium type, AgNO₃, sucrose and plant growth regulators have significantly influenced in vitro morphogenesis. The regeneration rate in regeneration media that MS supplemented with 0.5 mg·L⁻¹ TDZ and 0.1 mg·L⁻¹ NAA was acceptable, the induction rate of ‘Violet’ was 86.67%, and its proliferation coefficient was 5.27, while the induction rate of ‘Scarlet Bronze Leaf’ was 83.33%, and its proliferation coefficient 5.13. PIC could not induce clumped sprouts; however, it had a better effect on callus induction. We also included a shoot multiplication stage using regeneration New Guinea *Impatiens* medium that MS supplemented with 0.8 mg·L⁻¹ 6-BA, 0.5 mg·L⁻¹ TDZ and 0.05 mg·L⁻¹ NAA. Reducing sucrose concentration to 20 g·L⁻¹ or adding 1 mg·L⁻¹ AgNO₃ could alleviate the hyperhydricity phenomenon in the process of tufted bud proliferation. The optimal root culture medium for the regenerated seedlings of ‘Violet’ and ‘scarlet bronze leaf’ of New Guinea *Impatiens* was MS supplemented with 0.05 mg·L⁻¹ IBA, the rooting rate reached 100%. The study examined the micropropagation responses of New Guinea *Impatiens* in the presence of various growth regulators, which provide a simple and more suitable protocol adapted for the mass propagation of clones.

Key message

A protocol for high-frequency shoot induction from hypocotyls with some cotyledons has been standardized for New Guinea *Impatiens* (*Impatiens hawkeri* W. Bull) CV. ‘Violet’ and ‘Scarlet Bronze Leaf’.

Keywords

New Guinea *Impatiens* · High-efficiency regeneration system · In vitro · Tissue culture · Hyperhydricity

Abbreviations

| Abbreviation | Description                      |
|--------------|----------------------------------|
| MS           | Murashige and Skoogs medium       |
| 1/2 MS       | 1/2 Murashige and Skoog Medium    |
| B5           | Gamborg                          |
| N6           | Chu’s N-6 Medium                 |
| PGRs         | Plant growth regulators          |
| TDZ          | Thidiazuron                      |
| IAA          | Indole acetic acid               |
| IBA          | Indole butyric acid              |
| 2,4-D        | 2,4-Dichlorophenoxy acetic acid  |
| PIC          | Picloram                         |
| NAA          | 1-Naphthalene acetic acid        |
| ANOVA        | Analysis of variance             |

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Introduction

New Guinea *Impatiens* (*Impatiens hawkeri* W. Bull) is a perennial evergreen herb, which has the characteristics of a long flowering period, rich flower color, and high ornamental...
value, thus it has become an important flower in pots and flower beds in the world (Fu et al. 2007). Its discovery originated in 1884 when Kew Garden in England received an Impatiens herb specimen from Dr. Schomburgk, curator of the Adelaide Botanical Garden in Australia, and marked that the specimen had been collected by Lieutenant hawker (Staf-leu et al. 1976). It was introduced into Europe and the United States after 1886. Since the launch of the 'Circus' (circus) series in 1972, more than one hundred series have been successfully launched and distributed worldwide, and most of the varieties have obtained the right to breed (Linzi 2007).

The main breeding forms of New Guinea Impatiens were seed, rootless cuttage and cuttings. There is little literature about tissue culture and the transformation of New Guinea Impatiens. At present, the available articles on New Guinea Impatiens tissue culture are mainly involved in vitro propagation (Stephens et al. 1985; Han et al. 1987; Witomska et al. 2003), in vitro germination of immature ovules (Han et al. 1992), in vitro regeneration (Taha et al. 2009), callus culture (He et al. 1989; Josekutty et al. 1998), growth of cotyledon sections (Han et al. 1994), embryo and ovule culture in vitro (Arisumi et al. 1980), formation of secondary products in cell culture (Panichayupakaranant et al. 2001), etc. To the best of our knowledge, there is no report on establishing an efficient regeneration system of New Guinea Impatiens. Therefore, the establishment of an efficient regeneration system for New Guinea Impatiens is important.

Taking the seeds of New Guinea Impatiens ‘violet’ and ‘scarlet bronze leaf’ with good ornamental characteristics and widely used in the landscape recently as materials, this study used single factor and multi-factor orthogonal experiments to explore the suitable conditions for seed disinfection and sterilization, induction of cluster buds, the proliferation of cluster buds and rooting and strengthening seedlings of cluster buds. To establish an efficient regeneration system of New Guinea Impatiens, which provided a certain degree of technical support for solving the defects of traditional breeding methods and laid a foundation for the follow-up research of transgenic genetic transformation and molecular breeding.

Materials and methods

Plant materials and explants preparation

In this case, two varieties ‘violet’ and ‘scarlet bronze leaf’ of New Guinea Impatiens divine series were used as explants (Fig. 1). The seeds used in this experiment were purchased uniformly from Bauer (ball seed) Company in the United States.

Wrapped New Guinea Impatiens’ seeds in gauze, fixed them with a rubber band and rinsed for 30–40 min with clean water. Then, put the seeds after the above treatment into the ultra-clean worktable for sterilization operation. A total of 9 different disinfection treatments were set up and the disinfection scheme is shown in Table 1. Finally, the sterilized seeds were dried on the sterilized filter paper and then put into the pre-prepared MS medium for culture. Each disinfection treatment was inoculated with 10 bottles, each bottle was inoculated with two seeds, repeated 3 times. Two weeks later, the contamination rate and germination rate were counted. To establish an efficient regeneration system, enough clustered buds must be obtained, according to the results of previous induction of clustered buds of African Impatiens (Impatiens walleriana Hook.f).

The hypocotyls with partial cotyledons of the germinated New Guinea Impatiens ‘Violet’ and ‘Scarlet Bronze Leaf’ after disinfection were used as explants and cultured in MS medium with hormone concentration of 0.4mg·L−16-BA and 0.5mg·L−1TDZ (Dan et al. 2010).

Fig. 1 Pictures of plant materials used in this experiment. a and b ‘Violet’ flowers and seeds. c and d ‘Scarlet Bronze Leaf’ flowers and Seeds

Culture medium and culture conditions

Medium: MS (Murashige and Skoog 1962), 1/2MS, B5 (Gamborg et al. 1968) and N6 (Zhu et al. 1974) were used as the basic medium in this experiment. Various concentrations of plant growth regulators, 0.7% Agar and 3% sucrose were added to the culture medium before autoclaving. The pH value was adjusted to 5.8–6.0 using 1 mol·L−1 NaOH/1 mol·L−1 HCl and autoclaved at 121 °C for 20 min. PGRs were added to the culture medium after filter sterilization (0.22 µm). The inoculated cultures were maintained at 25 ± 2 °C with 16 h photoperiod under white fluorescent light and 8 h dark.
To examine the effects of PGRs on in vitro morphogenesis, the concentrations of 0.1 mg·L⁻¹ NAA and different concentrations of 6-BA, TDZ, and PIC (a novel synthetic hormone with high cytokinin activity) were added to the basic medium. Hypocotyls of ‘violet’ and ‘scarlet bronze leaf’ explants with partial cotyledons were inoculated in a basic medium (I1-I12), as listed in Table 2.

Fifteen bottles were inoculated per treatment, and two explants were placed per bottle. The sprouting of clumped buds was continuously observed every week, and the growth of clumped buds, induction rate and proliferation coefficient of different treatments were recorded after 4–5 weeks.

### Induction of clustered buds of New Guinea Impatiens

To explore the effects of PGRs on in vitro morphogenesis, the concentrations of 0.1 mg·L⁻¹ NAA and different concentrations of 6-BA, TDZ, and PIC (a novel synthetic hormone with high cytokinin activity) were added to the basic medium. Hypocotyls of ‘violet’ and ‘scarlet bronze leaf’ explants with partial cotyledons were inoculated in a basic medium (I1-I12), as listed in Table 2.

Fifteen bottles were inoculated per treatment, and two explants were placed per bottle. The sprouting of clumped buds was continuously observed every week, and the growth of clumped buds, induction rate and proliferation coefficient of different treatments were recorded after 4–5 weeks.

### Proliferation of cluster buds of New Guinea Impatiens

To explore a better proliferation of cluster buds, MS (P1-P4), 1/2MS (P5-P8), B5 (P9-P12) and N6 (P13-P16) were used as the basic medium. The orthogonal test of L16 (44) was designed by adding different concentrations of 6-BA (0.4, 0.8, 1.2, and 1.6 mg·L⁻¹), TDZ (0.25, 0.5, 0.75, and 1 mg·L⁻¹), NAA (0.01, 0.05, 0.1, and 0.2 mg·L⁻¹). The tufted buds of two varieties of New Guinea Impatiens with good growth and the same subculture times were selected, cut into small pieces of 0.5 cm × 0.5 cm. They were randomly inoculated in different multiplication medium combinations (P1-P16). Each treatment was inoculated with 15 bottles, and each bottle was inoculated with 2 pieces. After 4 weeks of culture, the proliferation of tufted buds was imaged, the proliferation coefficient and seedling height were calculated.

### Rooting of regenerated seedlings of New Guinea Impatiens

When the height of the stem segment of the single seedling with clustered buds reached more than 3cm, the well-growing clump buds were divided into individual seedlings and inoculated in the MS medium supplemented with NAA(R2-R5), IBA(R6-R9) and 6-BA(R10-R13). Each hormone was set with four gradients of 0.025, 0.050, 0.100 and 0.200 mg·L⁻¹, and the MS medium without any hormone served as the control (R1), as listed in Table 3.

### Statistical analysis

Contamination rate (%) = (total number of contaminated seeds/total number of seeds inoculated) × 100%

Germination rate (%) = (total number of germinated seeds/total number of seeds inoculated) × 100%

Induction rate of clustered buds (%) = (total number of germinated explants/total number of inoculated explants) × 100%

Proliferation coefficient = total number of buds produced after multiplication/number of buds inoculated

Rooting rate (%) = (total number of rooting explants/total number of inoculated explants) × 100%

The variance analysis of the data was conducted using SPSS statistics software. Multiple comparisons were made by the LSD method to test the significant differences of each factor (P < 0.05).
Results and discussion

Effects of different sterilization treatments on seed germination of New Guinea Impatiens

By controlling 70% alcohol sterilization for 15 seconds, the study demonstrates that different 2% NaClO disinfection lengths of time have significant impacts on both germination rate and contamination rate; these results also further highlight a decreasing tendency in contamination rate with an additional period of disinfection time, while seed germination rate exhibits an upward trend and follows by a downward trend at some point of time (Table 1). Moreover, various plant seeds tend to have different tolerances to different disinfection reagents, and this is consistent with the statement proposed by Jones, who suggests that the seed germination rate would decrease significantly despite an improvement in sterilization effect given an additional period of sterilization time (Jones et al. 1983). Moreover, under 2% NaClO treatment time, the seed germination rate of 'Violet' reaches the maximum value of 88.33% in 12 min (S5), while the seed germination rate of 'Scarlet Bronze Leaf' reaches the maximum value of 86.67% in 10 min (S4); meanwhile, the seed contamination rate reaches to the minimum value in 15 min (S6). Under the premise of 2% NaClO sterilization treatment for 10 min, both germination rate increases first and then decrease as 70% alcohol sterilization time goes by; in contrast, the contamination rate showed a downward trend over time, which indicates that 70% of alcohol has a good effect on seed sterilization, however, takes disadvantage in seed germination (Fig. 2 a, b). Therefore, regarding this finding, the treatment time of alcohol should be controlled. The similar results obtained from Ye Wei Yan further imply a certain degree of agreement in this experiment, where Ye Wei Yan conclude that the contamination rate of grapefruit seeds would not decrease significantly even if an extra sterilization time using 75% alcohol were provided (Ye et al. 2015); however, with a strikingly reduce in seed germination rate. Hence, the best disinfection scheme in this experiment is as follows: usage of 70% alcohol 15 s and 12 min disinfection time of 2%NaClO for the seeds of New Guinea Impatiens' Violet', the contamination rate of which is 10% and a maximum 88.33% of germination rate. For the seeds of New Guinea Impatiens' Scarlet Bronze Leaf', 70% alcohol 15 s and 10 min disinfection time of 2%NaClO are suggested, the contamination rate of which is 10% and the germination rate is 86.67%. They both present significant differences in this experiment. However, results here are inconsistent with Guo Yun gui's conclusion, which concerns that 70% alcohol and 15–20 min of 2%NaClO as the best disinfection scheme (Guo et al. 2012), the reasons for the differences in outcomes may be related to the variety, seed quality and pretreatment time of Impatiens balsamina.
Effects of different concentrations of PGR on tufted bud induction of ‘Violet’ and ‘Scarlet Bronze Leaf’ of New Guinea Impatiens

6-BA and TDZ are two types of cytokinins commonly used in tissue culture and plant regeneration of Impatiens. They can promote cell division, differentiation, elongation or regulation of endogenous hormones and metabolic synthesis of CTK by removing apical dominance (Wang et al. 1996). In this way, both can contribute to the induction of tufted buds (Miloševic et al. 2011). In this experiment, the effects of 0.1 mg·L⁻¹ NAA and different concentrations of 6-BA, TDZ and PIC on cluster bud induction of New Guinea Impatiens were compared. As listed in Table 2, for New Guinea Impatiens ‘Violet’ and ‘Scarlet Bronze Leaf’, no matter 6-BA or TDZ is used, the induction rate and increment coefficient of regenerated seedlings both show a trend from rising to decline as rising in hormone concentration. For the growth medium that only contains 6-BA, the induction frequency (‘Violet’ was 83.33%, ‘Scarlet Bronze Leaf’ was 80%) and proliferation coefficient (‘Violet’ was 2.73 ± 0.30, ‘Scarlet Bronze Leaf’ was 2.67 ± 0.30) reached the maximum value when the concentration of 6-BA is 0.8 mg·L⁻¹ (Fig. 2 c, d). Similarly, for the growth medium with only TDZ, the induction frequency (‘Violet’ was 86.67%, ‘Scarlet Bronze Leaf’ was 83.33%) and proliferation coefficient (‘Violet’ was 5.27 ± 0.59 ‘Scarlet Bronze Leaf’ was 5.13 ± 0.60) reached their maximum value when the concentration of TDZ approaches 0.5 mg·L⁻¹ (Fig. 2 e, f), which reveals a significant difference when comparing the results with the other three media with only TDZ concentration. In conclusion, the experiment shows that a combination of TDZ and NAA have a better induction ability of cluster bud than 6-BA and NAA, this outcome corroborates with the previous study on the induction of African Impatiens (I. walleriana) (Dan et al. 2010). Besides, Pavanichirumamilla et al. found that TDZ positively affected cluster bud induction in the Kashi eggplant regeneration system (chirumamilla et al. 2021). This is probably because the cytokinin activity of TDZ is more active than other cytokinins (Bhattacharyya et al. 2016), while other scholars conjecture that TDZ can induce explants to produce endogenous IAA (Guang et al. 2010). In this experiment, although the proliferation coefficient of tufted buds induced by 6-BA and NAA is low, the combination of both could induce root formation as well as help buds form a callus, this finding in our paper lends support to previous results of Taha A and Han who focus on in vitro regeneration of Impatiens cotyledons and cucurbit cotyledons. Whether the combination of 6-BA and NAA or TDZ and NAA, both induction rate and proliferation coefficient of ‘Violet’ are always slightly better than ‘Scarlet Bronze Leaf’, proving that genotypes have a certain effect on plant regeneration. Overall, the combination of MS with 0.5 mg·L⁻³ TDZ and 0.1 mg·L⁻¹ NAA have the best effect on the induction of clustered buds for the two varieties and exhibits a significant difference. Likewise, the results denote the 86.67% induction rate of ‘Violet’ and a value of 5.27 for its proliferation coefficient, while the induction rate of ‘Scarlet Bronze Leaf’ is 83.33%, and its proliferation coefficient is 5.13. Despite the amount of PIC concentration added to ‘Violet’ and ‘Scarlet Bronze Leaf’ of New Guinea Impatiens, the buds cannot be induced, yet with more culture time, the explants not

| Culture medium | PGR( mg·L⁻¹) | Induction frequency (%) | Proliferation coefficient |
|---------------|---------------|------------------------|--------------------------|
|               | NAA | BA | TDZ | PIC | Violet | Scarlet Bronze Leaf | Violet | Scarlet Bronze Leaf |
| I1            | 0.1 | 0.4 |   |     | 76.67 | 70 | 1.53 ± 0.20ab | 1.50 ± 0.21ab |
| I2            | 0.1 | 0.8 |   |     | 83.33 | 80 | 2.73 ± 0.30ab | 2.67 ± 0.30ab |
| I3            | 0.1 | 1.6 |   |     | 80 | 73.33 | 2.57 ± 0.29ab | 2.53 ± 0.34ab |
| I4            | 0.1 | 3.2 |   |     | 63.33 | 66.67 | 1.47 ± 0.27ab | 2.23 ± 0.37ab |
| I5            | 0.1 | 0.3 |   |     | 80 | 70 | 2.17 ± 0.25bc | 2.07 ± 0.30bc |
| I6            | 0.1 | 0.5 |   |     | 86.67 | 83.33 | 5.27 ± 0.59ab | 5.13 ± 0.60ab |
| I7            | 0.1 | 1   |   |     | 80 | 90 | 3.60 ± 0.50bc | 3.97 ± 0.43bc |
| I8            | 0.1 | 2   |   |     | 56.67 | 66.67 | 1.63 ± 0.31bc | 1.87 ± 0.30bc |
| I9            | 0.1 | 0.5 |   |     |     |     |             |             |
| I10           | 0.1 | 1   |   |     |     |     |             |             |
| I11           | 0.1 | 2   |   |     |     |     |             |             |
| I12           | 0.1 | 4   |   |     |     |     |             |             |

Each treatment was inoculated with 15 bottles, and two explants were placed in each bottle. The data represent the mean ± se. According to least significance difference, there was no significant difference in the mean after the same letter (P = 0.05).
only gradually turn yellow from the base to the leaves, but also forms callus; meanwhile, the degree of callus increase along with the additional increase in PIC concentration. In studying the tissue culture of Gasteria verrucosa haw and Haworthia fasciata haw (Beyl et al. 1983), Beyl and Sharma also discovered that pic was a good callus inducer, which is consistent with our results. Zhou Yin et al. (Yin et al. 2013) found that the proper increase of PIC concentration was beneficial to inducing Cymbidium clump buds, which contradicts experimental results. Hence, we suspect that the role of PIC in plant tissue culture depends on its ratio to cytokinins.

**Effects of different hormone concentrations and medium types on the proliferation of rosette buds of New Guinea *Impatiens***

In the proliferation process of tufted buds, the type and concentration ratio of cytokinin and auxin in culture medium is usually considered to play a key role in regulating the growth and differentiation of clustered buds (Mu et al. 2011). Xu et al. proposed that plant organ differentiation is regulated by two kinds of hormones (auxin and cytokinin) (Xu et al. 1996). All the treatments in this experiment can differentiate into clustered buds (Table 3), where treatment P2 is the best in terms of proliferation coefficient, seedling height and hyperhydricity rate, and there is a significant difference between treatment P2 and other treatments. ‘Violet’ proliferation coefficient reached 13.18, ‘Scarlet Bronze Leaf’ multiplication coefficient reached 11.52, the average plant height of ‘Scarlet Bronze Leaf’ reached 1.46 cm, and the average plant height of ‘Scarlet Bronze Leaf’ reached 1.19 cm. The hyperhydricity rate of ‘Violet’ is 13.33%. The hyperhydricity rate of ‘Scarlet Bronze Leaf’ is 20.00%. At the same time, the cluster buds proliferated by P2 were treated, the buds were dense, and the color was bright green. The effects of basic medium, 6-BA, TDZ, and NAA on the proliferation, seedling height, and hyperhydricity rate of ‘Violet’ and ‘Scarlet Bronze Leaf’ clustered buds were compared.

NAA and TDZ on the proliferation coefficient of New Guinea *Impatiens* clustered buds reached a significant level (Table 4), while the effect of 6-BA on the proliferation of clustered buds had no significant difference. The results showed that the basic medium, TDZ and NAA played a decisive role in the proliferation of rosette buds of New Guinea *Impatiens*, while the concentration of 6-BA did not play a decisive role in the proliferation of tufted buds. Among them, TDZ had a very significant effect on the proliferation of ‘Violet’ clustered buds, while it had a significant effect on the proliferation of ‘Scarlet Bronze Leaf’ clustered buds', indicating that Violet’ was more sensitive to TDZ than ‘Scarlet Bronze Leaf’ in the proliferation of clustered buds. This may have a relationship with the plant’s genes.

Among the four selected media, MS medium is significantly better than the other three media (Table 3), and the average proliferation coefficient of ‘Violet’ can reach 9.74 ± 0.41. The average increment coefficient of ‘Scarlet Bronze Leaf’ can reach 8.91 ± 0.39. Therefore, MS medium had a good effect on the proliferation of tufted buds, and similar results were also found in the proliferation culture of legumes (Siddique et al. 2007; Perveen et al. 2012). It shows that inorganic salts, large amounts and trace elements are indispensable hard nutrients in the tissue culture of *Impatiens balsamina* in New Guinea. Once there is a lack of nutrition, it is extremely disadvantageous to the proliferation and growth of clustered buds. The proliferation coefficient of clustered buds increased at first and then decreased with the increase of TDZ concentration. The average value-added coefficient of both New Guinea *Impatiens* reached the best when the concentration of TDZ was 0.5 mg·L⁻¹. The average value-added coefficient of ‘Violet’ can reach 5.83 ± 0.43, the average value-added coefficient of ‘Scarlet Bronze Leaf’ can reach 4.55 ± 0.36. Moreover, it was significantly different from the other three concentration levels. The results showed that in the proliferation process of clustered buds, the concentration of auxin and cytokinin should be appropriate, and too high or too low would inhibit its differentiation and be disadvantageous to its growth.

For ‘Violet’, there is a very significant difference between basic medium and TDZ on the seedling height of clustered buds (Table 5). For ‘Scarlet Bronze Leaf’, the basic medium has an extremely significant difference in the seedling height of clustered buds. In contrast, 6-BA and NAA have significant differences in the seedling height of clustered buds, indicating that the choice of basic medium plays an important role in the seedling height of clustered buds. At the same time, cytokinin and auxin may be different because of different varieties.

There are significant differences between MS medium and 1/2MS, B5, N6 for two kinds of New Guinea *Impatiens* (Table 3). The tufted buds treated with MS medium grow best. The average seedling height of ‘Violet’ and ‘Scarlet Bronze Leaf’ are 1.23±0.02 and 1.01±0.04 cm (Fig. 3i–l), respectively. The nitrogen content of N6 Medium is lower than that of MS medium, which has better ability of callus induction and maintenance (Bohorova et al. 1995). Rakshit's research results also show that N6 Medium is more conducive to inducing high-quality callus than MS medium (Rakshit et al. 2010). This paper speculates that the reason why MS medium is more conducive to the growth of cluster buds than the other three media caused by nitrogen content. For ‘Violet’ among the four concentrations of TDZ, the growth of clustered buds of 0.5 mg·L⁻¹TDZ was the best, average seedling height reached 0.94±0.02 cm, and there was a
Table 3  Effects of different hormone concentrations and medium types on the proliferation of clustered buds of 'Violet' and 'Scarlet Bronze Leaf' of New Guinea Impatiens

| Processing number | PGR(mg·L⁻¹) | Proliferation coefficient | Mean multiple shoots height (cm) | Hyperhydricity rate (%) |
|-------------------|-------------|---------------------------|----------------------------------|-------------------------|
|                   | 6-BA        | TDZ| NAA   | 'Violet' | 'Scarlet Bronze Leaf' | 'Violet' | 'Scarlet Bronze Leaf' | 'Violet' | 'Scarlet Bronze Leaf' |
| P1                | 0.40        | 0.25 | 0.01 | 10.50 ± 0.86ᵇ  | 9.22 ± 0.78ᵇ            | 1.27 ± 0.04ᵇ  | 1.05 ± 0.04ᵇ            | 16.67 ± 1.67ᵇ  | 21.67 ± 1.67ᵇ        |
| P2                | 0.80        | 0.50 | 0.05 | 13.18 ± 0.91ᵃ  | 11.52 ± 0.78ᵃ            | 1.46 ± 0.04ᵃ  | 1.19 ± 0.05ᵃ            | 13.33 ± 1.67ᵃ  | 20.00 ± 1.67ᵃ         |
| P3                | 1.20        | 0.75 | 0.10 | 8.48 ± 0.69ᶜ  | 6.37 ± 0.61ᶜ            | 1.14 ± 0.03ᵃᵈε | 0.90 ± 0.03ᵃᵈε         | 20.00 ± 1.67ᵇᵈ | 31.67 ± 3.34ᵇᵈ       |
| P4                | 1.60        | 1.00 | 0.20 | 6.80 ± 0.59ᵇ  | 7.27 ± 0.65ᶜ            | 1.04 ± 0.03ᵃᵉ | 0.94 ± 0.12ᵇᵈ           | 20.00 ± 1.67ᵇᵈ | 26.67 ± 3.34ᵇᵈ       |
| P5                | 0.80        | 0.25 | 0.10 | 2.23 ± 0.47ᵍ  | 2.23 ± 0.37ᶠ            | 0.92 ± 0.03ᵍᵈ | 0.87 ± 0.02ᵈᵉ           | 65.00 ± 1.67ᶠᵉ | 55.00 ± 3.33ᵇᵉ       |
| P6                | 0.40        | 0.50 | 0.20 | 2.53 ± 0.48ᶠ  | 2.53 ± 0.48ᶠ            | 1.04 ± 0.10ᵃᵉ | 0.71 ± 0.03ᶠᵉ           | 66.67 ± 1.67ᶠᵉ | 55.00 ± 1.67ᵇᵉ       |
| P7                | 1.60        | 0.75 | 0.01 | 1.82 ± 0.36ᵇᵉ | 2.33 ± 0.40ᶠ            | 0.97 ± 0.03ᵍᵈ | 0.74 ± 0.02ᶠᵉ           | 66.67 ± 3.34ᵇᵉ | 56.67 ± 3.34ᵇᵉ       |
| P8                | 1.20        | 1.00 | 0.05 | 2.28 ± 0.43ᶠᵉ | 2.57 ± 0.39ᶠ            | 0.89 ± 0.02ᵇᵉ | 0.70 ± 0.02ᵍᵉ           | 63.33 ± 3.34ᵇᵉ | 51.67 ± 5.00ᵇᵉ       |
| P9                | 1.20        | 0.25 | 0.20 | 5.95 ± 0.61ᵈᵉ | 4.22 ± 0.43ᵈᵉ           | 1.17 ± 0.04ᵇᵉ | 0.88 ± 0.04ᵈᵉ           | 30.00 ± 1.67ᵇᵉ | 28.33 ± 1.67ᵇᵉ       |
| P10               | 1.60        | 0.50 | 0.10 | 7.52 ± 0.52ᵉᵈ | 3.60 ± 0.34ᵈᵉ           | 1.16 ± 0.04ᵇᵉ | 0.87 ± 0.04ᵈᵉ           | 16.67 ± 1.67ᵇᵉ | 23.33 ± 3.34ᵇᵉ       |
| P11               | 0.40        | 0.75 | 0.05 | 4.70 ± 0.50ᶠᵉ | 5.05 ± 0.55ᵈᵉ           | 1.02 ± 0.03ᵈᵉ | 0.96 ± 0.04ᵇᵉ           | 26.67 ± 3.34ᵈᵉ | 36.67 ± 3.34ᵈᵉ       |
| P12               | 0.80        | 1.00 | 0.01 | 5.12 ± 0.53ᶠᵉ | 3.38 ± 0.37ᵈᵉ           | 1.03 ± 0.03ᵈᵉ | 0.90 ± 0.04ᵈᵉ           | 31.67 ± 1.67ᵈᵉ | 31.67 ± 1.67ᵇᵉ       |
| P13               | 1.60        | 0.25 | 0.05 | 0.42 ± 0.14ᵃᵉ | 0.62 ± 0.18ᵉ            | 0.90 ± 0.03ᵈᵉ | 0.90 ± 0.03ᵈᵉ           | 85.00 ± 5.00ᵃᵇ | 81.67 ± 6.67ᵃᵇ       |
| P14               | 1.20        | 0.50 | 0.01 | 0.37 ± 0.12ᵃᵉ | 0.53 ± 0.16ᵉ            | 1.03 ± 0.03ᵃᵉ | 0.81 ± 0.03ᵈᵉ           | 83.33 ± 1.67ᵃᵇ | 81.67 ± 3.34ᵃᵇ       |
| P15               | 0.80        | 0.75 | 0.20 | 0.20 ± 0.09ᵃᵉ | 0.63 ± 0.17ᵉ            | 0.89 ± 0.03ᵇᵉ | 0.84 ± 0.03ᵈᵉ           | 91.67 ± 1.67ᵃᵇ | 80.00 ± 1.67ᵃᵇ       |
| P16               | 0.40        | 1.00 | 0.10 | 0.75 ± 0.19ᵃᵉ | 0.83 ± 0.21ᵍᵉ           | 0.96 ± 0.02ᵍᵈ | 0.88 ± 0.03ᵈᵉ           | 76.67 ± 3.34ᵇᵉ | 75.00 ± 5.00ᵃᵇ       |

Each treatment was inoculated with 15 bottles, each bottle was connected with 2 pieces, and repeated twice. The data represent the mean ± se. According to least significance difference, there was no significant difference in the mean after the same letter (P = 0.05).
Table 4 Variance analysis of influencing factors of bud proliferation coefficient, average seedling height and vitrification rate of Impatiens in New Guinea

| Variance source | Proliferation coefficient | Significance | Average seedling height | Hyperhydricity rate |
|-----------------|--------------------------|--------------|-------------------------|---------------------|
|                 | ‘Violet’ | ‘Scarlet Bronze Leaf’ |  | ‘Violet’ | ‘Scarlet Bronze Leaf’ |  |
| Basic medium    | **      | **             | **   | **      | **             | **  |
| 6-BA            |         |                |      |         |                |      |
| TDZ             | **      | *             |   ** |         | *             |      |
| NAA             | *       |  *           |      |         |        |      |

Note:* stands for significant, ** stands for very significant

Table 5 Multiple comparative experiments on the effects of different factors on the average seedling height, increment coefficient and vitrification rate of New Guinea Impatiens cluster

| Basic medium | Average seedling height (cm) | Proliferation coefficient | Hyperhydricity rate (%) | Average seedling height (cm) | Proliferation coefficient | 6-BA (mg·L⁻¹) | Average seedling height (cm) | NAA (mg·L⁻¹) | Average seedling height (cm) |
|--------------|------------------------------|---------------------------|-------------------------|------------------------------|---------------------------|--------------|------------------------------|--------------|------------------------------|
| ‘Violet’     | ‘Scarlet Bronze Leaf’        | ‘Violet’                 | ‘Scarlet Bronze Leaf’    | ‘Violet’                    | ‘Scarlet Bronze Leaf’    | ‘Scarlet Bronze Leaf’ |
| MS           | 1.23 ± 0.02a 1.01 ± 0.04a     | 9.74 ± 0.41a 8.91 ± 0.39a | 17.50 ± 1.34 ± 0.25     | 1.04 ± 0.02b 4.76 ± 0.38b   | 4.07 ± 0.32a 0.4        | 0.90 ± 0.02a 0.01         | 0.87 ± 0.02ab |
| 1/2MS        | 0.96 ± 0.03c 0.73 ± 0.01c     | 2.03 ± 0.21c 2.42 ± 0.20f | 65.42 ± 1.12 ± 0.5      | 1.17 ± 0.03a 5.83 ± 0.43d   | 4.55 ± 0.36a 0.8        | 0.93 ± 0.02a 0.05         | 0.94 ± 0.02a  |
| B5           | 1.09 ± 0.02b 0.90 ± 0.02b     | 6.10 ± 0.28b 3.70 ± 0.20f | 26.25 ± 2.33b ± 0.75    | 1.01 ± 0.02b 3.98 ± 0.32bc  | 3.23 ± 0.25b 1.6        | 0.82 ± 0.02b 0.1          | 0.86 ± 0.02b  |
| N6           | 0.91 ± 0.02c 0.85 ± 0.02b     | 0.43 ± 0.07d 0.65 ± 0.09d | 84.17 ± 2.36d 1         | 0.95 ± 0.01c 3.74 ± 0.28c   | 3.51 ± 0.27b 3.2        | 0.86 ± 0.03ab 0.2         | 0.84 ± 0.03b  |

The data represent the mean ± se. According to least significance difference, there was no significant difference in the mean after the same letter (P = 0.05)
significant difference between 0.5 mg·L$^{-1}$ TDZ and the other three concentrations. For ‘Scarlet Bronze Leaf’, among the four concentrations of 6-BA, 0.4 and 0.8 mg·L$^{-1}$ were significantly different from 1.6 and 3.2 mg·L$^{-1}$, and the value-added coefficient increased at first and then decreased, when the concentration of 6-BA was 0.8 mg·L$^{-1}$, it reached the maximum value of 0.93±0.02 cm, indicating that too high concentration of 6-BA was not conducive to the growth of cluster buds, which should be controlled between 0.4 and 0.8 mg·L$^{-1}$. For the concentration of NAA, the average seedling height of 0.005 mg·L$^{-1}$ ‘Scarlet Bronze Leaf’ was the best, which was 0.94±0.02 cm, which was significantly higher than that of the other three levels, so the suitable concentration of NAA was beneficial to the increase of ‘Scarlet Bronze Leaf’ of New Guinea Impatiens.

The type of basic medium has a significant effect on the hyperhydricity rate of ‘Violet’ tufted buds (Table 5), indicating that the basic medium has an important effect on the hyperhydricity rate of ‘Violet’ clustered buds. However, the concentration of 6-BA, TDZ and NAA had no significant difference in the hyperhydricity rate of clustered buds. For ‘Scarlet bronze leaf’, the type of basic medium and the concentrations of 6-BA, TDZ and NAA showed no significant difference in the hyperhydricity rate of clumped sprouts.

Table 6 Effects of different sucrose and silver nitrate concentrations on hyperhydricity rate and proliferation coefficient of violet cluster buds

| Treatment | Medium composition | Hyperhydricity rate (%) | Multiplication coefficient |
|-----------|--------------------|-------------------------|---------------------------|
|           | Sucrose concentration (g·L$^{-1}$) | AgNO$_3$ (mg·L$^{-1}$) |                            |
| CK(1)     | 30                 | 0                       | 33.33±0.03$^b$            | 9.35±0.51$^a$ |
| O1        | 20                 | 1                       | 21.67±0.02$^a$            | 10.17±0.49$^a$ |
| O2        | 50                 | 2                       | 45.00±0.02$^c$            | 8.03±0.36$^b$ |
| O3        | 70                 | 5                       | 55.00±0.02$^d$            | 6.37±0.60$^d$ |
| CK(2)     | 0                  | 0                       | 33.33±0.03$^b$            | 9.35±0.51$^a$ |
| O4        | 30                 | 1                       | 23.67±0.02$^a$            | 9.74±0.63$^a$ |
| O5        | 50                 | 2                       | 31.67±0.00$^b$            | 9.40±0.49$^a$ |
| O6        | 70                 | 5                       | 36.67±0.00$^c$            | 8.55±0.48$^b$ |

Each treatment contained 30 explants, which were repeated twice. The data represent the mean±se. According to least significance difference, there was no significant difference in the mean after the same letter (P=0.05).
Kevers, Maene and Pâques reported that (Kevers and Gaspar 1986; Maene and Debergh 1985; Pâques et al. 1991), cytokinins in the culture medium were important for hyperhydricity. The results of this study may differ from the results of the current study, which may be due to different plant materials.

Most studies have shown that MS media are less prone to yield vitrified seedlings (Wang et al. 1990, 2009) consistent with the results of the current experiment. Among the four media used in this experiment, the MS medium has a significant difference in hyperhydricity rate compared with the other two types of media, and the hyperhydricity rate is the lowest, the hyperhydricity rate is only 17.50%, and the growth condition of clustered buds is the best (Table 3).

**Overcoming hyperhydricity seedlings**

The effects of sucrose on the hyperhydricity rate and proliferation coefficient of New Guinea Impatiens 'violet' are significantly different (Table 6). Among them, the O1 treatment reduced the sucrose concentration to 20 g·L⁻¹, significantly improving the hyperhydricity rate, its hyperhydricity rate reached the lowest value, only 21.67%. Meanwhile, in all treatments, the proliferation coefficient was the highest, reaching 10.17±0.49. However, with the continuous increase of sucrose concentration, the hyperhydricity rate increased. The hyperhydricity rate reached 55.00±0.02% when the sucrose concentration was 70 g·L⁻¹. The color became light green, the growth was slow, and the proliferation coefficient decreased significantly reaching the lowest value of 6.37±0.60 (Fig. 4a–c). Numerous studies have shown that increasing sucrose or other particle concentrations to increase media osmolality can reduce hyperhydricity rates (Xiao et al. 1997; He et al. 2008; Liu et al. 2013), which is not consistent with the results of this paper. We speculate that maybe because different plants have an optimum value for the tolerance of sucrose concentration, the excessive sucrose concentration may increase the metabolic burden of plants.

AgNO₃ is an ethylene inhibitor in plant tissue culture, which acts on ethylene sites to promote organogenesis, bud proliferation and plant regeneration frequency (Akasaka et al. 2005; Ozudogru et al. 2005). Vinoth and other studies have shown that the addition of silver ions can reduce the occurrence of hyperhydricity (Vinoth et al. 2015). In this paper, we also found that the effects of AgNO₃ on the hyperhydricity rate and proliferation coefficient of 'Violet' were significantly different (Table 6). Among them, the O4 treatment with 1 mg·L⁻¹AgNO₃ added to the culture medium had the best inhibitory effect on hyperhydricity (Fig. 4c), the hyperhydricity rate was only 23.67%, the growth condition of the seedlings was better, the leaf color was dark green, and the proliferation coefficient reached 9.70. However, with the increase of concentration, the hyperhydricity rate gradually increased and the proliferation coefficient continuously decreased. When the concentration of AgNO₃ increased to 5 mg·L⁻¹, the hyperhydricity rate reached the highest value of 36.67±0.00%, and the proliferation coefficient reached the minimum value of 8.55±0.48. Wu Li fang found that when AgNO₃ increased to a certain concentration, the proliferation rate
of buds began to decrease (Wu et al. 2020), consistent to the results in this paper (Table 6).

**Effects of different kinds and concentrations of hormones on rooting of regenerated seedlings of New Guinea Impatiens**

IBA and 6-BA have different effects on the rooting efficiency of New Guinea *Impatiens* 'Scarlet Bronze Leaf'. In all treatments (Table 7), although the rooting rate of 'Violet' and 'Scarlet Bronze Leaf' of New Guinea *Impatiens* in MS medium R1 without any hormone in the control group reached 100%, the mean rooting number of 'Violet' was 9.87 ± 0.53, and the mean rooting number of 'Scarlet bronze leaf' was 11.63 ± 0.85, the roots induced by them were so thin and weak that there were only a few hairy roots, which was not suitable for rooting of regenerated seedlings of New Guinea *Impatiens*. The rooting effect of adding IBA (R6-R9) and 6-BA (R10-R13) alone was better than adding NAA (R2-R5) alone, especially in terms of rooting rate, the rooting rates of both are 100% (Fig. 4e).

**Conclusion**

In this study, the hypocotyls with some cotyledons of 'Violet' and 'Scarlet Bronze Leaf' of New Guinea *Impatiens* were used as explants to study the disinfection and sterilization of seeds, the induction of clustered buds, the proliferation of clustered buds, the rooting of regenerates seedlings, and the effects of sucrose and AgNO₃ on the hyperhydricity of regenerated seedlings, the regeneration rate in...
regeneration media that MS supplemented with 0.5 mg·L\(^{-1}\) TDZ and 0.1 mg·L\(^{-1}\) NAA was acceptable, the addition of 0.8 mg·L\(^{-1}\)-6-BA, 0.5 mg·L\(^{-1}\)-TDZ and 0.05 mg·L\(^{-1}\)-NAA to MS had a good effect on the proliferation of Impatiens New Guinea, PIC was unable to induce clumped sprouts, but it had a better effect on callus induction. Reducing sucrose concentration to 20 g·L\(^{-1}\) or adding 1 mg·L\(^{-1}\)-AgNO\(_3\) could alleviate the hyperhydricity phenomenon, the optimal root culture medium for New Guinea Impatiens was MS supplemented with 0.05 mg·L\(^{-1}\)-IBA. The findings of this paper enable us to establish efficient and stable regeneration systems of 'Violet' and 'Scarlet Bronze Leaf' of New Guinea Impatiens.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are available in this published article (and its supplementary information files). The datasets generated during and/or analyzed during the current study are not publicly available due to [REASON(S) WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request. Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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