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BIOLOGY

Optimization of 'Zi Dieer' crabapple micropropagation through proliferation of axillary shoots

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ABSTRACT: *Malus* is an important ornamental plant around the world and widely used in commercial production due to its diversity and wide adaptability. 'Zi Dieer' crabapple has good adaptability to a wide temperature range (high and low), disease resistance, and high commercial value. To satisfy the market demand, we established 'Zi Dieer' crabapple micro-regeneration system for large-scale reproduction. Induced buds were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzylaminopurine (BAP: 0.5, 1.0, 1.5 mg/L) and 1-naphthaleneacetic acid (NAA: 0.05, 0.1, 0.3 mg/L) for 35 days. The highest bud induction rate (93.3%) was observed on MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA, with average micro-shoot length of 1.32 cm. The germinated buds (1 cm in length) were propagated and cultured on MS medium supplemented with BAP (1.0, 1.5, 2.0 mg/L) and NAA (0.1, 0.3, 0.5 mg/L), after 35 days, on the MS basal medium supplemented with 1.5 mg/L BAP and 0.3 mg/L NAA, the best average number of new shoots is 8.56±0.13 ($P<0.05$) and good shoot length (4.94±0.09 cm), reproduction coefficient higher than MS medium (0) without PGRs. In order to induce rooting, the shoots (length 2 cm) are inserted in half strength MS (1/2 MS) medium containing 0.1, 0.3 and 0.5 mg/L of 3-indole butyric acid (IBA) or NAA, and 1 g/L activated carbon (AC) was added on the basis of NAA 0.3 mg/L to study the effect of activated carbon on the rooting of crabapple. After 38 days of culture, compared with other treatments, among the explants treated with IBA, the rooting rate was higher in the 1/2 MS medium containing 0.1 mg/L (82.1%), and the highest root number (11.29±0.52) was observed, and the root length was the other treatments 2-3 times. Although, the highest rooting rate was observed on 1/2 MS medium containing 0.1mg/L NAA (87.8 %), but the bottom of these buds formed large callus, which was not conducive to survival, and the root length was short. In summary, a feasible and complete rapid propagation program for 'Zi Dieer' crabapple has been developed.
Key words: tissue culture, stem segment, rapid propagation.

Otimização da micropropagação de maçã silvestre 'Zi Dieer' através da proliferação de brotos axilares

RESUMO: *Malus* é uma planta ornamental importante no mundo e muito utilizada na produção comercial devido à sua diversidade e ampla adaptabilidade. A maçã silvestre 'Zi Dieer' apresentou boa adaptabilidade a altas e baixas temperaturas, resistência a doenças e maior valor comercial. Para satisfazer a demanda do mercado, estabelecemos o sistema de microrregeneração de maçã silvestre 'Zi Dieer' para reprodução em larga escala. Os botões induzidos foram cultivados em meio Murashige e Skoog (MS) suplementado com 6-benzilaminopurina (BAP) 0,5, 1,0, 1,5 mg/L e ácido 1-naftalenoacético (NAA) 0,05, 0,1, 0,3 mg/L por 35 dias. A maior taxa de indução de gemas foi em meio MS suplementado com 1,0 mg/L de BAP e 0,1 mg/L de NAA, que foi de 93,3% e o comprimento médio dos microbrotos foi de 1,32 cm. Os botões germinados (comprimento 1 cm) foram propagados e cultivados em meio MS suplementado com BAP e NAA de 1,0, 1,5, 2,0 e 0,1, 0,3, 0,5 mg/L, após 35 dias, em meio MS basal suplementado com 1,5 mg/L. L BAP e 0,3 mg/L ANA, o melhor número médio de brotos novos é 8,56±0,13 ($P<0,05$) e bom comprimento de brotos (4,94±0,09 cm), coeficiente de reprodução superior ao meio MS (0) sem PGRs. Para induzir o enraizamento, os brotos (comprimento 2 cm) são inseridos em meio MS de meia concentração (1/2 MS) contendo 0,1, 0,3 e 0,5 mg/L de ácido 3-indol butírico (IBA) ou ANA, e 1 g /L carvão ativado (CA) foi adicionado com base em ANA 0,3 mg/L para estudar o efeito do carvão ativado no enraizamento da maçã silvestre. Duas semanas depois, observando a geração de raízes. Após 38 dias de cultivo, comparado aos demais tratamentos, entre os explantes tratados com AIB, a taxa de enraizamento foi maior no meio 1/2 MS contendo 0,1 mg/L (82,1%) e o maior número de raízes (11,29±0,52) foi observado, e o comprimento da raiz foi igual aos demais tratamentos 2 a 3 vezes. Embora a maior taxa de enraizamento tenha sido observada em meio MS 1/2 contendo 0,1mg/L de NAA (87,8%), mas a parte inferior dessas gemas forma um grande calo, que não é propício à sobrevivência, e o comprimento da raiz é curto. Em resumo, foi desenvolvido um programa de propagação rápida viável e completo para a maçã silvestre 'Zi Dieer'.
Palavras-chave: cultura de tecidos, segmento de caule, propagação rápida.

INTRODUCTION

Malus (*Malus* spp.) is a deciduous shrub or small tree in the rose family (Rosaceae) with a distribution in the northern temperate zone, across the Eurasian and North America continents (YUAN, 2018). Ornamental crabapples had fruits with 5 cm or less in diameter, various bright colors, and long-lasting

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fruiting season. *Malus* are rich in varieties, having long viewing period, high ornamental, medicinal, and economic values, and are characterized by rapid growth and wide adaptability (GU, 2021; ZHAO et al., 1995). Crabapples tender leaves are rich in tea polyphenols, protein and other trace elements and their tea is known to enhancing physical fitness and lowering blood sugar (QU et al., 2000; WANG et al., 1999; ZHANG et al., 2000). According to "Food Herbs" and "Compendium of Materia Medica", Crabapple tea has been consumed by humans for more than 400 years (HUANG, 2019). 'Zi Dieer' crabapple is a new variety originated from open pollination and was approved by the China Forest Variety Certification Committee in 2018. 'Zi Dieer' crabapple, in Jiangsu, shows wide adaptability to temperatures (range: 37-40 to 4-6 °C). 'Zi Dieer' is free from apple aphids and resistant to red spiders and apple rust (ZHOU et al., 2020), a rare strong disease resistance among crabapple plants. It has a long-lasting flowering period with large flower buds, purple-red flowers, and red-green oval leaves, with excellent ornamental effect with great landscaping potential.

Malus asexual reproduction efficiency through cuttings is low, which greatly restricts the selection of superior trees and their large-scale production, thus, facing real difficulties meeting market demands (YUAN, 2018). Tissue culture is a well-known and effective tool for large-scale plant propagation with desired characteristics such as, high reproduction efficiency, fast speed, stable genetic traits, etc. 'Zi Dieer' crabapple has limited expansion; however, it can quickly reach large scale production through tissue to meet market demand. Plants of the genus Malus are generally established using anthers (SI et al., 2010), leaves (HOU et al., 2001; LIANG et al., 2009), pedicels (WANG et al., 2020), petals (LI et al., 2005; ZHENG et al., 2003), with leaves and stems being the most common. However, leaves often show high pollution and severe browning rates. Also, regeneration protocols involving the callus stage are considered to be the least reliable for clonal propagation, whereas seedlings regenerated by branching from axillary buds or direct somatic embryos are considered to be the most genetically homogeneous (ESPINOSA-LEAL et al., 2018). Here, we used stems with axillary buds as explants for tissue culture, which not only maintain excellent woody traits, and clonal fidelity (genetic characteristics), but also offers avenues for environment control and avoiding unsuccessful cultivation caused by seasonal factors (GU, 1996), thus large amount of materials can be obtained in a short time (ZHAO et al., 1995).

To date, there is no report on 'Zi Dieer' crabapple tissue culture research, therefore, we used tissue culture methods to evaluate the effects of plant growth regulators on the induction, proliferation and rooting of shoots including the axillary buds, and establishment of a systematic and efficient tissue culture protocol. The rapid propagation system is expected to lay the foundation for fine varieties selection, introduction and cultivation, and promoting the utilization of 'Zi Dieer' in the market (LI, 1989).

MATERIALS AND METHODS

'Zi Dieer' crabapple explants collection and sterilization

Four-year-old 'Zi Dieer' crabapple trees growing in the Yangzhou Crabapple National Forest Tree Germplasm Bank (E119°54′, N32°42′) provided the explants for this study. On May 4, 2019, unbranched young branches were collected, soaked in 5% washing powder (Tide, Guangzhou, China) solution for 30 minutes, rinsed with running tap water for 2 hours, and placed on sterilized work-bench with 70% ethanol for surface disinfection for 35 s. The branches were rinsed again with sterile distilled water one to three times, immediately transferred into 0.1% mercury chloride ($HgCl_2$) (The first Chemical Plant, Huaihua, China) for 8 minutes, and then rinsed with sterile distilled water for 6 to 8 times. Sterilized branches were cut into 1 to 2cm long segments containing a single bud (Figure 1A). These segments were transplanted into a 30 mL glass tube containing MS basic medium (Haibo Biotechnology Co., Qingdao, China), plus 30 g/L sucrose (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), and 7 g/L agar (Saiguo Biological Co., Ltd., Guangzhou, China). The glass tubes were sealed with a sterile parafilm (Jiafeng Gardening Products Co., Ltd., Shanghai, China) for shoot sprouting. Before adding the agar, medium pH was adjusted to 5.8±0.02 with NaOH or HCl. A total of 10 mL of culture medium was transferred to a glass tube, lid was closed, and autoclaved at 121 °C for 20 minutes. All test tubes with explants were placed in a culture room at a temperature of 25±2 °C, with 16 h photoperiod at 2000-4000 lx light intensity.

Bud induction

The sterilized stem explants were cultured in a basic MS (Murashige and Skoog) medium containing 7.0 g/L agar and 30 g/L sucrose, adjusted to pH 5.8. On this medium, nine combinations were added representing 6-benzylaminopurine (BAP)

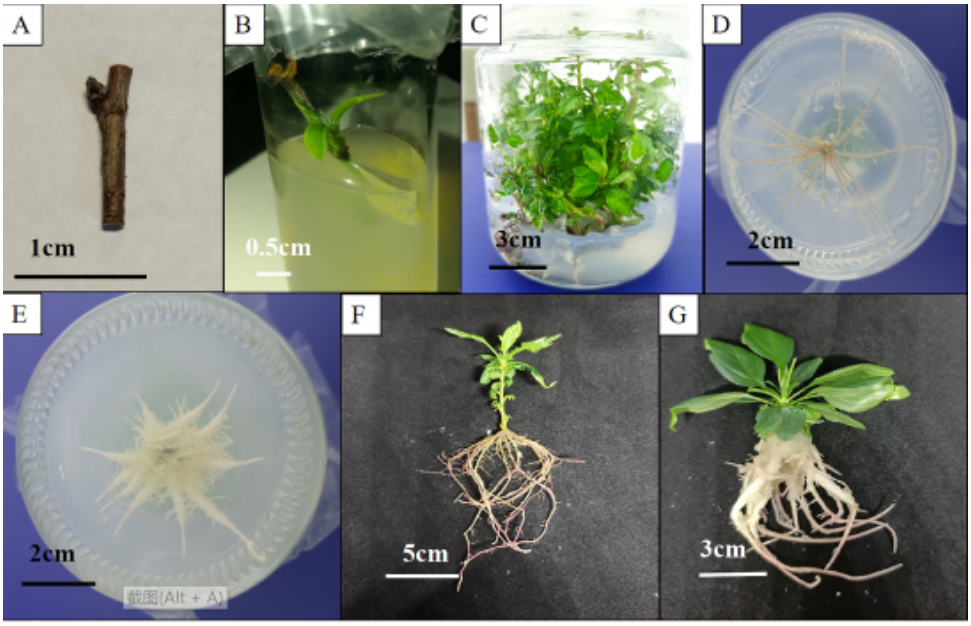

Figure 1 - The morphogenesis of 'Zi Dieer' crabapple in the stages of establishment (A), induction (B), multiplication (C), and rooting (D-H). ((A) A segment containing an axillary bud as an explant; (B) Induction of explants on MS medium; (C) shoot multiplication on MS medium supplemented with 1.5 mg/L 6-benzylaminopurine (BAP) and 0.3 mg/L 1-naphthaleneacetic acid (NAA); (D, F) Rooting and seedling on 1/2 MS medium and 0.1 mg/L 3-indolebutyric acid (IBA); (E, G) Rooting and seedling on 1/2 MS medium and 0.1 mg/L 1-naphthaleneacetic acid (NAA)).

(Jintan Maosheng Fine Chemicals, Changzhou, China) with 0.5, 1.0, 1.5 mg/L and 1-naphthaleneacetic acid (NAA) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) with 0.05, 0.1, 0.3 mg/L, with MS medium without Plant growth regulators (PGRs) as a control (Table 1). A total of 30 bottles (30 mL) of each treatment, each bottle contained one explant and each treatment was repeated three times, and the shoot induction rate was recorded. After 35 days, the axillary bud grew to 3 cm (Figure 1B). A segment (1 cm long) was cut from the axillary bud and used for the following experiment.

Bud proliferation

A 1 cm long section was cut and cultured on MS medium containing 30 g/L sucrose and 7.0 g/L agar. In order to determine the best conditions for axillary bud proliferation and shoot elongation, nine different culture combinations were tested, MS medium without PGRs was used as a control (Table 2). All cultures were incubated under the same physical conditions as described previously. A total of 30 bottles of each treatment, one explant per bottle, and each treatment were repeated three times. After 35 days, the number of shoots and average shoot length (cm) were recorded.

Induction of adventitious roots from adventitious buds

Terminal bud (length 2.0 cm) was inserted in half strength MS (1/2 MS) medium, plus 30 g/L sucrose and 7.0 g/L agar, containing 3-indolebutyric acid (IBA) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) or NAA at 0.1, 0.3 and 0.5 mg/L. Buds were also placed on 1/2 MS medium without PGRs as a control (Table 3). A total of 30 bottles (30 mL) of each treatment, each bottle contained one explant and each treatment was repeated three times. All cultures were incubated under the same physical conditions as above except for adding 20 g/L sucrose. After 35 days of cultivation, the number of explants with roots, number of roots per plant, and average root length (cm) on each plant were recorded.

Statistical Analysis

A completely random design was used in all cases. A total of 30 explants were cultured per treatment. The treatments were arranged randomly on the growth room shelves. For each PGRs combination,

Table 3 - Effect of 1-naphthaleneacetic acid (NAA) and 3-indolebutyric acid (IBA) on in vitro rooting of 'Zi Dieer' crabapple after 35d.

Treatment	----------Concentrations of PGRs----------		Rooting (%)	Number of roots	Length of roots/cm
	NAA（mg/L）	IBA（mg/L）			
1	0	0	34.6	6.04±0.25d	1.54±0.32b
2	0.1	0	63.2	14.73±1.01 a	2.02±0.78b
3	0.3	0	78.5	12.13±0.51b	1.53±0.19b
4	0.5	0	76.8	11.00±0.82 bc	1.49±0.12b
5	0	0.1	82.1	11.29±0.52bc	4.21±0.45a
6	0	0.3	67.3	11.17±1.19 bc	2.08±0.16b
7	0	0.5	74.7	9.83±0.38 c	2.33±0.19b
8	0.3+ 1 g/L AC	0	87.8	8.05±1.14c	3.65±0.43a

Means ± SD with different letters in the columns differ significantly by Duncan's test ($P \leq 0.05$).

the growth parameters were recorded and average ± SD value were calculated in three analyses. Since PGRs was not added, the data of full-strength MS medium and half-strength MS (1/2 MS) medium were also excluded from the analysis. A one-way analysis of variance was performed on the obtained data, and then Waller-Duncan analysis was performed using SPSS 23.0 (IBM, USA) to determine significant differences ($P < 0.05$, is considered significant). Excel 2010 was used to analyze the linear trend of bud induction rate.

RESULTS AND DISCUSSION

Primary culture is mainly to establish the vitro growth system of tissue culture of sterile plants. Therefore, whether adventitious buds can be induced is the key to tissue culture (YANFEN et al., 2002).

Table 2 - Effects of 6-benzylaminopurine (BAP) and 1-naphthoacetic acid (NAA) on the proliferation of 'Zi Dieer' crabapple buds after 35d.

Treatment	----------Concentrations of PGRs----------		Number of shoots per explant	Length of shoots(cm)
	BAP（mg/L）	NAA（mg/L）		
1	0	0	0	9.03±1.56a
2	0	0.1	0	7.89±1.02ab
3	0	0.3	0	8.90±0.93a
4	0	0.5	0	8.12±1.05ab
5	1.0	0.1	3.65±0.09c	5.43±0.19cd
6	1.0	0.3	2.32±0.21e	6.77±0.34bc
7	1.0	0.5	2.81±0.14d	8.06±0.21ab
8	1.5	0.1	3.62±0.15c	4.74±0.30d
9	1.5	0.3	8.56±0.13a	4.94±0.09cd
10	1.5	0.5	5.21±0.11b	5.11±0.23cd
11	2.0	0.1	3.91±0.11c	7.92±0.28ab
12	2.0	0.3	2.44±0.09e	3.84±1.06d
13	2.0	0.5	2.33±0.09e	4.09±0.95d

Data represent means ± standard error (SE). Means followed by the same letter(s) within a column are not significantly different ($P > 0.05$) according to Duncan's test.

Table 3 - Effect of 1-naphthaleneacetic acid (NAA) and 3-indolebutyric acid (IBA) on in vitro rooting of 'Zi Dieer' crabapple after 35d.

Treatment	----------Concentrations of PGRs----------		Rooting (%)	Number of roots	Length of roots/cm
	NAA（mg/L）	IBA（mg/L）			
1	0	0	34.6	6.04±0.25d	1.54±0.32b
2	0.1	0	63.2	14.73±1.01 a	2.02±0.78b
3	0.3	0	78.5	12.13±0.51b	1.53±0.19b
4	0.5	0	76.8	11.00±0.82 bc	1.49±0.12b
5	0	0.1	82.1	11.29±0.52bc	4.21±0.45a
6	0	0.3	67.3	11.17±1.19 bc	2.08±0.16b
7	0	0.5	74.7	9.83±0.38 c	2.33±0.19b
8	0.3+ 1 g/L AC	0	87.8	8.05±1.14c	3.65±0.43a

Means ± SD with different letters in the columns differ significantly by Duncan's test ($P \leq 0.05$).

In tissue culture, PGRs are essential components in the culture media to define the pathway of plant cells and tissues development (JAFARI et al., 2011). To this end, different combinations and concentrations of basal media and PGRs were tested. Supplementing the medium with PGRs can promote axillary buds formation (Table 1). No bud induction was detected on MS medium lacked PGRs (i.e., induction rate of 0.0). Supplementing 1.0 mg/L BAP and 0.1 mg/L NAA to the basal medium gave the highest bud induction rate of 93.3% (Table 1). Axillary buds of explants begin to germinate two weeks after inoculation. Four weeks after inoculation, buds grow vigorously (Figure 1B). The average length of the buds was 1.32 cm, which was longer than most other treatments (Table 1). High concentration of auxin in combination with high concentration of cytokinin was detrimental to adventitious shoot formation. At lower auxin concentration an increase of cytokinin stimulated adventitious shoot formation. Just like the results reported in this experimental study, at 0.05 mg/L NAA, regeneration tends to increase with the increase of BAP concentration, but when the concentration of BAP increased to a certain extent, the length of adventitious buds decreased rather than increased. As in HIRAKAWA & TANNO, 2022, cytokinins inhibited axillary bud formation and bud development. HERNANDEZ-GARCIA et al. (2021) also suggested that shoot regeneration was significantly lower in treatments with higher BAP concentrations (>1.0 mg/L). Moreover, LI et al. (2008) showed that generally high-concentration of auxin and low-concentration of cytokinin were beneficial to callus induction. Just as in our experiment, when BAP was at 0.5 mg/L, adventitious buds induction rate was low and callus was produced. Comprehensive analysis of the results showed that the best treatment method included adding 1.0 mg/L BAP and 0.1 mg/L NAA to MS medium to bud induction.

On all tested media, bud proliferation changed to varying degrees (Table 2). The concentration ratio of cytokinin significantly affected the number of buds and each explants height. ANOVA also demonstrated a significant interaction between these two factors. The explants (control) on MS medium without PGRs did not proliferate, and there was a small number of adventitious roots. Compared with treatments containing both BAP and NAA, only 0.1, 0.3 and 0.5 mg/L NAA did not stimulate bud proliferation. Compared with the control (without cytokinin), the combination of 9 PGRs containing 1.0, 1.5, 2.0 mg/L BAP and 0.1, 0.3, 0.5 mg/L NAA significantly increased the number of explants shoots (Table 2). On MS basal medium supplemented with 1.5 mg/L BAP and 0.3 mg/L NAA, the average number of best buds was 8.56±0.13 ($P<0.05$) and good bud length (4.94±0.09 cm) (Figure 1C). In fact, BAP was found in most Solanaceae plants induction (e.g., potatoes) (MOKHTARZADEH et al., 2018). It is reported that the interaction between BAP and NAA is beneficial to bud reproduction of various species (FANG, 2012), results supporting the present study. In contrast, the tallest bud length (9.03± 1.56 cm) was obtained without PGRs ($P<0.05$). As in WANG et al. (2020) who suggested that cytokinins (such as BAP) can induce axillary bud formation (LI, 2002), and have a certain inhibitory effect on cell elongation. Among the 9 treatments containing PGRs, the sprout length (8.06±0.21 cm) of 1.0 mg/L BAP and 0.5 mg/L NAA was longer, but the number of proliferations was small (2.81±0.14) and could not be applied. Although, shoots grown on media containing 1.5 mg/L BAP and 0.3 mg/L

NAA were 4.94 ± 0.09 cm long, but the number of buds proliferated was large (Table 2). According to the above results, it is recommended to use 1.5 mg/L BAP and 0.3 mg/L NAA for 'Zi Dieer' crabapple proliferation.

After 9 days of cultivating buds on all 1/2 MS medium supplemented with NAA, adventitious roots began to produce white or red radiated adventitious roots (Figure 1). After 10 days, root formation was observed in 1/2 MS medium with IBA or without PGRs. Studies have shown that all 8 treatments can produce roots (Table 3). The root number of roots on the medium containing 0.1 mg/L NAA was the highest (14.73±1.01), but there was more callus formation at the bottom of the bud (Figure 1E and G), which was not conducive to planting survival. On the contrary, in the explants treated with IBA, the rooting rate was higher in the medium containing 0.1 mg/L (82.1 %), and the highest number of roots (11.29±0.52) was observed. In general, IBA was the most commonly used auxin for rooting in different apple genotypes, and rooting percentage varied from 18 to 100% (TEIXEIRA DA SILVA et al., 2019). IBA is the most common auxin to induce root formation because it is more potent than IAA or synthetic auxins (HERNANDEZ-GARCIA et al., 2021). In addition, there was basically no callus formation at the bottom of the buds, accompanied by many fibrous roots (Figure 1D and F). In 1/2 MS medium without PGRs, the number of roots was low, but weret longer in length. According to BLAKESLEY & CHALDECOTT (1993), the existence of roots in the basal medium may be caused by endogenous auxin. The rooting rate of 1/2 MS medium containing 1 g/L activated carbon (AC) and 0.3 mg/L NAA was high (87.8%), and the root length was significantly different from the other seven treatments (3.65±0.43 cm), but the roots were lower in quantity. It is known that low-light environment is beneficial to rooting, and the rooting time is short. This may be because the low light environment is conducive to the differentiation of callus into roots (GUAN et al., 2016). There are also studies (NAOGAN, 2023; BOUDABOUS et al., 2010) that recommended adding activated carbon to the rooting medium can absorb the endogenous cytokinins present in the explants and promote the activity of auxins. However, low light limits photosynthesis and is not conducive to the growth of its aerial parts. Therefore, after successfully inducing rooting under low light conditions, tissue culture seedlings should be cultivated immediately under normal light (ELIASSON & BRUNES, 1980). Based on the above results, it is recommended to use 0.1 mg/L IBA for 'Zi Dieer' crabapple rooting.

CONCLUSION

The effect of inducing axillary buds of stem segment of 'Zi Dieer' crabapple was best on MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L NAA, resulting on induction rate of 93.3%, and average length of buds reaching 1.32 cm.

The effect of germinated buds (length 1 cm) on MS medium supplemented with 1.5 mg/L BAP and 0.3 mg/L NAA was the best, with average number of shoots of 8.56±0.13 ($P<0.05$), and good bud length (4.94 ± 0.09 cm).

The shoots (length 2 cm) were cultured on 1/2 MS medium supplemented with IBA 0.1 mg/L, resulted in a rooting rate of 82.1%, average root number of 11.29±0.52, and average root length of 4.21±0.45 cm.

The study demonstrated a successful method for the micropropagation of 'Zi Dieer' crabapple with the potential for large-scale production of plants.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

Na Li: Methodology, Software, Writing–original draft preparation. Ting Zhou: Data curation & supervision. Donglin Zhang, Wangxiang Zhang and Guangping Li: Review & editing.

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