



A Rapid and Efficient Plant Regeneration Protocol using Leaves and Stems with Buds of *Euphorbia pulcherrima* Willd. ex Klotzsch (Euphorbiaceae)

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ABSTRACT

Background: *Euphorbia pulcherrima* is highly ornamental value of poinsettia flower, which possesses colorful bracts and low temperature resistant leaves. Its embryos or adventitious buds were cultivated mainly from stems, petioles and tender buds. The cultivation of seedlings is studied firstly for improving the quality of Poinsettia.

Methods: A rapid and reproducible regeneration system of leaves and stems with buds has been established. There is a comparative analysis of the results of tissue culture of different varieties of Poinsettia.

Result: The best medium component of hormones about leaf callus of poinsettia inducing cluster buds formation comprise 6-BA 1.0 mg/L, KT 0.4 mg/L, NAA 0.1 mg/L and 2,4-D 0.05 mg/L. Its medium component inducing cluster buds proliferation include 6-BA 1.2 mg/L and NAA 0.05 mg/L. The medium component about stem with bud inducing cluster buds to germinate comprise 6-BA 1.5 mg/L and NAA 0.1 mg/L. Its medium component inducing cluster buds proliferation include 6-BA 1.0 mg/L and NAA 0.05 mg/L. The different hormones and different addition level of Ac have effect on the length of the root and quantity of the first level root and the second level root.

Key words: Cluster buds, Organogenesis, Plant regeneration, Poinsettia (*Euphorbia pulcherrima*).

INTRODUCTION

Poinsettia *Euphorbia pulcherrima* Willd. ex Klotzsch belongs to *Euphorbia* Linn., Euphorbiaceae Juss. (Ma and Michael, 2000), which possesses some typical characteristics including 30~50 cm of the plant height, compact plant-type, strong branches, numerous buds, big and colorful bracts and the powerful low temperature resistance as well as not easy-falling leaves (Jiao 2001). It is highly ornamental value of the upmarket pot flower, which bracts begin to change color in December and colorful flowers bloom during the large festival.

The propagation of poinsettia is mainly through tissue culture, which has been used in production since the early 1970s (Nataraj 1975). Its embryos or adventitious buds were cultivated mainly from poinsettia stems, petioles and tender buds (Grotkass *et al.*, 1995). The cultivation of seedlings is studied firstly for improving the quality of Poinsettia (Vilperte *et al.*, 2019), thus we have established regeneration system of leaves and stem with bud. The tissue culture and rapid reproduction research of poinsettia play a key role in exogenous hormone of plant growth regulators (Cheng *et al.*, 2016) and this study has involved the ratio of auxin and cytokinin in exogenous hormones to comprehensive experiments. There is a comparative analysis of the results of tissue culture of different varieties of Poinsettia, and how to reduce costs and simplify tissue culture in the use of exogenous hormones for rapid propagation of poinsettia (Dole and Wilkins, 1992; Horn *et al.*, 2014). The vegetative propagation experiment and

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establishment research of poinsettia have strong theoretical value and practical significance.

MATERIALS AND METHODS

This experiment was carried out in the Tissue Culture Laboratory of Yangtze River Rare Plant Research Institute, China Three Gorges Corporation from 2018-3 to 2020-10. 6-BA (6-Benzyladenine), KT (Kinetin), NAA (1-Naphthaleneacetic acid), 2, 4-D (2, 4-Dichlorophenoxyacetic acid) four-factor and three-level (Table 1) L₉ (3⁴) coaxial experiment was designed and each treatment was 10 bottles, with each bottle was 2 callus mass of 0.8~1 cm², repeated 3 times. After 30 days, the number of induced cluster buds and the length of buds were observed and the number of buds were counted.

The experiment used 6-BA, different auxin types and different concentrations of L_8 (4×2^3) mixed orthogonal experimental design (Table 2) and a total of 8 treatments. The single buds were respectively inoculated on the medium, each treatment was 10 bottles, each bottle had 2 single buds, and repeated 3 times. After 30 days of inoculation, the number of effective, the length of buds and the proliferation rate were calculated.

Using a two-factor completely random design, the differentiated cluster buds were divided into 3 levels, according to the height of the seedlings (The height of the first seedling is 1.2~1.6 cm, the height of the second seedling is 0.8~1.2 cm and the third level is 0.4~0.8 cm). The method of adding or not adding Ac during the culture process was carried out in a total of 6 treatments, each treatment was 10 bottles and each bottle was inoculated with 2 ex-plants, repeated 3 times. Respectively, they were inoculated in Murashige and Skoog medium (MS + Sucrose: 30 g/L + Agar: 6.0 g/L) for strong seedling culture. The seedling height, inter-nodes, stem thickness and leaf color after 30 days.

Table 1: L_9 (3^4) orthogonal table.

No.	6-BA	KT	NAA	2,4-D
P ₁	0.5	0	0.1	0
P ₂	0.5	0.2	0.2	0.05
P ₃	0.5	0.4	0.3	0.1
P ₄	1	0	0.2	0.1
P ₅	1	0.2	0.3	0
P ₆	1	0.4	0.1	0.05
P ₇	1.5	0	0.3	0.05
P ₈	1.5	0.2	0.1	0.1
P ₉	1.5	0.4	0.2	0

Table 2: L_8 (4×2^3) orthogonal table of cluster buds reproduction.

No.	Leaves			Stems with buds		
	6-BA	2,4-D	NAA	6-BA	2,4-D	NAA
P ₁	0.8	0.1	0.1	0.6	0.05	0.05
P ₂	0.8	0.15	0.15	0.6	0.1	1
P ₃	1	0.1	0.1	0.8	0.05	0.05
P ₄	1	0.15	0.15	0.8	0.1	1
P ₅	1.2	0.1	0.15	1	0.05	1
P ₆	1.2	0.15	0.1	1	0.1	0.05
P ₇	1.4	0.1	0.15	1.2	0.05	1
P ₈	1.4	0.15	0.1	1.2	0.1	0.05

Table 3: L_4 (2^3) orthogonal table.

No.	Leaves			Stems with buds		
	IBA	NAA	6-BA	IBA	6-BA	Ac
P ₁	0.2	0.5	0	0.8	0.1	100
P ₂	0.2	1	0.1	0.8	0.2	200
P ₃	0.8	0.5	0.1	1.2	0.1	200
P ₄	0.8	1	0	1.2	0.2	100

6-BA, IBA (Indolebutyric acid), NAA or Ac (Activated carbon) three-factor and two-level L_4 (2^3) coaxial experiment was designed (Table 3). A total of 4 treatments, 10 bottles for each treatment, 2 for each bottle, we repeated 3 times. The root length, seedling height, and number of first-level roots and second-level roots were observed after 30 days.

The data obtained were statistically analyzed by SPSS (Statistical Product and Service Solutions) software package which the least significant difference test set at a 5% level of significance (Du and Pijut, 2008). The percentage data were first transformed by arc-sine and then analyzed by variance and multiple comparisons.

RESULTS AND DISCUSSION

Leaves regeneration

The different hormones have effect on leaf callus induction with the frequency and size of callus formation as the main index and the color and quality of callus as a reference. Finally, the results have revealed that the best medium component about leaves of poinsettia inducing callus formation contain MS, NAA 1.0 mg/L, 6-BA 0.1 mg/L, sucrose 30 mg/L, agar 6.0 g/L and its PH value is 5.8 (Fig 1a; Table 4). The different hormones have influence on callus inducing cluster buds formation with the germination rate as the main index and the quantity and length of buds and the better growth vigor as references, which have indicated that the best medium component of hormones about leaf callus inducing cluster buds formation comprise 6-BA 1.0 mg/L, KT 0.4 mg/L, NAA 0.1 mg/L and 2,4-D 0.05 mg/L. The different 6-BA concentration and the different kind of auxin have impact on the cluster buds proliferation with proliferation rate as the main index, referencing the quantity and length of effective buds and better growth vigor. In conclusion, the medium component about leaf callus inducing cluster buds proliferation include 6-BA 1.2 mg/L and NAA 0.05 mg/L (Fig 1b; Table 5). The fractional culture of different budling and Ac addition have effect on strong seedling cultivation of cluster buds (Firoozabady and Gutterson, 2003). The different hormones have influence on rooting with length of the root and quantity of the first level root and the second level root deemed to the main index, referencing the height of seedling and the better growth vigor. Lastly, the medium component about leaf inducing cluster buds to take root contain IBA 0.8 mg/L, NAA 0.5 mg/L and 6-BA 0.1 mg/L (Fig 1c; Table 6).

Stems with buds regeneration

The different concentrations of 6-BA and NAA have influence on inducing cluster buds of stem with bud to germinate (Jiao *et al.*, 2017). The medium component about stem with bud inducing cluster buds to germinate comprise 6-BA 1.5 mg/L and NAA 0.1 mg/L (Fig 1d). The different 6-BA concentration and the different kind of auxin have effect on the cluster buds proliferation, which the medium component inducing cluster buds proliferation include 6-BA 1.0 mg/L and NAA 0.05 mg/L (Fig 1e; Table 5). The different addition level of

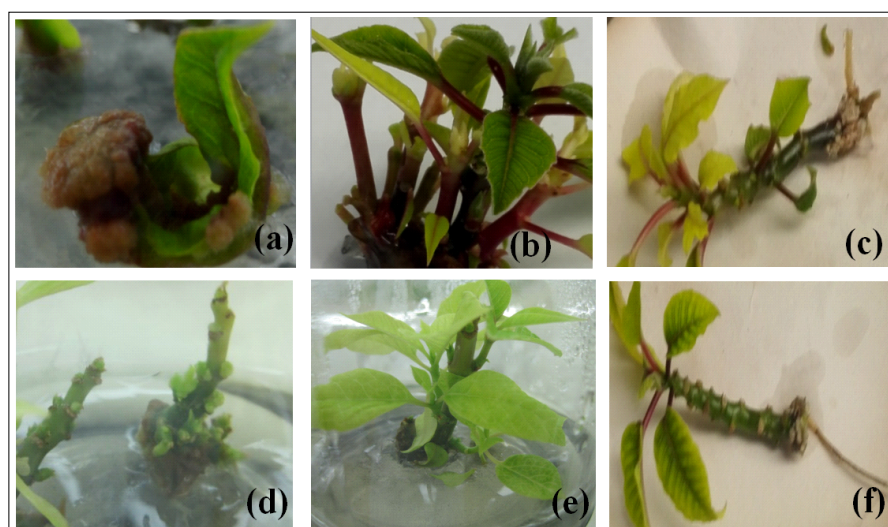


Fig 1: Establishment of poinsettia leaf and stem with buds regeneration (a) Leaf callus induction; (b) Cluster buds proliferation; (c) Callus rooting at the base of cluster buds; (d) Bud enlargement; (e) Bud germination and vigorous growth; (f) Clustering callus rooting at bud base.

Table 4: Multiple comparison of callus inducing cluster buds between combinations affected by the different hormone.

Leaves	6-BA (mg/L)	KT (mg/L)	NAA (mg/L)	2,4-D (mg/L)	Germination rate (%)	Number of buds	Bud length (cm)
P ₁	1(0.5)	1(0)	1(0.1)	1(0)	30.0±0.05Bd	15.7±1.5Ff	0.7±0.2Gf
P ₂	1(0.5)	2(0.2)	2(0.2)	2(0.05)	31.7±0.07Bd	16.7±1.5EFf	0.7±0.1Gf
P ₃	1(0.5)	3(0.4)	3(0.3)	3(0.1)	40.0±0.10Bdc	20.7±2.0Ee	1.0±0.1FGe
P ₄	2(1.0)	1(0)	2(0.2)	3(0.1)	46.7±0.07Bc	35.3±2.5Cc	1.6±0.1DEd
P ₅	2(1.0)	2(0.2)	3(0.3)	1(0)	50.0±0.05Bc	32.7±1.5Cc	2.2±0.2Cc
P ₆	2(1.0)	3(0.4)	1(0.1)	2(0.05)	83.3±0.07Aa	51.3±1.5Aa	3.3±0.1Aa
P ₇	3(1.5)	1(0)	3(0.3)	2(0.05)	46.7±0.07Bc	32.7±1.5Cc	1.8±0.1DCd
P ₈	3(1.5)	2(0.2)	1(0.1)	3(0.1)	71.7±0.07Ab	43.3±2.0Bb	2.6±0.1Bb
P ₉	3(1.5)	3(0.4)	2(0.2)	1(0)	43.3±0.07Bdc	27.0±2.6Dd	1.3±0.1EFf

Table 5: Multiple comparison of cluster buds reproduction between combinations.

Leaves	1 (6-BA)	2 (type)	3 (mg/L)	Proliferation rate (%)	Effective bud number	Bud length (cm)
P ₁	1(0.8)	1(2,4-D)	1(0.05)	33.3±0.12Bc	1.0±0.57Ab	1.41Ab
P ₂	1(0.8)	2(NAA)	2(0.1)	53.3±0.06Bb	2.0±0.51Ab	1.65Ab
P ₃	2(1.0)	1(2,4-D)	1(0.05)	50.0±0.1Bbc	2.0±0.47Ab	1.64Ab
P ₄	2(1.0)	2(NAA)	2(0.1)	60.0±0.17Bb	2.0±0.36Ab	1.55Ab
P ₅	3(1.2)	1(2,4-D)	2(0.1)	60.0±0.1Bb	4.0±0.06Aa	2.68Aa
P ₆	3(1.2)	2(NAA)	1(0.05)	93.3±0.06Aa	5.0±0.30Aa	3.10Aa
P ₇	4(1.4)	1(2,4-D)	2(0.1)	60.0±0.1Bb	2.0±0.48Ab	1.29Ab
P ₈	4(1.4)	2(NAA)	1(0.05)	53.3±0.16Bb	2.0±0.31Ab	1.15Ab
Stems with buds						
P ₁	1(0.6)	1(2,4-D)	1(0.05)	33.3±0.12Bc	3.3±0.57Dd	1.0±0.07C
P ₂	1(0.6)	2(NAA)	2(0.1)	53.3±0.06Bb	3.3±0.57Dd	1.5±0.10B
P ₃	2(0.8)	1(2,4-D)	1(0.05)	50.0±0.1Bb	6.3±0.57Cc	1.7±0.15B
P ₄	2(0.8)	2(NAA)	2(0.1)	60.0±0.17Bb	10.0±1.0Bb	1.8±0.20B
P ₅	3(1.0)	1(2,4-D)	2(0.1)	60.0±0.1Bb	7.3±0.57Cc	1.7±0.24B
P ₆	3(1.0)	2(NAA)	1(0.05)	80.0±0.1Aa	13.7±0.57Aa	2.6±0.18A
P ₇	4(1.2)	1(2,4-D)	2(0.1)	60.0±0.1Bb	7.7±0.57Cc	1.1±0.03C
P ₈	4(1.2)	2(NAA)	1(0.05)	53.3±0.16Bb	11.3±1.57Bb	1.7±0.07B

Table 6: Multiple comparison of different hormones impacting rooting between combinations.

Leaves	IBA	NAA	6-BA	Root length (cm)	Seedling height (cm)	First-level roots	Second-level roots
P ₁	1(0.2)	1(0.5)	1(0)	14.7±1.4Cc	1.9±0.2Bc	1.7±0.5Bb	5.7±1.5Bc
P ₂	1(0.2)	2(1.0)	2(0.1)	21.0±1.6Bb	2.6±0.2ABb	2.3±0.5ABb	6.7±1.1Bbc
P ₃	2(0.8)	1(0.5)	2(0.1)	26.9±2.0Aa	3.2±0.4Aa	4.3±0.5Aa	13.0±1.7Aa
P ₄	2(0.8)	2(1.0)	1(0)	22.3±2.0ABb	2.0±0.2Bc	3.0±1.0ABb	9.7±2.0ABb

Table 7: Multiple comparison of the different hormones and addition level of activated carbon having influence on rooting between combinations.

Stems with buds	IBA	6-BA	Ac	Root length (cm)	Seedling height (cm)	First-level roots	Second-level roots
P ₁	1(0.8)	1(0.1)	1(100)	35.4±0.85B	1.9±0.15Cc	1.7±0.57Cc	11.3±0.57Cc
P ₂	1(0.8)	2(0.2)	2(200)	22.7±1.44C	2.2±0.20BCbc	2.3±0.57BCbc	16.3±1.52Bb
P ₃	2(1.2)	1(0.1)	2(200)	43.8±4.25A	3.5±0.10Aa	5.7±0.57Aa	25.3±1.52Aa
P ₄	2(1.2)	2(0.2)	1(100)	31.9±1.40B	2.6±0.20Bb	3.3±0.57Bb	18.3±1.52Bb

Ac and fractional culture of different budling have influence on strong seedling cultivation of cluster buds. The different hormones and different addition level of Ac have effect on rooting with the length of the root and quantity of the first level root and the second level root deemed to the main index, and the height of seedling and the better growth vigor as references. Lastly, the results have showed that the medium component inducing cluster buds to take root include IBA 1.2 mg/L, 6-BA 0.1 mg/L, Ac 200 mg/L (Fig 1f; Table 7).

The tender leaves which selected as ex-plants can easily induce callus (Grotkass *et al.*, 1995). This experiment found that the callus induction rate of the leaves at the top of the shoots was high and the callus induction time was about 7 days earlier than that of other leaves. The callus was induced after being cut on the leaves of the Poinsettia, and was easy to grow from the wound with the strong differentiation (Wang *et al.*, 2013; Jantasorn *et al.*, 2021). This experiment found that cutting 2 horizontally along the middle of the main vein of the leaf has a higher rate of producing callus than other parts of the wound. The callus was pale yellow and granular. This callus was more prone to dedifferentiation to produce cluster buds.

In the process of multiplication and cultivation of cluster buds, the appropriate addition of Ac can inhibit the proliferation of a large number of weak buds and play a role in seedlings (Jiao 2001). Combining the proliferation of cluster buds and the amount of Ac, it can not only save the cultivation time, but also reduce the investment and achieve the purpose of reducing costs and increasing efficiency (Mali and Chavan, 2016). In the further study, as the Poinsettia leaves induce the cluster buds to conduct outdoor hydroponic rooting, it is hoped that this will simplify the tissue culture procedure, reduce the indoor culture time and production costs, and enhance the ability of the tissue culture seedlings, which adapt to the external environment and improve the survival rate of tissue cultured seedlings (Cheng *et al.*, 2016; Thakuria *et al.*, 2018).

Poinsettia shoots with buds induce cluster buds. If the culture time is too long, the ex-plants and the medium are prone to produce secretions. And the secretions affect the differentiation, proliferation and growth of the cluster buds (Huang *et al.*, 2014; Kenasa *et al.*, 2021). This experiment found that in the process of cultivating shoots with buds, especially during the transfer process, the clustering buds were easy to flow out of white jelly from the wound after being cut. If the jelly has been absorbed in time, it was easy to cause endophytic infection, which affected the growth of sterile seedlings.

The appropriate addition of Ac can inhibit the proliferation of a large number of weak buds and overcome the growth of endophytic bacteria and adsorb substances that inhibit the growth of seedlings (Moustaka *et al.*, 2018). This is the first report of an efficient plant regeneration protocol for poinsettia through direct organogenesis using ex-plants. Hence, this provides a more efficient basis for the genetic transformation than previously available (Moustaka *et al.*, 2020; Trang *et al.*, 2021).

CONCLUSION

We have developed a stable and efficient plant regeneration system for Poinsettia using leaves and stem with bud as the original ex-plants. The medium component about leaf of Poinsettia inducing cluster buds to take root contained MS, IBA 0.8 mg/L, NAA 0.5 mg/L, 6-BA 0.1 mg/L, sucrose 30 mg/L, agar 6.0 g/L and its pH value was 5.8. The best way of stem with bud of Poinsettia inducing strong seedling cultivation of cluster buds was that the optimum height of seedling was the first level budding with 1.2~1.6 cm. The medium component which pH value was 5.8, which contained MS, sucrose 30 mg/L, agar 6.0 g/L and Ac 200 mg/L. The medium component about stem with bud of Poinsettia inducing cluster buds to take root included MS, IBA 1.2 mg/L, 6-BA 0.1 mg/L, Ac 200 mg/L, sucrose 30 mg/L, agar 6.0 g/L and its pH value was 5.8. This regeneration

system provides the necessary basis for genetic transformation procedures and we are currently making progress towards an mediated transformation protocol (Pooja *et al.*, 2021).

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