

The Biomass Dosage Influences the Effects of Diethyl Aminoethyl Hexanoate on Micropropagation of *Echinacea purpurea* (L.) Moench

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Abstract

The plant growth regulator diethyl aminoethyl hexanoate (DA-6) has proved highly effective on micropropagation of the medicinal plant purple coneflower (*Echinacea purpurea* (L.) Moench), however, sharp variation of the effects existed among explants in the same treatment, making the application of DA-6 in micropropagation difficult. In order to clarify factors that influencing the treating results of DA-6, explants with different biomass dosage were prepared and inoculated onto medium supplemented with different concentrations of DA-6. It was found that among the three kinds of biomass dosage explants, the lowest biomass explants required the lowest concentration of DA-6, and the highest biomass explants required the highest concentration of DA-6 for the best results on adventitious buds regeneration. Similar results were obtained when regenerated buds of three different biomass dosages were cultured. It could be concluded from the above experimental results that for achieving better DA-6 application results, the concentration of DA-6 should be determined not only by the types but also by the biomass dosage of the explants. The present finding might help to improve the micropropagation efficiency in *E. purpurea*, and might be applicable for other species

Keywords

Plant Growth Regulator, DA-6, Micropropagation, Biomass Dosage, *Echinacea purpurea*

1. Introduction

With the confirmed non-specific immunostimulant bioactivities, purple coneflower (*Echinacea purpurea* (L.) Moench) has gained much attention for decades [1] [2] [3]. Traditionally, people use it as prevention or a treatment of upper respiratory infections in common cold in nations across Europe and North America [4] [5]. In recent years, five purple coneflower products have been certified as the new veterinary drugs in China (Certification Nos. 2012-23, -24, -25, and 2014-44, -45) [6] [7]. These promulgations promote the opening of a brand-new market of biological feed additives. It is foreseeable that the scale of commercial cultivation of this plant is going to expand rapidly and steadily. However, this plant is highly cross-pollinated [8] and the commercial plantations were still growing this medicinal plant with seeds. Consequently, the yield and the quality varied because of segregation of genetic characters. In the aspect of breeding, both transgenic [9] [10] [11] and ploidy breeding technique [12] [13] [14] were applied on breeding of new variety. Some kinds of the newly created materials were more valuable than the normal ones, because they grew with higher biomass and contained higher concentration of high-value secondary metabolites [15] [16]. All these studies were performed by *in vitro* culture.

To obtain qualified regenerated plants for commercial production and breeding studies, many efforts have been made on *in vitro* culture in the past fifteen years. Leaf [9], petiole [17], and root [18] were the most frequently used explant for inducing adventitious buds. Various Regular Plant Growth Regulators (PRGs) had been tested, such as 6-benzylaminopurine (BA), naphthylacetic acid (NAA), thidiazuron (TDZ), Kinetin (KT), 3-indolebutyric acid (IBA), and 3-indolylacetic acid (IAA) [9] [17] [19] [20].

However, the efficiency of the micropropagation of purple coneflower was still not high enough. As the breeding researches processed, there were many individuals with special genotypes created [12] [14] [21] [22]. Part of them were found to be sterility or with low fertility, and hard to be reproduced and maintained by performing the present *in vitro* culture protocols.

The diethyl aminoethyl hexanoate (DA-6), was normally used in field [23] [24] and was believed to be safe [25], but uncommon used in tissue culture. Previously, our researches indicated that this PGR was highly effective in *in vitro* cultures of the purple coneflower [26]. Later, the effects of DA-6 on enhancement of microalgae growth and high-value biocompounds produced were tested, and the results were impressive as well [27] [28]. It was even been considered an effective PGR to alleviate salinity stress [29].

Despite of the high efficiency, the effect of DA-6 seems uncontrollable under former propagation strategy. Unlike the BA or NAA [22] [30], the effect of DA-6 is insensitive to gene dosage difference. The 0.08 mg·L⁻¹ of DA-6 may increase the regeneration rate of an explant, but strongly decreased the regeneration rate of another explant with same or higher gene dosage; the 0.16 mg·L⁻¹ of DA-6 may promote the growing of a shoot, but severely inhibit the growing of another shoot [26]. The affecting factors may contain the treatment concentration, explant source, or something else, which had not being counted in in those studies. As in the process of tissue culture plantlet production,

the medium was usually mass-produced, this fact severely restrain the practical application of the DA-6.

No researches have studied the influence of biomass dosage of explants on the effects of DA-6. To address this problem, we did the following researches. Results showed that the biomass dosage influenced the effects of DA-6 on *in vitro* culture of the purple coneflower plants. For explants with less biomass, the optimum concentrations of DA-6 to improve the regeneration or growing situations were lower, for tissues and buds with more biomass, higher. This finding communicated in this manuscript may help people applied the DA-6 better in culture of this plant and other creatures.

2. Materials and Methods

2.1. Plant Materials

Diploid *E. purpurea* were grown up from seeds from the Company of Plantation Products (Norton, MA, USA). Tetraploid was obtained by treating diploid explants with colchicine [31]. We got the triploid plants by crossing diploid and tetraploid plants, and growing the seeds. The ploidy levels plants were confirmed by counting chromosome numbers in at least three root tips in at least five cells [26]. Plantlets of different genotypes were cloned by stimulating axillary bud proliferation [32] from one individual plant.

2.2. Preparation of Explants

Leaves, petioles, and roots were isolated from *in vitro* maintained clones of diploid plants. All these explants were prepared by cutting: leaf explants: 4×4 , 7×7 , and 10×10 mm², petiole and root explants: 4, 9, and 25 mm in length. We identified these leaf, petiole, and root explants with different sizes, from small, medium, to large, as the low, medium, and high biomass explants, respectively.

Buds of diploid, triploid, and tetraploid were proliferated on agar-gelled medium containing MS basal elements, 3% sucrose, $0.01 \text{ mg}\cdot\text{L}^{-1}$ NAA and $0.5 \text{ mg}\cdot\text{L}^{-1}$ BA [32]. Bud explants were isolated from these proliferated shoots and buds. Buds with two to four leaves and 15 mm in height were identified as low biomass bud explants; four to six leaves and 25 mm in height, medium biomass bud explants, six to eight leaves and 40 mm in height, high biomass bud explants. If a bud had grown root(s) when it was isolated for the experiment, we cut off the root(s) before inoculating it onto the medium.

2.3. Preparation of the Medium

Glass jars of about 250 ml inside volume, filled with 40 mL of medium and covered with a polycarbonate screw cap were used. The medium for inducing adventitious bud formation from explants contained Murashige and Skoog (MS) medium [33] containing 3% sucrose, $0.3 \text{ mg}\cdot\text{L}^{-1}$ BA, $0.01 \text{ mg}\cdot\text{L}^{-1}$ NAA [34], and DA-6 of different concentrations. The essential components of the medium for culture of the regenerated buds included MS medium including 3% sucrose, $0.05 \text{ mg}\cdot\text{L}^{-1}$ NAA, and DA-6 of different

concentrations. All the media used were adjusted to a pH 6.0, gelled with 0.45% agar prior to autoclaving at $1.4 \text{ kg}\cdot\text{cm}^{-2}$ for 20 min.

2.4. Maintenance of the Cultures

For seed germination, cultures were first kept in darkness for 10 days, then shift to 12-hours photoperiod (about $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). For regeneration of adventitious buds, and rooting of the isolated buds, cultures were kept directly under 12-hours photoperiod (about $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

2.5. Data Collection and Analysis

Data for adventitious bud formation were recorded 30 days after initiation of the bud regeneration cultures. The regeneration rate was calculated by the number of buds per explant. Buds of at least 1 cm in height, without hyperhydricity [35] and at least two visual leaves were considered as valid. The values of the shoots per explant, and the roots number, as well as those of height were expressed as means \pm SE.

Statistical analysis of the data was carried out by using the Statistical Product and Service Solution (SPSS) 19.0 software. The significant differences among the means were determined by the one-way ANOVA and then Duncan's multiple range tests for pairwise comparison judgment of more than two data sets, or by the independent sample *t*-test for two sets of data. The differences were considered significant when $P < 0.05$. Graphs were draw by using Origin 8.5 software. Data-fitting curves were carried out in b-spline style. Photographs were recorded by D90 digital camera (Nikon) with Speedlight SB-26 flash (Nikon), and arranged by the Adobe Photoshop CC software.

3. Results

3.1. Effects of DA-6 on Bud Regeneration in Explants with Different Biomass Dosage

Leaf, petiole, and root explants with different biomass dosage were inoculated onto medium with various concentrations of DA-6. Effects of DA-6 on the regeneration of adventitious buds were evaluated.

As shown in **Figure 1(a)** and **Figure 2**, demonstrated that DA-6 could stimulate the regeneration of adventitious buds in leaf explants when used at suitable concentrations, which varied for biomass dosage. The medium and high biomass explant required higher concentration of DA-6 ($0.08 \text{ mg}\cdot\text{L}^{-1}$) in order to maximize the number of regenerate buds.

The DA-6 affected the regeneration of petiole explants with different biomass differently. In **Figure 1(b)** and **Figure 3**, for small petiole explants, $0.08 \text{ mg}\cdot\text{L}^{-1}$ DA-6 significantly inhibited the regeneration. For large petiole explants, $0.08 \text{ mg}\cdot\text{L}^{-1}$ of DA-6 increased the regeneration.

As well as to leaf and petiole explants, the DA-6 also affected the regeneration of root explants with different biomass differently. In **Figure 1(c)** and **Figure 3**, for small root explants, $0.01 \text{ mg}\cdot\text{L}^{-1}$ and higher concentrations of DA-6 significantly inhibited the

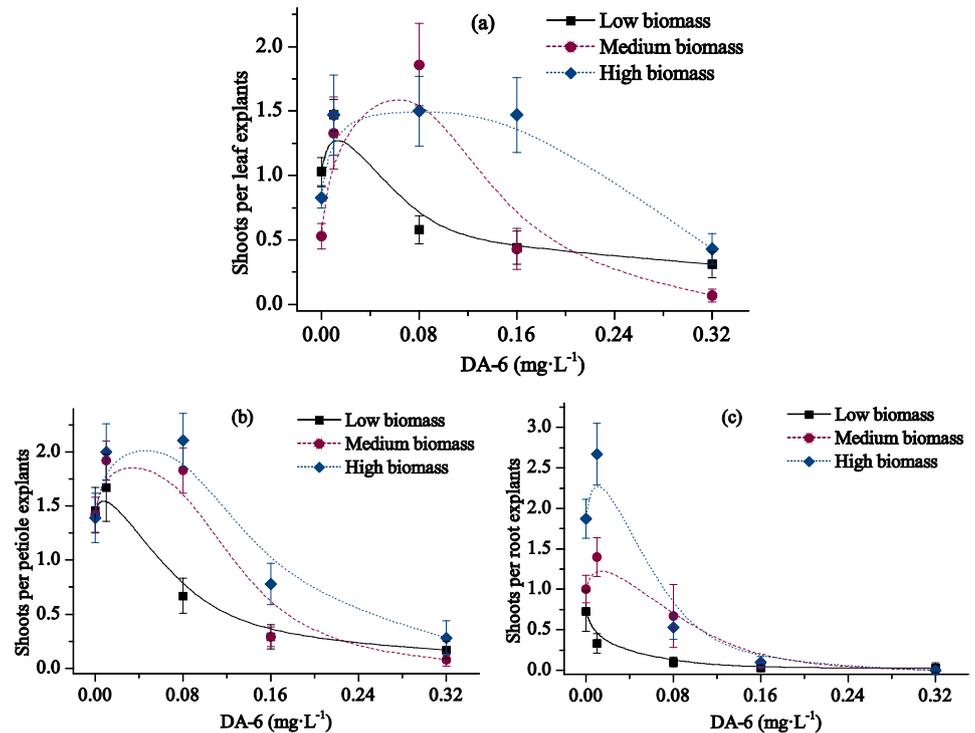


Figure 1. Effects of DA-6 on the regeneration of buds from leaf (a), petiole (b), and root (c) explants with different biomass demonstrated by scatter points with standard error (SE) bars and b-spline curve fittings.

regeneration, while for large ones, the same concentration of DA-6 significantly increased the regeneration.

3.2. Effects of DA-6 on Rooting of Bud Explants with Different Biomass

The bud explants with the height of 15 and 25 mm were defined as the low, and medium biomass bud explants. The former grew two to four leaves, and the later, four to six. As well as to regeneration of leaf, petiole, and root explants, the DA-6 affected the growth of bud explants with different biomass differently. In **Table 1** and **Figure 5**, for small bud explants, 0.16 mg·L⁻¹ DA-6 significantly inhibited the formation of the adventitious roots, while for large ones, the same concentration of DA-6 significantly increased the rooting.

3.3. Effects of DA-6 on Growing of Bud Explants with Different Gene Dosage

The diploid, triploid, and hexaploid bud explants with the height of 40 mm were tested. The selected buds grew six to eight leaves. In **Table 2** and **Figure 6**, the optimal concentrations of DA-6 to improve the height of diploid, triploid, and hexaploid bud explants were 0.32, 0.32, and 0.64 mg·L⁻¹. The optimal concentration of DA-6 to improve the rooting of the explants was 0.64 mg·L⁻¹. When the concentrations of DA-6 were rose to be higher than the optimal one, the promotional effect decreased. Even so, when

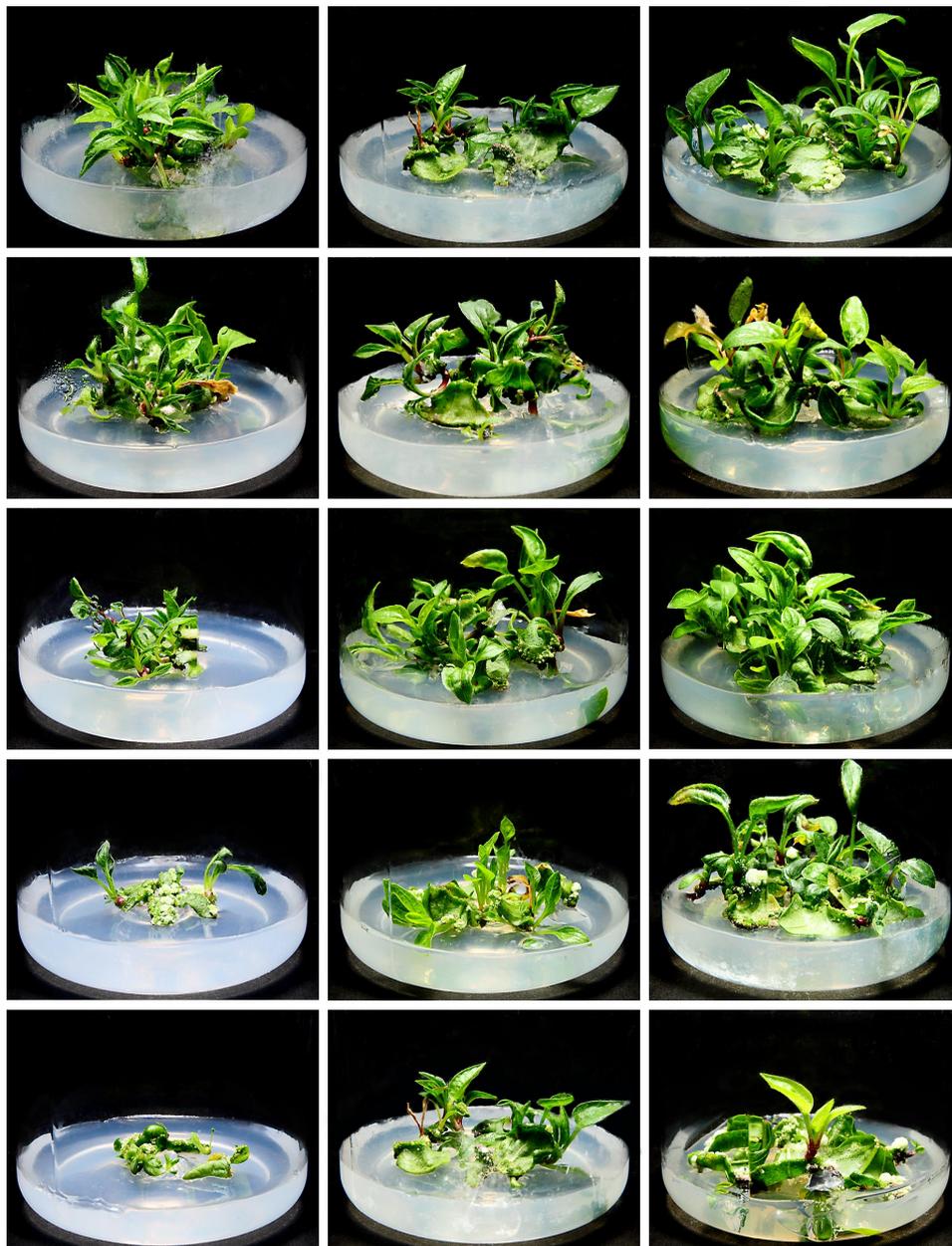


Figure 2. The effect of DA-6 on regeneration of leaf explants with different biomass Leaf explants from left to right were low-, medium-, and high-biomass; concentrations of DA-6 from top to bottom were 0, 0.01, 0.08, 0.16, and 0.32 mg·L⁻¹.

the concentration of DA-6 was three times higher (1.28 mg·L⁻¹) than the optimal one (0.32 mg·L⁻¹), the situations of the plants were still better than the plants grown in the medium with no DA-6.

4. Discussion and Conclusion

Previous studies have proved that certain concentrations of DA-6 improved the regeneration and the growth situation of adventitious buds in purple coneflower. However,

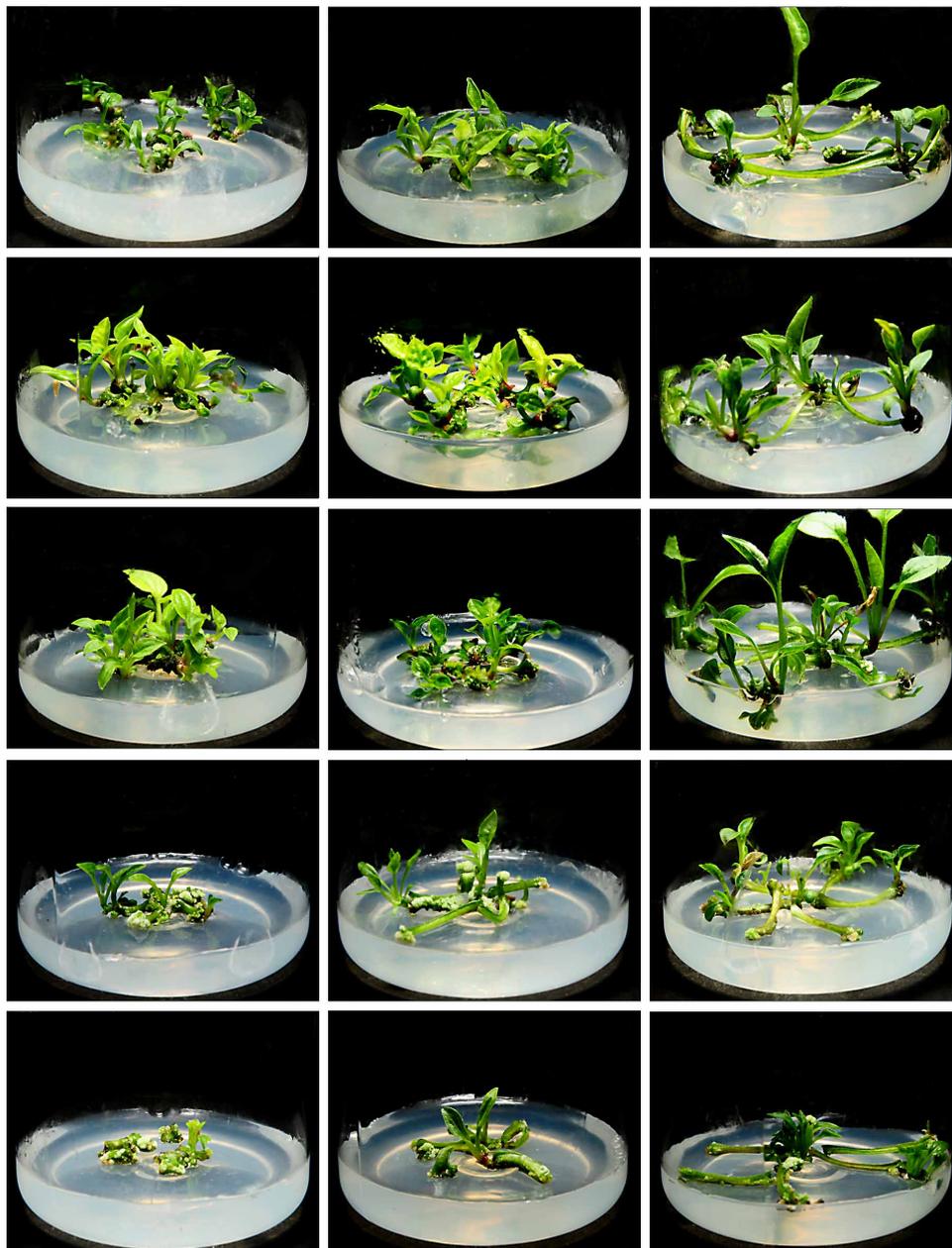


Figure 3. The effect of DA-6 on regeneration of petiole explants with different biomass. Petiole explants from left to right were low-, medium-, and high-biomass; concentrations of DA-6 from top to bottom were 0, 0.01, 0.08, 0.16, and 0.32 $\text{mg}\cdot\text{L}^{-1}$.

the effects varied. For example, the optimal concentrations for the diploid and triploid leaf explants to achieve the maximal regeneration rate were higher than that for the tetraploid, and hexaploid ones [26]. In another words, the gened dosage may not be the critical factor influenced the effects of DA-6.

In this study, we investigated the effects of DA-6 on *in vitro* culture of the purple coneflower leaf, petiole, root, and bud explants with different biomass dosages. The results showed that explants with lower biomass were more sensitive to DA-6. On one

Table 1. Effects of DA-6 on rooting of bud explants (initial height 15 and 25 mm).

Initial size of shoots (mm)	DA-6 (mg·L ⁻¹)	Primary roots No. per shoot	Secondary roots No. per Shoot	Total Root tips No. per shoot
15	0.00	2.00 ± 0.30a*	0.20 ± 0.13b	2.20 ± 0.39b
15	0.08	1.90 ± 0.31a	4.30 ± 0.90a	6.20 ± 0.92a
15	0.16	2.40 ± 0.48a	3.00 ± 0.84a	5.40 ± 1.07a
15	0.32	2.20 ± 0.51a	0.40 ± 0.22b	2.60 ± 0.67b
15	0.64	0.70 ± 0.40b	0.00 ± 0.00b	0.70 ± 0.40b
25	0.00	2.30 ± 0.21c	2.40 ± 0.78b	4.70 ± 0.87b
25	0.08	3.10 ± 0.28c	3.40 ± 0.58b	6.40 ± 0.73b
25	0.16	4.20 ± 0.44b	8.40 ± 0.83a	12.60 ± 0.99a
25	0.32	5.90 ± 0.35a	0.30 ± 0.10c	6.20 ± 0.44b
25	0.64	5.20 ± 0.47ab	0.10 ± 0.32c	5.30 ± 0.45b

*The values of the root numbers are expressed as the mean ± SE. The data in the same column followed by different letters are significantly different, as determined by the one-way ANOVA and then the Duncan's multiple range test at $P < 0.05$ level.

Table 2. Effect of DA-6 on growth of diploid, triploid, and tetraploid shoots with larger biomass (initial height 40 mm).

DA-6 (mg·L ⁻¹)	Diploid		Triploid		Tetraploid	
	Height (mm)	Root tips No.	Height (mm)	Root tips No.	Height (mm)	Root tips No.
0.00	43.80a*	6.40c	39.00b	12.33b	40.50a	4.30b
0.16	58.50a	18.75bc	44.67ab	16.50ab	48.50a	13.50ab
0.32	67.25a	38.00ab	52.50a	20.50ab	43.50a	15.00ab
0.64	64.00a	44.50a	42.00ab	22.00a	64.00a	24.50a
1.28	62.67a	36.00ab	45.80ab	12.50b	55.50a	18.00ab

*The values of the height and root tip numbers are expressed as the mean ± SE. The data in the same column followed by different letters are significantly different, as determined by the one-way ANOVA and then the Duncan's multiple range test at $P < 0.05$ level.

hand, they needed very low concentration of DA-6 to reach their best regeneration rates or growth situation. For example, the leaf and petiole explant with low biomass needed only 0.01 mg·L⁻¹ of DA-6 to increase their regeneration rates (**Figure 1(a)** and **Figure 2**). On the other hand, though no studies have demonstrated the application of DA-6 may inhibit the plant growth, the regeneration of adventitious buds was easily inhibited by DA-6 when the concentrations were higher than the optimal ones. Sometimes, a high concentration of DA-6 can be fatal for a low-biomass explant. Such as 0.08 mg·L⁻¹ of DA-6, it was fatal for low-biomass petiole and root explants (**Figure 1(b)**, **Figure 1(c)**, **Figure 2** and **Figure 3**). As a contrast, the explants with high biomass needed higher concentrations of DA-6 to obtain the best regeneration or growth states.

Meanwhile, the explants with high biomass were more tolerant to high concentration of DA-6. For instance, regeneration rates of the high-biomass leaf, petiole, and root explants decreased slower than the low-biomass ones as the concentrations of DA-6 increased over the optimal ones (**Figures 1-4**).

During the growth of regenerated buds, the suitable concentration for bud explants with 4 cm in height and 6 - 8 leaves was 0.32 or 0.64 mg·L⁻¹. Either of these was higher than the optimal concentrations for the low and the medium biomass bud explants (0.01, 0.08 mg·L⁻¹ respectively) (**Table 1 and Table 2, Figure 5 and Figure 6**).

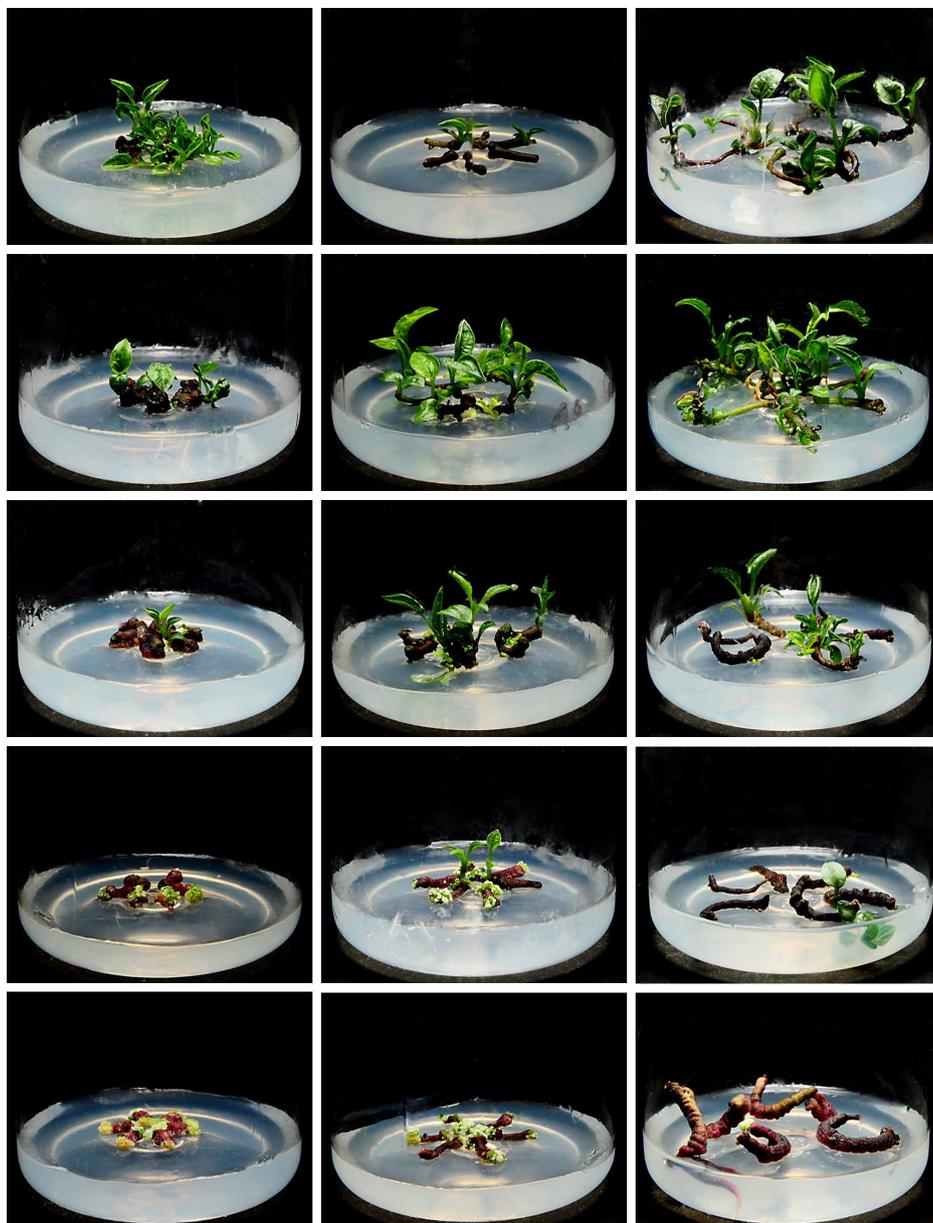


Figure 4. The effect of DA-6 on regeneration of root explants with different biomass. Root explants from left to right were low-, medium-, and high-biomass; concentrations of DA-6 from top to bottom were 0, 0.01, 0.08, 0.16, and 0.32 mg·L⁻¹.

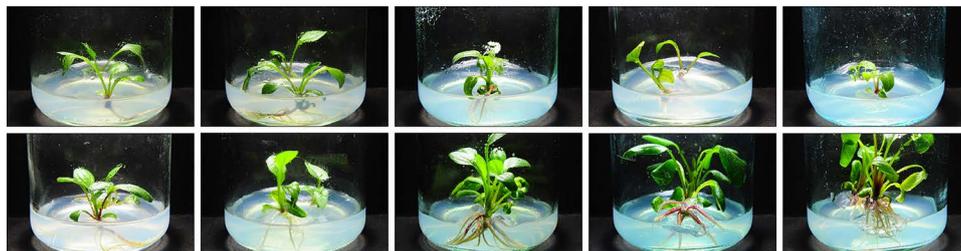


Figure 5. The effect of DA-6 on rooting of bud explants with different biomass. Initial biomass of buds were small (upper), and large (lower); concentrations of DA-6 from left to right were 0, 0.01, 0.08, 0.16, 0.32, and 0.64 $\text{mg}\cdot\text{L}^{-1}$.



Figure 6. The effect of DA-6 on growth of shoots of the diploid, triploid, and tetraploid plants with larger biomass. From left to right: concentrations of DA-6 were 0, 0.16, 0.32, 0.64, and 1.28 $\text{mg}\cdot\text{L}^{-1}$; from top to bottom: diploid, triploid, and tetraploid.

The present results evidenced that the effects of DA-6 on micropropagation of this medicinal plant were related with the initial biomass dosages of the explants, which had not been considered as an influence factor in earlier studies. It is understandable because this *novi* PGR has a very short history of being applied on plant micropropagation [26], and/or soon later on microalgae [27]. As it had been practically used in field [23] [24] and had already been proved to be safe [25], more studies of this PGR on plants and other creatures were worth exploring.

BA and NAA are commonly used plant growth regulators in *in vitro* culture for the

induction of adventitious buds formation. Many researchers used them and obtained high regeneration rates on purple coneflowers [9] [17]. In the present experiments, we did not obtain high regeneration rates compared with those reported by Choffe *et al.* and Koroch *et al.* Even so, the effects of *novo* PGRs are still worth study, because we found significant variations in adventitious bud regeneration abilities genotypes, and for some genotypes, the effect of BA was limited [36]. Present results proved the application of DA-6 at certain concentration as a supplement to the culture medium was able to enhance the stimulating effects BA on bud regeneration.

As we known, the gened dosage influenced the effects of BA [30] [32] and NAA [22] on micropropagation of purple coneflower significantly. The way that DA-6 worked on the explants seems to be different from either BA or NAA. The biomass dosage influenced the effects of DA-6 on the micropropagation of purple coneflower. The working mechanism of this *novo* PGR is still unclear. As the synthetic technology of DA-6 has being well studied and applied [37] [38] [39] [40], the application of DA-6 on purple coneflower may help who produce and grow purple coneflower clonal plants improve their productivity with low cost.

To make good use of DA-6, the explants inoculated on the medium with DA-6 should be cut into standard size. For example, prepare the medium contains $0.08 \text{ mg}\cdot\text{L}^{-1}$ for leaf explants at least $7 \times 7 \text{ mm}^2$ in areas, petiole and root explants at least 9 mm at length, and bud explants at least 25 mm in height and with four to six leaves. For leaf explants smaller than $4 \times 4 \text{ mm}^2$ in areas, petiole and root explants shorter than 4 mm, and bud explants smaller than 15 mm in height and with less than four leaves, no DA-6 should be added into the medium in case of the culture be strongly restrained.

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Author Contributions

XLC: Experiments, interpretation of data, wrote the manuscript, references management, and obtained founding. DLL: Experiments, interpretation of data, and modify the manuscript. JJZ and QLL performed the experiments. YSY: Conceived and designed the experiments, revised the manuscript critically for important intellectual content, and obtained founding. HW: Obtained founding; conceived the experiments; and collected the purple coneflower seeds used in the experiments.

Conflicts of Interest

The authors declare no conflict of interest.

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Abbreviations

DA-6: Diethyl Aminoethyl Hexanoate

BA: 6-Benzyladenine

MS: Murashige and Skoog (1962)

NAA: Naphthalene acetic acid



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