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Mannitol and Sorbitol Improve Uniformity of Adventitious Shoots Regeneration in *Echinacea purpurea* L. Moench

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Abstract

Mannitol or sorbitol was added into the Murashige and Skoog (MS) medium containing certain concentrations of 6-Benzyladenine (BA) which was used to induce adventitious buds of *Echinacea purpurea* L. Results showed that the induced adventitious buds growing from medium added with 15 g·L^{-1} mannitol or sorbitol of the same concentration were more consistent in height. The regeneration rates in MS medium containing 0.2 mg·L^{-1} BA and 15 g·L^{-1} mannitol were increased, while in MS medium containing 0.2 and 0.5 mg·L^{-1} BA, and 15 g·L^{-1} sorbitol, the regeneration rates were suppressed. On the other hand, genotype of explants and the concentration of BA influenced the incidence of hyperhydricity, and the hyperhydricity of regenerated buds was more severe when the petiole explants were inoculated on medium with 15 g·L^{-1} mannitol or 15 g·L^{-1} sorbitol. The present study offers new possibility to the production of uniform plantlets for commercial cultivation in this important medicinal plant.

Keywords

Echinacea purpurea, Micropropagation, Tissue Culture, Mannitol, Sorbitol, Osmotic Pressure

1. Introduction

Purple coneflower (*Echinacea purpurea* L. Moench) has been used in North American over centuries and had been traded worldwide for decades because of its immunoregulation capability [1] [2]. However, plantations are still growing *E. purpurea* plants from

wild seeds [3], indicating the presently available *in vitro* propagation protocols are yet to be upgraded to meet the demands of large scale cultivation practice. One of the problems in the cloning techniques is that the *in vitro* induced adventitious buds from each petiole explant were not of uniform size [4], which resulting in the lack of uniformity of the consequent *in vitro* plantlets and the plants after transplantion. The lack of uniformity makes it difficult to perform efficient plantation management.

Mannitol and sorbitol were usually used as osmotic pressure regulators [5] [6] in plant *in vitro* cultures, and were considered not to be absorbed as plant carbon sources. The aim of the present experiments was to ascertain the effects of mannitol and sorbitol in *in vitro* adventitious bud regeneration cultures in *E. purpurea*.

2. Materials and Methods

2.1. Materials

The original *E. purpurea* plants were grown from seeds bought from the company of Plantation Products (Norton, MA, USA) [7]. Seeds were sterilized with 0.1% mercury bichloride for 30 s, 10% sodium hypochlorite for 60 s, and then washed with sterile water for three times [8]. The aseptic seeds were inoculated on Murashige and Skoog (MS) medium with half-strengthmineral salts, 0.01 $mg{\cdot}L^{-1}$ Naphthalene Acetic Acid (NAA) and 30 $g{\cdot}L^{-1}$ sucrose. The *in vitro* plants were propagated by inducing axillary buds [9] with medium consisting of full MS mineral salts, 0.5 $mg{\cdot}L^{-1}$ BA, 0.01 $mg{\cdot}L^{-1}$ NAA, and 30 $g{\cdot}L^{-1}$ sucrose. After tests, the petioles cut from the cloned seedlings A9, A13, and A54, with different hyperhydricity ratios were selected for use in the present study.

2.2. Methods

- Preparation of the Medium

Each glass jar was filled with 40 mL of medium and then covered with a polycarbonate screw cap. The medium for testing hyperhydricity contained Murashige and Skoog (MS) basal elements [10], 3% sucrose, 0.4 $mg{\cdot}L^{-1}$ BA and 0.01 $mg{\cdot}L^{-1}$ NAA [11]. The medium for inducing adventitious bud formation from explants contained MS basal elements, 3% sucrose, 0.2, and 0.5 $mg{\cdot}L^{-1}$ BA, 0.01 $mg{\cdot}L^{-1}$ NAA and 15 $g{\cdot}L^{-1}$ man- nitol, or 15 $g{\cdot}L^{-1}$ sorbitol. All the media used were adjusted to a pH value of 6.0, gelled with 0.45% agar prior to autoclaving at 1.4 $kg{\cdot}cm^{-2}$ for 20 min.

- Maintenance of the Cultures

The cultures were stored in a culture room at 25˚C - 27˚C. The cultures used for seed germination were first kept in darkness for 10 days, and all the other cultures were kept under a photoperiod of 12 h light (about 50 $\mu mol{\cdot}m^{-2}{\cdot}s^{-1}$)/12h darkness.

- Data Collection and Analysis

Data for adventitious bud formation were recorded after 40 days of culture. For the regeneration rate of adventitious buds, at least 1 cm in height, without hyperhydricity [12] and at least two visual leaves were considered as valid. For the height of buds, every bud was picked out from the bottle and the length from rhizome to leaf tip was measured. Statistical analysis of the data was carried out by using the SPSS 19.0 soft-

ware. The results were expressed as mean ± SD (standard deviation). Data were analyzed by one-way ANOVA followed by Duncan's multiple range tests or the independent sample *t*-test using Statistical Package for Social Sciences 19.0 (SPSS, Chicago, IL, USA). A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Hyperhydricity Ratio Tests of Petiole Explants from Different Seeds

Three groups of petiole explants from different seeds were inoculated on the same medium consisting of full MS mineral salts, 0.5 mg·L^{-1} BA, 0.01 mg·L^{-1} NAA, and 30 g·L^{-1} sucrose. Results were showed in **Table 1**. Petiole explants from seed clone A9, A13, and A54 were tested. All three groups of explants share similar regeneration efficiency (3.29, 2.92, and 3.04 shoots per explant), but hyperhydricity ratio in clones A9 and A3 were higher.

3.2. Effects on Regeneration of Explant with Low Hyperhydricity Ratio

Results showed in **Figure 1** and **Table 2** indicate that in MS medium with 0.2 mg·L^{-1} BA and 0.01 mg·L^{-1} NAA, adding 15 g·L^{-1} mannitol increased the regeneration rate of explants of A54, with low hyperhydricity ratio from 1.77 to 2.37 shoots per explant. In MS medium with 0.2, and 0.5 mg·L^{-1} BA, and 0.01 mg·L^{-1} NAA, adding 15 g·L^{-1} sorbitol decreased the regeneration rates for all the clones. But, supplementing 15 g·L^{-1} mannitol or 15 g·L^{-1} sorbitol to any of the media decreased the mean value and standard deviation of shoot height. In other words, both of them increased the uniformity.

3.3. Effects on Regeneration of Explant with High Hyperhydricity Ratio

Results showed in **Figure 2** indicate that in MS medium with 0.3 mg·L^{-1} BA and 0.01 mg·L^{-1} NAA, adding 15 g·L^{-1} mannitol increased the hyperhydricity rate, the regeneration rate, and the uniformity of height of shoots in clone A13, which was with higher hyperhydricity ratio. Meanwhile, it decreased the mean height of shoots.

Another group of explants with high hyperhydricity ratio were collected from seed clone A13. The explants were inoculated on MS medium with 0.2, and 0.5 mg·L^{-1} BA, 0.01 mg·L^{-1} NAA, and 15 g·L^{-1} mannitol or 15 g·L^{-1} sorbitol. The hyperhydricity ratios increased with the addition of mannitol or sorbitol. Some leave of shoots in medium

Table 1. Hyperhydricity ratio tests of petiole explants from different seed clones.

Clone code.	No. Explants	Normal shoots per explant	No. Hyperhydricity shoots per explant	% hyperhydricity	Hyperhydricity level
A9	24	3.29 ± 0.50a*	1.33 ± 0.29a	40.51	High
A13	24	2.92 ± 0.43a	0.92 ± 0.24a	31.43	High
A54	24	3.04 ± 0.48a	0.13 ± 0.07b	4.11	Low

*Values are expressed as mean ± SE. Data in the same column followed by different letters are significantly different by Duncan's test at $P < 0.05$ level.

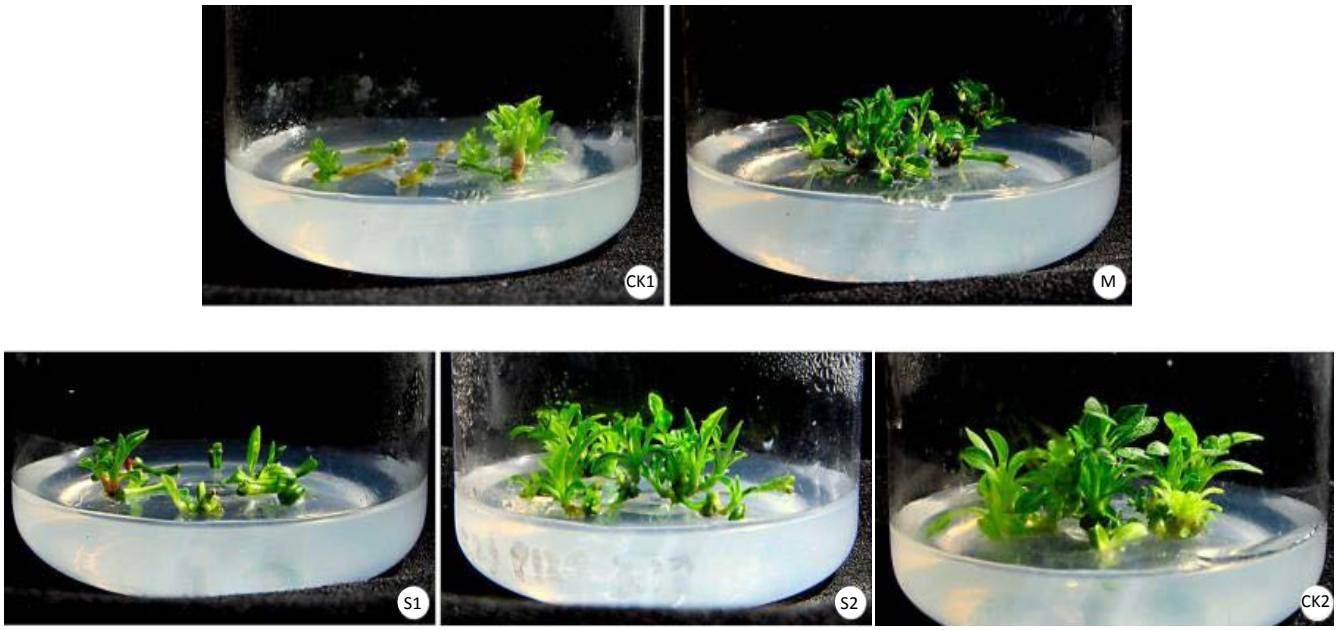

Figure 1. Effect of mannitol or sorbitol on regeneration of explants of a low hyperhydricity ratio clone (A54). CK1: 0.2 mg·L^{-1} BA; M: 0.2 mg·L^{-1} BA + 15 g·L^{-1} mannitol; S1: 0.2 mg·L^{-1} BA + 15 g·L^{-1} sorbitol; S2: 0.5 mg·L^{-1} BA + 15 g/L sorbitol; CK2: 0.5 mg·L^{-1} BA.

Table 2. Effect of mannitol or sorbitol on regeneration rate and height of shoot.

Treatment	Content of BA (mg/L)	Supplement and concentration	No. shoots per explant	Height mm)	No. shoots surveyed	Standard deviation of height
CK1	0.2	--	1.77 ± 0.34b*	8.69 ± 0.58a	54	4.27
M	0.2	Mannitol, 15g·L^{-1}	2.37 ± 0.41b	4.61 ± 0.26b	56	1.94
S1	0.2	Sorbitol, 15 g·L^{-1}	0.78 ± 0.19c	4.93 ± 0.35b	28	1.84
S2	0.5	Sorbitol, 15 g·L^{-1}	2.77 ± 0.34b	5.81 ± 0.27b	83	2.50
CK2	0.5	--	4.83 ± 0.47a	8.94 ± 0.39a	165	4.95

*Values are expressed as mean ± SE. Data in the same column followed by different letters are significantly different by Duncan's test at $P < 0.05$ level.

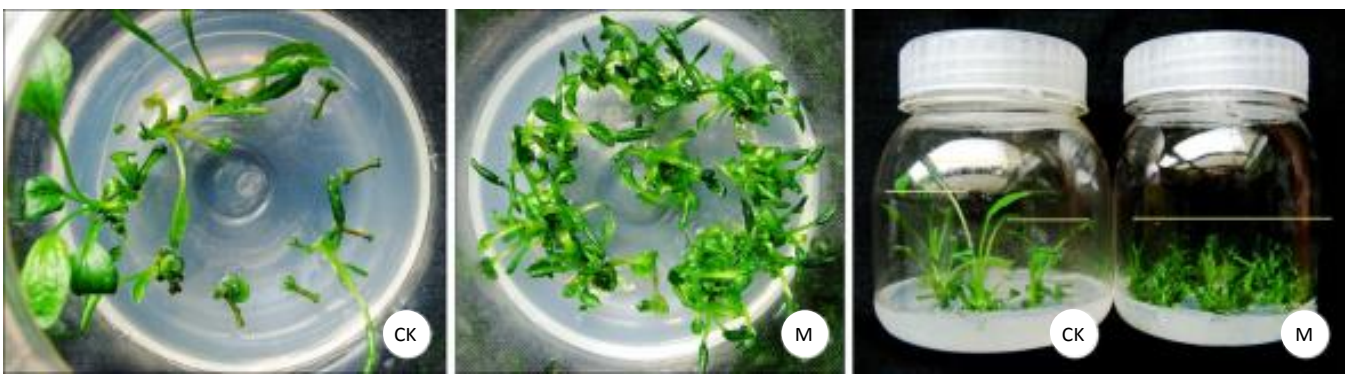

Figure 2. Effect of mannitol on regeneration of explants of a high hyperhydricity ratio clone (A9). CK: MS medium with 0.3 mg·L^{-1} BA and 0.01 mg·L^{-1} NAA; M: MS medium with 0.3 mg·L^{-1} BA, 0.01 mg·L^{-1} NAA, and 15 g·L^{-1} mannitol.

with mannitol turned red, other leaves of shoots in medium with sorbitol were small, twist, and red (**Figure 3**).

4. Discussion and Conclusions

Normally, mannitol and sorbitol were used as osmotic pressure regulators [5] [6]. Osmotic pretreatment has been considered conducive to the axillary shooting from cauliflower curd pieces by acting through internal cytokinin level modifications [13].

The effects of sorbitol and mannitol on adventitious shoots inducement of three genotypes of explants with different hyperhydricity ratios were studied in the present paper. Firstly, the explants of three selected seed clones were test, and the results showed that among them, A54 had low hyperhydricity ratio, while A9 and A13 had high hyperhydricity ratio. Secondly, the explants from clone A54 were found to yield more shoots in MS medium with 0.2 mg·L^{-1} of BA and 15 g·L^{-1} mannitol than in the one without mannitol. However, in MS medium with BA and 15 g·L^{-1} mannitol and sorbitol, the regeneration was restrained, indicating the effects of 15 g·L^{-1} mannitol and 15 g·L^{-1} sorbitol were different. Thirdly, the explants from seed clones A9 and A13 were all weaker and the hyperhydricity was serious in medium with 15 g·L^{-1} mannitol or 15 g·L^{-1} sorbitol. Since sorbitol has been proved to increase shoot proliferation and reduce hyperhydricity in Japanese pear [14], the concentration or chemicals used in the present experiments may be not suitable.

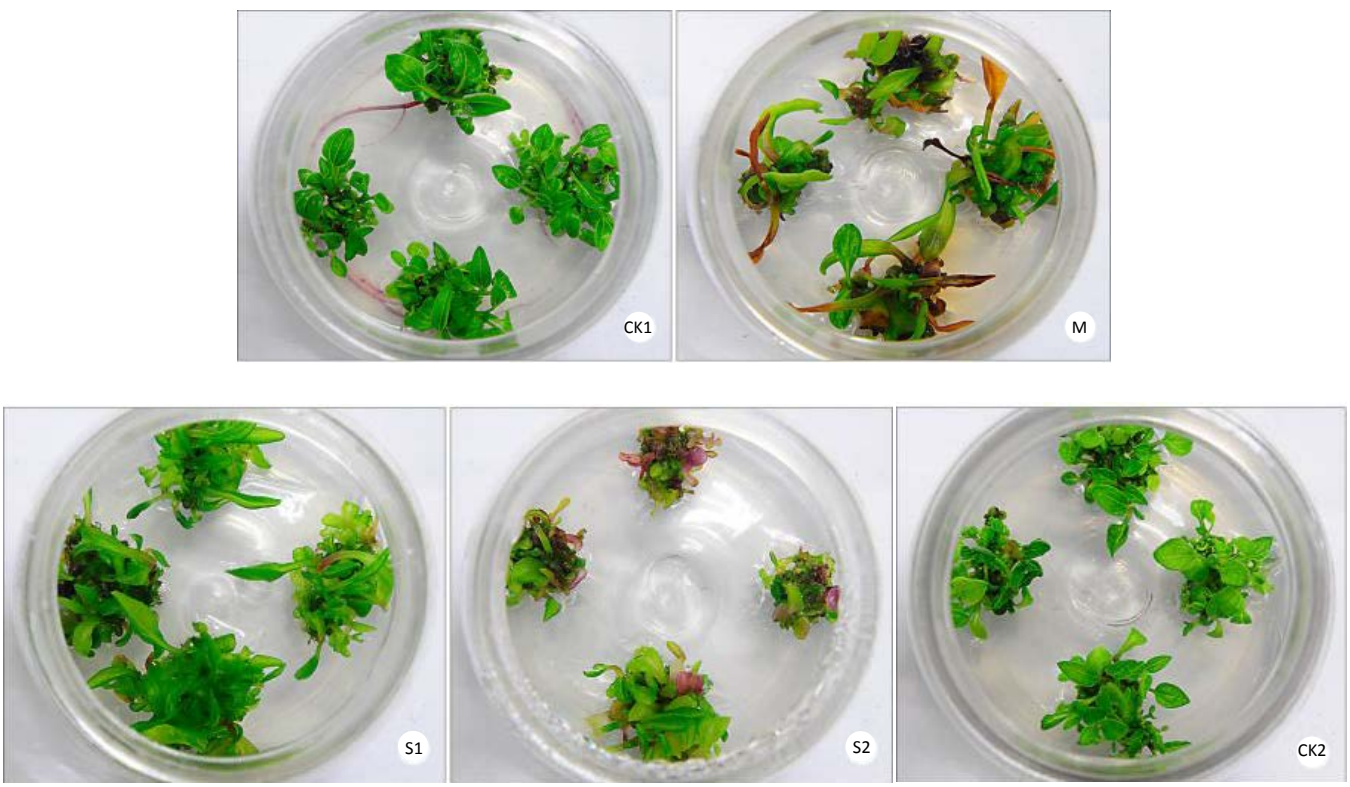

Figure 3. Effect of mannitol or sorbitol on regeneration of a seed clone (A13). CK1: 0.2 mg·L^{-1} BA; M: 0.2 mg·L^{-1} BA + 15 g·L^{-1} Mannitol; S1: 0.2 mg·L^{-1} BA + 15 g·L^{-1} sorbitol; S2: 0.5 mg·L^{-1} BA+15 g·L^{-1} sorbitol; CK2: 0.5 mg·L^{-1} BA.

In all experiments, uniformities of adventitious shoots were all increased. Whether the hyperhydricity of regenerated buds will become more severe when a petiole explant was inoculated on medium with mannitol or sorbitol depends, maybe to the difference of genotype. The genetic diversity [15] was showed again in the present study investigating the effects of sorbitol and mannitol in *E. purpurea*, and the results obtained in the present study may offer new insights for increasing the cloning efficiency of this important medicinal plant.

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