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# Anther Culture and Plant Regeneration of Tetraploid Purple Coneflower (*Echinacea purpurea* L.)

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## Abstract

Anthersisolated from tetraploid purple coneflowerplants were cultured *in vitro*. The highest callus induction rate was obtained when the medium was consisted of N6 basal elements, 4% sucrose, 0.5 mg·L$^{-1}$ BA, and 0.10 mg·L$^{-1}$ NAA. Various morphogenesis such as globular, heart-shape, torpedo-shapeand final state embryos as well asvarious texture calluses around were observed. Out of 110 plantlets regenerated, 104 were confirmed as diploid and the rest were as tetraploid. Plants of one diploid offspring strain presented aspecialcharacter in pot: unlike the original tetraploid plants, it grown tubular, bisexual ray florets. The results obtained in the present studies indicated that although the tetraploid purple coneflower plants produced only diploid microspores, the recovery of some useful mutants through *in vitro* anther cultures might be reasonably expected.

## Keywords

Purple Coneflower, *Echinacea purpurea*, Anther Culture; Tetraploid

## 1. Introduction

As a medicinal plant with functional compounds accumulated in the vegetative parts [1], polyploidization has been carried out successfully [2] [3] [4], and has been proved a breeding strategy worth tryingforpurple coneflower [5] [6] [7]. As anther culture has been reported in purple coneflower which resulted in the regeneration of haploid plants [8], it is foreseeable that aneuploidy plants may occurthrough culturing anthers isolated from the multiploid plants. Aneuploidy triticale has been obtained from regenerants in

*in vitro* anther cultures [9]. Wild purple coneflower plants are diploids [10], and tetraploid purple coneflower plantsrecovered from colchicine-treated diploid explants have been proved with high value because they contain higher concentrations of cichoric acid than the natural diploid plants do [11]. Asabnormal chromosome behaviors have been found in tetraploid purple coneflower plants after the first meiotic diplotene and after the second meiotic telophase, the present study is to certify if aneuploidy could be induced through culturing anthers from tetraploid purple coneflower plants.

## 2. Materials and Methods

### 2.1. Materials

Original diploid purple coneflower plants were grown from seeds provided by the Company of Plantation Products (Norton, MA, USA). Tetraploid plants were obtained by *in vitro* treatment of diploid explants with colchicine, and plant regeneration was induced in these colchicine-treated explants [4]. The tetraploid plants were transplanted to pots with soil.

### 2.2. Methods

#### 2.2.1. Observation on Morphological Change of Florets and Microspores

A capitulum in which the first few florets were open was cut from the inflorescence stem. Florets with different sizes were picked up and the photos were taken in sequence. Each picked floret was placed on a glass slide. Pelea, perianth and pistil were removed with a tweezers. The anthers were cut into pieces with a blade. The pollen mother cells or microspores were squeezed out of the anther wall. The slides with cells were stained with Carbol fuchsin, observed under an optical microscope, and photos were taken.

#### 2.2.2. Accessing Microspore Develop Process and Determining the Callus Induced Rate

A capitulum in which the first few florets were open was cut from the inflorescence stem. Thecapitulum was surface-sterilized by immersingin 70% ethanol for 1 minute, soaking in a 0.1% mercuricchloride solution for 10 minutes and followed by 1% sodiumhypochlorite solution containing one drop of Tween 20 per50 mL for 10 minutes. The surface-sterilizedcapitulumwasrinsed three times in sterile deionized water. Each floret was pulled out and the pelea, perianth, and pistil were removed. Each 20 anthers were inoculated onto medium in one bottle. Each jar was filled with 40 mL of medium and covered with a polycarbonate screw cap. The medium was consisted of N6 basal elements, 5% sucrose, 0.5 $mg{\cdot}L^{-1}$ BA, and 0.10 $mg{\cdot}L^{-1}$ NAA. The cultures were kept in light conditions with a 12-hour photoperiod under cool-white light (about 50 μmol $m^{-2}{\cdot}s^{-1}$), and all thecultures were kept in a room with temperature of 25˚C - 27˚C. Numbers of callus were counted 30 days after initiation of the cultures.

#### 2.2.3. Investigation of the Effect of Genotype on Callus Induction

Anthers isolated from plants with different genotypes were cultured under same condi-

tions according to the reported methods [8]. Numbers of callus were counted 30 days after initiation of the cultures.

#### 2.2.4. Investigation of the Effect of Basic Medium on Callus Induction

The surface-sterilized anthers were prepared by the same way given in 2.2.2 and then inoculated onto medium with 5% sucrose, 0.5 mg·L$^{-1}$ BA, 0.10 mg·L$^{-1}$ NAA, and four groups of different basal elements, N6, C17, W14, and Potato II. Numbers of callus were counted 30 days after initiation of the cultures.

#### 2.2.5. Investigation of the Effect of Sucrose Concentration on Callus Induction Rate

The surface-sterilized anther was prepared by the same way given in 2.2.2 and then inoculated onto medium consists N6 basal elements, 0.5 mg·L$^{-1}$ BA, and 0.10 mg·L$^{-1}$ NAA, and different concentration of sucrose, 4%, 5%, 6%, and 7% respectively. Numbers of callus were counted 30 days after initiation of the cultures.

#### 2.2.6. Induction of Adventitious Buds and Roots, and Counting of the Chromosome Number

Induction of adventitious buds and roots, and counting of the chromosome were performed by the reported methods [8].

#### 2.2.7. Data Collection and Analysis

Callus induction rate (%) was calculated by dividing the No. of callus with the No. of anthers cultured. Statistical analysis of the data was carried out with ANOVA analyses using the SPSS 19.0 software, and the significant differences among the means were determined by the Duncan's multiple range tests for more than two data sets, or by the independent sample *t*-test for two sets of data. The differences were considered significant when the *P* values were less than 0.05.

## 3. Results

### 3.1. Relationship between Morphological Change of Florets and Microspores

As showed in **Figure 1**, the color of floret was closely related with the stage of microspore.

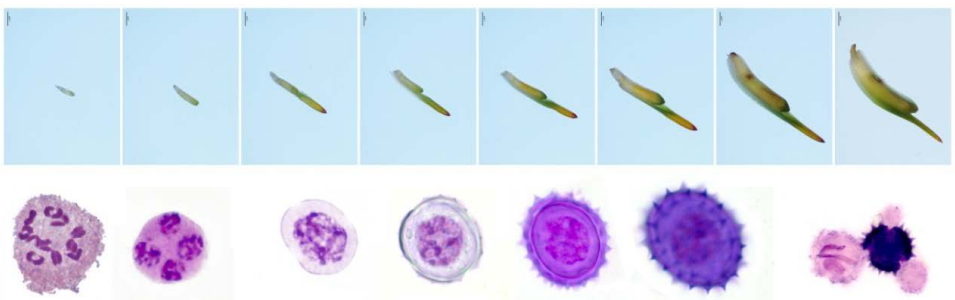

**Figure 1.** Relationship between morphological change of florets and microspores. From left to right: meiosis phase, tetrad, early uninucleate, early-middle uninucleate, middle uninucleate, late uninucleate, mature pollen; Bar = 1 mm.

The appearing of yellow color was almost simultaneous with the formation of pollen wall. The pollens in brown color floret were mature. A maturemicrosporeconsists of one vegetative nucleus and two generative nucleuses.

### 3.2. Relation between Microspore Development Process and Callus Induced

The callus induction rate was connected directly to the development state of microspores. Microspores from early uninucleate phase to late uninucleatephase represented a high potential in callus induction rate (**Table 1**).

### 3.3. Effect of Genotype on Callus Induction

Callus induction rate of anthers isolated from plants with different genotype were evidently different from each other (**Table 2**).

### 3.4. Effect of Basic Medium on Callus Induction

Basal medium elementary affected the callus induction rate strongly. Callus induction rates for C17, W14, and Potato Ⅱ basal elements were significantly lower than that for the control (**Table 3**).

### 3.5. Effect of Sucrose on Callus Induction

Callus induction rate on medium with 4%, 5%, 6% and 7% sucrose were not significant

**Table 1.** Relation between microspore development state and callus induction rate.

| Length of floret (mm) | Color of floret | State of microspore | Callus induction rate (%) |
|---|---|---|---|
| >6.0 | Brown | Mature pollen | 1.25 ± 1.25e* |
| 5.9 - 6.0 | Deep yellow | Mature pollen | 33.75 ± 6.57d |
| 3.8 - 5.9 | Yellow | Mature pollen | 45.00 ± 2.04c |
| 2.6 - 3.8 | Faint yellow | Late uninucleate | 83.75 ± 5.15b |
| 1.8 - 2.6 | Faint yellow | Middleuninucleate, late uninucleate | 96.25 ± 1.25a |
| 1.2 - 1.8 | Colorless or pale green | Middleuninucleate | 98.75 ± 1.25a |
| 0.8 - 1.2 | Colorless or pale green | Early-middle uninucleate | 96.67 ± 1.67a |
| 0.6 - 0.8 | Colorless or pale green | Early uninucleate | 96.25 ± 2.39a |

*Data in the same column followed by different letters are significantly different by Duncan's test at $P < 0.05$ level.

**Table 2.** Effect of genotype on callus induction rate.

| Genotypecode | No. of anther | No. of callus | Callus induction rate (%) |
|---|---|---|---|
| T1 | 580 | 77 | 13.28 |
| T2 | 480 | 119 | 24.79 |
| T3 | 160 | 77 | 44.38 |
| T4 | 640 | 143 | 22.34 |

different, while the highest induction rate was obtained when the concentration of sucrose was of 4% (**Table 4**).

### 3.6. Observation of Cultivation

Some anthers did not yield callus and died on callus induction medium, some anthers recovered hyperhydricitycallus and/or adventitious buds, and some produced healthy shoots which initiated roots afterward (**Figure 2**). Moreover, some calluses did not regenerate plants, but formed on the surface numerous globularembryos, heart-shapeembryos, torpedo-shape embryos (**Figure 3**). Among the 110 plantlets investigated,

**Table 3.** Effect of basic medium on callus induction rate.

| Basic medium | Callus inductionrate (%) |
|---|---|
| N6 (CK) | 65.00 ± 6.21a* |
| C17 | 31.67 ± 6.06b |
| W14 | 32.50 ± 7.50b |
| Potato II | 41.25 ± 5.54b |

*Data in the same column followed by different letters are significantly different by Duncan's test at $P < 0.05$ level.

**Table 4.** Effect of sucrose on callus induction rate.

| Sucrose concentration (%) | Callus inductionrate (%) |
|---|---|
| 4.0 | 70.00 ± 13.53a* |
| 5.0 | 68.75 ± 12.81a |
| 6.0 | 66.43 ± 14.99a |
| 7.0 | 66.88 ± 13.59a |

*Data in the same column followed by different letters are significantly different by Duncan's test at $P < 0.05$ level.

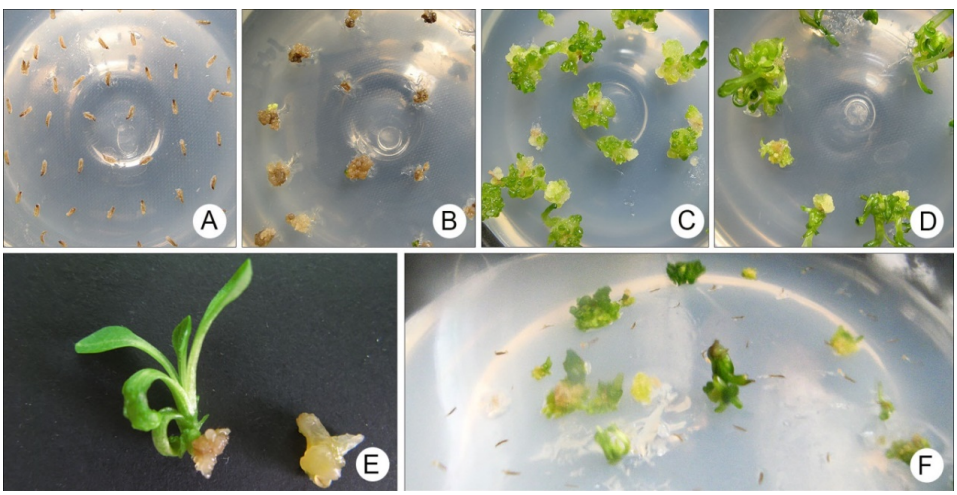


**Figure 2.** Responses of anthers cultured on callus induction medium. (A): inoculated anthers; (B): browning callus; (C): shoot-buds induced from callus; (D): adventitious buds induced from callus; (E): a normal regenerated bud induced from a piece of callus; (F): embryoids and shoots induced directly from tetraploid anthers.

six were confirmed as tetraploid and the rest were diploid. None of aneuploid was detected in present study. One abnormal phenotype (diploid) was detected. It grew tubular, bisexual ray florets (**Figure 4**).

## 4. Discussion and Conclusions

The development of microspores was related to appearance of floret in many plants, for instance, triticale (*XTriticosecale Wittmack*) [9]. The present study revealed that the microspores may be suitable for inducing callus when the floret was faint yellow, colorless, or pale green. The appearing of color on florets and the microspores develop process were linked to the callus induction rates. Large variation in the callus induction rates was observed among the four genotypes. These results coincide with those reported in literatures [8] [12].

In comparison to C17, W14, and Potato II, N6 was more suitable for culturing anther from tetraploid purple coneflower plants. Concentration of sucrose, from 4% to 6%, seems influenced the culture non-significantly. The callus, which induced from tetraploid anthers, seems weaker compared to those from diploid anthers [8]. Some advent-

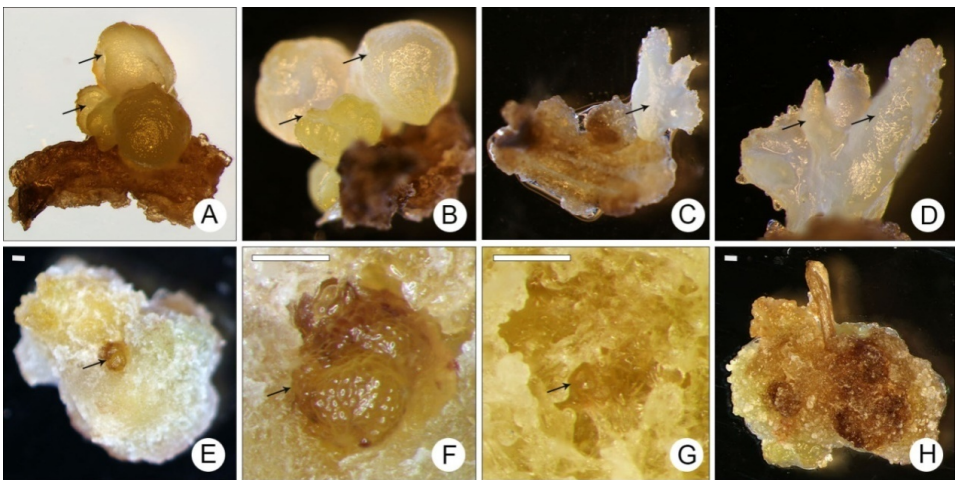


**Figure 3.** Morphogenesisof callus induced from anthers. (A) globularembryo; (B) heart-shapeembryo; (C) and (D), torpedo-shape; (E)-(G): embryo and callus around; H: callus, bar = 200 μm.


**Figure 4.** An inflorescence andits floret of adiploid regenerant recovered from tetraploid plant anther cultures. (A) inflorescence in 2012; (B) inflorescence in 2013; (C) a tubular ray floret, arrow shows top end of the floret which doesn't cracks to ovary like others; (D) inner sight of a tubular ray floret, arrow shows pistil and stamens. Bar = 1 mm.

tious buds regenerated from the callus were hyperhydric, failed to develop roots and died finally. As a result the counting of chromosome number did not perform on this group of buds. Some newly reported strategies may help solve this problem [13].

Out of 110 plantlets regenerated in the cultures were investigated. Among them 104 were diploid and the rest were tetraploid. This result proved that most of the plantlets were androgenic progeny, rather than adventitious buds of somatic cells, such asanther wall cells. The abnormal phenotype, which grown tubular, bisexual ray florets, indicated that though the tetraploid purple coneflower plants produced diploid microspore, the recovery of some useful mutants through *in vitro* anther cultures might be reasonably expected.

In conclusion, the present study implicated that the meiosis process in tetraploid purple coneflower plants was quite orderlysince so far there have been no aneuploid recovered. For this reason it might be considered that the reductional division of chromosomes in purple coneflower is not controlled by base complementation pairing rule only but also by genes [14] [15].

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## Conflicts of Interest

The authors declare no conflict of interest.

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