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Establishment of Callus Induction and Cell Suspension Cultures of *Dendrathema Indicum* var. *Aromaticum* a Scented Chrysanthemum

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Abstract

Dendranthema indicum var. *aromaticum* is an important aroma plant in genus *Dendrathema*, and the establishment of callus cultures and cell suspension cultures is the basement of further protoplast fusion studies, which make it possible to breed new fragrant chrysanthemum. In this study, the effects of different plant growth regulating substances in different concentrations on callus induction were investigated with stem segments, leaves, petioles as explants. The results showed that the optimal explants were lower stem segments according to the percentage of callus formation, callus hardness, growth potential and shoot differentiation. The optimal induction mediums were MS supplemented with 1.0 $mg\cdot l^{-1}$2.4 D and 0.2 $mg\cdot l^{-1}$ 6-BA. The cell suspension culture system was established by using the subculture calli. The results showed that the suitable inoculum size was 2g and the suitable cell suspension culture medium was MS supplemented with 0.2 $mg\cdot l^{-1}$ 6-BA and 0.5 $mg\cdot l^{-1}$ 2,4-D. The time course of cell growth showed that the greatest cell fresh weight appeared on day 14 and the highest cell viability on day 3.

Keywords: scented *Chrysanthemum*, in vitro, explants, callus formation, cell culture

1. Introduction

1.1 Introduce the Problem

Chrysanthemum *(Dendrathema×grandiflora)*, one of the famous traditional flowers in China, is widely cultivated in the world. Flowers of Chrysanthemum with brightly colored flowers and varied flower types are not fragrant or with a slightly scent. Thus, the improvement of Chrysanthemum's aroma is an important topic in chrysanthemum breeding.

Dendranthema indicum var. *aromaticum* is an herbaceous perennial plant characterized by special scent, which is the important aroma resource in genus *Dendrathema* and found in Shennongjia, Hu Bei province, P. R. China (Liu, Jia, & Yang, 1983). Previous research has proved that *Dendranthema indicum* var. *aromaticum* was diploid (2n=2x=18) and *Dendrathema* × *grandiflora* was polypoild (2n=6x=54) (Zhu, Liu, & Dai, 2011) .As we know that it is difficult to cross breed if one species is diploid while the other is a higher ploidy level. In order to breed scented chrysanthemum, somatic hybridization will be applied. Somatic hybridization is to hybrid plants through the fusion of somatic protoplasts of two different plant species/varieties, with which some interspecific hybrids have been obtained in family *Compositae*, for example the hybrids between *Helianthus annuas* and *H. giganteus* (Henn, Wingender, & Schnabl, 1998; Krasnyanski, & Menczel, 1995), *Tanacetum vulgare* and *T. cinerariifolium* (Keskitalo, Angers, Earle, & Pehu,1999), *Dendrathema×grandiflorum* and *Artemsia sieversiana*(Lee, Paek, & Hwang, 1995),ect.

The objective of this project was to establish callus cultures and cell suspension cultures of *D. indicum* var. *aromaticum* for further protoplast fusion studies, which make it possible to breed new fragrant chrysanthemum.

2. Materials and Methods

2.1 Plant Material and Callus Induction

Seedlings of *D. indicum* var. *aromaticum* were obtained in 2008 from Shennongjia in the Hu Bei province, P. R. China and were maintained in the garden nursery, College of Landscape Architecture and Horticulture, Northeast Forestry University, Harbin, P. R. China. Shoots were surface sterilized first by washing under running tap water and laundry bleach for 10 min, then disinfected in 2% or 5% sodium hypochlorite for 10 min and rinsed with sterile distilled water 6-8 times. Surface sterilized shoots were trimmed, prior to inoculation on the basal medium under sterile conditions. The subcultures were done at regular intervals incubated at 25±2℃ with a 16-h photoperiod and illumination of 2000 lx. Basal nutrient medium contained MS(Murashige & Skoog,1962) salts and vitamins and sucrose (30 g· l^{-1}).Nutrient medium pH was adjusted to 5.8 prior to addition of 8 g· l^{-1} Agar and the autoclaved 121℃ for 20min.Fresh and healthy leaves(2×2mm from the central plate),stem segments(4mm in length) and petioles were obtained from in vitro grown plants, then transferred on MS basal medium supplemented with different concentrations of 2,4-D(0.0,0.5,1.0,1.5,2.0,2.5,3.0 mg· l^{-1}), BA(0.2 mg· l^{-1}), sucrose(30 g· l^{-1}) and agar(8 g· l^{-1}).The pH of medium was adjusted to 5.8±0.1.Each treatment combination consisted of three replications and maintained in the presence or absence of light.

2.2 Cell Suspension Cultures and Cell Growth Determination

Suspension cultures were established from friable calli. 1-4g fresh weight friable calli was transferred to 100 ml Erlenmeyer flasks containing 50 ml MS basal medium with BA 0.2 mg· l^{-1}, 2,4-D 0.5 mg· l^{-1}， 30 g· l^{-1} sugar in order to optimize the suitable inoculum size. The research of effect of exogenous plant growth regulators on cell suspension cultures were carried out by using 2 g fresh weight friable calli, the best inoculum size, with different concentrations of 2,4-D,6-BA and NAA, which was designed by orthogonal design L9 (3^3).All the cell suspension cultures were placed on a rotary shaker with a speed of 90 rpm at 25 ±2℃ under a 16-h photoperiod and illumination of 1000 lx. The FWt was measured after 18 days of culture. Five flasks were used per treatment and each experiment was repeated third. Anovas and Duncan comparison tests for each evaluated factor were carried out using the SPSS 12.0 software.

3. Results and Discussion

3.1 Effect of 2, 4-D and the Types of Explants on Callus Induction

Different types of explants were placed on MS basal medium supplemented with different concentrations of 2,4-D and 0.2 mg· l^{-1} BA for 60 days of cultivation. The percentage of callus formation, growth potential, callus hardness and shoots per callus were evaluated according to the method of Fadi Chen (Chen, Jiang, & Guo, 2003).

Some related research indicated that 2, 4-D played a more important role in callus formation from leaf and stem explants compared to BA (Satyavani, Ramanathan, & Gurudeeban, 2011).But in this experiment, Calli were successfully generated in all treatments regardless of 2, 4-D concentration and different types of explants (Table 1).These results showed that leaf and stem explants were suitable for callus induction. Similar results were reported in callus induction of *Corydalis saxicola and Orthosiphon stamineus*, in which it were described that callus could be induced successfully from leaf, petiole, stem (Cheng, Yu, Hu, Chen, & Sun, 2006).

The callus hardness was classified into level 1 to level 5 according to the visual observation. The higher hardness level showed the callus was more hardness, which was bad to cell suspension culture. The callus potential growth was also classified into level 1 to level 4 according to the growth from slow to fast. The percentage of callus formation was the highest with the leaf as exlants (Table 1), but the callus was more hardness and the potential growth was lower. As for further cell suspensions, the friable calli were better than the compact calli. Thus we thought that lower stem segments were the best explants because the induced calli hardness was smaller and the growth potential was the biggest although the percentage of callus formation was not the highest. And the optimum medium was MS containing 0.2 mg· l^{-1}6-BA and 1.0 mg· l^{-1} 2,4-D.

3.2 Effects of Inoculum Size on Cell Suspension Cultures

Suspension cultures were initiated by transferring 1-4 g fresh friable calli into 50 ml MS medium in 100 ml flasks. The inoculum size had a positive effect on biomass. Cell fresh weight reached the lowest value of 1.71g at the inoculum size of 1g, and increased to the highest value of 5.88g at 4g. But the highest proliferation ratio and the best cell growth status were observed at the inoculum size of 2g（88.5）(Table 2), and its cell cluster was small round and well dispersed, cell suspension liquid was clear.

3.3 Effects of Exogenous Plant Growth Regulators on Cell Suspension Cultures

The effect of these plant growth regulators on cell suspension culture was presented in Table 3. There existed significant differences among all the treatments which were designed by orthogonal design L9 (3^3) (α=0.05).In case of Treatment 4 and 7 the cell fresh weights (Fwt) were significantly higher than other treatments, and cell suspension liquids were clear the cell cluster was small and high dispersion. Therefore, the most suitable cell suspension culture medium for cell growth in *D. indicum* var. *aromaticum* was MS+0.2 mg·l^{-1} 6-BA+0.5 mg·l^{-1} 2, 4-D.

3.4 Time Course of Cell Growth

The time course of cell growth has been studied by growing suspension cells in 100 ml flasks, containing 40ml liquid MS medium with 0.2 mg· l^{-1} 6-BA , 0.5 mg· l^{-1} 2,4-D and 30 g· l^{-1} sugar. As shown in Fig. 1, cell growth was slow during initial 6 d of cultivation. From day 6, biomass accumulated rapidly and reached the greatest value on day 14. Then a slow decrease of biomass was observed in the later stage of cultivation. These results showed that some cells which couldn't adapt to the change from solid to liquid medium and gradually went to death. After that, biomass increased rapidly and reached the highest value. Similar results were reported in *Cleistocalyx operculatus* (Zhou, Wang, & Xiao, 2007), *Dendrathema grandiflora* (Jiang, 2002), *Morus alba* (Li Yong et al., 2007) .Cell viability was measured by TTC method（Steponkus & Lanphear, 1967）using a Unic spectrophotometer, model UV-2102C, at 485 nm wavelength. The bigger OD value indicates the greater cell viability. The TTC test results showed cell viability increased in the initial 3 d followed by a gradual decrease. The growth patterns of cultures showed that cell viability was lower in its rapid growth, and higher in its slow growth stage, which was the same with *Xanthoceras sorbifolia* (Liu,2009)and *Taxus cuspidata*(Liu,2002).

In conclusion, the friable callus of *D. indicum* var. *aromaticum* could be efficiently introduced with lower stem segments as explants on MS medium supplemented with 1.0 mg· l^{-1}2.4 D and 0.2 mg· l^{-1} 6-BA. 2 g fresh weight friable calli were the best inoculum size. The most suitable cell suspension culture medium was MS+0.2 mg· l^{-1} 6-BA+0.5 mg· l^{-1} 2,4-D. The growth patterns of cell cultures showed S-curve and the greatest cell fresh weight appeared on day 14, the highest cell viability on day 3.

Table 1. Effect of 2,4-D and different types of explants on the callus induction

Treat-ment code	2,4-D	Explants' types	Percentage of Callus formation	Callus potential growth classification	callus hardiness classification	Shoots per callus
T1	0	leaves	100±0.00	0.80±0.18	5.00±0.00	0.90±0.00
T2	0	peridol	73.3±2.40	1.20±0.00	5.00±0.00	1.19±0.01
T3	0	Upper stem	100±0.00	1.00±0.14	5.00±0.00	1.62±0.02
T4	0	Lower stem	86.7±2.40	1.80±0.13	5.00±0.00	1.19±0.01
T5	0.5	leaves	100±0.00	1.80±0.16	3.50±0.11	1.70±0.12
T6	0.5	peridol	83.3±1.40	2.00±0.14	4.30±0.15	1.10±0.11
T7	0.5	Upper stem	76.7±2.40	1.80±0.28	3.20±0.10	1.30±0.01
T8	0.5	Lower stem	100±0.00	3.60±0.22	1.50±0.08	1.10±0.16
T9	1	leaves	100±0.00	2.20±0.15	4.00±0.10	0.80±0.11
T10	1	peridol	76.7±2.40	1.20±0.31	3.90±0.12	0.56±0.06
T11	1	Upper stem	70.0±3.30	2.60±0.11	2.10±0.10	0.26±0.01
T12	1	Lower stem	96.7±2.40	3.80±0.15	1.00±0.00	0.79±0.01
T13	1.5	leaves	100±0.00	1.60±0.21	4.00±0.14	0.32±0.08
T14	1.5	peridol	93.3±2.53	2.40±0.14	4.10±0.23	0.60±0.01
T15	1.5	Upper stem	63.3±2.33	1.80±0.14	3.10±0.11	0.12±0.01
T16	1.5	Lower stem	90±1.33	2.80±0.13	1.50±0.11	0.60±0.01
T17	2	leaves	96.7±2.40	2.20±0.23	3.50±0.15	0.42±0.02
T18	2	peridol	100±0.00	2.60±0.14	2.90±0.07	0.16±0.02
T19	2	Upper stem	83.3±2.33	2.80±0.18	2.90±0.21	0.00±0.00
T20	2	Lower stem	93.3±2.53	2.20±0.28	2.00±0.24	0.54±0.04
T21	2.5	leaves	100±0.00	2.80±0.18	3.50±0.11	0.30±0.01
T22	2.5	peridol	80±2.73	2.20±0.14	2.20±0.18	0.30±0.02
T23	2.5	Upper stem	73.3±4.40	2.20±0.13	1.80±0.22	0.30±0.01
T24	2.5	Lower stem	96.7±2.40	3.20±0.14	1.00±0.14	0.86±0.01
T25	3	leaves	100±0.07	2.00±0.07	3.50±0.07	0.80±0.07
T26	3	peridol	60±0.08	1.60±0.08	3.20±0.08	0.10±0.08
T27	3	Upper stem	100.0±0.00	1.00±0.00	3.80±0.00	0.00±0.00
T28	3	Lower stem	90±0.02	2.20±0.02	2.00±0.02	0.28±0.02

Table 2. Effect of inoculum size on cell growth in *Dendranthema indicum* var. *aromaticum* suspension cultures

Inoculum mass(g)	Cell fresh weight(g)	proliferation ratio	Cell Growth status
1	1.71±0.48	71.0 B	Good dispersion, cell cluster small and pale yellow, medium clear
2	3.77±0.53	88.5 A	Excellent dispersion, cell cluster small and pale yellow, clear
3	4.59±0.78	53.0 C	Bad dispersion, cell cluster big and dark yellow, a little turbidity
4	5.88±0.49	47.0 D	Bad dispersion, cell cluster big and dark yellow, turbidity

The suspension cultures were cultivated in MS medium containing 0.5 $mg \cdot l^{-1}$ 2,4-D and 0.2 $mg \cdot l^{-1}$ BA on a rotary shaker. Data are expressed as means e S.D. (n = 3).

Note: A~D is the variance analysis of LSD letter notation, α=0.01, All values are T statistical data after 18 d culture.

Table 3. Effects of exogenous plant growth regulators on cell suspension cultures in *Dendranthema indicum* var. *aromaticum*

Treatment Code	exogenous plant growth regulators (mg. l-1)			FWt (g)
	2,4-D	NAA	6-BA	
1	0.0	0.0	0.0	1.30±0.52 bc
2	0.0	0.2	0.2	1.33±0.32 bc
3	0.0	0.5	0.5	0.90±0.27 c
4	0.5	0.0	0.2	2.19±0.35 a
5	0.5	0.2	0.5	1.54±0.33 b
6	0.5	0.5	0.0	1.62±0.40 b
7	1.0	0.0	0.5	2.06±0.41 a
8	1.0	0.2	0.0	0.99±0.41 c
9	1.0	0.5	0.2	1.12±0.33 c

The suspension cultures were cultivated on a rotary shaker and collected on day 18 of cultivation. Data indicate means of three independent experiments (means e S.D.)

Note: a~c is the variance analysis of LSD letter notation, α=0.05, All values are T statistical data after 18 d culture.

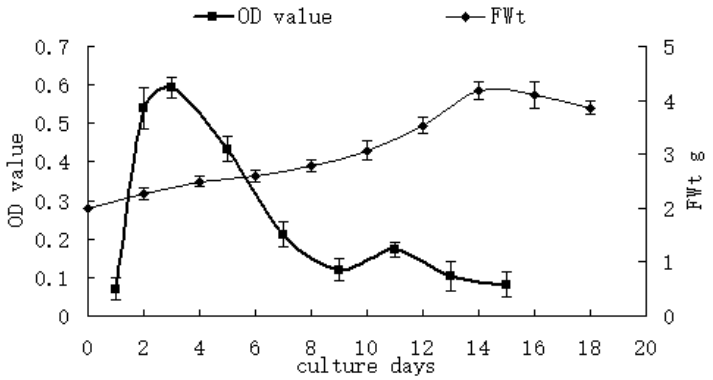

Figure 1. Time course of cell growth and viability in *D. indicum* var. *aromaticum* suspension culture

The suspension cells were grown in liquid MS medium containing 0.5 mg· l^{-1} 2,4-D and 0.2 mg· l^{-1} BA and incubated on a rotary shaker at (25±2)℃ under a 16-h photoperiod and illumination of 1000 lx. Data indicate means of three independent experiments (means e S.D.).

Abbreviations: MS, Murashige and Skoog medium; 6-BA, 6-Benzylaminopurine;2,4D,2,4-Diclorophenoxyacetic acid; 2,3,5-triphenyltetrazolium chloride. I am deeply grateful to Dr. Shunsuke Naruto for his invaluable guidance and advice.

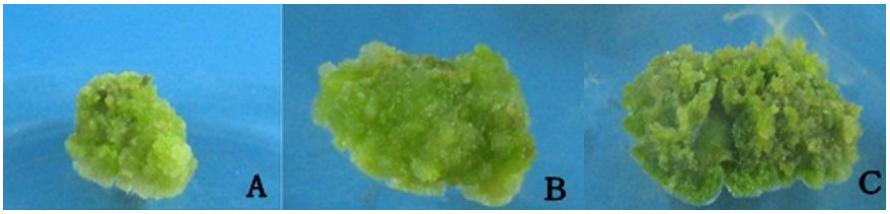

Figure 2. Different types of explants of *D. indicum* var. *aromaticum* on the callus induction

A.The callus induction of stem segments.B.The callus induction of leaves .C.The callus induction of petioles.

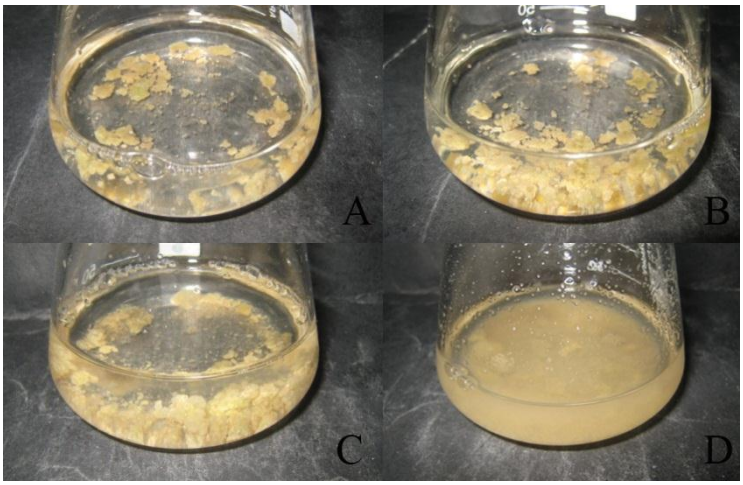

Figure 3. Cells suspension culture growth in different initial quantity of *Dendranthema indicum* var. *aromaticum*

A. The initial quantity is 1g.B. The initial quantity is 2g.C. The initial quantity is 3g.D. The initial quantity is 4g.

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