

Regeneration of Haploid Plantlet through Anther Culture of *Chrysanthemum (Dendranthema grandiflorum)*

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Abstract

To observe the possibility of producing haploid plants of Chrysanthemum, anthers of three Korean cultivars 'Yes Morning', 'Hi-Maya', and pot cultivar 'Peace Pink' were cultured. Callus induction among cultivars differed little, but equally good results were obtained with the basal MS medium supplemented with 1 mg/L of 2,4-D, 2 mg/L of BA, 250 mg/L of casein hydrolysate, 45 g/L of sucrose; solidified by 2.75 g/L gelrite. A pretreatment of anthers in media at 4 °C for 48h enhanced the callus induction. Calli were allowed to differentiate on basal MS medium supplemented with 2 mg/L of BA, 0.1 mg/L of NAA, 30 g/L of sucrose; solidified by 2.75 g/L gelrite. Shoot formation from calli in that media slightly differed among cultivars. Multiple shoots elongated from calli were shifted to basal MS medium supplemented with 0.1 mg/L of NAA, 30 g/L of sucrose; solidified by 3 g/L gelrite for rooting. The plantlets with sufficient roots thus obtained were acclimatized and transferred to the soil. Fifty regenerated plantlets from each cultivar were randomly selected for ploidy observation by chromosome counting and haploid plantlet was detected for the garden cultivar 'Yes morning'.

Keywords: callus inductions, haploid, rooting, regenerated plantlets, shoot formation

Introduction

Chrysanthemum is one of the most popular ornamentals in the world, includes about 40 species of perennial flowering plants in the family Asteraceae. It is preferred particularly for its wide range of shapes and colors of flowers, long lasting vase life, and diversity in height and growth habit (Mukherjee *et al.*, 2013). These plants have both aesthetic and medicinal value and have economic importance in many countries including Korea. Chrysanthemum is also a source of various valuable secondary metabolites, biologically active compounds and essential oils (Schwinn *et al.*, 1994). The genome of Chrysanthemum is composed of multiple sets of chromosomes. The considerable variations in morphology and ploidy level (from $2n = 2x=18$, to $2n= 36, 54, 72$, up to 90) are exhibited (Chen *et al.*, 2008; Liu *et al.*, 2012). Intra-species and even infra-population variations in ploidy have been found (Yang *et al.*, 2006; Zhmyleva and Kondo,

2006). Generally, marketable varieties are vegetatively propagated with cuttings and suckers. Breeding and molecular techniques have been applied for development of Chrysanthemum ornamental value (Rout and Das, 1997; Gion *et al.*, 2012). Although some desirable traits have been introduced by classical breeding, there are some limitations in this technique due to parental ploidy differences and the polygenic nature of growth and flowering. The rate of successful crosses between related and unrelated cultivars is low, and selfing is generally not possible, although some pseudo self-incompatible plants have been discovered (Zagorski *et al.*, 1983; Mukherjee *et al.*, 2013).

The ability to regenerate whole plants from tissue culture has been achieved in Chrysanthemum (*Dendranthema grandiflora*) by a number of groups using various species and cultivars, basal media, different plant growth regulator (PGR) and media additive combinations and concentrations, resulting organogenesis from a number of explant sources including: stems (node and internode),

axillary buds, leaves, shoot tips or apical meristems, protoplasts, roots, pedicels and florets (Teixeira da Silva, 2003a; Rout *et al.*, 2006; Teixeira da Silva *et al.*, 2012). In anther culture technique the developing anthers at a precise and critical stage are excised aseptically from unopened flower bud and are cultured on a nutrient medium where the microspores within the cultured anther develop into callus tissue or embryoids that can give rise to haploid plantlets either through organogenesis or embryogenesis. Compared with conventional inbreeding, the *in vitro* androgenesis technique enables a faster generation of virtually fully homozygous lines but until now, to the best of our knowledge, there are few published reports about anther culture of Chrysanthemum (Watanabe *et al.*, 1972; Yang *et al.*, 2005; Gao *et al.*, 2011).

Present study has been undertaken for growing plants from anther culture of Chrysanthemum (*Dendranthema grandiflorum*) in order to observe *in vitro* response and the possibility of producing haploid plants.

Materials and methods

Chrysanthemum (*D. grandiflorum*) garden cultivars 'Yes Morning', 'Hi-Maya', and pot cultivar 'Peace Pink' were used as plant materials for anther culture. The donor plants were grown in the experimental field using standard agronomic practices in Chrysanthemum Experiment Station, Yesan, Republic of Korea. Flower buds were collected as donor material when most microspores were at the late-uninucleate to early-binucleate stage of development. Anthers were isolated from the buds and placed on glass slides, stained with acetocarmine solution, mashed, and observed under a microscope (Olympus BX51, Japan). The microspores in the anthers were at that stage when the flower buds diameter were 0.5 cm ~ 0.8 cm, center sepal was closed, transparent, and the light yellow florets on the outside.

Based on previous reports (Yang *et al.*, 2005; Gao *et al.*, 2011), four induction media labeled A-1, A-2, A-3, A-4 were prepared using MS (Murashige and Skoog, 1962) as the basic culture medium combined with different plant growth regulators (Tab. 1). All media were adjusted to pH 5.8 and contained 2.75 gm/L of gelrite. Following 20 min of autoclaving at 121°C and 1.1 kg/cm² pressure, 25 ml aliquots of media were poured into 100×15 mm Petri dishes. The Petri dishes were sealed with parafilm while cooling.

Flower buds were rinsed thoroughly under running tap water for 15-20 min. Then they were surface-sterilized by immersion in 70% of ethanol for 30 second followed by sodium hypochlorite (1.5% active chlorite) for 15 min, and then rinsed three times with sterile distilled water (5 min each time). Under aseptic conditions, anthers were removed gently with the help of sterile scalpel. Then 25 anthers were placed immediately onto a single plate of induction medium. Then Petri dishes were sealed and placed at 4 °C for 24h and 48h in dark prior to incubate at 24 ± 1 °C with a 14 hour daily illumination under fluorescent 20W lamps (30-40 μmol/m²/s). Each treatment was applied to 200 anthers and different media were tested using a completely randomized design. The anthers were sub-cultured 2 times

for one month each round on the same medium used for callus induction.

The differentiation medium D-1 was prepared according to Gao *et al.* (2011) with modification (Tab. 1). D-1 contained basal MS medium supplemented with 2 mg/L of BA, 0.1 mg/L of NAA, 30 g/L of sucrose. The pH was adjusted to 5.8 and 2.75 g/L Gelrite was used as solidifying agent. 150 ml aliquot of media was poured into glass vessels of 5×5×13 cm dimensions, sealed with cap and autoclaved at 121 °C and 1.1 kg/cm² pressure for 20 min. The sub-cultured calli from Petri dishes were transferred into glass vessels containing sterile differentiation medium to proliferate and differentiate under a 14 hour photoperiod provided by fluorescent 20W lamps (30-40 μmol/m²/s) at 24 ± 1 °C. Differentiating calli were sub-cultured for 2-3 times each round on the same medium used for differentiation.

Tab. 1. Composition of media used for anther culture of *Chrysanthemum*

Compounds	Induction medium				Differentiation medium	Rooting medium
	A-1	A-2	A-3	A-4	D-1	R-1
Basal medium	MS	MS	MS	MS	MS	MS
6-BA ^z	2	2	2	2	2	-
2,4-D ^z	1	1	1	1	-	-
NAA ^z	-	-	-	-	0.1	0.1
Cascin hydrolysate ^z	-	250	-	250	-	-
Sucrose ^x	90	90	45	45	30	30
Gelrite ^x	2.75	2.75	2.75	2.75	2.75	3

^xg/L; ^zmg/L

Healthy shoots differentiated from green calli with 8-10 true leaves were rescued aseptically from the cultured vials and were separated from each other and again cultured on glass vials with freshly prepared medium to induce root in rooting medium R-1. R-1 consisted of basal MS medium supplemented with 0.1 mg/L of NAA and 30 g/L of sucrose. The pH was adjusted to 5.8 and 2.75 g/L Gelrite was used as solidifying agent and subjected to autoclaving as previously described. When the plantlets reached 6-7cm in length with sufficient root system then they were taken out from the vials.

Fifty regenerated plantlets from each cultivar were randomly selected for ploidy observation by chromosome counting. Traditional chromosome counting was conducted from root tips of regenerated plants. Root tips were pretreated in a saturated water solution of alpha-bromonaphthalene for 2 h and then fixed in a solution of ethanol: acetic acid (3:1) for one day (Liu *et al.*, 2012). Roots were then hydrolyzed in 1N HCl at 60 °C for 2 min, and then stained with Feulgen solution. Squash preparation was made in acetocarmine. Chromosomes were stained with acetocarmine and counted under a microscope.

Results and discussion

In this study, the structures of the anthers were found to be similar in all of the three cultivars. Chrysanthemum anthers were inflated after two weeks on the induction medium (Fig. 1A). The anthers started callus induction from 20 to 22 days of incubation, and it took about 35 to 45 days for completion. The induction of callus was quick and

high on MS basal medium supplemented with 1.0 mg/L of 2, 4-D + 2.0 mg/L of 6-BA + 4.5% W/V sucrose. Induction potential was slightly increased in medium containing 250 mg/L casein-hydrolysate. A pretreatment of anthers at 4 °C for 48h enhanced the induction ratio. The rate of callus formation differed slightly between the cultivars. Soft green colored calluses were suitable for shoot initiation. Multiple shoots were initiated from most of the calluses in differentiation medium (Fig. 1B), and were easily rooted on

rooting medium R1 (Fig. 1C, D). Regenerated plants showed slow growth. Callus inductions, shoot regeneration performance of all the varieties in each treatment were evaluated (Tabs. 2-3-4). After sufficient development of root system, the small plantlets were taken out from the culture vessels without damaging roots. Medium around the roots was washed off by running tap water to prevent microbial infection and transplanted in small plastic pots containing the potting mixture. The pots were then transferred into the growth chamber for proper hardening of the plantlets (Fig. 1E, F). To reduce sudden shock, the pots were kept in growth room for 2 weeks under controlled environment. Fifty regenerated plantlets from each cultivar were randomly selected for ploidy observation by chromosome counting and three haploid plantlets were detected for the garden cultivar 'Yes morning'. The variety 'Yes Morning' used as donor in this study has a number of 54 chromosomes (Fig. 2A). The chromosome number of the regenerated haploid plant of 'Yes Morning' was 27 as shown in Fig. 2B. The survival rate of plantlet in soil was the highest in the pot 'Peace Pink' followed by the genotype 'Yes Morning' and pot cultivar 'Hi-Maya' respectively (data not shown).

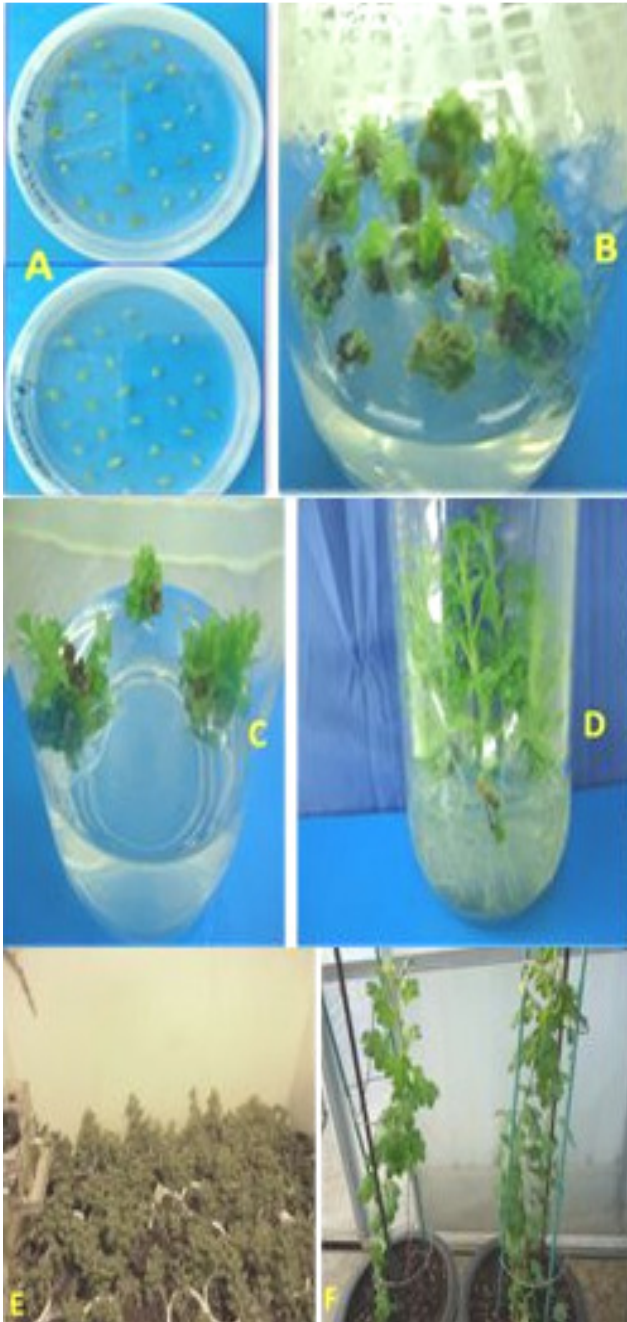


Fig. 1. Anther culture of *Chrysanthemum* (*D. grandiflorum*). A) Callus induction in medium. B) Shoot initiation in differentiation medium D-1. C) Shoots placed in rooting medium R-1. D) Anther culture derived plants rooting. E) Hardening of the Anther culture derived plants. F) Anther culture derived plants growing in outside

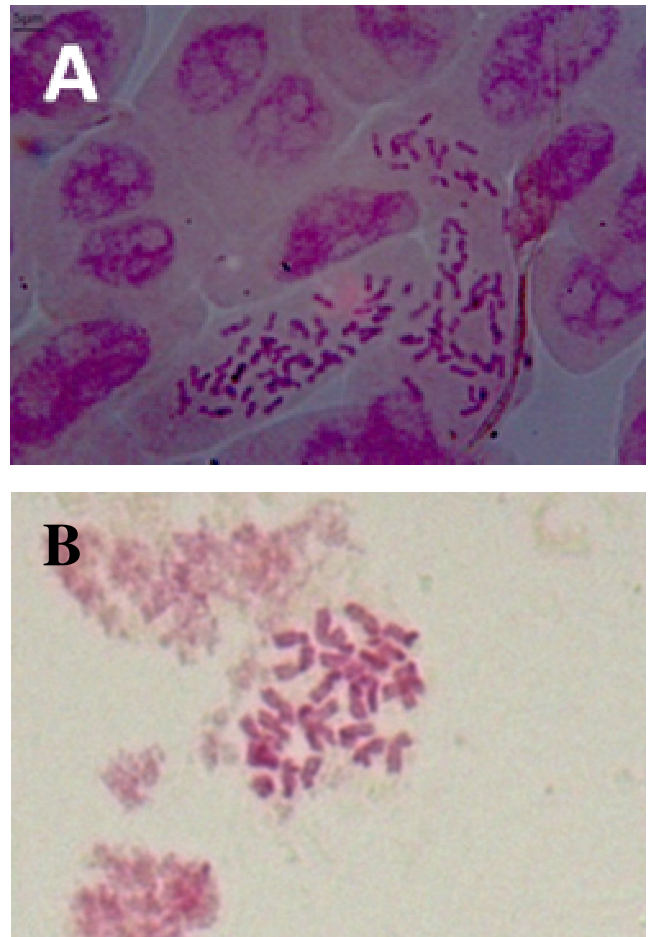


Fig. 2. (A) Chromosomes in root tip cell of the donor plant of 'Yes Morning' ($2n = 54$). B) Chromosomes in root tip cell of haploid regenerated from anther culture of 'Yes Morning' ($2n = 27$)

Tab. 2. Effects of media and cold pretreatments on anther culture of *Chrysanthemum* cv. 'Yes Morning'

Induction medium	Treatments	Anthers inoculated	CIR (%) ^a	SIR (%) ^b
A-1	T-1: 4°C 24h (dark)	200	74	61
	T-2: 4°C 48h (dark)	200	79	64
	T-3: Without cold treatment	200	64	56
A-2	T-1: 4°C 24h (dark)	200	79	66
	T-2: 4°C 48h (dark)	200	85	69
	T-3: Without cold treatment	200	68	60
A-3	T-1: 4°C 24h (dark)	200	86	69
	T-2: 4°C 48h (dark)	200	89	73
	T-3: Without cold treatment	200	72	67
A-4	T-1: 4°C 24h (dark)	200	89	76
	T-2: 4°C 48h (dark)	200	92	79
	T-3: Without cold treatment	200	78	73

^aCallus Induction Rate from anthers (%) = No. of callus formed / No. of anthers inoculated) × 100.^bShoot Induction Rate from anthers (%) = No. of callus that generating shoots in differentiation medium / No. of anthers inoculated) × 100Tab. 3. Effects of media and cold pre-treatments on anther culture of *Chrysanthemum* cv. 'Hi Maya'

Induction medium	Treatments	Anthers inoculated	CIR (%) ^a	SIR (%) ^b
A-1	T-1: 4°C 24h (dark)	200	66	44
	T-2: 4°C 48h (dark)	200	72	52
	T-3: Without cold treatment	200	60	40
A-2	T-1: 4°C 24h (dark)	200	75	55
	T-2: 4°C 48h (dark)	200	78	58
	T-3: Without cold treatment	200	64	41
A-3	T-1: 4°C 24h (dark)	200	84	69
	T-2: 4°C 48h (dark)	200	85	72
	T-3: Without cold treatment	200	77	60
A-4	T-1: 4°C 24h (dark)	200	89	85
	T-2: 4°C 48h (dark)	200	95	90
	T-3: Without cold treatment	200	80	76

^aCallus Induction Rate from anthers (%) = No. of callus formed / No. of anthers inoculated) × 100^bShoot Induction Rate from anthers (%) = No. of callus that generating shoots in differentiation medium / No. of anthers inoculated) × 100Tab. 4. Effects of media and cold pre-treatments on anther culture of *Chrysanthemum* cv. 'Peace Pink'

Induction medium	Treatments	Anthers inoculated	CIR (%) ^a	SIR (%) ^b
A-1	T-1: 4°C 24h (dark)	200	72	46
	T-2: 4°C 48h (dark)	200	75	54
	T-3: Without cold treatment	200	62	42
A-2	T-1: 4°C 24h (dark)	200	75	57
	T-2: 4°C 48h (dark)	200	78	60
	T-3: Without cold treatment	200	68	43
A-3	T-1: 4°C 24h (dark)	200	84	71
	T-2: 4°C 48h (dark)	200	89	74
	T-3: Without cold treatment	200	72	62
A-4	T-1: 4°C 24h (dark)	200	89	87
	T-2: 4°C 48h (dark)	200	91	89
	T-3: Without cold treatment	200	79	73

^aCallus Induction Rate from anthers (%) = No. of callus formed / No. of anthers inoculated) × 100.^bShoot Induction Rate from anthers (%) = No. of callus that generating shoots in differentiation medium / No. of anthers inoculated) × 100

Demand for *Chrysanthemum* production, including cut flowers, gardens, potted plants, and ground-cover types, has increased worldwide. In addition to ornamental and medicinal properties, *Chrysanthemum* extract can also be used as raw material in dye and tea production. In particular, simple *Chrysanthemum* flower tea is a very common beverage in China and Korea. Although *chrysanthemums* have endless uses, the genetics of *Chrysanthemum* has not yet been completely understood

and relatively few systematic genetic analyses have been performed compared with other crops. Histologically confirmed reports of somatic embryogenesis in *Chrysanthemum* are few, and have been developed in only selected *D. grandiflora* cultivars (Pavingerová *et al.*, 1994; Urban *et al.*, 1994; Teixeira da Silva, 2003b). Advances in biotechnology of members of the *Chrysanthemum*-complex are possible due to improvements and new and significant findings in regeneration protocols. Chromosome-doubled

plants, produced by colchicine treatment, were used to produce breeding parents with improved pollen fertility (Endo *et al.*, 1997), while para-fluorophenylalanine was used to successfully produce chromosome-reduced plants (Endo *et al.*, 1994). Confirmation of hybrids and of ploidy (aneuploidy and euploidy) levels continues to be achieved by the use of chromosome counts (Aoyama *et al.*, 1997; Liu *et al.*, 2012). To date, over 200 crop and horticultural plant varieties have been developed using various haploid and DH methods (Thomas *et al.*, 2003). Most haploid or DH lines have originated from anther and microspore cultures, and anther culture is effective and widely used method (Li *et al.*, 2010; Parra-Vega *et al.*, 2013). Although it is emphasized that anther culture technique is very simple but its use is still limited in ornamentals. In an experiment, Watanabe *et al.* (1972) used two varieties of *Chrysanthemum* for anther culture and reported high frequency callus formation using Miller's solution supplemented with plant growth hormones (PGH) but regenerated plants showed the same chromosome numbers with parents indicated that plants were derived from the somatic tissue of the anthers. Yang *et al.* (2005) examined different level of callus formation from anthers of six cultivars using MS medium with PGH and similar number of chromosomes observed for the regenerated plants. Gao *et al.* (2011) first stated about haploid plants derived from anther culture of garden chrysanthemum. The production of haploid plants from anther culture could provide *Chrysanthemum* breeders with a means of accelerating cultivar development. The anther wall development in the *Chrysanthemum* is of the dicotyledonous type, the anthers are tetrasporangiate, the endothecium has a thickened wall, and simultaneous cytokinesis during microspore-mother-cell meiosis leads to the formation of mainly tetrahedral tetrads. Thus, *Chrysanthemum* shares a number of embryological features with other Compositae species (Li *et al.*, 2010). The florets inside the young inflorescence buds are already sterile, and callus formation is readily obtained. The use of high concentrations of sucrose is commonly reported in papers on anther culture where the addition of 5-20% sucrose to the culture medium is found to assist the development of somatic embryos from pollen microspores (Thorpe *et al.*, 2008) and this appears to be due to an osmotic regulation of morphogenesis (Sunderland and Dunwell, 1977), for once embryoid development has commenced, such high levels of sucrose are no longer required, or may be inhibitory. The temporary presence of high sucrose concentrations is said to prevent the proliferation of callus from diploid cells of the anther that would otherwise swamp the growth of the pollen-derived embryoids (Thorpe *et al.*, 2008).

Conclusion

The present study reports the successful production of haploid lines of *Chrysanthemum* variety "Yes Morning" via anther culture. In our study, we found that the cold pretreatment of flower buds was increased the induction rate of callus. This protocol can be followed for genetic manipulation for improvement of *Chrysanthemum* species. Considering the findings, further investigation is required for the callus induction and subsequent haploid production

of different varieties of *Chrysanthemum* by changing the type of media, hormonal composition and by trying additional growth regulators rather than those were used.

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