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 BA, 0.1 mg/L of NAA, 30 g/L of sucrose; solidified by 2.75 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). Infraspecies and even infra-population variations in ploidy have been found (Yang et al., 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 though 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 (Dendrantha 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 aceticarmine 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 g/L of Gelrite. Following 20 min of autoclaving at 121 ºC 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 ºC for 24h and 48h in dark prior to incubate at 24 ± 1 ºC 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 ºC 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 ºC. 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 |
| 2,4-D | 1 | 1 | 1 | 1 | - | - |
| NAA | 0.1 | 0.1 | 0.1 | 0.1 | - | - |
| CaCO₃ | 90 | 90 | 45 | 45 | 30 | 30 |
| Gelrite | 2.75 | 2.75 | 2.75 | 2.75 | 2.75 | 3 |
| g/L | mg/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 ºC for 2 min, and then stained with Feulgen solution. Squash preparation was made in aceticarmine. Chromosomes were stained with aceticarmine 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 pre-treatments on anther culture of Chrysanthemum cv. 'Yes Morning'

| Induction medium | Treatments | Anthers inoculated | CIR (%)<sup>a</sup> | SIR (%)<sup>b</sup> |
|------------------|------------|--------------------|---------------------|---------------------|
| 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 |
|                  | 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 |
|                  | 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 |
|                  | 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 |

<sup>a</sup>Callus Induction Rate from anthers (%) = No. of callus formed / No. of anthers inoculated) × 100.

<sup>b</sup>Shoot Induction Rate from anthers (%) = No. of callus that generating shoots in differentiation medium / No. of anthers inoculated) × 100

Tab. 3. Effects of media and cold pre-treatments on anther culture of Chrysanthemum cv. 'Hi Maya'

| Induction medium | Treatments | Anthers inoculated | CIR (%)<sup>a</sup> | SIR (%)<sup>b</sup> |
|------------------|------------|--------------------|---------------------|---------------------|
| 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 |
|                  | 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 |
|                  | T-1: 4°C 24h (dark) | 200 | 84 | 69 |
|                  | T-2: 4°C 48h (dark) | 200 | 77 | 60 |
|                  | T-3: Without cold treatment | 200 | 80 | 76 |
|                  | 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 |

<sup>a</sup>Callus Induction Rate from anthers (%) = No. of callus formed / No. of anthers inoculated) × 100.

<sup>b</sup>Shoot Induction Rate from anthers (%) = No. of callus that generating shoots in differentiation medium / No. of anthers inoculated) × 100

Tab. 4. Effects of media and cold pre-treatments on anther culture of Chrysanthemum cv. 'Peace Pink'

| Induction medium | Treatments | Anthers inoculated | CIR (%)<sup>a</sup> | SIR (%)<sup>b</sup> |
|------------------|------------|--------------------|---------------------|---------------------|
| 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 |
|                  | 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 |
|                  | 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 |
|                  | 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 |

<sup>a</sup>Callus Induction Rate from anthers (%) = No. of callus formed / No. of anthers inoculated) × 100.

<sup>b</sup>Shoot 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-fluorophenylaneline 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 tetradsporangiate, 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 embryos (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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References

Aoyama M, Ikeda H, Suzuki A, Shimizu A (1997). Studies on the variation of chromosome numbers in hybrids between Dendranthema shiwogiku (Kitam.) Kitam. and spray type cultivars of D. x grandiflorum (Ramat.) Kitam. J Japan Soc Hortic Sci 66:401-407.

Chen FD, Zhao HB, Li C, Chen SM, Fang WM (2008). Advances in cytology and molecular cytogenetics of the genus Dendranthema. J Nanjing Agric Univ 31:118-126 (in Chinese).

Endo M, Kawaraya M, Inada I (1994). Creation of mutants through tissue culture of Chrysanthemum, Dendranthema x grandiflorum (Ram.) kitam: An attempt to create chromosome-reduced plants in a liquid medium treated with para-fluorophenylalanine. J Fac Agric Iwate Univ 21:231-243.

Endo M, Kim J, Inada I (1997). Production and characteristics of chromosome-doubled plants of small-flowered garden chrysanthemum, Dendranthema x grandiflorum (Ramat.) Kitam. cv. YS by colchicine treatment of cultured shoot tips. J Japan Soc Hortic Sci 65:825-833.

Gao Y, Chen B, Zhang J (2011). Anther culture of garden Chrysanthemum. Acta Hort 923:103-110.

Gion K, Suzuki R, Ishiguro K, Katsumoto Y, Tsuda S, Tanaka Y, Mouradova E, Brugliera F, Chandler S (2012). Genetic engineering of floricultural crops: modification of flower colour, flowering and shape. Acta Hort 953:209-216.

Li F, Chen S, Chen F, Teng N, Fang W, Zhang F, Deng Y (2010). Anther wall development, microsporogenesis and microgametogenesis in male fertile and sterile Chrysanthemum (Chrysanthemum morifolium Ramat., Asteraceae). Sci Hortic 126:261-267.

Liu PL, Wan Q, Guo YP, Yang J, Rao GY (2012). Phylogeny of the genus Chrysanthemum L.: evidence from single-copy nuclear gene and chloroplast DNA sequences. PLoS One 7(11): e48970.

Mukherjee AK, Dek A, Acharya L, Palai SK, Panda PC (2013). Studies on genetic diversity in elite varieties of Chrysanthemum using RAPD and ISSR markers. Indian J Biotechnol 12:161-169.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:472-497.
Parra-Vega V, Renau-Morata B, Sifres A, Seguí-Simarro J (2013). Stress treatments and in vitro culture conditions influence microspore embryogenesis and growth of callus from anther walls of sweet pepper (Capsicum annuum L.). Plant Cell Tiss Organ Cult 112:353-360.

Pavingerová D, Dostál J, Bísková R, Benetka V (1994). Somatic embryogenesis and Agrobacterium-mediated transformation of Chrysanthemum. Plant Sci 97:95-101.

Rout GR, Das P (1997). Recent trends in the biotechnology of Chrysanthemum: a critical review. Sci Hortic 69:239-257.

Rout GR, Mohapatra A, Jain SM (2006). Tissue culture of ornamental pot plant: A critical review on present scenario and future prospects. Biotechnol. Adv 24:531-560.

Schwinn KE, Markham KR, Given NK (1994). Floral flavonoids and their potential for pelargonidin biosynthesis in commercial Chrysanthemum cultivars. Phytochemistry 35:145-150.

Sunderland N, Dunwell JM 1977. Anther and pollen culture, p. 223–265. In: Street HE (Eds.). Plant Tissue and Cell Culture. Blackwell Oxford, London.

Teixeira da Silva JA (2003a). Chrysanthemum: advances in tissue culture, cryopreservation, postharvest technology, genetics and transgenic biotechnology. Biotechnol Adv 21:715-766.

Teixeira da Silva JA (2003b). Control of Chrysanthemum organogenesis by thin cell layer technology. Asian J Plant Sci 2:505-514.

Teixeira da Silva JA, Shinoyama H, Aida R, Matsushita Y, Raj SK, Chen F (2012). Chrysanthemum Biotechnology: Quo vadis? Crit Rev Plant Sci 32: 21-52.

Thomas W, Forster B, Gertsson B (2003). Doubled haploids in breeding. p. 337-349. In: Doubled Haploid Production in Crop Plants. Springer, Dordrecht, Netherlands.

Thorpe T, Stasolla C, Yeung EC, de Klerk GJ, Roberts A, George EF (2008). The Components of Plant Tissue Culture Media II: Organic Additions, Osmotic and pH Effects, and Support Systems. p. 115-174. In: George EF, Hall MA, De Klerk DJ (Eds.). Plant Propagation by Tissue Culture, 3rd Edition, Springer, Dordrecht, Netherlands.

Urban LA, Sherman JM, Moyer JW, Daub ME (1994). High frequency shoot regeneration and Agrobacterium-mediated transformation of Chrysanthemum (Dendranthema grandiflora). Plant Sci 98:69-79.

Watanabe K, Nishii Y, Tanaka R (1972). Anatomical observations on the high frequency callus formation from anther culture of Chrysanthemum. Japan J Genet 47(4):249-255.

Yang J, Endo M, Inada I (2005). Anther and microspore culture of Chrysanthemum (Dendranthema grandiflorum (Ramat.) Kitam.). J Japan Soc Hortic Sci 74(1):78-86.

Yang W, Glover BJ, Rao GY, Yang J (2006). Molecular evidence for multiple polyploidization and lineage recombination in the Chrysanthemum indicum polyploid complex (Asteraceae). New Phytologist 171:875-886.

Zagorski J, Ascher P, Widmer RE (1983). Multigenic self incompatibility in hexaploid Chrysanthemum. Euphytica 32:1-7.

Zhmyleva AP, Kondo K (2006). Comparison of somatic chromosomes in some species of Chrysanthemum sensu lato in Russia. Chromosome Bot 1:13-22.