

Primary and secondary somatic embryogenesis in *Chrysanthemum* cv. Euro

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Abstract Somatic embryogenesis was induced from in vivo grown leaf explants of *Chrysanthemum* cv. Euro incubated on Murashige and Skoog (MS) medium supplemented with 2.0 mg/L 2,4-dichlorophenoxyacetic acid and 2.0 mg/L Kinetin, yielding the highest mean number of embryos (42 ± 5.97) per explant after 5 weeks of culture. We evaluated the effects of basal medium, various concentrations of sucrose, and timentin on the proliferation of secondary somatic embryos. MS medium was observed to be the more effective in promoting the proliferation of somatic embryos than half-strength Murashige and Skoog (1/2MS). In addition, timentin was also more efficient in induction of secondary embryogenesis than sucrose. Whole plantlets were obtained by culturing of secondary embryos on hormone-free MS medium and successfully acclimated in the green house.

Keywords *Chrysanthemum* · Plant growth regulators (PGRs) · Somatic embryogenesis · Secondary somatic embryogenesis · Sucrose · Timentin

Abbreviations

MS	Murashige and Skoog (1962)
1/2MS	Half-strength Murashige and Skoog
2, 4-D	2, 4-Dichlorophenoxyacetic acid
TDZ	Thidiazuron
BA	6-Benzyladenine
NAA	α -Naphthalene acetic acid
PGRs	Plant growth regulators

KN Kinetin
FAA Formalin acetic acid

Introduction

Chrysanthemum is one of the most important cut flowers, pot plants and herbaceous landscape plants grown worldwide. In breeding programs, desirable traits have been introduced by conventional breeding; however, there are limitations to the techniques due to a limited gene pool and cross incompatibility (Rout and Das 1997). Application of biotechnology in plant breeding programs requires efficient in vitro regeneration procedures. Somatic embryogenesis is a desirable and fastest method of plant regeneration. Somatic embryogenesis may be induced via a direct or indirect pathway. For direct somatic embryogenesis, embryos develop directly on the surface of organized tissue. Alternatively, indirect somatic embryogenesis may occur via an intermediate step involving callus formation or a cell suspension culture. Somatic embryos have a bipolar morphology and the regenerated plants possess complete root-tip tissue systems. Thus, such a regeneration pathway provides a more integrated and resilient material than organogenesis-obtained plants (Rout et al. 1991).

Somatic embryogenesis in *Chrysanthemum* has been reported (May and Trigiano 1991; Pavingerova et al. 1994; Tanaka et al. 2000; Shinoyama et al. 2004; Mandal and Datta 2005). However, the capacity of somatic embryogenesis and number of somatic embryos per explant were still low. May and Trigiano (1991) reported that only 4.1 % of leaves showed embryogenesis in *Chrysanthemum* (*Dendranthema grandiflora*). Pavingerova et al. (1994) did not mention the capacity of somatic embryogenesis. Tanaka et al. (2000) noted that 58 % of somatic

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embryogenesis was obtained from ray florets of *Chrysanthemum* (*Dendranthema grandiflora*). Mandal and Datta (2005) also reported direct somatic embryogenesis from ray florets of different *Chrysanthemum* cultivars, however, embryogenic response (30 %) with number of somatic embryos per responding explant (15.8 ± 1.2) was the best. To date, the number of somatic embryos per explant (21.3) reported by Shinoyama et al. (2004) was the highest in *Chrysanthemum*. Furthermore, there is no information about secondary somatic embryogenesis in *Chrysanthemum*. Secondary somatic embryogenesis offers advantages over primary somatic embryogenesis, notably a high multiplication rate and increased level of uniformity, and this process is independent of the original explant source. Recently, secondary somatic embryogenesis has been reported in some crops including *Carnation* (Karami et al. 2008), and *Rosa* hybrid “Samantha” (Bao et al. 2012) using BA, 2,4-D, different kinds of carbohydrate.

Application of somatic embryogenesis in *Agro-bacterium* mediated transformation has been reported in *Chrysanthemum* (Pavingerova et al. 1994), in walnut (Tang et al. 2000), and *Bixa orellana* (Parimalan et al. 2011). Moreover, timentin has been reported as an effective antibiotic for elimination of *Agro-bacterium* (Cheng et al. 1998; Tang et al. 2000). However, effect of timentin on secondary embryogenesis is very limited. To our best knowledge, there is only one study that reported the effect of timentin on somatic embryogenesis in walnut (Tang et al. 2000). Thus, we investigated the effect of timentin on secondary somatic embryogenesis in *Chrysanthemum* to know whether it has inhibitory or stimulatory effect on the process.

In this study, we investigated the effect of plant growth regulators on primary somatic embryogenesis from leaf segment, and several factors including basal medium, sucrose, and timentin on secondary somatic embryogenesis in *Chrysanthemum*. We expect that our report would be helpful for the *Agro-bacterium* mediated genetic transformation studies of the cultivar.

Materials and methods

Plant materials

Leaves of *Chrysanthemum* cv. Euro were collected from plants grown in the greenhouse. Leaves were then thoroughly washed under running tap water with 5 % liquid detergent (Sando, Korea). Thereafter, they subsequently were surface sterilized with a 70 % (v/v) ethanol solution for 30 s and a 1 % NaOCl plus Tween 20 for 5 min, followed by five rinses with sterilized deionized water.

Induction of somatic embryogenesis

Four or five 0.5–1 cm long midrib explants were excised and abaxially cultured on Murashige and Skoog (MS) (1962) medium supplemented with various combinations and concentrations of plant growth regulators, such as 6-benzyladenine (BA), thidiazuron (TDZ), KN and 2,4-D, and with 3 % sucrose and 7 g/L Bacto-Agar to evaluate the capacity of somatic embryogenesis. Plant growth regulators were added to the medium before autoclaving. Each treatment consisted of 10 explants, and there were three replicates. The cultures were incubated at 25°C under fluorescent lights ($60\text{--}100 \mu\text{mol m}^{-2} \text{s}^{-1}$) with a 16 h light and 8 h dark photoperiod. After 5 weeks, number of embryos induced per explant was recorded. As this study mainly focused on capacity of somatic embryogenesis and number of somatic embryos per explant, total number of all various staged somatic embryos per explant was counted.

Secondary somatic embryogenesis

Effects of different basal media and sucrose on secondary somatic embryogenesis

To examine the effects of basal media and sucrose on somatic embryogenesis, somatic embryo clumps (about 5 embryos) obtained from primary somatic embryogenesis experiment were transferred onto MS and 1/2MS basal medium supplemented with combination of 2 mg/L 2,4-D and 2 mg/L KN that showed the best result of somatic embryogenesis in primary somatic embryogenesis, 7 g/L Bacto agar, and different concentrations of sucrose (3, 6, 9, and 12 %). All cultures were incubated at 25°C under fluorescent lights ($60\text{--}100 \mu\text{mol m}^{-2} \text{s}^{-1}$) with a 16 h light and 8 h dark photoperiod. Ten somatic embryo clumps were considered for each treatment and there were three replicates. The numbers of secondary embryos induced on primary embryos clumps were recorded after 5 weeks culture. As this study mainly focused on capacity of somatic embryogenesis and number of somatic embryos per explant, total number of all various staged somatic embryos per explant was counted.

Effects of different basal media and timentin on secondary somatic embryogenesis

To examine the effects of media and timentin on somatic embryogenesis, somatic embryo clumps (about 5 embryos) obtained from primary somatic embryogenesis experiment were transferred onto MS and 1/2MS basal medium supplemented with combination of 2 mg/L 2,4-D and 2 mg/L KN that showed the best result of somatic embryogenesis in primary somatic embryogenesis, 3 % sucrose, 7 g/L Bacto

agar, and different concentrations (10, 20, 30, 50 and 100 mg/L) of timentin (Duchefa, the Netherlands). Timentin was dissolved in distilled water and filter sterilized before adding to the medium after autoclaving. All cultures were incubated at 25°C under fluorescent lights ($60\text{--}100\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$) with a 16 h light and 8 h dark photoperiod. Ten somatic embryo clumps were considered for each treatment and there were three replicates. The numbers of secondary embryos induced on primary embryos clumps were recorded after 5 weeks culture. As this study mainly focused on capacity of somatic embryogenesis and number of somatic embryos per explant, total number of all various staged somatic embryos per explant was counted.

Plant regeneration and acclimatization

Secondary somatic embryos isolated from the clumps were placed onto hormone-free MS medium containing 3 % sucrose for plant generation. Four weeks after culture when plantlets reached in size of 4–5 cm, they were removed from culture vessel and planted into a cell tray with vermiculite soil and covered with a plastic sheet for acclimation to the greenhouse at 25°C. After 3 weeks, the plantlets were transferred to the pots containing soil mixture of peat and perlite (1:1) and grown in the greenhouse.

Scanning electron microscope

Somatic embryo at globular and cotyledonary stages were fixed in FAA for 24 h. Thereafter, samples were dehydrated in ethanol concentrations of 25, 50, 70, 85, and 100 % for each 10 min. Dehydrated samples were then dried at room temperature until critical point dry. Samples were coated with gold–palladium on a Quick Cool Coater (Sanyu-Denshi, Japan) and examined using scanning electron microscope (JOEL, Japan).

Results and discussion

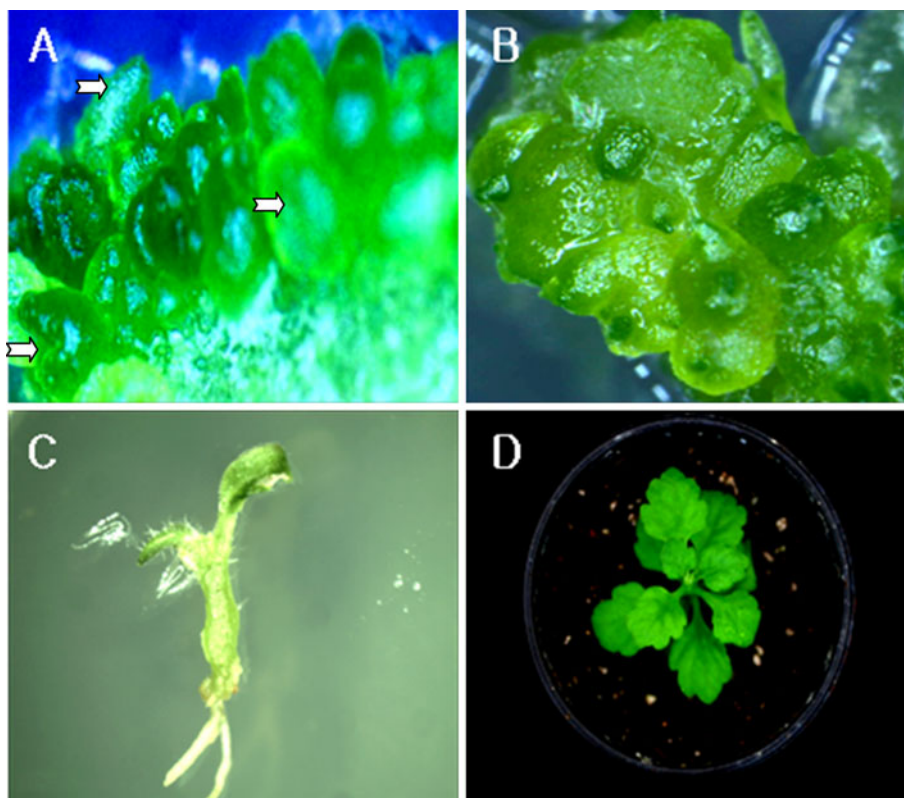
Effects of plant growth regulators on embryo induction from leaf segment

Developing somatic embryogenic culture systems with reliable regeneration capacity from ornamental plants is a prerequisite for mass propagation and their genetic improvement. Studies were being intensively made to develop a protocol for somatic embryogenesis. The process can be induced in tissue cultures of *Chrysanthemum* either directly from the epidermal cells of explants (Mandal and Datta 2005) or indirectly via intervening callus (Shinoyama et al. 2004). In this study, somatic embryogenesis was induced directly from the leaf segment.

Combinations of 2,4-D and the cytokinins (BA, or KN) have been reported for somatic embryogenesis of *Chrysanthemum* (May and Trigiano 1991; Pavingerova et al. 1994; Shinoyama et al. 2004; Mandal and Datta 2005). However, TDZ has not been reported so far. When Leaf explants of cv. Euro were cultured on the medium containing the plant growth regulators, they became swollen and turned yellow 4–5 days after culture initiation. Somatic embryogenesis was noticed after 20–25 days, mostly in the cut margin of the explants. Within 30 days of culture initiation, different developmental stages of somatic embryos were detected on the explants. Although somatic embryo development was asynchronous in nature, at the same time, globular stage, heart-shaped, and early cotyledonary stage (Fig. 1a) were observed. As this study mainly focused on capacity of somatic embryogenesis and number of somatic embryos per explant, total number of all various staged somatic embryos per explant was counted.

Somatic embryogenesis was observed upon the addition of cytokinins (BA, TDZ, and KN) in the presence of 2,4-D. No somatic embryogenesis was observed on the media containing 2,4-D only. A similar result has been reported by Tanaka et al. (2000). Some other combinations were also failed to induce somatic embryogenesis. Furthermore, ratios between concentrations of auxin and cytokinins were significantly associated with percentages of somatic embryogenesis and number of somatic embryos. Among the cytokinins used, KN was found to be the best for number of somatic embryos per explant followed by BA and TDZ. Shinoyama et al. (2004) mentioned the KN was found to be better responsive for somatic embryogenesis than BA. However, they reported that the best results of somatic embryogenesis was obtained on the medium containing 2 mg/L 2,4-D and 1 mg/L KN. In the present study, maximum embryogenic response (100 %) with maximum number of somatic embryos per responding explant (42.1 ± 5.97) was observed in 2.0 mg/L KN with 2.0 mg/L 2,4-D (Table 1). Thus, the combination of 2.0 mg/L KN with 2.0 mg/L 2,4-D was noted as optimal concentration for somatic embryogenesis of the cultivar. The combinations higher or lower concentrations of the optimal concentration showed distinctly inhibition of number of somatic embryos per explant. In addition, combination of 2 mg/L 2,4-D and 1 mg/L KN recommended by Shinoyama et al. (2004) in this study could induce only 8 somatic embryos per explant. Similarly, Shinoyama et al. (2004) reported combination of 2 mg/L 2,4-D and 2 mg/L KN that was observed as optimal concentration in this study gave only 6 somatic embryos per explant. In this study, a combination of 3.0 mg/L 2,4-D with 1.0 mg/L BA also responded maximum embryogenic response (100 %) with a considerable number of somatic embryos per explant (34.7 ± 2.1). Effects of 2,4-D and BA combinations on

Fig. 1 Plant regeneration via direct somatic embryogenesis from leaf explants of *Chrysanthemum* cv. Euro. **a** Primary somatic embryogenesis. **b** Proliferation of secondary somatic embryos. **c** Plant regenerated from somatic embryo cultured on hormone free MS medium. **d** A potted plant in greenhouse



somatic embryogenesis in *Chrysanthemum* has been reported by May and Trigiano (1991) and Mandal and Datta (2005). May and Trigiano (1991) firstly reported that combination of 1.0 mg/L 2,4-D with 0.2 mg/L BA induced embryogenesis from leaf explants, however, responding percentage was relatively low. Mandal and Datta (2005) also previously reported direct somatic embryogenesis from ray florets of different *Chrysanthemum* cultivars, however, maximum embryogenic response (30 %) with maximum number of somatic embryos per responding explant (15.8 ± 1.2) was observed in 4.0 mg/L 2,4-D with 2.0 mg/L BA. We found the optimal concentration that is efficient for one cultivar is not easily adapted to other cultivars.

TDZ among the cytokinins seemed to be less responsive for somatic embryogenesis, however, combination of 3 mg/L 2,4-D and 3 mg/L TDZ exhibited a reasonable number of somatic embryos (18 embryos) per explant. Tanaka et al. (2000) reported that no formation of embryo or adventitious shoot or root was formed when ray floret of *Chrysanthemum* was cultured on the medium containing TDZ alone. It seemed that using TDZ alone was not appropriate for embryogenesis or organogenesis in *Chrysanthemum*. So, it is interesting to study effect of TDZ alone on somatic embryogenesis or organogenesis from different explants of *Chrysanthemum*. This is the first report that TDZ like other cytokinins (BA and KN) can be used for somatic embryogenesis of *Chrysanthemum* when

2,4-D was combined. Recently, effect of TDZ on somatic embryogenesis has been reported in some crops, such as *Merwillia plumbea* (Baskaran and Van Staden, 2012), and *Primulina tobacum* Yang et al. (2012).

May and Trigiano (1991) reported that 4.1 % of leaves showed embryogenesis; these were cultured in the dark for 1 week followed by culture in 10 days of light and 35 days of darkness. Similarly, Tanaka et al. (2000) also reported 58 % of somatic embryogenesis that were first cultured in the dark for 1 week followed by culture under a photoperiod of 12 h light and 12 h dark for 1 month. In the present study, somatic embryogenesis (100 %) was observed under a light condition (16 h). We could not explain clearly the different results because the genotypes of *Chrysanthemum* used in their study were different from those we used, the response to plant growth regulators might be also different.

Secondary somatic embryogenesis

Effects of basal media and sucrose on secondary somatic embryogenesis

A proliferation of somatic embryos was observed on primary somatic embryos when they were transferred onto the tested media. However, newly induced secondary embryos were compact and greenish yellow (Fig. 1b). The effect of basal media and sucrose concentrations on the percentage of responding explants and on mean number of secondary

Table 1 Effects of phytohormones on callus induction from leaf segments of *Chrysanthemum* cv. Euro after 5 weeks of culture

PGRs (mg/L)		Somatic embryogenesis %	Mean no. of embryos/explant
0	0	0	0
BA	2,4-D		
	1	0 h	0.1
	2	0 h	0.1
	3	0 h	0.1
	1	90 b	5.5 ± 0.5 ijk
1	2	80 c	3.7 ± 0.29 k
	3	100 a	34.7 ± 2.1 b
	1	90 b	3.7 ± 0.2 k
2	2	100 a	10.7 ± 0.58 f
	3	80 c	5.7 ± 0.58 hijk
	1	100 a	13.7 ± 1.53 e
3	2	0 h	0.1
	3	0 h	0.1
TDZ			
	1	50 f	6.3 ± 0.58 hij
1	2	70 d	4.3 ± 0.64 jk
	3	30 g	1.3 ± 0.1
	1	0 h	0.1
2	2	60 e	7.7 ± 1.15 hi
	3	50 f	5.7 ± 0.58 hijk
	1	70 d	3.7 ± 0.58 k
3	2	0 h	0.1
	3	90 b	18.3 ± 1.53 d
KN			
	1	50 f	4.3 ± 0.58 jk
1	2	60 e	14.0 ± 1.0 e
	3	0 h	0.1
	1	100 a	8.0 ± 1.0 gh
2	2	100 a	42.1 ± 5.97 a
	3	0 h	0.1
	1	100 a	15.3 ± 0.58 e
3	2	100 a	10.0 ± 0.1 f g
	3	100 a	23.0 ± 1.0 c

Means within the same letter are not significantly different by DMRT ($P > 0.05$). Ten somatic embryo clumps were considered for each treatment and there were three replicates

embryos formed on each primary embryo is shown in Table 2. It shows that percentages of somatic embryogenesis and the number of embryos were strongly determined by sucrose concentrations and types of basal medium used.

On 1/2MS medium, only lower concentrations of sucrose (3 or 6 %) induced secondary somatic embryos, however, the latter gave the higher percentage of somatic embryogenesis and number of somatic embryos per

Table 2 Effects of different basal media and sucrose on secondary somatic embryogenesis on primary embryo clump after 5 weeks of culture

Basal medium	Sucrose (%)	Secondary embryogenesis (%)	Mean no. of embryos per explant
1/2MS	3	40 e	4.2 ± 0.76 d
	6	50 d	12.0 ± 0.2 b
	9	0 f	0 e
MS	12	0 f	0 e
	3	80 c	12.0 ± 1.0 b
	6	100 a	15.5 ± 0.5 a
	9	90 b	11.2 ± 0.76 b
	12	90 b	10.0 ± 0.2 c

Means within the same letter are not significantly different by DMRT ($P > 0.05$). Ten somatic embryo clumps were considered for each treatment and there were three replicates

explant. Suppression of secondary somatic embryogenesis occurred in the presence of sucrose either 9 or 12 %, and explants became brown in color and finally died. In contrast to 1/2MS medium, secondary embryogenesis was observed in all treatments when embryos were cultured on MS medium. Moreover, frequency of somatic embryogenesis and number of somatic embryos per explant were definitely better as compared to those on 1/2MS medium. Explants cultured on the medium with sucrose 6 % responded the highest percentage of somatic embryogenesis and number of somatic embryos per explant, which is same to those of 1/2MS medium where sucrose 6 % was found to be an optimal concentration for secondary embryogenesis. Medium containing higher concentrations of sucrose (9 or 12 %) linearly reduced secondary embryogenesis. According to our findings, although concentration of sucrose significantly affected the secondary embryogenesis, it is important to note that types of basal medium used was also highly supporting for induction of secondary embryogenesis.

Although secondary somatic embryogenesis has been reported for several plant species such as peanut (Baker and Wetzstein 1995; Little et al. 2000), *Medicago truncatula* (Neves et al. 1999), tea (Akula et al. 2000), *Helianthus maximiliani* (Vasic et al. 2002), and Carnation (Karami et al. 2008), this is the first report of secondary somatic embryogenesis in *Chrysanthemum*. In this study, we found concentration of sucrose (6 %) was more effective than 3 % for induction of secondary embryogenesis in cv. Euro. This finding is concordant with the results of similar studies in Rosa hybrid (Bao et al. 2012) and in *Morus alba* L. (Agarwal et al. 2004). However, we observed concentrations of sucrose above 6 % linearly reduced somatic embryogenesis on MS medium, whereas quite suppression

of secondary embryogenesis occurred on 1/2MS medium in spite of optimal PGR concentrations. This finding is not in agreement with Karami et al. (2008) who reported increasing sucrose concentration in culture medium enhanced secondary embryogenesis. The suppression by sucrose 9 or 12 % could be due to a higher osmolarity in the culture medium. Karami et al. (2008) reported the highest secondary somatic embryogenesis was observed when combination of sucrose and mannitol was added to the culture medium. Previous reports on regulation of osmotic pressure in culture medium by mannitol have been done by many researchers (Robert 1991; Kamada et al. 1993; Ikeda-Iwai et al. 2002). Therefore, it would be interesting to study the influence of sucrose combined with an osmotic agent such as mannitol and/or of a sequential supply of high concentration.

Recently, Naing et al. (2011) reported that the capacity of somatic embryogenesis observed on MS medium was extremely low as compared to that on 1/2 MS medium. However, to our best knowledge, there was no information about effect of basal medium on secondary embryogenesis so far. The medium strength in this study strongly influenced the formation of secondary embryogenesis in cv. Euro. Formation of secondary embryogenesis on MS medium was higher than that on 1/2MS. The higher concentration of sucrose totally suppressed secondary embryogenesis on 1/2MS medium; however, they produced some embryos on MS medium. It might be that higher concentration of organic salts in culture medium was conducted to secondary embryogenesis.

Effects of basal media and timentin on secondary somatic embryogenesis

Somatic embryo-to-plant regeneration system is one of the most important systems used for genetic transformation; however, it is important to evaluate effect of antibiotics for somatic embryogenesis, particularly for repetitive somatic embryogenesis. In the present work, we have evaluated the effects of timentin and basal medium on secondary embryogenesis of *Chrysanthemum* cv. Euro. In general, it was observed a non-detrimental effect of timentin on secondary embryogenesis. On both culture media, concentration of timentin at 10–30 mg/L linearly increased secondary embryogenesis. However, when concentrations of timentin above optimum level such as 50 or 100 mg/L were added to the media, number of embryos linearly decreased. Thus, 30 mg/L was noted as optimal concentration for secondary embryogenesis, however, the mean number of embryos on both culture media containing either 30 mg/L or other concentrations significantly differed (Table 3), in which number of embryos obtained on MS medium considerably higher than those on 1/2 MS medium.

Table 3 Effects of different basal media and timentin on secondary somatic embryogenesis on primary embryo clump after 5 weeks of culture

Basal medium	Timentin (mg/L)	Secondary embryogenesis (%)	Mean no. of embryos per explant
1/2MS	10	10 e	9.0 ± 0 g
	20	10 e	11.0 ± 1.0 f
	30	90 a	14.3 ± 1.15 e
	50	50 c	7.0 ± 1.0 h
	100	30 d	5.3 ± 0.58 i
MS	10	80 ab	11.0 ± 0 f
	20	80 ab	23.7 ± 1.15 b
	30	90 a	31.0 ± 1.0 a
	50	80 ab	21.3 ± 0.58 c
	100	70 b	19.0 ± 0 d

Means within the same letter are not significantly different by DMRT ($P > 0.05$). Ten somatic embryo clumps were considered for each treatment and there were three replicates

Timentin has been used for plant regeneration in many species. However, to our best knowledge, there was no report about effect of timentin on secondary embryogenesis of *Chrysanthemum*, but effect of timentin on secondary embryogenesis in walnut has been reported by Tang et al. (2000). They mentioned that non-detrimental effect of timentin was observed but its higher concentration reduced secondary embryogenesis. It was similar to the finding of our result. In addition, we found timentin was more efficient for secondary embryogenesis as compared to sucrose. There are several reports that timentin can effectively eliminate *Agrobacterium* without inhibiting plant regeneration (Yepes and Aldwinckle, 1994; Hammerschlag et al. 1997; Shackelford and Chlan 1996; Ling et al. 1998; Tang et al. 2000). Although there have been many reports concerning the toxicity of antibiotics to callus growth and shoot regeneration, but only a few on their toxicity to its somatic embryogenesis (Nakano and Mii 1993; Sarma et al. 1995).

When timentin was added to both media, somatic embryogenesis was responded in all treatments. However, formation of secondary embryogenesis on MS medium was higher than that on 1/2MS. It was similar to the finding of above result tested by different sucrose concentrations. It has been discussed that higher concentration of organic salts in culture medium was conducted to secondary embryogenesis. According to the finding of the experiment, we could have confirmed that concentration of organic salts in culture medium was playing as a critical role for secondary embryogenesis in *Chrysanthemum*.

Plant regeneration and acclimatization

When somatic embryos were cultured on hormone-free MS medium containing 3 % sucrose, 30 % of somatic embryos

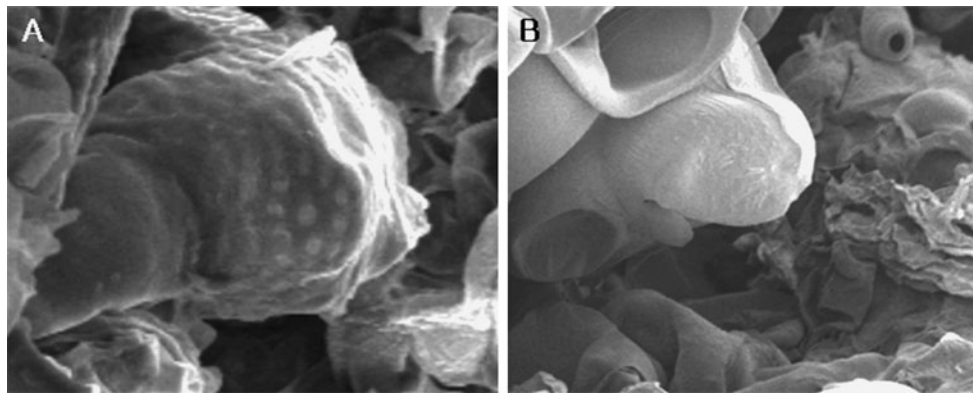


Fig. 2 Scanning electron microscopy of somatic embryogenesis of *Chrysanthemum* cv. Euro. **a** Globular-stage somatic embryo developed from leaf segment. **b** Cotyledonary-stage embryo developed from leaf segment

was germinated. On the media, shoot was formed at the apex of somatic embryo and the root was allowed to develop into plantlet (Fig. 1c). Four weeks after culture when plantlets reached in size of 4–5 cm, they were removed from culture vessel and planted into a cell tray with vermiculite soil and covered with a plastic sheet for acclimation to the greenhouse at 25°C. After 3 weeks, the plantlets were transferred to the pots containing soil mixture of peat and perlite (1:1). More than 80 % of the plants were well survived in the greenhouse (Fig. 1d).

Scanning electron microscopy

Different stages of embryos appeared on the surface of leaf segment. To confirm the appearance of embryo-like structures in greater detail, we observed the structure microscopically by scanning electron microscopy. These structures revealed typical early stage of globular shape (Fig. 2a) and mid stage of cotyledonary embryo (Fig. 2b).

We have successfully established an efficient and reliable plant regeneration system via somatic embryogenesis for the commercial *Chrysanthemum* cv. Euro. In this system, somatic embryos were proliferated by secondary somatic embryogenesis. This is the first report on *Chrysanthemum* secondary embryogenesis. The protocol described in this study provides an important resource which should facilitate genetic transformation of the cultivar.

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