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High Frequency Shoot Regeneration from Leaf Explants of Some Chrysanthemum Cultivars

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Abstract

This study was conducted to examine differences in shoot regeneration among chrysanthemum cultivars. Leaf explants of chrysanthemum cultivars 'Sulhwa', 'Puma', 'Geummokseo' and 'Sulpoong' were used. Explants cultured on the medium for 2 weeks formed calli at the cut surfaces. Shoots regenerated on MS basal medium supplemented with various concentration combinations of NAA and BAP. Explants were cultured under cool-white fluorescent lamps with a light intensity of 40 $\mu Mm^{-2} \cdot s^{-1}$ for 16 $hday^{-1}$, at 25℃ and 70-80% relative humidity. 'Geummokseo' and 'Sulpoong' were the most responsive cultivars in shoot regeneration. Most effective medium for 'Sulhwa' and 'Puma' was MS basal medium supplemented with 10.0 μM NAA and 5.0 μM BAP and for 'Geummokseo' MS supplemented with 10.0 μM NAA and 20.0 μM BAP. Regeneration of multiple shoots was observed on MS basal medium supplemented with 1.0 μM or 10.0 μM NAA and 5.0 μM BAP. High frequency regeneration of adventitious shoots from leaf explants and efficient induction of root from these regenerated shoots were obtained.

Key words: adventitious shoot, callus, cultivar difference, plant growth regulator, responsive cultivar

Introduction

Chrysanthemum is one of the three most important cut flower crops in the world market today. A great deal of effort has gone into improving chrysanthemum by using conventional breeding, selection and mutagenesis. *In vitro* culture of chrysanthemum has been possible for several years. *In vitro* culture was first of interest as a means of eliminating virus diseases from chrysanthmum stock plants (Hakkaart and Quak, 1964). Later workers investigated the potential of *in vitro* culture as a method of propagation. At the present time, gene transfer mediated by *Agrobacterium* has become possible in chrysanthemum. This approach can potentially be used to add specific characteristics such as novel flower color, increased vase life, and disease and insect resistance to existing commercial cultivars (Yang et al. 1995).

In order to apply the recombinant DNA technology to chrysanthemum, an efficient shoot regeneration system is a prerequisite. Successful regeneration has been reported using different explants such as shoot tip (Ben-Jaacov and Langhans, 1972; Earle and Langhans, 1974), flower bud (Dabin et al. 1983), receptacle (Hill, 1968), stem (Annadana et al. 2000), and leaf (Bhattacharya et al. 1990). However, the genus chrysanthemum showed large differences in its response depending on types, cultivars and concentrations of plant growth regulators.

Therefore, this experiment was conducted to determine the appropriate concentrations of plant growth regulators for shoot regeneration from leaf explants of four chrysanthemum cultivars.

Materials and Methods

Plant materials and surface sterilization

Stock plants of *Dendranthema grandiflorum* 'Sulhwa', 'Puma', 'Geummokseo' and 'Sulpoong' were grown in the greenhouse. Terminal shoot cuttings were collected and 8 cm long cuttings were prepared by removing top and basal ends. Leaves were carefully removed avoiding damages to

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the epidermis of stem.

The materials were washed thoroughly under running tap water for 15 min. These materials were surface-sterilized by washing in 70% ethanol for 30 sec. and rinsing 2~3 times with distilled water. And then, the materials were surface-sterilized with a 1.5% sodium hypochlorite solution for 15 min. and rinsed several times with sterile distilled water to avoid further tissue damage (Lee et al. 1979). Single node cuttings used as the explant were planted on the MS medium.

Maintenance and culture environment

The MS medium (Murashige and Skoog, 1962) was used as the basal medium. Sucrose (3%, w/v), B5 vitamins and agar (0.8%, w/v) were added to the medium, and medium pH was adjusted to 5.80 prior to autoclaving at 121C for 15 min. Plant material was multiplied and maintained by subculturing of nodal cuttings regenerated from the original stem nodes at a six-week interval on the MS medium. Cultures were incubated at 25℃ under a 16 h per day photoperiod and 70-80% relative humidity. The light source used was cool-white fluorescent lamps with a light intensity of 40 $mol \cdot m^{-2} \cdot s^{-1}$.

Shoot regeneration

1. Explants. As a part of the effort to optimize culture condition for shoot regeneration from cultured tissues of chrysanthemum, leaf disc explants excised from young and mature leaves on six week-old *in vitro*-grown plants were cultured on the regeneration media. The leaves were cut about 5 mm away from the midrib. The slices were cut across their length to produce explants of about 25 mm^2 (Annadana et al. 2000).

2. Methods. Regeneration experiments were conducted in petri dishes using 25 mL MS basal medium supplemented with various combinations of plant growth regulators (PGR). Regeneration media with 0.0, 1.0 or 10.0 μM NAA and 0.0, 2.5, 5.0, 10.0 or 20.0 μM BAP were tested. The MS basal medium with 3.0% (w/v) sucrose, and 0.8% (w/v) agar (Yakuri Pure Chemicals Co. Ltd., Japan) was used and medium pH was adjusted to 5.80 before autoclaving. Prepared leaf explants were placed abaxial side up on the regeneration medium. Four leaf segments were placed in each petri dish. After positioning explants, petri dishes were sealed with para film and were transferred to a growth chamber.

3. Measurements. Callus and shoot formation from explants and average number of shoots per explant were determined after five weeks in culture. Shoots shorter than 0.3 cm were not counted, because they were too small to count and were considered not being able to produce plantlets.

Results

The first morphogenetic change, observed after one week in culture in all cultivars, was the development of callus at the cut surfaces. After 2~3 weeks in culture, the first adventitious shoots were visible. Shoots elongated rapidly over the next 3~4 weeks followed by the initiation and development of many additional shoot primordia. The explants cultured on the medium without PGR turned brown or died in 3~4 weeks.

Shoot regeneration from leaf disc explants was highly variable depending on the concentrations of auxin and cytokinin. The first experiment on PGR concentration was conducted in 'Puma' (Table 1). When the concentration of BAP was higher than that of NAA, such as 1.0 μM NAA and 5.0 μM BAP, only callus was formed without shoot formation. On the other hand, in the combination of 10.0 μM NAA and 5.0 μM BAP, approximately two shoots per explant were formed. Table 2 shows shoot regeneration in 'Sulhwa', and the hightest number of shoots was observed on the medium supplemented with 10.0 μM NAA

Table 1. Effect of NAA and BAP on shoot regeneration from leaf explants in *Dendranthema grandiflorum* 'Puma' after five weeks in culture.

Plant growth regulator (μM)		Callus formation (%)[a]	Shoot formation (%)	Average number of shoots per explant
NAA	BAP			
0.0	0.0	0[b]	0	0
1.0	5.0	100	0	0
10.0	5.0	80	20	2.0±0.0[b]

[a] Percentage of shoot formation and callus formation was calculated based on number of explants forming shoot or callus per total number of explants after five weeks in culture.

[b] Each value represents the mean ± S.E. of five replications, each with four explants.

Table 2. Plantlet regeneration from leaf explants in *Dendranthema grandiflorum* 'Sulhwa' as affected by growth regulator and their concentrations after five weeks in culture.

Plant growth regulator (μM)		Regeneration[a]
NAA	BAP	
0	0	-
1	2	++
1	5	-
1	10	-
10	5	+++
10	10	++

[a] - , no callus and shoots formed; +, callus formed but no shoots formed; ++, 1~2 shoots formed per explants; and +++, ≥3 shoots formed per explants.

Table 3. Effects of NAA and BAP on the formation of callus and shoots from leaf explant, and average number of shoots per explant in *Dendranthema grandiflorum* 'Geummokseo' after five weeks in culture.

Plant growth regulator (μM)		Callus formation (%)[a]	Shoot formation (%)	Average number of shoots per explant
NAA	BAP			
0.0	0.0	75[b]	25	2.0±0.50
10.0	2.0	75	25	1.0±0.60
10.0	5.0	100	0	0
10.0	10.0	100	0	0
10.0	20.0	50	50	7.5±2.50

[a] Percentage of callus and shoot formation was determined as described in Table 1.
[b] Each value represents the mean ± S.E. of five replications, each with four explants.

Table 4. Effects of NAA and BAP on shoot regeneration from leaf explant in *Dendranthema grandiflorum* 'Sulpoong' after five weeks in culture.

Plant growth regulator (μM)		Shoot formation (%)[a]	Average number of shoots per explant
NAA	BAP		
0.0	0.0	0[b]	0
1.0	5.0	0	0
1.0	10.0	0	0
10.0	5.0	50	2.0±0.08
10.0	10.0	75	3.3±1.50

[a] Percentage shoot formation was determined as described in Table 1.
[b] Each value represents the mean ± S.E. of five replications, each with four explants.

and 5.0 μM BAP.

Based on this result, an experiment on regeneration of 'Geummokseo' was conducted on the medium containing 10.0 μM NAA and various concentrations of BAP (Table 3). On the medium with 10.0 μM NAA and 5.0 μM BAP, only a few calli were formed with less number of shoots as compared with 'Puma'. On the medium with 10.0 μM NAA and 10.0 μM BAP, only callus was formed, but shooting did not occur. On the other hand, explants grown on the medium containing 10.0 μM NAA and 20.0 μM BAP gave rise to most abundant shoots (7.5 ± 2.5 shoots per explant).

Table 4 shows shoot formation and average number of shoots per explant as affected by PGR in 'Sulpoong'. When the medium was supplemented with the same concentrations of auxin and cytokinin, the number of shoots per explant was highest showing approximately 3.3. However, hyperhydric or abnormal shoots were produced. Therefore, the regeneration of 'Sulpoong' was most successful on medium with 10.0 μM NAA and 5.0 μM BAP.

Discussion

'Sulhwa', 'Puma', 'Geummokseo' and 'Sulpoong' successfully regenerated shoots from leaf explants in two weeks by *in vitro* culture and regeneration percent increased with culture period. According to Arabaud et al. (1994), bud differentiation from leaf explants of chrysanthemum started on the 11th day and the first leaf was observed on the 17th day.

Beginning of shoot regeneration in this experiment was observed two or three weeks after initiation of culture, but there were differences among cultivars.

Concentrations of suitable PGR for shoot regeneration from leaf explants were dependent on cultivars. Except 'Geummokseo', 'Sulhwa', 'Puma' and 'Sulpoong' gave most abundant shoot regeneration when concentrations of BAP were higher than those of NAA. On these media, multiple shoots were regenerated in 'Sulhwa'. The optimum ratio between auxin and cytokinin for the shoot organogenesis in chrysanthemum was contradictory among investigators. Lu et al. (1990) reported that 0.5 mg·L^{-1} BAP and 1.0 mg·L^{-1} NAA were most effective in 'Royal Purple', while Khehra et al. (1995) reported that the best results were obtained with 1.0 mg·L^{-1} BAP and 0.5 mg·L^{-1} NAA in 'Early Charm'.

Results of this study show that shoot regeneration from leaf was very much affected by cultivar. Some cultivars needed more auxin, while others needed more cytokinin. There were also cultivars which gave best result with similar ratio of auxin and cytokinin (Kaul et al. 1990). These results might have been caused by genetic differences of various chrysanthemum cultivars.

Lu et al. (1990) reported that the hightest percentages shoot regeneration and number of shoots per explant in chrysanthemum were obtained from stem explants. However, leaf explants also can regenerate shoots under an appropriate condition. Furthermore, most protocols used for the transformation were set on the basis of culture of leaf explants. Therefore, it is advisable to use the leaf explants for shoot regeneration. Further research is required to obtain more effective regeneration system.

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