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Research Article

Comparison of chrysanthemum cultivars based on direct shoot regeneration rates in tissue culture

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Abstract Direct shoot regeneration from leaf or internode or petiole segments was conducted in 33 cultivars of chrysanthemum. Shoot regeneration rates varied according to cultivars, culture media, and explant types. The high shoot regeneration rate of more than 70% in 15 cultivars ('Pink Pangpang', 'Orange Memory', 'Relance', 'Zinba', 'Beakma', 'Innocence', 'Sunny Pangpang', 'Euro Yellow', 'Dublin', 'Boramae', 'Peak', 'Euro White', 'Vesuvio White', 'Linneker Salmon' and 'Pink Pride') and 2 ones ('Forward' and 'Agason') was obtained from the segments of leaves and internodes, respectively, cultured on MS medium containing 1.0 mg·L^{-1} BAP, 0.5 mg·L^{-1} IAA and 30 g·L^{-1} sucrose. That in 6 cultivars ('Shuhonochikara', 'Hakunosen', 'Whitney Pangpang', 'Plaisir D'Amour', 'Grace' and 'Kumsu') was observed from the segments of leaves or internodes cultured on 1/2 MS medium 1.0 mg·L^{-1} BAP, 0.5 mg·L^{-1} IAA and 15 g·L^{-1} sucrose In case of 3 cultivars ('Ilweol', 'Puma White' and 'Sharon'), when internode explants excised from mother plants, which were pre-cultured on MS medium containing 2 g·L^{-1} activated charcoal and 30 g·L^{-1} sucrose for two months in the dark, and cultured on MS medium containing 1.0 mg·L^{-1} BAP, 0.5 mg·L^{-1} IAA and 30 g·L^{-1} sucrose, that was shown. Seven cultivars including 'Puma Yellow', 'Argus', 'Yes Morning', 'Whiparam', 'Hakunohikari', 'Charming Eye' and 'Moon light' requires more improved culture conditions. Tissues with the highest shoot regeneration rates were in descending order, leaf, petiole, and internode segments.

Keywords browning, *Dendranthem grandiflorum* (Ramat.) Kitamura, explants type

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Introduction

Generally, the traditional breeding of chrysanthemum has been conducted by crossing. Today, the molecular transformation technique has become an alternative approach to introduce specific characteristics, because it is advantageous especially in 'one point' crop improvement (Ledger et al. 1991). Since chrysanthemum plants are propagated mainly by cutting and suckering, any desirable lines obtained through transformation can be easily developed as useful cultivars (Han et al. 2007). Over the last several decades, many studies on transformation of chrysanthemum have been reported (Ledger et al. 1991; Renou et al. 1993; De Jong et al. 1994; Fukai et al. 1995; Takatsu et al. 1999; Mitiouchkina and Dolgov 2000; Kubo et al. 2006; Han et al. 2007). *Agrobacterium*-mediated transformation is an ideal technique for producing plants that are valuable for agronomic and scientific purposes, because it is very efficient in yielding desirable changes in cultivars without disturbing their important ornamental traits (Saito et al. 1992; John et al. 1998; Chung and Park 2005). However, to successfully introduce useful and interesting foreign genes into plant genomes via *Agrobacterium*, a reproducible plant regeneration system shall be necessary (Chung and Park 2005). Regeneration of various cultivars of *D. grandiflora* in vitro achieved by using various species and cultivars, different basal media, different plant growth regulators, media additive combinations and concentrations through organogenesis from a number of explant sources

was reviewed by Teixeira da Silva (2004). There were a few reports on direct shoot regeneration from leaf or internode explants mostly applied in transformation studies of chrysanthemum (Chung and Park 2005; Kubo et al. 2006; Han et al. 2007), although studies on shoot regeneration from leaf or internode explants have been quite scarce. Annadana et al. (2000) reported shoot regeneration efficiency varied among cultivars. And moreover, cultivars in chrysanthemum have changed and evolved as a result of customer demands or the latest trends. Hence, this study was conducted to screen shoot regeneration from leaf and internode explants in a number of chrysanthemum cultivars, which have been widely cultivated in Korea, to obtain data applicable for mutation breeding and genetic transformation of chrysanthemum.

Materials and methods

Plant material and surface sterilization

Thirty-three cultivars of chrysanthemum [*D. grandiflorum* (Ramat.) Kitamura] were selected for shoot regeneration because they have been widely cultivated in Korea. Young new shoots were taken from the donor plants in a greenhouse of National Institute of Horticultural & Herbal Science, and their surfaces were sterilized with 70% ethyl alcohol for 30 s and 1.0% sodium hypochlorite for 15 min, and rinsed three times with sterile distilled water. Then, they were incubated on Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) containing 2 $g \cdot L^{-1}$ activated charcoal and subcultured at eight weeks interval. The second and third leaves (from the top), which were excised from the shoots and cut into leaf segments of 5 mm length containing midrib, the internodes (from the second and third position), which were cut into 2 mm segments in length, and petioles were used as explants in the experiments.

Culture media

To screen shoot regeneration from leaf and internode explants of 33 cultivars (listed in Table 1), MS basal medium containing 1.0 $mg \cdot L^{-1}$ 6-benzyl amino purine (BAP) and 0.5 $mg \cdot L^{-1}$ indole-3-acetic acid (IAA), 30 $g \cdot L^{-1}$ sucrose and 7 $g \cdot L^{-1}$ plant tissue culture agar (Ducheffa, The Netherlands) was used in the initial shoot regeneration experiment. In the second experiment, leaf and internode segments of 16 cultivars (with low shoot regeneration ratio in the first experiment) (listed in Table 2) were cultured on medium comprising 1/2 MS medium salts, 1.0 $mg \cdot L^{-1}$ BAP, 0.5 $mg \cdot L^{-1}$ IAA, 15 $g \cdot L^{-1}$ sucrose and 7 $g \cdot L^{-1}$ plant tissue culture agar. In the third experiment, we let mother plantlets of the rest recalcitrant 10 cultivars (listed in Table 3) showing low shoot regeneration rate even in the second experiment cultured on MS medium containing 2 $g \cdot L^{-1}$ activated charcoal and 30 $g \cdot L^{-1}$ sucrose for two months in the dark, and then the white internode sections (2 mm) were cut from the mother plantlet and cultured on the same medium as in the first experiment. Furthermore, to compare shoot regeneration efficiency according to explant sources, the segments from leaf, internode and petiole of 4 cultivars (listed in Table 4) were cultured on the same medium as in the first experiment.

Culture conditions

Each experiment had four replications on four petri dishes with ten explants. All cultivation for shoot regeneration was performed in the dark. All cultures were incubated at 25 ±2°C. Data on percentage of explants regenerating shoots and number of shoots per explants was collected after 6 weeks in culture.

Results and discussion

As MS medium with high concentration (0.5 to 1.0 $mg \cdot L^{-1}$) of BAP and low concentration (0.2 to 0.5 $mg \cdot L^{-1}$) of auxin was used generally for direct shoot regeneration in chrysanthemum (Aswath et al. 2004; Chung and Park 2005; Han et al. 2007), the segments of leaves or internodes of 33 chrysanthemum cultivars were cultured on MS medium containing 1.0 $mg \cdot L^{-1}$ BAP, 0.5 $mg \cdot L^{-1}$ IAA, 30 $g \cdot L^{-1}$ sucrose and 7 $g \cdot L^{-1}$ plant tissue culture agar to regenerate shoots for 6 weeks. High regeneration ratio of more than 70% were obtained from the explants of leaves of 15 cultivars including 'Pink Pangpang', 'Orange Memory', 'Relance', 'Zinba', 'Beakma', 'Innocence', 'Sunny Pangpang', 'Euro Yellow', 'Dublin', 'Boramae', 'Peak', 'Euro White', 'Vesuvio White', 'Linneker Salmon' and 'Pink Pride' and from those of internodes of two cultivars ('Forward' and 'Agason'). These cultivars produced more than 1.1 shoots per explant favorably. The remaining cultivars showed very low shoot regeneration efficiency from the segments of leaves or internodes, and 6 of them produced brown callus, 5 cream callus growing vigorously and 2 white friable callus, and 3 cultivars did not produce any callus on the edges of explants (Table 1). As Takatsu et al. (1998) reported that shoot regeneration capacity was different in cultivars owing to variations in their genetic characteristics, which had already been verified in earlier research activities (Sherman et al. 1998, Annadana et

Table 1 Shoot regeneration from leaf and internode explants cultured on MS medium containing 1.0 mg·L^{-1} BAP and 0.5 mg·L^{-1} IAA according to cultivars of chrysanthemum after 6 weeks in culture

Cultivar	Leaf		Internode		Callus color[y]
	Regeneration (%)	No. of shoots/explant	Regeneration (%)	No. of shoots/explant	
Pink Pangpang	92 ± 0.9[z]	1.1 ± 0.2	10 ± 2.0	0.1 ± 0.0	Cream
Orange Memory	100 ± 0.0	5.6 ± 0.1	100 ± 0.0	2.5 ± 0.0	Cream
Relance	74 ± 2.3	1.7 ± 0.1	82 ± 3.3	2.2 ± 0.1	Cream
Zinba	86 ± 2.3	2.1 ± 0.2	58 ± 3.0	0.8 ± 0.0	Cream
Beakma	96 ± 1.8	1.7 ± 0.0	58 ± 3.0	1.0 ± 0.1	Cream
Innocence	98 ± 0.9	1.7 ± 0.1	-	-	Cream
Sunny Pangpang	90 ± 2.5	3.2 ± 0.2	66 ± 5.2	1.3 ± 0.1	LB
Euro Yellow	82 ± 2.2	3.2 ± 0.2	76 ± 2.3	2.1 ± 0.1	LB
Dublin	76 ± 1.1	1.8 ± 0.1	24 ± 1.8	0.5 ± 0.1	LB
Boramae	100 ± 0.0	2.8 ± 0.1	62 ± 2.6	0.9 ± 0.1	LB
Peak	100 ± 0.0	1.7 ± 0.1	82 ± 2.6	1.6 ± 0.1	-
Euro White	94 ± 1.8	5.3 ± 0.1	60 ± 3.7	1.5 ± 0.1	-
Vesuvio White	78 ± 1.7	1.3 ± 0.0	30 ± 3.7	0.4 ± 0.0	-
Linneker Salmon	70 ± 2.5	1.5 ± 0.1	88 ± 1.7	2.4 ± 0.2	-
Pink Pride	90 ± 2.8	2.8 ± 0.1	30 ± 3.5	0.6 ± 0.1	-
Forward	22 ± 2.2	0.2 ± 0.0	74 ± 4.1	2.0 ± 0.1	LB
Agason	30 ± 1.4	0.4 ± 0.0	74 ± 2.3	1.8 ± 0.1	LB
Puma White	0 ± 0.0	0 ± 0.0	46 ± 3.6	0.9 ± 0.1	Brown
Plaisir D'Amour	40 ± 1.4	0.9 ± 0.0	22 ± 3.0	0.3 ± 0.0	Brown
Puma Yellow	0 ± 0.0	0 ± 0.0	2 ± 0.9	0 ± 0.0	Brown
Argus	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	Brown
Ilweol	14 ± 2.3	0.3 ± 0.1	44 ± 4.0	1.1 ± 0.1	Brown
Yes Morning	32 ± 3.8	0.4 ± 0.1	50 ± 2.0	0.7 ± 0.0	Brown
Whitney Pangpang	4 ± 1.8	0.1 ± 0.0	0 ± 0.0	0 ± 0.0	Cream
Charming Eye	14 ± 1.8	0.2 ± 0.0	18 ± 2.6	0.2 ± 0.0	Cream
Hakunosen	4 ± 1.1	0.1 ± 0.0	8 ± 1.7	0.1 ± 0.0	Cream
Whiparam	16 ± 3.0	0.4 ± 0.1	40 ± 2.0	0.7 ± 0.1	Cream
Grace	18 ± 3.0	0.3 ± 0.1	44 ± 2.3	1.0 ± 0.1	Cream
Hakunohikari	4 ± 1.8	0.1 ± 0.0	4 ± 1.1	0.1 ± 0.0	White
Shuhonochikara	22 ± 3.3	0.3 ± 0.0	0 ± 0.0	0 ± 0.0	White
Kumsu	54 ± 3.6	0.7 ± 0.0	58 ± 2.6	1.3 ± 0.1	-
Sharon	40 ± 4.0	0.9 ± 0.1	6 ± 1.1	0.1 ± 0.0	-
Moon Light	10 ± 0.0	0.1 ± 0.0	12 ± 2.2	0.1 ± 0.0	-

[z] Mean ± Standard error
[y] LB: light brown

al. 2000), shoot regeneration rates varied according to cultivar and explant type, and regeneration rates were generally higher from leaf explants than internode ones. Especially, the cultivars generated vigorously growing brown or cream color callus and friable white ones showed very low regeneration rates. Faisal et al. (2006) reported that the highest number of multiple shoots and maximum length were obtained on half-strength MS medium. Han et al. (2004) also reported that callus growth of *Philodendron* was inhibited by reduction of sucrose in the medium to 20 g·L^{-1}. To inhibit vigorous growth of callus and stimulate shoot regeneration of the 16 cultivars (showing low shoot regeneration rate in the first experiment)

Table 2 Shoot regeneration from leaf and internode explants cultured on 1/2 MS medium containing 1.0 mg·L^{-1} BAP, 0.5 mg·L^{-1} IAA and 15 g·L^{-1} sucrose according to cultivars of chrysanthemum after 6 weeks in culture

Cultivar	Leaf		Internode		Callus color
	Regeneration (%)	No. of shoots/explant	Regeneration (%)	No. of shoots/explant	
Shuhonochikara	70 ± 2.0 [z]	1.0 ± 0.0	12 ± 0.9	0.1 ± 0.0	Cream
Hakunosen	82 ± 3.3	1.2 ± 0.1	62 ± 1.7	0.9 ± 0.1	Cream
Whitney Pangpang	86 ± 5.2	1.4 ± 0.1	70 ± 1.4	1.2 ± 0.0	Cream
Plaisir D'Amour	84 ± 4.1	1.6 ± 0.1	54 ± 3.3	0.6 ± 0.0	-
Grace	100 ± 0.0	2.8 ± 0.1	-	-	-
Kumsu	92 ± 1.7	1.4 ± 0.1	-	-	-
Charming Eye	0 ± 0.0	0 ± 0.0	18 ± 1.7	0.2 ± 0.0	Cream
Ilweol	8 ± 1.7	0.1 ± 0.0	26 ± 3.9	0.3 ± 0.0	Brown
Puma White	0 ± 0.0	0 ± 0.0	26 ± 2.3	0.4 ± 0.0	Brown
Puma Yellow	0 ± 0.0	0 ± 0.0	12 ± 2.2	0.1 ± 0.0	Brown
Hakunohikari	16 ± 3.3	0.2 ± 0.0	2 ± 0.9	0.0 ± 0.0	-
Sharon	24 ± 3.3	0.4 ± 0.1	18 ± 1.7	0.2 ± 0.0	-
Argus	0 ± 0.0	0 ± 0.0	0 ± 0.0	0 ± 0.0	-
Whiparam	8 ± 1.2	0.1 ± 0.0	-	-	-
Yes Morning	8 ± 1.2	0.1 ± 0.0	-	-	-
Moon Light	0 ± 0.0	0 ± 0.0	-	-	-

[z] Mean ± Standard error

(listed in Table 2), segments of leaves or internodes were cultured on medium supplemented with 1/2 MS salts, 1.0 m g·L^{-1} BAP, 0.5 mg·L^{-1} IAA, 15 g·L^{-1} sucrose. The callus growth was inhibited significantly and shoot regeneration ratio of more than 70% was obtained from leaf explants of 6 cultivars including 'Shuhonochikara', 'Hakunosen', 'Whitney Pangpang', 'Plaisir D'Amour', 'Grace', and 'Kumsu'. These cultivars produced more than 1.0 shoots per explant (Table 2). The similar result was reported for in vitro culture of carnation (Schnapp and Preece 1986). Explants coming from most of the recalcitrant 10 cultivars appeared to have brown callus growth even in the second experiment. In castor (*Ricinus communis* L.), the pre-treatment of explants in the dark increased shoot regeneration (Ahn et al. 2007). In the third experiment, to inhibit browning, in vitro mother plants of the recalcitrant 10 cultivars were pre-cultured on MS medium containing 2 g·L^{-1} activated charcoal and 30 g·L^{-1} sucrose for two months in the dark, and then the white internode sections were cultured on the same medium as in the first experiment. Three cultivars including 'Ilweol', 'Puma White' and 'Sharon' showed high shoot regeneration rates of more than 70% from internode sections (Table 3). The rest 7 cultivars including 'Puma Yellow', 'Argus', 'Yes Morning', 'Whiparam', 'Hakunohikari', 'Charming Eye' and 'Moon light' remained at low shoot regeneration rate condition, and so seem to require more improved culture conditions. Explant browning is a major problem for regeneration. Light regime induced browning of explant, embryo necrosis and eventually low plantlet conversion rate. In this study, pre-incubation of mother plants in the dark led to an increase in shoot regeneration rate of 3 cultivars. GA3 (0.5 to 5.0 mg·L^{-1}) was supplemented into regeneration medium to stimulate the shoot regeneration of the rest recalcitrant 7 cultivars showing low shoot regeneration rate even in the third experiment, but it did not show any effect on increasing the rate of shoot regeneration (data was not shown). To compare shoot regeneration efficiency according to explant sources, the segments from leaf, internode, and petiole of 4 cultivars (listed in Table 4) were cultured on the same medium as in the first experiment. There was a marked difference in shoot regeneration rates according to cultivars and explant sources. Shoot regeneration rate was high in descending order, leaf, petiole and internode segments. High shoot regeneration rates of more than 80% were recorded in the culture of leaf explants (Table 4).

In conclusion, direct shoot regeneration rate from leaf or internode or petiole segments of 33 cultivars of chrysanthemum widely cultivated in Korea was investigated. Until now, there were few studies that used leaf disk (Chung and Park 2005) and internode sections (Takatsu et al. 1999),

Table 3 Shoot regeneration from internode explants (excised from the mother plants cultured under dark) cultured on MS medium containing 1.0 mg·L^{-1} BAP and 0.5 mg·L^{-1} IAA according to cultivars of chrysanthemum after 6 weeks in culture

Cultivar	Internode		Callus color
	Regeneration (%)	No. of shoots/explant	
Ilweol	78.0 ± 2.2[z]	1.4 ± 0.1	Light brown
Puma White	70.0 ± 4.2	1.4 ± 0.1	Cream
Sharon	70.0 ± 2.6	1.1 ± 0.1	-
Puma Yellow	6.0 ± 1.8	0.1 ± 0.0	Dark Brown
Argus	0.0 ± 0.0	0.0 ± 0.0	Dark Brown
Yes Morning	24.0 ± 2.3	0.4 ± 0.0	Brown
Whiparam	14.0 ± 1.1	0.2 ± 0.0	White
Hakunohikari	0.0 ± 0.0	0.0 ± 0.0	White
Charming Eye	8.0 ± 1.7	0.2 ± 0.0	Cream
Moon Light	26.0 ± 3.9	0.3 ± 0.0	-

[z] Mean ± Standard error

Table 4 Shoot regeneration from different explants on MS medium containing 1.0 mg·L^{-1} BAP and 0.5 mg·L^{-1} IAA in chrysanthemums after 6 weeks in culture

Cultivar	Leaf		Internode		Petiole	
	Regeneration (%)	No. of shoots /explant	Regeneration (%)	No. of shoots /explant	Regeneration (%)	No. of shoots /explant
Pink Pangpang	94 ± 4.0[z] a[y]	2.0 ± 0.2 a	8 ± 3.7 c	0.1 ± 0.0 c	64 ± 6.8 b	0.9 ± 0.1 b
Zinba	84 ± 5.1 a	2.1 ± 0.1 a	42 ± 7.3 b	0.6 ± 0.1 c	66 ± 6.8 a	1.0 ± 0.1 b
Linneker Salmon	86 ± 2.4 a	1.8 ± 0.2 a	82 ± 5.8 a	1.2 ± 0.1 a	86 ± 2.4 a	1.6 ± 0.2 a
Orange Memory	96 ± 2.4 a	3.9 ± 0.3 a	52 ± 17.4 b	2.2 ± 0.2 b	96 ± 2.4 a	3.9 ± 0.2 a

[z] Mean±Standard error
[y] Different letters in same line represent significant differences by the Duncan's multiple range test, $P \leq 0.05$

whereas the majority used leaf segments (Ledger et al. 1991, Kudo et al. 2002, Aida et al. 2005, Han et al. 2007) for regeneration of chrysanthemum. Results obtained in this study were the same, too. Therefore, these results suggest that leaf explant might be more effective than either leaf disks or internodes in shoot regeneration applicable for mutation breeding and genetic transformation of chrysanthemum.

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