J. Plant Biotechnology(2004) Vol. 6(4). pp. 253~258

Journal of Plant Biotechnology

Plant Regeneration Depending on Explant Type in *Chrysanthemum coronarium* L.

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Abstract

Plant regeneration depending on explant type was investigated with cotyledon, hypocotyl, and leaf explants of garland chrysanthemum (*Chrysanthemum coronarium* L.) cultured on MS basal medium supplemented with various concentrations of BAP and NAA combination. Among the three different types of explants, hypocotyl explants grown on MS medium containing 1.0 μM NAA, 1.0 μM BA and 6 μM $AgNO_3$ produced the highest adventitious shoots (4.67 per explant). Hypocotyl explants not only produced more vigorous shoots, which regenerated faster than the cotyledon and leaf explants. An efficient root formation was observed in MS medium containing 3% sucrose. The concentration of NAA did not show significant effects on root formation. Results from this experiment suggested that hypocotyl explants were efficient for the regeneration of garland chrysanthemum.

Key words: Explant type, garland chrysanthemum, plant growth regulator, plant regeneration

Introduction

Chrysanthemum family includes 96 genera and more than 20,000 species of annual and perennial herbs and shrubs worldwide. Garland chrysanthemum, one of the *Chrysanthemum* species, has been cultivated as a floral crop in Europe and as a vegetable crop in India, Southeast Asia, China, Japan, and Korea. It is a self-pollinated crop and propagated by seeds. Its optimum temperature for germination and growth ranges from 15 to 20℃.

Tissue culture has been applied for mass propagation and efficient breeding in floral chrysanthemum. Relatively numerous successful organogenesis through shoot tip culture (Ben-Jacov and Langhans 1972; Earl and Langhans 1974; Hill 1968), ovule culture (Watanabe 1977), protoplast culture (Sauvadet et al. 1990; Schum and Preil 1981) and somatic embryogenesis (May and Trigiano 1991; Pavingerova et al. 1994; Tanaka et al. 2000) were reported in horticultural chrysanthemums.

Tissue culture systems are important for crop and vegetable improvement using genetic engineering. In horticultural chrysanthemum, plant transformation system has been well developed and used practically (Jong et al. 1993; Kim et al. 1998; Sherman et al. 1998). Although tissue culture and transformation system have been well developed in various horticultural chrysanthemums, only a few regeneration studies were conducted with garland chrysanthemum (Amagasa and Kameya 1989; Lee et al. 1997). The plant regeneration through tissue culture in garland chrysanthemum still needs to be improved and made efficient. Besides, genetic transformation system to introduce foreign genes into garland chrysanthemum is yet to be standardized. Accordingly, as a part of effort to develop an efficient transformation system, this study was carried out to improve the tissue culture system in garland chrysanthemum by determining proper explant types and optimum culture medium compositions for shoot and root regeneration, and examining the effect of $AgNO_3$ on shoot regeneration.

Materials and Methods

Preparation of plant materials

Seeds of garland chrysanthemum (*Chrysanthemum coronarium* L.) were sterilized by dipping in 70% ethanol for 1 min followed by immersing in the solution of 3% sodium

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Received Sep. 20, 2004; Accepted Dec. 10, 2004

hypochlorite for 20 min, and then rinsed 3 to 5 times with sterile distilled water. Sterilized seeds were sown into autoclaved Mazenta box (Magenta Co., size: 60×60×100 mm, volume: 400 mL, quality: body-polycarbonate, cap-polypropylene) containing 1/2 MS medium (Murashige and Skoog 1962) supplemented with 0.8% agar and 1.5% sucrose. Seeds were germinated and maintained in a tissue culture room under 16 h light and 8 h dark cycles at 21±2℃. Hypocotyl and cotyledon explants were excised from seedlings grown for 1 week and leaf explants were excised from seedlings grown for 4 weeks after seeding.

Induction of callus and adventitious shoot

To optimize the shoot regeneration condition in garland chrysanthemum, about 1 cm long explants from leaf, hypocotyl, and cotyledon were cultured on MS medium containing 3% sucrose and 0.8% agar and supplemented with 0, 0.5, 1.0, 2.5, and 10.0 μM 6-benzylaminopurine (BAP) in combination with 0, 0.25, 0.5, 1.0, and 2.5 μM α-naphthalene acetic acid (NAA). Plant growth regulators and media were autoclaved at 1.2 kg/cm^2 and 121℃ for 20 min.

To investigate the influence of $AgNO_3$ on shoot regeneration, leaf explants were placed on 95 mm × 20 mm Petri dish containing MS basal medium supplemented with 2.5 μM BAP and NAA, respectively, and 0, 6, 18, and 30 μM $AgNO_3$, and hypocotyl explants were placed on 95 mm × 20 mm Petri dish containing MS medium supplemented with 1.0 μM BAP and NAA, respectively, and 0, 6, 18, and 30 μM $AgNO_3$. $AgNO_3$ was filter-sterilized and added to the autoclaved medium.

Root induction and acclimatization

To investigate the effect of sucrose on rooting, regenerated shoots from hypocotyl explants were transferred to a Mazenta box containing MS medium supplemented with 0, 1, 3, and 5% sucrose. The optimum sucrose concentration determined was applied to examine the effect of NAA (0, 0.5, 1.0, and 2.5 μM) on rooting.

To acclimatize regenerated plantlets, they were washed thoroughly to remove adhering agar. The regenerated plantlets were then transferred into 12 cm wide vinyl pots containing 'Baroker' (Seoul agricultural materials Co. Ltd) and three pots were wrapped in one vinyl bag (50 cm × 50 cm). These potted plantlets were subjected to acclimatization under 16 h light and 8 h dark cycle at 24±2℃. For adaptation to the atmospheric conditions of relatively low humidity, the vinyl bags containing plantlets were widely opened once a day for 1 h during the first week and three times a day for 1 h during the second week of acclimatization.

Data collection and analysis

All the experiments were replicated 3 times. Callus formation was observed and the diameter of callus was measured. The number of adventitious shoots longer than 3 mm was counted. Shoot regeneration data were collected twice after 6 and 10 weeks of culture and analyzed with Duncan's multiple range test. For the root regeneration data, the number of roots was counted after 5 weeks of culture and statistically analyzed using standard error.

Results and Discussion

Determining the medium conditions for callus and adventitious shoot induction

To establish optimum medium conditions for callus and adventitious shoot regeneration in garland chrysanthemum, a series of experiments were conducted using hypocotyl, cotyledon, and leaf explants.

Effects of plant growth regulators on callus and adventitious shoot regeneration from 3 different types of explants in garland chrysanthemum are shown in Table 1. After 2 weeks of culture, callus was generated from the cut surface of the explants used, and the media containing both BAP and NAA produced callus. However, even though the shoot organogenesis was completed through callus formation, it was not affected by the degree and size of callus in the three types of explants.

The highest mean number of regenerated shoots per explant was 4.08, which is obtained from hypocotyl explants in medium containing 1.0 μM BAP and NAA, respectively. In case of leaf explants, the medium supplemented with 2.5 μM BAP and 2.5 μM NAA was the best for number of shoots regenerated per explant. These results are similar to previous study, which demonstrated that medium containing 2.5 μM BAP and 2.5 μM NAA was the most effective for shoot regeneration from leaf explants (Lee et al. 1997). However, another study conducted with 11 floral chrysanthemum cultivars showed that stem explants were superior to leaf explants in shoot regeneration (Kaul et al. 1990). Although it is difficult to compare two studies, results are in conformity with those of present experiment, suggesting that hypocotyl explants could be used for plant regeneration in garland chrysanthemum.

Effect of $AgNO_3$ on callus and adventitious shoot induction

To investigate the effect of $AgNO_3$ on callus and adventitious shoot regeneration in garland chrysanthemum, hypocotyl and leaf explants were placed on the culture media supplemented with various concentrations of $AgNO_3$. Although callus formation from both explants was not affected by the presence of $AgNO_3$, adventitious shoot regeneration was significantly affected in both hypocotyl and leaf explants (Table 2). By supplementing 6 μM of $AgNO_3$, hypocotyl explants produced 1.47 shoots per explant after 6 weeks and 4.67 shoots per explant after 10 weeks of culture on the regeneration media containing 1.0 μM BAP and 1.0 μM NAA. This difference between hypocotyl and leaf explants might be caused by the difference in shoot induction rate between two types of explants. Leaf explants placed on the regeneration media containing 2.5 μM BAP and 2.5 μM NAA supplemented with 18 μM of $AgNO_3$ produced 1.47 shoots per an explant after 10 weeks of culture. These results are similar to the earlier studies, suggesting that 1~50 μM of $AgNO_3$ stimulated shoot regeneration in garland chrysanthemum (Lee et al. 1997) and in *Cichorium intybus* L. (Bais et al. 2001).

Table 1. Effects of combined treatments of NAA and BAP on callus and shoot induction from cotyledon, hypocotyl, and leaf explants in garland chrysanthemum (*Chrysanthemum coronarium* L.)

Plant growth regulators (μM)		Callus formation/explant[a]			No. of shoot/explant[b]		
NAA	BAP	Cotyledon	Hypocotyl	Leaf	Cotyledon	Hypocotyl	Leaf
0	0	-	-	-	0.00 c[c]	0.17 c	0.00 b
0	0.5	-	-	++	0.00 c	0.00 c	0.08 b
0	1.0	+	-	+	0.00 c	0.00 c	0.00 b
0	2.5	+	-	+	0.00 c	0.00 c	0.00 b
0	10	+	+	+	0.00 c	0.17 c	0.00 b
0.25	0	+	-	+	0.00 c	0.00 c	0.00 b
0.25	0.5	+	++	++	0.00 c	0.67 c	0.00 b
0.25	1.0	+	++	++	0.00 c	0.00 c	0.00 b
0.25	2.5	++	+	++	0.00 c	0.00 c	0.00 b
0.25	10	-	++	++	0.00 c	0.19 c	0.00 b
0.5	0	-	+	+	0.00 c	0.00 c	0.00 b
0.5	0.5	+	+	++	0.13 b	0.19 c	0.00 b
0.5	1.0	+	++	+++	0.00 c	0.00 c	0.00 b
0.5	2.5	+	++	++	0.00 c	2.42 b	0.00 b
0.5	10	+	++	+++	0.00 c	0.17 c	0.25 b
1.0	0	+	+	-	0.00 c	0.17 c	0.00 b
1.0	0.5	++	+++	++	0.53 a	0.17 c	0.00 b
1.0	1.0	+++	+++	++	0.00 c	4.08 a	0.00 b
1.0	2.5	+++	++	++	0.00 c	0.00 c	0.33 b
1.0	10	+++	++	+	0.00 c	0.00 c	0.00 b
2.5	0	+	-	+	0.00 c	0.00 c	0.00 b
2.5	0.5	-	-	+	0.00 c	0.00 c	0.00 b
2.5	1.0	+	+	+	0.00 c	0.00 c	0.17 b
2.5	2.5	+	-	+	0.00 c	0.00 c	0.92 a
2.5	10	+	++	++	0.00 c	0.00 c	0.00 b

[a]Mean diameter of callus: - = no callus formation; + = callus under 1 cm; ++ = callus from 1 to 1.99 cm; +++ = callus above 2 cm.
[b]Total number of regenerated shoots/total number of explants. The number of regenerated shoots longer than 3 mm in length was counted. Cotyledon and hypocotyl explants were excised from 1 week-old seedlings and leaf explants were excised from 4 week-old seedlings. 11 to 15 explants per treatment were used and three independent experiments were conducted. Data were collected after 10 weeks of culture.
[c]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$.

Table 2. Effects of $AgNO_3$ on callus and shoot regeneration from hypocotyl and leaf explants in garland chrysanthemum (*Chrysanthemum coronarium* L.).

$AgNO_3$ (mM)	6 weeks of culture				10 weeks of culture			
	Hypocotyl explant		Leaf explant		Hypocotyl explant		Leaf explant	
	Callus formation[a]	No. of shoots /explant[b]	Callus formation	No. of shoots/ explant	Callus formation	No. of shoots/ explant	Callus formation	No. of shoots/ explant
0	++	0.60 b[c]	++	0.13 a	++	3.60 b	++	0.93 b
6	++	1.47 a	++	0.20 a	++	4.67 a	++	0.87 b
18	++	1.27 ab	++	0.67 a	++	4.40 a	++	1.47 a
30	++	0.87 ab	++	0.27 a	++	3.33 b	++	1.00 b

[a]Mean diameter of callus: - = no callus formation; + = callus under 1 cm; ++ = callus from 1 to 1.99 cm; +++ = callus above 2 cm in diameter.
[b]Total number of regenerated shoots/total number of explants. The number of regenerated shoots longer than 1 mm in length was counted. Hypocotyl explants were excised from 1 week-old seedlings and leaf explants were excised from 4 week-old seedlings. Fifteen explants per treatment were used and three independent experiments were conducted. Data were collected after 6 and 10 weeks of culture.
[c]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$.

Effect of sucrose and NAA on root induction

To establish an efficient medium condition for rooting, MS medium supplemented with 0, 1, 3, and 5% sucrose was used for rooting from regenerated shoots. After 10 days of culture, regenerated shoots from hypocotyl explants began to form root primordia, and 5 weeks later, shoots grown on MS medium supplemented with 3% sucrose formed the maximum roots (Figure 1A). A similar concentration of sucrose was found effective for root induction in *Dendranthema grandiflorum* cvs Nam-chon (Hyung 1999) and Royal Purple (Lu et al. 1990).

To evaluate the effect of NAA on root formation in addition to sucrose, media containing 3% sucrose with 0, 0.5, 1.0, and 2.5 μM NAA were tested. As shown on Figure 1B, more vigorous shoot and root growth was observed from the rooting medium containing only 3% sucrose. This result clearly indicated that NAA did not affect the root formation from regenerated shoots when it was supplemented to the medium containing 3% of sucrose. In contrast to this result, Lee et al. (1979) showed that the addition of 0.5 mg/L and 1 mg/L NAA promoted root formation in *D. grandiflorum* cv Shin Dong Ah, whereas regenerated shoots of 11 chrysanthemum cultivars were rooted in hormone free medium containing 3% sucrose only (Kaul et al. 1990). Besides, the results of root induction in garland chrysanthemum 'Tong-wo' also suggested that various auxins, except IBA, did not show additional effect on root formation (Lee et al. 1997). Furthermore, the shoots also failed to regenerate roots at higher concentration of auxin, 17.1 μM IAA (Lu et al 1990). From the previous and the studies present, it is evident that regenerated shoots of garland chrysanthemum formed normal roots regardless of the presence of auxin in the medium.

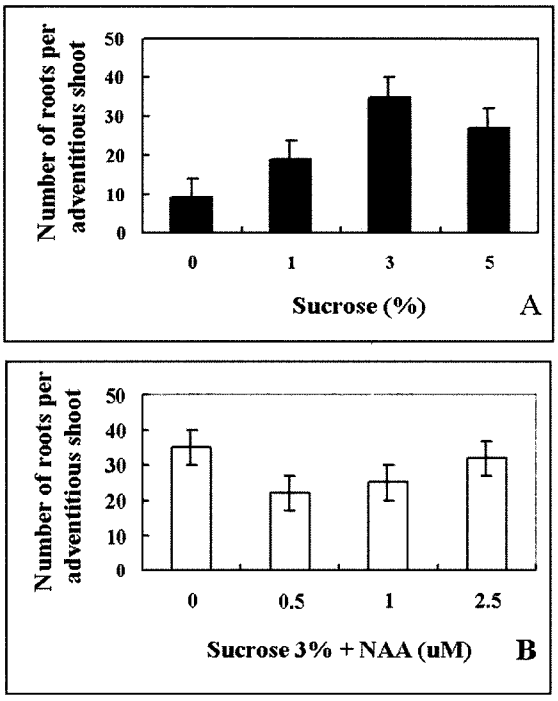

Figure 1. Effects of sucrose (A) and NAA (B) on rooting of shoots regenerated from hypocotyl explants in garland chrysanthemum (*Chrysanthemum coronarium* L.). Bars represent mean ±SE. Five shoots per treatment were used. Regenerated shoots from leaf and hypocotyl explants used in this experiment were 3 to 5 cm in length. Data were collected after 5 weeks of culture.

Plant regeneration and acclimatization

Plant regeneration using hypocotyl, leaf, and cotyledon explants are shown in Figure 2. In all three types of explants, adventitious shoots were formed after generating

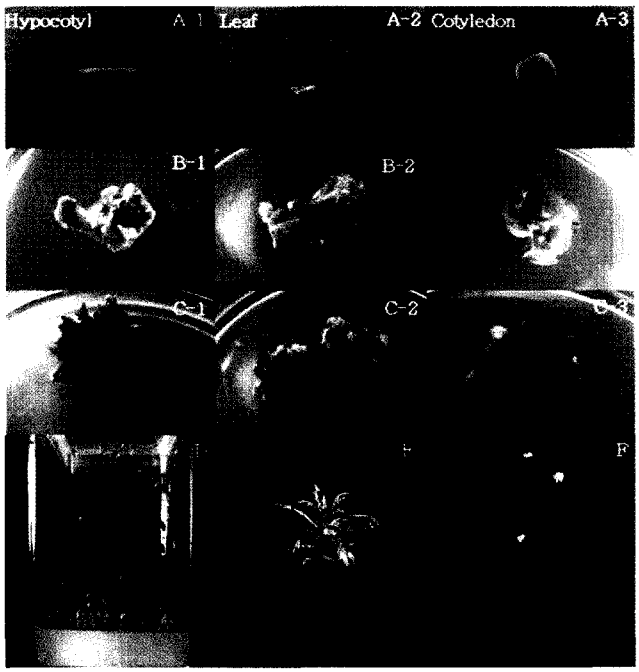

Figure 2. Plant regeneration from three types of explants in garland chrysanthemum (*chrysanthemum coronarium* L.) A: hypocotyls (A1), leaf (A2), and cotyledon (A3) explant on MS medium with 1.0 μM BAP and 1.0 μM NAA, 2.5 μM BAP and 2.5 μM NAA, and 0.5 μM BAP and 1.0 μM NAA, respectively; B: Calli formed from hypocotyl (B1), leaf (B2), and cotyledon (B3) after 2~3 weeks of culture. Multiple shoots were induced from the three types of explants tested after 5~6 weeks of culture (C1: hypocotyl, C2: leaf, C3: cotyledon). A regenerated shoots (D) was rooted on the medium containing 3% sucrose after 5 weeks of culture. A regenerant (E) was transferred into a vinyl pot for acclimatization. After 2 weeks of acclimatization, an adult regenerant (F) having four flowers was obtained after 4 weeks of culture under green house conditions.

abundant calli. A little difference in adventitious shoot regeneration rate was observed. Adventitious shoots from hypocotyl explants were induced within 4 weeks. However, in case of leaf and cotyledon explants, this duration was 6 weeks. Due to this difference, adventitious shoots from hypocotyl explants tended to be more vigorous. These shoots were more responsive to rooting with reduced by one week as compared to those from adventitious shoots obtained from leaf or cotyledon explants.

Among 40 plantlets, 38 showing normal phenotype survived and were maintained till flowering. There was no problem for acclimatization. Previous studies have shown successful acclimatization of transplanted regenerants in pots with more than 80% survived (Hyung 1999; Kaul et al. 1990; Lee et al. 1979; Lee et al. 1997). Acclimatized plants grew vigorously, especially in the greenhouse conditions, and achieved adequate growth for flowering after 3 weeks of culture in the greenhouse. The phenotypically normal yellow flowers in all the adult plants bloomed within 3 to 5 weeks in the greenhouse and produced seeds.

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