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High Frequency Shoot Regeneration from Petal Explants of *Chrysanthemum morifolium Ramat. in vitro*

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Abstract: An efficient and novel method was developed to initiate multiple shoots from the petal which is indispensable for normal and transgenic plant of *Chrysanthemum morifolium*. The study indicates that high frequency of plant regeneration that would be crucial for the application of genetic transformation methods. The 7 mm petal explants were cultured on a Murashige and Skoog's (MS) basal nutrient medium containing plant growth regulators (cytokinin or auxin-cytokinin) with various combinations and concentrations for the study of callus formation and shoot induction. The highest callus formation (96%) in 4037 genotype cultured in MS medium supplemented with 2 mg L^{-1} BA and 0.1 mg L^{-1} NAA. The highest number of shoots was obtained from genotype 89 and 4037 in MS medium supplemented with 2 mg L^{-1} BA and 0.1 mg L^{-1} kinetin, 1 mg L^{-1} kinetin and 0.1 mg L^{-1} NAA, respectively. A significant difference in regeneration capacity was observed among five genotypes. Genotypes × growth regulators interaction caused considerable variation in the expression of regeneration responses, suggesting that determination of specific level of growth regulator concentration in the medium is necessary for a particular genotype to obtain optimum response. Strong seasonal variation in plantlet regeneration frequency was observed for every genotype. The elongated shoots were multiplied on a multiplication medium, rooted and acclimatized in a green house.

Key words: Callus induction, *Chrysanthemum morifolium*, kinetin, plant regeneration, genotypes

INTRODUCTION

Chrysanthemum morifolium is a perennial composite plant, globally important cut flower and pot plant species usually cultivated by vegetative cuttings (Jaime and Teixeiria, 2004). Breeding programmes have focused on important characteristics of ornamental value, including flower colour, size and form, production quality and reaction to the environment (Broertjes *et al*., 1980). Although desirable traits have been introduced using classical breeding, there are limitations to this technique.

Firstly, there is the limited gene pool of any species, e.g., no plant species possesses the genes required for producing varieties with the full spectrum of colors (Mol *et al*., 1989).

Secondly, distant crosses may be limited by incompatibility or differences in ploidy level between putative parents (Lowe *et al*., 1993).

Thirdly, characteristics such as uniform growth and synchronous flowering are polygenic. Hence, sexual crossing may alter the delicate balance of factors determining plant growth and shape (Mol *et al*., 1989).

But this method (cuttings) has a low multiplication rate and often the plants are low quality. Because cuttings are obtained repeatedly from mother plants, there may be subjected to any virus infection and degeneration, there by increasing production costs (Hahn *et al*., 1998; Kim *et al*., 2003).

These problems have been solved by applying micropropagation methods, which are routinely applied to the clonal propagation of a variety of horticultural plants including Chrysanthemum (Ben-Jacov and Langhans, 1972). Due to the global importance, both culturally and economically, a successful regeneration protocol is indispensable for the development of physiologically and morphologically normal control and transgenic plant of Chrysanthemum. In Chrysanthemum petals are another candidate as a source for organogenesis and plant regeneration (Bush *et al*., 1976). The regeneration of whole plants from tissue culture, a prerequisite for any transformation system, has been previously achieved in Chrysanthemum petal culture via callus from various cultivars (Bush *et al*., 1976; De jong and Clusters, 1986; Malaure *et al*., 1991; Bajaj *et al*., 1992; Mizutani and

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Tanaka, 1994; Tanaka *et al*., 1998). But it was not reported distinctly about successive shoot regeneration in *C. morifolium* petal culture. There were some successive shoot regeneration reports through TCL (Thin cell layer technology) from petal culture in some Chrysanthemum cultivars (Chakrabarty *et al*., 2000; Dwivedi *et al*., 2000; Datta *et al*., 2001). Although TCL solves many of preanalytical problems, however, it introduces some difficulties of its own. There is additional labour, additional time, another machine in the laboratory and the significant cost of reagent (Kurtyez and Hoerl, 2000). Moreover, success of *in vitro* response of these explants does not only depend on the genotype but it is strongly affected by the quality of the donor plants and environmental conditions like temperature, photoperiod, light intensity, light quality etc.

As part of our effort to develop an efficient transformation system for Chrysanthemum morifolium, we describe a tissue culture system for efficient plant regeneration from petal explants and efficient root induction from these regenerated shoots of this species. This study, introduced a new procedure for high frequency of plantlet regeneration from petal culture of several *C. morifolium* genotypes.

MATERIALS AND METHODS

Plant materials and surface sterilization: Five chrysanthemum genotypes namely 89, 4038, 4037, 4040, 78 were grown in a green house under natural light condition of the Plant Genetics and Breeding Laboratory, Faculty of Bioagricultural Sciences, Nagoya University, Japan. This research was conducted from April, 2005 to October, 2006. After 14 days of flowering, petals were collected from different Chrysanthemum genotypes. Flowers head were surface sterilized with 70% ethanol for 1 min, followed by immersing in a sterilization solution containing 10 mL KH_2PO_4 and 2 mL antiformine (5% sodium hypochlorite solution for 100 mL) distilled water. After 20-25 min, the flowers heads were rinsed four to five times with sterile distilled water.

Callus induction and shoot regeneration: To optimize the type and concentration of growth regulators in culture medium for Callus induction, petals were transversely cut into two pieces (7 mm in length) upper and basal and cultured on the MS medium (Murashige and Skoog, 1962) containing MS basal salts 4.29 g L^{-1}, MS vitamins, several concentrations of NAA in a combination with BA or Kinetin and sucrose 30 g L^{-1}. Callus induction was observed in every 7 days. The percentage of callus induction was recorded.

After 1-2 weeks, when the explants had produced callus and the cultures were transferred to shoot regeneration medium containing neither several concentrations of kinetin in a combination with BA or NAA. The percentage of explants on which shoot developed were recorded. Explants were subcultured after 2 weeks interval. Shoots cultured on the multiplication medium multiplied and elongated. All media was supplemented with 3% sucrose, 0.4% agar and pH was adjusted to 5.8-6 before autoclaving. Cultures were incubated at 28±1°C under 24 h photoperiod.

Rooting and acclimatization: After 1-2 weeks, the shoots were cultured for rooting. Isolated shoots about 3-4 cm. long and were rooted on half-strength MS medium with 20% sucrose, 0.2% agar, 0.1 mg L^{-1} NAA or without growth regulator medium. The percentage of root initiation per shoot was also recorded. After 3-4 weeks, well rooted plantlets were transferred to plastic pot containing sterilized vermiculite soil and kept covered with small glass bottle for 1-2 weeks. After 2-3 weeks, plants were transferred to field conditions for flowering.

Statistical analysis: Means and standard errors were calculated for frequency of callus induction, shoot regeneration as well as number of shoots per explant. A total of ten Petri dishes per treatment was used and five segments were cultured per petridish. Each experiment was conducted three times and the statistical differences among the means were analyzed Duncan's multiple range test at p = 0.05. To explain the main effects and interactions among the treatments an Analysis of variance table is also included.

RESULTS AND DISCUSSION

Effect of explants size on callus formation and shoot initiation: It was found that the 7 mm size of the petal showed a higher frequency of callus induction (96%) and adventitious shoot regeneration (83.33) as well as shoot number per explants (1.24) than other (3,5 mm and 1cm) size (Table 1) (Fig. 1A).

Table 1: Effect of explants size on callus induction, shoot regeneration and shoot number per explants of Chrysanthemum genotype 4037

Explants size	Callus induction (%)[a]	Shoot regeneration (%)[b]	No. of shoots per explants
3 mm	41.00±1.07c	0.66±0.37d	0.05±0.58c
5 mm	63.00±0.87b	55.66±0.79b	0.18±0.78b
7 mm	96.00±0.48a	83.33±0.91ba	1.24±1.54a
1 cm	38.66±0.94c	3.33±0.40c	0.05±0.58c

Each value represents the mean±SD of three replicates, each with fifty explants; Values in a column followed by a common letter(s) are significantly different at the 0.05 level (Duncan's multiple range test); [a]Percent callus induction was calculated based on number of explants induced callus as a percentage of the total number of explants grown on MS medium in culture. [b]Percent shoot formation was calculated based on number of explants forming shoots as percentage of the total number of explants grown on MS medium after 1-2 weeks in callus culture

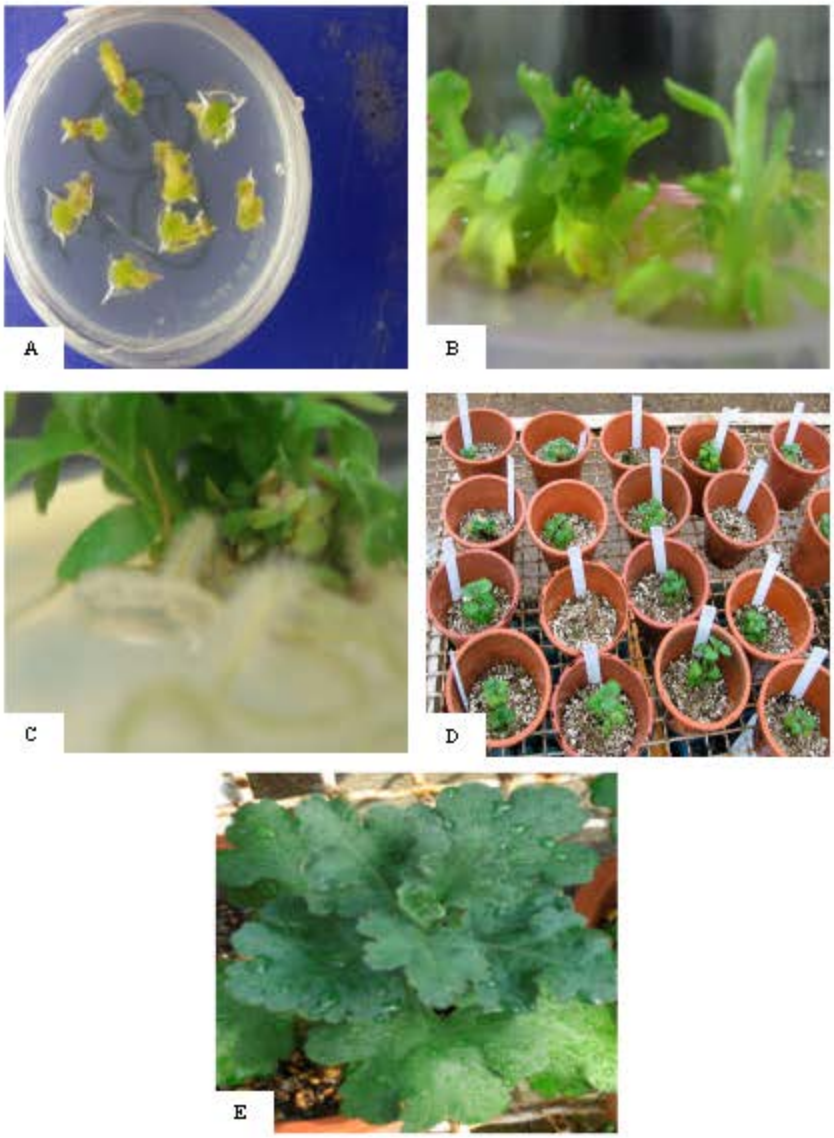

Fig. 1: Plantlet regenerated from *in vitro* petal culture of *Chrysanthemum morifolium*. (A) Callus induction from petal culture (B) Shoot regeneration and multiple shoot formation (C) Root initiation (D) Regenerated plant in the Green house (E) *in vitro* plant

Effect of plant growth regulators on callus formation and shoot induction: The maximum frequency of callus formation was obtained in MS medium supplemented with 2 mg L^{-1} BA and 0.1 mg L^{-1} NAA. Appropriate auxin and cytokinin levels are required for callus induction from each species or variety (Rout and Das, 1997). In present experiment, significance difference was observed among the different treatments and Callus formation varied from 24 to 96% (Table 2). BA combined with kinetin or alone had no effect on callus growth, suggesting that BA combined with NAA may be crucial for callus induction of Chrysanthemum. Obukosia *et al*. (2005) also reported the influence of auxins on callus production from both leaf and stem segments of Chrysanthemum cultivars. NAA acts as an auxin to induce cell division and enlargement at low concentration. Cell enlargement has been associated with an increase in activities of autolytic and synthetic enzymes, which effect cell wall plasticity and synthesis of new wall materials (Cleland, 1971). Similar results were found in achenes and petals of *C. coccineum* (Fuji and Shimizu, 1990).

We have tested the different combinations of BA, NAA and Kinetin and the adventitious shoots were observed on petal explants after 1-2 weeks of callus

Table 2: Effects of plant growth regulators on callus induction and shoot regeneration were evaluated 14 days old explants cultured on MS medium of Chrysanthemum genotype 4037

BA	NAA	Kinetin	Callus induction (%)	Shoot regeneration (%)
1	0.1	0.0	76.66±1.08b	58.33±1.07b
2	0.1	0.0	96.00±0.48a	59.33±1.11b
2.5	0.1	0.0	92.66±0.71a	0.00
3	0.1	0.0	75.33±1.16bc	0.00
5	0.1	0.0	24.00±0.99e	3.33±0.37d
0.5	1.0	0.0	0.00	0.00
0.5	0.0	0.5	0.00	0.00
1	0.0	1.0	0.00	5.33±0.44c
2	0.0	0.1	0.00	0.00
3	0.0	2.0	0.00	0.00
3	0.0	3.0	0.00	5.33±0.44c
0	0.1	1.0	42.00±0.99d	83.33±0.91a

Values are mean of three replicates±SD. Values in a column followed by a common letter(s) are significantly different at the 0.05 level of Duncan's multiple range test. Means followed by the same letter(s) are not significantly different at p = 0.05

culture. Present results indicate that kinetin plays a key role on adventitious shoot regeneration in petal culture. Among the different treatments shoot regeneration frequency varied significantly (Table 2). The regeneration frequency could be improved by manipulating the composition of hormones in the culture medium. The reason is that juvenility played an important role in regeneration is not clear and the number of regenerated shoot buds depends on the composition of culture medium, especially on level of PGRS (Rout and Das, 1997). The presence of 1 mg L^{-1} kinetin combined with 0.1 mg L^{-1} NAA significantly promoted shoot induction compared to those grown at higher concentration of BA (5 mg L^{-1}), Kinetin (3 mg L^{-1}) or NAA (1 mg L^{-1}).

Present results indicate that low concentration of NAA are good for shoot regeneration and kinetin which is a chemical analogue of cytokinin was most effective to regenerate shoot tissues from callus. Present results also agree with the result of Mok and Mok (2001) where BA and Kinetin act as a cytokinin that have long been recognized as an essential plant hormone that are involved in diverse process of plant growth and development including cell division, shoot initiation etc.

Effect of genotype on callus induction and shoot regeneration: There were significant differences among the five genotypes at all concentrations of growth regulator in both percentage of callus induction and shoot regeneration. The highest callus induction 96% and shoot regeneration 86% had observed in genotype 4037 and 89, respectively whereas lowest percentage was observed in genotypes 89 (44%) for callus induction and 4040 (42%) for shoot regeneration (Table 5). Present results indicate that shoot regeneration and callus induction ability are

Table 3: The effects of growth regulators (BA, NAA and Kinetin), genotypes and their interaction on shoot induction in different Chrysanthemum genotypes

	Shoot induction % (Mean±SD)		
Genotype	G1 medium	G2 medium	G3 medium
89	27.33±0.61	86.00±0.87	12.00±0.50
4038	82.66±0.97	0.00	60.66±1.12
4037	83.33±0.91	0.00	59.33±1.11
78	44.66±0.62	0.00	34.00±1.05
4040	42.00±0.60	0.00	30.66±1.00

Here three growth regulators medium were used. G1 = NAA (0.1 mg L^{-1}) and Kinetin (1 mg L^{-1}); G2 = BA (2 mg L^{-1}) and Kinetin (0.1 mg L^{-1}); G3 = NAA (0.1 mg L^{-1}) and BA (2 mg L^{-1}); Three replications were used for each genotype in one growth regulator medium. A total of ten petridises per replication were used and five segments were cultured per Petridis. Values are mean of three replicates±SD

strongly influenced by the genotype. Kaul *et al.* (1990) also reported that there were wide variations between the 11 cultivars in their shoot regeneration frequency from leaf and stem explants in Chrysanthemum.

Present results also suggested that determination of specific concentration of growth regulator in the medium was necessary for a particular genotype to obtain optimum response to regeneration were biased due to the presence of significant effect on genotype and growth regulator interaction (Table 3 and 4). It could also be implied that growth regulator concentration in the medium had effect on the degree of gene expression responsible for regeneration in Chrysanthemum petal culture. The effectiveness of BA, Kinetin and NAA depends on the concentration of the active ingredient which reaches the active sites for biochemical action. This in term depends on the interrelated process, including efficiency of uptake, translocation within the plant and accumulation at the active sites. It is also possible that the abilities of uptake, translocation and metabolism of NAA, BA and Kinetin among the different Chrysanthemum genotypes may be different. Obukosia *et al.* (2005) also observed such result from leaf petioles of different Chrysanthemum genotypes.

Effect of season on callus induction and shoot regeneration: Despite growth under standardized and controlled environmental conditions the effect of time of season was observed had a pronounced effect on callus induction and shoot regeneration frequency of Chrysanthemum. Petal explants of Chrysanthemum showed an increase in the frequency of plant regeneration from October to March, reaching the highest value in December/January followed by a decrease in April and a continuous and strong decrease from May to September (Table 5). The physical effects of the donor plants which depends on the growth environment effects on the response of explants under *in vitro* conditions (Morrish and Vasil, 1987).

Table 4: Analysis of variance for data in Table 3

Sources	df	SS	MS	f-calculated	f-probability (0.1%)
Total	449	1325.04			
Genotypes	4	118.48	29.62	35.26**	2.37
Growth regulator	2	284.14	142.07	169.13**	3.00
Genotypes × growth regulator	8	648.53	81.06	96.5**	1.94
Between replication within location	116	4.26	0.03	0.04^{NS}	1.32
Pooled error	319	269.63	0.84		

df = Degrees of freedom, SS = Sum of squares, MS = Mean of squares; ** Stand for the significance difference at the 0.01 level. NS: Indicate no significance difference between block within location. For evaluating genotype and growth regulator interaction three different growth regulator medium were used for five genotypes in Table 3

Table 5: Seasonal effect on days of callus induction, callus induction percentage and shoot regeneration percentage of Chrysanthemum

	Callus induction (%)		Shoot regeneration (%)	
Genotypes	Winter	Summer	Winter	Summer
89	44.00±1.27e	23.33±0.69e	86.00±0.87a	38.00±0.40b
4037	96.00±0.48a	46.66±0.47a	83.33±0.91b	24.00±0.40f
4038	91.33±0.67b	43.33±0.37c	82.66±0.97b	30.66±0.67e
4040	86.00±0.70c	47..30±0.49b	42.00±0.60e	18.00±0.30g
78	69.33±1.45d	30.66±0.62d	44.66±0.62d	0.00

Each value represents the mean±SD of three replicates, each with fifty explants; Values in a column followed by a common letter(s) are significantly different at the 0.05 level (Duncan's multiple range test)

Table 6: Genotype × growth regulator interaction on root induction of different Chrysanthemum genotypes

	Root induction (%)	
Genotypes	Without growth regulator	0.1 mg L^{-1} NAA
89	15.00±0.59bc	95.00±0.52a
4038	96.25±0.48a	67.50±0.80c
4037	95.00±0.52a	63.75±0.75d
78	16.25±0.48b	93.75±0.55a
4040	95.00±0.52a	80.00±0.48b

Values for root induction are percentage of three replicates±SD; Values in a column followed by a common letter(s) are significantly different at the 0.05 level (Duncan's multiple range test)

Due to the strong seasonal variation in the sun height, the way of the light passed through the atmosphere differs leading to a different spectral composition of global irradiance. Thus in winter time and the months close to winter, natural light contains more red light and the short waved parts were filtered out during the long way through the atmosphere. Besides that atmospheric turbidity also influences the spectral composition because of absorption and diffraction, interestingly this seasonal curve shows an optimum in June (Becker *et al*., 1994).

Our experiments showed that season may be a crucial factor for adventitious shoot regeneration of Chrysanthemum and winter season regenerated more shoots than those taken during spring or summer. Same results have been observed Miyazaki and Tashiro (1978) from stem segments of *Chrysanthemum morifolium*.

Root induction: The induction of roots in regenerated shoots depends on the composition of mineral nutrients and growth regulators in the medium (Rout and Das, 1997). It was observed that Growth regulator × genotype interaction had a significant effect on root induction of different Chrysanthemum genotypes (Table 6). Better rooting was achieved when the shoots were transferred to sterile pots containing ½ MS medium without growth regulator for genotypes 4037, 4038, 4040. But for genotypes 89, 78 required 0.1 mg L^{-1} NAA in Ms Media for better rooting after 2-3 weeks (Fig. 1B-C, Table 6). This result also indicates genotype × growth regulator interaction on root initiation of Chrysanthemum genotypes. Regenerated plants were acclimatized in the green house and then transferred to the field (Fig. 1D-E).

We can conclude that this efficient shoot regeneration system of Chrysanthemum will contribute to the production of transgenic plants as well as to be a practical tool for Chrysanthemum improvement and the study of its physiology.

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