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Improvement of Plating Efficiency on the Mesophyll Protoplast Culture of Chrysanthemum, *Dendranthema* × *grandiflorum* (Ram.) Kitam.

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There are several reports on chrysanthemum mesophyll protoplast culture, even though their regeneration frequency is low in general. In addition, the cultivars in which plant regeneration from protoplasts has been achieved are restricted to specific cultivars, Shuho-no-chikara [1-4] or clone, No. 42 [5]. Therefore, there is an expectation about further improvement of plating efficiency. This paper reports the physiological conditions of protoplast donor plants and several factors in the primary culture media intended to improve the isolation and plating efficiency in the chrysanthemum mesophyll protoplast culture.

Two kinds of cvs., Shuho-no-chikara (strain No. 4) and Kayo-no-sakura, grown *in vitro* were used. Cv. Kayo-no-sakura, which regenerates plants easily from *in vitro* cultures of stem segments [6], was used to investigate varietal difference from cv. Shuho-no-chikara. The first to third fully expanded leaves were chopped into 1 to 2 mm wide strips. The leaf tissue (1 g fwt.) was incubated in the dark for 4 h at 25°C without shaking in 10 m*l* of an enzyme solution which consisted of 2% Cellulase Onozuka RS, 0.5% Cellulase Onozuka R 10, 0.05% Macerozyme R 10, 0.02% Pectolyaze Y 23, 0.1% MES and 0.5 M sorbitol at pH 5.8. Thereafter, it was incubated for 30 min. on a reciprocal shaker (25 rpm). In the primary culture, isolated protoplasts were suspended in a MS liquid medium modified to contain one-fourth the normal strength of NH_4NO_3 (pH 5.8) supplemented with 2 mg/*l* NAA, 1 mg/*l* BA, 0.41 M sorbitol and 10 g/*l* each of sucrose and glucose. In the subculture, the composition of the medium was the same as primary one execpt that 10 g/*l* glucose and 0.41 M sorbitol were modified to 5 g/*l* glucose and 0.2 M sorbitol as osmoticums. 2.0 m*l* of this modified medium was added to every 6 cm Petri dishes four times at one week intervals after plating. Purified protoplasts were cultured in 6.0 cm Petri dishes with 2.5 m*l* of medium per dish and were plated at the density of 1×10^5/m*l*. The cultures were maintained at 25°C in the dark for one month, then transferred to continuous illumination (2,500 lux) conditions. Because of aggregation of divided protoplasts in liquid culture it was difficult to examine % colony formation (CF) per protoplast, so the % CF per plate was examined after Otsuka *et al.* [1].

As for the test plants, the young and old phases of axenic plants were examined; the former after 30~45 days of subculturing, and the latter after 70~90 days. Four kinds of strains of cv. Shuho-no-chikara that differed among themselves in their morphological and ecological characteristics were used.

In both the isolation of protoplasts and plating efficiency, the young plants proved superior to old ones for both cultivars. There were particularly remarkable differences for the percentage of normal protoplasts and plating efficiencies. In addition, sufficient results were obtained in cv. Shuho-no-chikara but not in cv. Kayo-no-sakura (**Table 1**, **Fig. 1**). On the other hand, when we used the greenhouse-grown plants, the normal protoplasts were scarcely isolated and their percentage was under 1% for both cultivars. As for the age of test plants, Ostuka *et al.* [1] used fully expanded leaves of axenic plants grown for about fifty days after shoot-tip culture, and Sauvadet *et al.* [5] selected the fourth uppermost fully expanded leaves of axenic plants subcultured every six weeks. However, Fukai *et al.* [2] and Iwai [3] did not refer to the relationship between the age of axenic plants and protoplast isolation.

There are no reports of trials with respect to the difficulty of plating efficiency in the strains of chrysanthemum. The yield of protoplasts in No. 5 strain of cv. Shuho-no-chikara was about twice as heavy as in the other three strains. However, we couldn't obtain a clear difference among the strains in the percentage of normal protoplasts, plating efficiency or colony formation (**Table 2**).

The effect of primary culture media was examined. The concentration of NH_4NO_3 in MS standard medium ranged from one half to zero. The concentration of dimethylsulfoxide (DMSO) was arranged to be from 0 to 1%. Four kinds of osmoticums, sorbitol, mannitol, glucose and sucrose were added after adjustment to the same mol concentration, 0.41 M.

On the effect of primary culture media, the percentage of plating efficiency was related to the concentration of NH_4NO_3 in MS medium, *i.e.*, that percentage tended to decrease in proportion to the NH_4NO_3

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Table 1.
Effect of age of explants on isolation and plating efficiency in chrysanthemum mesophyll protoplast culture.

Cultivar	Age*1	Yield*2 (10^5/g FW) (Mean ±S.D.)	% of normal protoplasts*2,*3 (Mean ±S.D.)	PE(%)*2,*4 (Mean ±S.D.)
Shuho-no-chikara	Young	4.45±1.94	87.93± 3.93	9.86±3.96
	Old	3.00±0.44	44.20±23.26	0.36±0.28
Kayo-no-sakura	Young	1.83±0.56	78.75± 3.93	5.04±0.62
	Old	1.20±8.63	17.28± 9.73	0

*1 The materials for cultures were used from the first to third fully expanded leaves. Young; sampled 30-45 days after subculturing, Old; after 70-90 days.

*2 DMSO was not added to the medium.

*3 (No. of normal protoplasts during purification/No. of protoplasts and cells obtained after purification)×100.

*4 PE; plating efficiency, (No. of protoplasts divided/No. of protoplasts obtained after purification)×100, examined 7 days after plating.

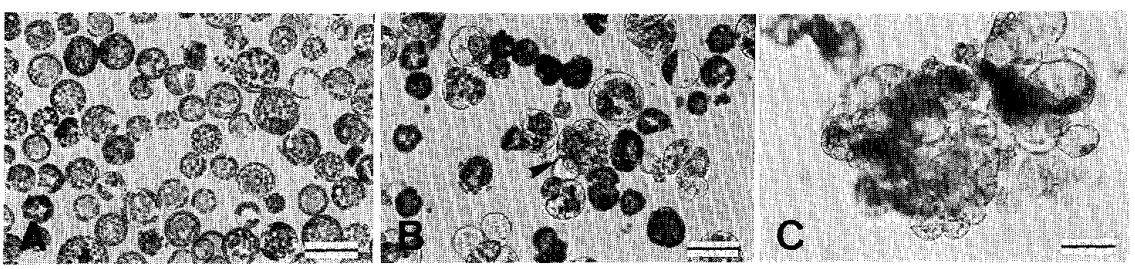

Fig. 1 Mesophyll protoplast culture of cv. Shuho-no-chikara.
A: Freshly isolated protoplasts; B: Early cell division of protoplasts, 7 days after plating. Arrow indicates cell division; C: Protoplast-derived colony, 30 days after plating. Bars=50 μm.

Table 2.
Effect of strains of cv. Shuho-no-chikara on isolation, plating efficiency and colony formation of chrysanthemum mesophyll protoplasts.*1

Strain No.	Yield (10^5/g FW) (Mean±S.D.)	% of normal protoplasts*2 (Mean±S.D.)	PE (%)*2 (Mean±S.D.)	% of CF*3
No. 4	4.45±1.94	87.93±3.93	22.09±7.10	80 (28/35)
5	9.25±3.20	83.38±2.83	26.42±3.75	83.33 (5/6)
8	4.40±1.06	87.38±3.22	22.90±3.22	100 (7/7)
9	4.80±1.08	74.74±9.71	22.77±5.18	100 (4/4)

*1 Added NH_4NO_3 to the medium in the conc. of 1/4 as standard MS medium, 1% of DMSO and 0.41 M sorbitol.

*2 See the footnote in **Table 1.**

*3 CF; colony formation, (No. of plates colony-formed/No. of plates cultured)×100, examined 30 days after culturing.

concentration, which went from a maximum in a one-fourth plot to a minimum in a full plot. Furthermore, the percentage of plating efficiency in each concentration of NH_4NO_3, cv. Shuho-no-chikara was higher than in cv. Kayo-no-sakura (**Table 3**). The most suitable concentration of NH_4NO_3 differed from previous reports by one-half [1], one-eighth [7], one-thirty second [8] and one-fourth [4]. Under the reduction of inorganic substances in MS medium to one fourth [2, 3] and the addition of all kinds of micro elements and vitamins [5], good results were obtained in the culture on NH_4NO_3-free MS medium. Putting together the results in this study and those in the above reports, the best concentration of NH_4NO_3 for plating efficiency would be from one-fourth to one-thirty second of a standard MS medium.

According to previous reports on the effect of DMSO in mesophyll protoplast culture, it has been recognized that DMSO promoted plating efficiency and colony formation in hyacinth bean and soybean [9], *Raphanus sativus* L. and *Brassica oleracea* L. [10] and *Hibiscus rosa-sinensis* [11]. In this study, the

Table 3.
Effects of the concentration of NH_4NO_3 and DMSO, and the osmoticums on the plating efficiency and colony formation in chrysanthemum mesophyll protoplast culture.

Cvs.*1	NH_4NO_3*2			DMSO*4			Osmoticum*5		
	Conc.*3	PE(%)*6 (Mean±S.D.)	% of CF*6	(%)	PE(%)*6 (Mean±S.D.)	% of CF*6	Name	PF(%)*6 (Mean±S.D.)	% of CF*6
S.C.	1	3.90±1.02	0(0/3)	0	9.86±3.96	0(0/14)	sorbitol	22.09± 7.10	80 (28/35)
	1/2	7.41±3.99	0(0/6)	0.5	13.93±4.37	40(2/5)	mannitol	11.45± 1.16	0 (0/3)
	1/4	9.86±3.96	0(0/14)	1.0	22.09±7.10	80(28/35)	glucose	15.77±13.38	3.3(1/3)
	0	0.72±0.26	0(0/3)				sucrose	0.61±0.26	0 (0/3)
K.S.	1	1.36±0.30	0(0/3)	0	5.04±0.62	0(0/12)	sorbitol	10.29±2.02	0 (0/14)
	1/2	3.44±2.10	0(0/5)	0.5	7.08±1.99	0(0/3)	mannitol	3.02±0.36	0 (0/3)
	1/4	5.04±0.62	0(0/14)	1.0	10.29±2.02	0(0/14)	glucose	3.73±1.05	0 (0/3)
	0	0.29±0.11	0(0/3)				sucrose	0.13±0.23	0 (0/3)

*1 S.C.; Shuho-no-chikara (strain No. 4), K.S.; Kayo-no-sakura.
*2 0.41 M sorbitol used as osmoticum in the medium.
*3 Magnification shows 1 as standard MS medium.
*4 Added NH_4NO_3 to the medium in the conc. of 1/4 as standard MS medium and 0.41 M sorbitol.
*5 Added NH_4NO_3 to the medium in the conc. of 1/4 as standard MS medium and 1% of DMSO. Conc. of all kinds of osmoticums is 0.4 M
*6 See the footnote in **Table 2.** Examined 30 days after culturing.

percentage of plating efficiency was improved considerably in 1% plots over the control plots for both cultivars (**Table 3**). This result agreed with the above reports. Especially,the percentage of colony formation was improved in cv. Shuho-no-chikara and was considerably higher (80%) than that obtained in DMSO-free medium [1] (about 66.7%). We only know of the report of Otsuka *et al.* [1], in which the % CF in chrysanthemum protoplast culture was exhibited. Therefore, it can be considered that addition of DMSO to MS medium would be effective in improving plating efficiency.

There are no reports on the effect of different kinds of osmoticums on the medium in the mesophyll protoplast culture of chrysanthemums. It was ascertained that the percentage of plating efficiency and colony formation differed among the osmoticums for both cultivars. The best was sorbitol, followed by glucose, mannitol and sucrose (**Table 3**). In addition, colony formation was recognized only in sorbitol and glucose plots in cv. Shuho-no-chikara. Up to now, mannitol was used as the osmoticum in protoplast culture of chrysanthemums [2, 3]. It is now clear that sorbitol will be effective osmoticum to improve plating efficiency.

We clarified various conditions for colony formation at high frequency on the mesophyll protoplast culture of chrysanthemum. We now plan to induce callus formation from these colonies.

References

[1] Otsuka, H., Suematsu, N., Toda, M., 1985. Bull. Shizuoka Agr. Exp. Stn., **30**: 25-33.
[2] Fukai, S., Shibata, M., Amano, M., Yamasaki, N., Oe, M., 1988/1989. Bull. Osaka Agr. For. Res. Ctr., **25**; 25-30.
[3] Iwai, T., 1993. Bull. Hyogo Pref. Agr. Inst. (Agriculture), **41**: 17-20.
[4] Furuta, H., Nomura, Y., Maeda, M., Makara, K., 1996. Bull. Fukui Pref. Agr. Exp. Stn., **33**: 7-13.
[5] Sauvadet, M.-A., Brochard, P., Boccon-Gibod, J., 1990. Plant Cell Reports., 8: 692-695.
[6] Miyazaki, S., Tashiro, Y., Shimada, T., 1976. Agr. Bull. Saga Univ., **40**: 31-44.
[7] Amagasa, K., Kameya, T., 1989. J. Japan Soc. Hort. Sci., **57**: 620-625.
[8] Shibata, M., 1990. In "Exploitation of the new biotic resources by cell fusion, nuclear transplantation. Kenkyu-Seika No. 1", p. 98-102, Norin-Suisan-Kaigi Jimu-Kyoku, Tokyo.
[9] Sano, H., Ono, K., Suzuki, Y., 1988. J. Japan. Soc. Hort. Sci., **57**: 399-407.
[10] Yamanaka, H., Kuginuki, Y., Kanno, T., Nishio, T., 1992. Japan J. Breed., **42**: 329-339.
[11] Yang, L. J., Hidaka, M., Masaki, H., Uozumi, T., 1994. Plant Tissue Culture Lett., **11**: 233-236.