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Formation of Somatic Hybrid Callus between *Chrysanthemum coccineum* and *Chrysanthemum coronarium* L. war. Spatiosum Bailey (CHUUBA)

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Chrysanthemum coccineum is one of the pyrethrum plants which produce insecticide, pyrethrins. We have already reported the regeneration of plants from achenes and petals of *C. coccineum*[1] and callus formation from mesophyll protoplasts of *C. coccineum*[2]. *Chrysanthemum coronarium*

Table 1. Division frequency and cluster formation of fused and non-fused protoplasts of *C. coccineum* (A) and *C. coronarium* (B).

protoplasts		Division frequency (%) 3 days	6 days	Formation of cell clusters 3 weeks
non-fused	mesophyll(B)	0.0	1.5	−
	culture(A)	< 1.0	10.3	−
	mixture *1			
	mesophyll(B)	0.0	0.0	−
	culture(A)	0.0	0.0	−
fused	homo *2			
	mesophyll(B)	0.0	0.0	−
	culture(A)	0.0	8.1	−
	homo-mix. *3			
	mesophyll(B)	0.0	0.0	−
	culture(A)	0.0	0.0	−
	hybrid-2 *4	12.5	10.9	+
non-fused	mesophyll(A)	0.0	5.7	+
	culture(B)	1.0	19.1	−
	mixture *1			
	mesophyll(A)	0.0	1.0	−
	culture(B)	2.6	21.0	−
fused	homo *2			
	mesophyll(A)	0.0	0.0	−
	culture(B)	< 1.0	18.1	−
	homo-mix. *3			
	mesophyll(A)	< 1.0	0.0	−
	culture(B)	< 1.0	21.0	−
	hybrid-1 *4	24.3	21.8	+

*1 : Two species of protoplasts were mixed and cultured without fusion.
*2 : Each species of protoplasts were fused and cultured separately.
*3 : Each species of protoplasts were fused separately and then mixed.
*4 : Two species of protoplasts were mixed and then fused (Somatic hybrid). Fused protoplasts were incubated in the medium of 1/2 MS medium (pH 5.7), 1/4 NH_4^+, 1% glucose, 0.4 M mannitol, 0.1 mg/*l* BA and 0.5 mg/*l* 2, 4-D.

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(Shungiku) is one of the most commonly grown vegetables. We have also extracted pigments from a callus culture of *C. coronarium*[3]. In this paper, we report somatic hybrid callus formation between *C. coccineum* and *C. coronarium*.

C. coronarium mesophyll protoplasts were prepared from the leaves of green house-grown plants. Leaves were surface sterilized by immersion in 3% NaOCl, and washed three times with sterile distilled water. *C. coccineum* mesophyll protoplasts were isolated as described previously[2]. Petiols of *C. coronarium* and petals or achenes of *C. coccineum* were used for callus initiation. Suspension cultures of *C. coronarium* and *C. coccineum* were established from each callus in MS medium supplemented with 0.5 mg/*l* kinetin and 2.0 mg/*l* 2, 4-D. Protoplasts were isolated from 3-day old cultured cells by the treatment of 1.5% Driserase, 0.05% Pectryase Y-23, 1.5% Cellulase Onozuka RS and 0.1% $CaCl_2$ in 0.4 M mannitol at pH 5.6.

Freshly isolated protoplasts were mixed (2×10^4 protoplast/m*l* each) and fused in a chamber with 40 V DC pulse of 40 μs duration at a field voltage of 0.75 kV/cm. After 1 min, treated cells were collected by centrifugation at 100X g for 2 min and incubated in the medium consisting of 1/2 MS medium (pH 5.7), 1/4 NH_4NO_3, 1% glucose, 0.4 M mannitol, 0.1 mg/*l* BA and 0.5 mg/*l* 2, 4-D.

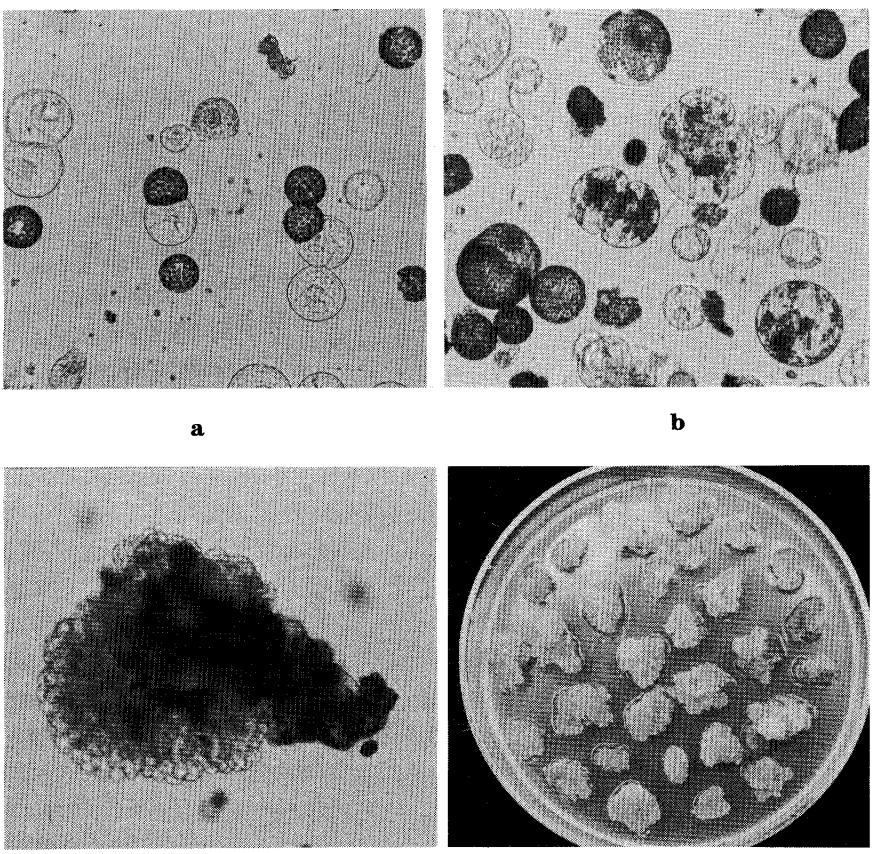
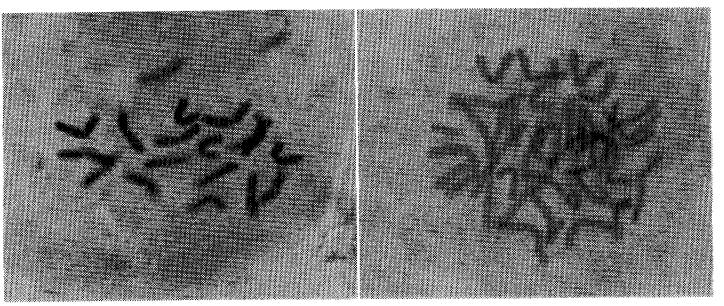


Fig. 1a-d Culture of fused protoplasts of *C. coccineum* mesophyll and *C. coronarium* culture cell (hybrid 1).
a: 1 min after electrofusion.
b: First and second cell division 5 days after fusion.
c: Cell cluster.
d: Subcultured calli on MS agar medium.

Frequencies of heterokaryons formation (**Fig. 1a**) after electrofusion of mesophyll and suspension culture protoplasts were estimated at approximately 4% of total protoplasts.

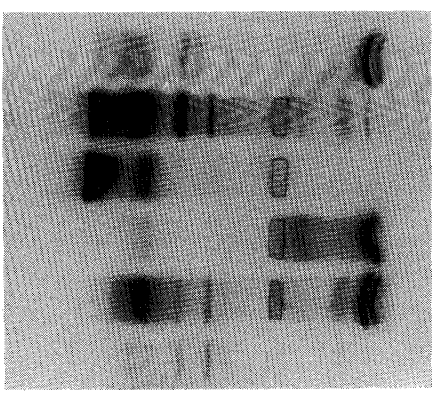
As shown in **Table 1**, *C. coronarium* mesophyll protoplasts rarely divided and formed no cell clusters in any of the test conditions. *C. coccineum* cultured protoplasts divided for 1 week, but they did not didvide with *C. coronarium* protoplasts. Cultured cell protoplasts of *C. coronarium* divided vigorously during the first 1 week culture, but they did not form any cell clusters. Non-fused mesophyll protoplasts of *C. coccineum* formed cell clusters and grew further to form calli as reported previously[2]. But if the protoplasts were cultured with *C. coronarium* cultured protoplasts they aggregated and no cell division occured. Furthermore, when the mesophyll protoplasts of *C. coccineum* were fused, there were many damaged cells after fusion treatment and no cell division was observed.

Rapidly dividing cells were obtained from the fused products of *C. coccineum* mesophyll protoplasts and *C. coronarium* cultured protoplasts (hybrid 1, **Fig. 1b**), and *C. coronarium* mesophyll protoplasts and *C. coccineum* cultured protoplasts (hybrid 2). Cultured cell protoplasts were larger than mesophyll protoplasts, so the mesophyll protoplasts were fused with cultured cell protoplasts just like an endocytosis. It may diminish damage to the mesophyll protoplasts on fusion treatment. These cells showed first cell divisions within 3 days, in spite of the other protoplasts dividing after 5 days, at the earliest. In the case of *Brassica* species[4] and *Datura* species[5], hybrid calli grew faster than the calli derived from their parent protoplasts. It was considered that the hybrid vigor made



(a) **(b)**

Fig. 2. Chromosome number of (a) parental plant (*C. coccineum*) and (b) hybrid calli (hybrid 1).



1: leaves of *C. coronarium*

2: hybrid calli between 1 and 3 (hybrid 2)

3: culture cells of *C. coccineum*

4: leaves of *C. coccineum*

5: hybrid calli between 4 and 6 (hybrid 1)

6: culture cells of *C. coronarium*

Fig. 3. Peroxidase isozyme patterns of *C. coccineum*, *C. coronarium* and somatic hybrids. Enzymes were extracted from 1: leaves of *C. coronarium*, 2: hybrid calli between 1 and 3, 3: culture cells of *C. coccineum*, 4: leaves of *C. coccineum*, 5: hybrid calli between 4 and 6, and 6: culture cells of *C. coronarium*.

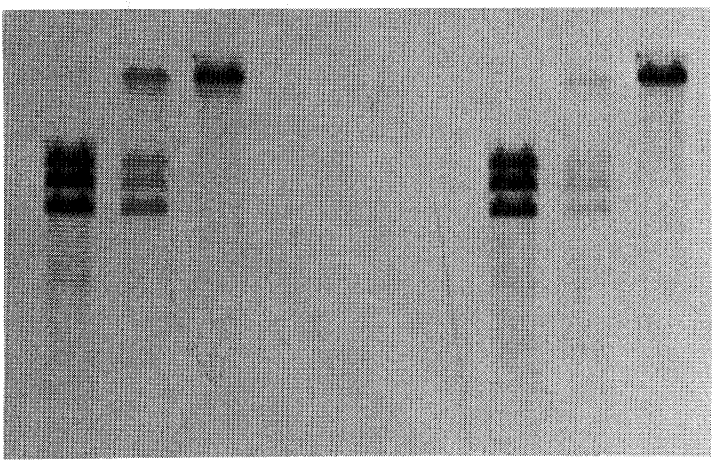


Fig. 4. Autoradiography of a blot-hybridization of ^{35}S-labelled pRR217 (rice rDNA) to EcoRV digests of nuclear DNA from leaves of *C. coccineum* (a), *C. coronarium* (b) and hybrid calli (h-1, 2).
h-1: *C. coccineum* mesophyll x *C. coronarium* culture cell protoplasts
h-2: *C. coccineum* culture cell x *C. coronarium* mesophyll protoplasts

the cell growth rapid. When the developing cell clusters grew 0.5 to 1 mm in diameter 3 weeks after electrofusion (**Fig. 1c**), they were transferred to MS medium and or supplemented with 2 mg/*l* BA and subcultured every month (**Fig. 1d**) for regeneration. We got 4 and 3 hybrid lines for hybrid 1 and hybrid 2, respectively, and they were analized for their hybrid nature.

Chromosomes were stained with orcein-acetic acid, and the chromosome number was counted in squash preparations. Both hybrid calli had the amphiloid chromosome number (2 n=36), which is the summation of the two parent species (2 n=18) (**Fig. 2a, b**).

Isozyme analysis of peroxidase in fused calli, parental leaves and calli was performed by isoelectrofocusing using a 1% agarose gel (IEF) containing 6.3% (v/v) ampholyte (pH 3-10). The bands of peroxidase in the fused calli were a combination of both parents' bands with the addition of some typical bands (**Fig. 3**).

DNA was isolated from 10 g leaves of each parent plant and from 40 g calli of each of the two fusion products by the method described by Uchimiya[6]. DNA (0.5 μg) was digested with 20 units of restriction enzyme. After electrophoresis, DNA was blotted and hybridized to 35S-labelled rice ribosomal DNA (pRR217)[7,8,9]. *C. coccineum* DNA digested with EcoRV gave DNA fragments of 3.7, 4.3 and 4.8 kbp hybridizing to rice ribosomal DNA, while, *C. coronarium* DNA gave a signal 8.5 kbp fragment. The DNA from the two fusion products contained all of these fragments (**Fig. 4**). Showing that the calli obtained by the electrofusion between *C. coccineum* and *C. coronarium* were somatic hybrids.

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References

1) Fujii Y., K. Shimizu, 1980. Plant Cell Rep., 8: 625-627
2) Fujii Y., K. Shimizu, 1980. Plant Tissue Culture Lett., **7**(2): 111-113

3) Fujii Y., Japan Patent Applied No: 1989-0141631
4) Schneck H. R., G. Robbelen, 1982. Z. Pflanzenzuchg, **89**: 278
5) Schider O., 1980. Z. Pflanzenphysiol., **98**: 119-127
6) Uchimiya H., Y. Tanaka, M. Sugiura, 1984. "Plant Gene Tecpnology Manual" Kodansha Japan
7) Oono K., M. Sugiura, 1980. Chromosoma **76**: 85-89
8) Takaiwa F., K. Oono, M. Sugiura, 1985. Plant Mol. Biol. **4**: 355-364
9) Uchimiya H., T. Ohgawara, H. Kato, T. Akiyama, H. Harada, 1983. Theor. Appl. Genet. **64**: 117-118

《和文要約》

赤花除虫菊と春菊の細胞融合による体細胞雑種カルスの作出とその解析

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赤花除虫菊葉肉細胞プロトプラストと春菊培養細胞プロトプラストならびに春菊葉肉細胞プロトプラストと赤花除虫菊培養細胞プロトプラストを電気融合法により細胞融合させた．非融合処理の赤花除虫菊葉肉細胞プロトプラストだけがカルス化する培地で，2系統のカルスを得た．両カルスの染色体数は両親植物の和に等しかった．Peroxidase isozymeの等電点電気泳動は両カルスの雑種性を示した．両カルスから抽出した全DNAをリボゾームRNA遺伝子をプローブとしたサザンブロッテイング法で解析した結果，カルス中に両親植物のリボゾームRNA遺伝子が存在し，2系統のカルスはいずれも体細胞雑種であることが確認された．