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Callus Formation from Mesophyll Protoplasts of Pyrethrum (*Chrysanthemum coccineum*)

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The family Compositae contains economically important crop species, therefore, plant regeneration from protoplasts is a prerequisite for the utilization of protoplast technology. Plant regeneration from protoplasts has been described by Binding[1,2] for many Compositae, and by Suzuki[3] and Brown[4] for *Lactuca sativa* L. *Chrysanthenum coccineum* is one of the commercially grown pyrethrum plants which produce pyrethrins. It is neccessary for the genetic improvement of pyrethrum plants to establish an ideal method of protoplast culture.

This report describes a method of successful isolation and culture of protoplasts from mesophyll of green house grown plants and axenic shoot cultures of *C. coccineum*, which produce insecticide pyrethrins.

Axenic shoot cultures were established from the achenes and petals of *C. coccineum* by the method described in the previous paper.[5]

Protoplasts were isolated from fully expanded leaves of shoot cultures (6-12 months old). Lower epidermis were removed by peeling, and incubated in an enzyme mixture which consisted of 0.1% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, Japan), 1.5% (w/v) Cellulase Onozuka RS (Yakult, Japan) and 2.5 mM $CaCl_2$ in 0.4 M mannitol at pH 5.6. Incubation was for 2-3 hr on a shaker (90 rpm), in room light at 30°C. Incubation mixture was passed through a 63 μm nylon sieve. Released protoplasts were washed twice with 0.4 M mannitol by centrifugation for 1 min at $100\times g$.

Purified protoplasts were suspended in half strength MS medium[6] (pH 5.7) supplemented with 0.1 mg/l BA, 0.5 mg/l 2,4-D, 1% (w/v) glucose and 0.4 M mannitol (HMS medium). NH_4NO_3 content was reduced to 0-1/4. Protoplasts were plated in 1 ml aliquots in each of 12 well dishes. Cultures were maintained in the dark during first 2 weeks, then 16 hr light and 8 hr dark photo-period at 24°C.

During culture, a progressive reduction in the osmoticum was initiated after 14 days, and thereafter every week till the colonies grew. This was achieved by a removal of aliquots of the original culture medium, which was replaced with an equal volume of osmoticum reduced (0.2 M mannitol) HMS medium.

After 2 months in culture, microcalli (1.5-2 mm) were transferred onto 0.8% agar embedded medium for callus proliferation. The calli were subsequently subcultured onto MS agar medium (3% sucrose) supplemented with various concentrations of growth hormones for morphogenesis.

Viable protoplasts were obtained from green house grown plant leaves and axenic leaves of shoot cultures after 2-3 hr incubation with Pectolyase Y-23 and Cellulase Onozuka RS at the yields of about 5×10^6 protoplast/g fresh weight. Size of freshly isolated protoplasts ranged from 30-50 μm (**Fig. 1**). Protoplasts were cultured in various cell densities (1.5×10^3-10^4). The results demonstrated that the optimum density for cell division was 3.7-7.3×10^3 protoplasts/ml (**Table 1**). Cell divisions were observed in the HMS medium after 5 days culture. Calf serum and coconut water promoted protoplast division at lower cell density in 7% glucose containing medium (**Fig. 1 b**).

Cell divisions were also observed after 5 days in culture in the medium of Nitch and Nitch[7] and

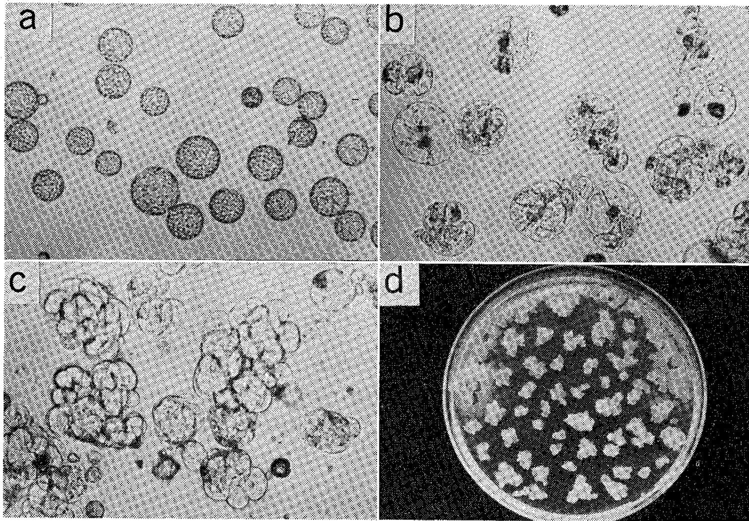

Fig. 1. (a) Freshly isolated mesophyll protoplasts from axenic shoot culture of *C. coccineum*. (b) Seven-day-old protoplasts in liquid HMS medium. (c) Clusters obtained from protoplasts of *C. coccineum* in osmoticum reduced HMS medium after 3 weeks. (d) Greening calli obtained from protoplasts of *C. coccineum* after 3 months culture on MS agar medium supplemented with 1 mg/*l* BA.

Table 1. Effect of additives and cell density on division of mesophyll protoplast of *C. coccineum*.

Additives	Sugars	Cell density ($\times 10^3$ cell/m*l*) 1.5	2.2	3.7	7.3	15
		% (divided cells/living cells)				
	1S	18.9	21.3	33.8	38.6	32.0
	1G	36.5	39.0	45.5	44.3	22.1
	7G	0	1.0	56.8	59.2	47.5
Coconut water 2%	1S	0	12.9	36.4	51.7	31.5
	1G	25.7	27.0	32.3	62.5	37.5
	7G	34.8	40.0	55.7	55.4	22.4
Calf serum 1%	1S	8.1	15.7	15.8	12.5	18.1
	1G	39.5	28.0	38.2	66.7	18.9
	7G	50.0	25.3	68.9	55.7	20.9

Protoplasts were cultured in half strength of MS liquid medium without ammonium ion supple mented with 0.1 mg/*l* BA and 0.5 mg/*l* 2,4-D for 5 days. 1S : 1% sucrose 0.4 M mannitol, 1G : 1% glucose 0.4 M mannitol, 7G : 7% glucose.

White[8] which contains no ammonium ion and in the medium of ammonium ion ommitted MS and Nagata and Takebe[9] medium. But no cell division occured in MS, B 5[10] and Kao[11] medium (data not shown). No protoplast-derived cell colonies were recovered in full strength MS and Nagata and Takebe medium or sucrose containing MS, Nitch and Nitch and White medium. In the half strength MS medium containing glucose, cell clusters were obvious after 3 weeks culture (**Fig. 1c**). Inhibiting effect of ammonium ion in protoplast culture was reported by Okamura[12] for Asteraceae plants and some Chrysanthemum species also by Suzuki[3] for *Lactuca sativa* L. The sequential addition of fresh medium during the culture period was essential for sustained colony formation. Efficiency of reduction in osmoticum and replacement of the culture medium for proliferation of microcalli was also reported

in *Malus xdomestica*[13] and in *Lactuca sativa* L.[3] Replacement of the culture medium probably removed phenolic compounds which may inhibit cell growth.

When the microcalli were transferred to MS agar medium, approximately 70% of transferred microcalli were able to survive. They grew rapidly on MS medium and greened under 16 hr light 8 hr dark photo-period at 3,000 lux on MS medium supplemented with 1.0 mg/l BA or zeatin and without hormones (**Fig. 1 d**).

We report here for the first time a sucessful isolation and colony formation of Pyrethrum mesophyll protoplasts. Further study is underwent for plantlets regeneration.

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Abbreviations BA : 6-benzyladenine, 2, 4-D : 2, 4-dichlorophenoxyacetic acid, NAA : 1-naphthalene acetic acid.

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≪和文要約≫

赤花除虫菊葉肉細胞プロトプラストからのカルス形成

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赤花除虫菊の瘦果と花弁から再分化させた植物体と種子から生育させた植物体の葉肉細胞から 0.1% ペクトリアーゼ Y-23 と 1.5% セルラーゼオノズカ RS を含む酵素液でプロトプラストを分離した．プロトプラストはグルコースを糖源としアンモニウム塩を減じた MS 培地に 0.1 mg/l BA と 0.5 mg/l 2, 4-D を添加した液体培地で培養した．培養後 5～7 日で分裂が起こり，新鮮培地を 1 週間ごとに添加して浸透圧をさげていくことで一カ月後にコロニーを得た．高濃度の BA あるいは zeatin を含有する MS 培地とホルモン無添加の MS 培地で緑化カルスが得られた．