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The Effects of Per-treatments on Cell Division and Colony Formation from Cotyledon Protoplasts of Tomato

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Summary

The effects of pre-treatments of cotyledons from tomato young seedlings (*Lycopersicon esculentum* cv. 'Kyoryokutoko') on yield, rates of cell division and colony formation of protoplasts were studied. The most efficient results were obtained by low temperature treatment, and the combination of dark and low temperture treatment when the same solution was used for the low temperature treament as the protoplast culture medium. The all pre-treamtments, dark treament, lwo temperature teratment, or their combination gave improved results compared with none pre-treatment. Liquid culture gave generally more efficient results in plating than double-layer system. TM-2 medium containing 0.5 mg/*l* NAA and 0.5mg/*l* BA gave the highest rates of cell division in either liquid culture or double-layer culture.

Abbreviations

BA : Benzyladenine, 8E : ZAPATA *et al.* (1977), MS : MURASHIGE and SKOOG (1962), NAA : Naphthaleneacetic acid, 2, 4-D : 2, 4-dichlorophenoxyacetic acid, TM : SHAHIN (1985). ZR : Zeatin riboside.

Ⅰ. Introduction

In recent years, protoplast manipulation shows a powerful potential as a new way of overcoming breeding barriers, so, numerous experiments have been performed to obtain efficient cell division, colony formation and plant regeneration from tomato protoplasts (IMANISHI 1980, ZAPATA et al. 1981, SHAHIN 1985). Especially, much attention has been paid to the effects of treatments before protoplast isolation (SHAHIN 1985, TAN et al. 1987 a, b). The authors have reported the effects of the temperatures for growing seedlings, the methods for protoplast isolation, and protoplast culture on plating efficiency (CHEN et al. 1987, 1988). In this study, we report the effects of pre-treatments necessary to obtain high yield, efficent cell division and colony formation of *L. esculentum* cotyledon protoplasts. These pre-treatments are : dark treatment, low temperature treatment, and their combination of dark and low temperature treatment of tomato cotyledons. In addition, the effects of protoplast culture medium and plant hormone combinations, seedling growth medium, and methods of protoplast culture on plating efficiency were also investigated.

Ⅱ. Materials and Methods

Plant materials

The *L. esculentum* cultivar used in Experiment Ⅰ and Ⅱ was 'Kyoryokutoko', while in Experiment Ⅲ, 3 cultivars, 'Kyoryokutoko', 'VF-36' and 'Hagoromo', and 2 races, 'L-125' and 'L-3692', were used. Seeds were surface sterilized by dipping in aqueous sodium hypochlorite solution (0.3%) following washing with sterilized water 5 times. About 30 seeds were incubated in the dark, at 25℃, on 2 layer of sterilized filter paper wetted

with about 4 m*l* sterilized distilled water in 9 cm Petri dish for germination. After 3 ～ 4 days, young seedlings were transplanted and grown in 200 m*l* Erlenmeyer flasks which containing about 50m*l*/flask TM-1 medium with 0.6% agar or MS basal medium with 0.8% agar in a controlled environment chamber (16 h photoperiod, 3000～4500 *l*ux), the first two days at 25℃, and then at 20℃.

Table 1. Combinations of plant hormones in mg/1 used in TM-2 or 8E media for protoplast culture

	TM-2				8E
Hormone	(1)	(2)	(3)	(4)	
2,4-D	1.0	0.0	0.0	0.0	1.0
NAA	0.5	1.0	0.5	0.5	0.5
BA	0.5	0.0	0.5	2.0	0.5
ZR	0.0	0.5	0.0	0.0	0.0

Protoplast isolation

The lower epidermis of cotyledons of about 10 day-old seedlings was pealed off and preplasmolyzed in a solution of CPW salts[5)] with 9 % mannitol (CPW 9 M), at 25℃, for one hour. And then, the CPW 9 M medium was replaced by a enzyme solution containing 3.0% (w/v) Meicelase P ; 0.3% (w/v) Macerozyme R 10 ; 0.25% (w/v) Driselase ; in CPW 9 M. The incubation was conducted in the dark at 27℃ for 16 h. After the enzyme treatment, protoplasts were collected as described CHEN et al., (1987). The final density of protoplasts was adjusted at 10^5 cells/m*l*.

Protoplast culture

The protoplasts were cultured in TM-2 medium by two methods : 1) liquid culture as 2 m*l* suspension of protoplasts (10^5 cells/m*l*) in a 5 cm Petri dish ; 2) double-layer culture as 1 m*l* suspension of protoplasts (10^5 cells/m*l*) layered onto 4 m*l* TM-2 agar medium in a 5 cm Petri dish. The plant hormones shown in Table 1 were used for protoplast culture. In TM-2 medium, protoplasts were cultured at 300～500 *l*ux, 16 h photoperiod, 25℃. On the other hand, culture of the protoplasts in 8 E medium was conducted as described by ZAPATA et al. (1981).

Pre-treatments

The media used for seedling growth and pre-treatments in Experiments Ⅰ, Ⅱ and Ⅲ are shown in Table 2. Pre-treatments were performed as follows : for dark treatment, half or 1/3 of the flasks where containing growing seedlings, were wrapped with a sheet of aluminium foil for 24 h before protoplast isolation, and the other were used as a control ; for low temperature treatment, the lower epidermis of cotyledons which did not undergo the dark treatment, were pealed off and im-

Table 2. Protocols for pre-treatments

	Experiment					
	Ⅰ			Ⅱ		Ⅲ
Seedling growth medium	MS	TM-1	TM-1	MS	TM-1	MS
Pre-treatments[a]	none	none	d.	none d.+low(2)[b]	none d. low(2) low(3)[c] d.+low(2) d.+low(3)	none

[a] d.=dark treatment ; low=low temperature treatment ;
d.+low=the combination of dark treatment+low temperature treatment.
[b] low(2) shows TM-2(2) used in low temperature treatment.
[c] low(3) shows TM-2(3) used in low temperature treatment.
For other details to refer to Tables 4, 5 and 6.

mediately floated on CPW 9 M solution for one hour. The CPW 9 M solution was then changed by the low temperature treatment solution TM-2 (2) or TM-2 (3) liquid medium (Table 1). After 10 h of incubation, at 10～15℃, protoplasts were isolated; for their combination of dark treatment and low temperature treatment, after the dark treatment (24 h), the cotyledons were treated again by low temperature treatment, that both the treatments were conducted as described as the above, respectively. Plating efficiency was observated as mentioned by CHEN et al. (1988).

Ⅲ. Results and Discussion

In Experiment Ⅰ, the width, weight and length of cotyledon in MS medium were more excellent than those in TM-1 medium in either none or dark treatment (Table 3). The growth of young seedlings in dark treatment was restricted compared with that in none treatment. The yield of protoplasts from seedlings grown on MS agar medium was higher than that from seedlings grown on TM-1 under both none and dark treatment (Table 4). Cell division and colony formation of protoplasts showed the same trend as the yield of protoplasts. The dark treatment was most effective in medium TM-2(2), TM-2(3) andTM-2(4) in plating efficiency.

The liquid culture method was more efficient than the double-layer culture method on the cell division and colony formation. Regarding the effect of plant hormones in the liquid culture, as shown in Table 4, TM-2 basal medium supplemented with 0.5 mg/l NAA and 0.5 mg/l BA, i. e. TM-2(3), gave the best rates of cell division and colony formation, ranging from 3.1～5.2% and 2.5～4.1%, respectively. TM-2(3) also produced the highest rates in the double-layer culture. In the present study, we also obtained a higher rates of cell division and colony formation of protoplasts in liquid culture than in double-layer culture, which is similar to the other reports (SHAHIN 1985).

In Experiment Ⅱ, the effects of dark treatment, low temperature treatment, and the combination of dark and low temperature treatment on cell division and conlony formation of cotyledon protoplasts are shown in Table 5. Different trend of cell division appeared between the protoplast culture media and the pre-treatments, i. e. the rates of cell division with pre-teratments except dark+low(3) were higher than that of none treatment, when using TM-2 (2) for protoplast culture and TM-1 agar medium for seedling culture. On the other hand, the rates of cell division of the pre-treatments except low(2) were higher than that of none treatment, when using TM-2(3) medium.

When TM-2(2) was used as pre-treatment solution, the rates of cell division in two pre-treatments, i. e. dark+low(2) and low(2), were 4.9%, 4.0% in culture medium TM-2(2) and these were higher than 3.1%, 1.3% in culture medium TM-2(3). On the other hand, when TM-2(3) was used as pre-treatment solution, the rates of cell division in two pre-treatments, i. e. dark+low(3) and low(3), were 3.1%, 4.6% in culture medium TM-2(3) and these were higher than 1.7%, 4.1% in culture medium TM-2 (2).

Between dark+low(2) and low(2), the rates of cell division of the former were higher than that of the latter in TM-2(2) and TM-2(3); between dark+low(3) and low(3),

Table 3. Cotyledon and hypocotyl characteristics of young seedlings on different growth media[a]

Medium	Cotyledon				Hypocotyl
	Length (mm)	Width (mm)	Thickness (mm)	Weight (mg)	Length (mm)
TM-1	20.05±3.18	3.90±0.52	0.45±0.05	13.30	39.80±2.59
TM-1 (d.)	17.80±3.01	3.51±0.46	0.45±0.06	10.00	48.00±3.74
MS	20.90±1.72	4.65±0.41	0.41±0.05	14.17	42.00±2.74

[a]: Ten seedlings were measured, respectively.

Table 4. Effects of hormones, culture methods and seedling media on the protoplast culture[a]

		Seedling medium		
		MS	TM-1	TM-1(d.)[b]
Protoplast yield per flask		5.7×10^5	5.2×10^5	4.7×10^5
TM-2(1)	C. D.[c]	1.5±0.2	0.6±0.1	0.1±0.0
L. C.[e]	C. F.[d]	0.8±0.1	0.4±0.1	0.1±0.0
TM-2(1)	C. D.	0.3±0.0	0.1±0.0	0.1±0.0
D. L. C.[f]	C. F.	0.2±0.0	0.1±0.0	0.1±0.0
TM-2(2)	C. D.	2.8±0.3	0.9±0.1	1.3±0.2
L. C.	C. F.	1.9±0.2	0.5±0.1	1.1±0.1
TM-2(2)	C. D.	0.7±0.1	0.1±0.0	0.1±0.0
D. L. C.	C. F.	0.3±0.0	0.1±0.0	0.1±0.0
TM-2(3)	C. D.	5.2±0.5	3.1±0.3	3.5±0.4
L. C.	C. F.	4.1±0.6	2.5±0.3	3.0±0.3
TM-2(3)	C. D.	0.8±0.1	2.0±0.3	3.4±0.4
D. L. C.	C. F.	0.8±0.1	1.4±0.2	2.9±0.3
TM-2(4)	C. D.	3.8±0.4	0.4±0.1	3.4±0.4
L. C.	C. F.	2.5±0.3	0.2±0.0	2.6±0.3
TM-2(4)	C. D.	0.8±0.1	0.1±0.0	2.6±0.3
D. L. C.	C. F.	0.7±0.1	0.1±0.0	2.1±0.2

[a] The observation was conducted 10 days after protoplast plating. [b] (d.) = dark treatment ; [c] C. D. = rate of cell division of protoplasts (%). [d] C. F. = rate of colony formation of protoplasts (%). [e] L. C. = Liquid culture. [f] D. L. C. = Double-layer culture.

the former were lower than the latter in the same media.

Using 8E medium for protoplast culture and MS medium for seedling culture, the combination of dark and low temperature treatment in TM-2(2) solution improved the culture efficiency compraed to none treatment.

In Experiment Ⅲ, the yield of protoplasts of 'VF-36' was the highest among the 5 cultivars tested (Table 6). However, the rate of cell division of 'Kyoryokutoko' (2.9%) was the highest, and the rate of colony formation of 'VF-36' (1.9%) was the highest. Generally, the rates of cell division and colony formation in any cultivars were more efficient in liquid culture than in double layer culture.

'L-125, and 'L-3692' are the self-fertile races close to wild species. From their results, it is clear that these races are not advantageous than 'Kyoryokutoko' in cell division and colony formation of cotyledon protoplasts (Table 6).

SHAHIN (1985) reported that the highest rate of cell division was 50% and the plantlet regeneration reached 60%, as well as genotypic differences appeared in the response to the TM culture system. On the other hand, TAN,et al. (1987) reported a low rate (4 %) of cell division when TM-2 medium and different tomato varieties were used for protoplast culture, which is similar to that reported in the present study. An universal culture medium can certainly not be adequate for each genotype. It is, therefore, necessary to screen the target plant mate-

Table 5. Effects of pre-treatments and culture media on protoplast culture[a]

Seedl. pre-growth media	treatments	TM-2(2) C. D.[f] (%)	TM-2(2) C. F.[g] (%)	TM-2(3) C. D. (%)	TM-2(3) C. F. (%)	8 E C. D. (%)	8 E C. F. (%)
MS	none					2.5±0.3	0.6±0.1
	d.+low(2)[b]					4.3±0.5	0.5±0.0
TM-1	none	3.0±0.4	2.1±0.3	3.0±0.3	2.7±0.3		
	d.[d]	3.1±0.3	2.7±0.2	3.5±0.4	3.2±0.3		
	d.+low(2)	4.9±0.5	2.1±0.2	3.1±0.3	2.2±0.2		
	d.+low(3)[c]	1.7±0.2	1.2±0.1	3.1±0.3	2.1±0.2		
	low(2)[e]	4.0±0.4	1.7±0.2	1.3±0.1	1.0±0.1		
	low(3)	4.1±0.3	2.6±0.4	4.6±0.4	3.7±0.4		

[a] The observation were conducted after 10 days and 7 days in TM-2 medium and 8E medium, respectively.
[b] low(2) shows TM-2(2) used in low temperature treatment.
[c] low(3) shows TM-2(3) used in low temperature treatment.
[d] d.=dark treatment. [e] low=low temperature treatment.
[f] C. D.=rate of cell division of protoplasts.
[g] C. F.=rate of colony formation of protoplasts.

Table 6. Effects of different cultivars and culture methods on protoplast culture cotyledon[a]

Cultivars or races	Protoplast yield per flask	TM-2(2) C. D.[d] (%)	L. C.[b] C. F.[e] (%)	TM-2(2) C. D. (%)	D. L. C.[c] C. F. (%)
Kyoryokutoko	6.3×10^5	2.9±0.4	1.5±0.1	1.0±0.1	0.5±0.1
L-125	4.8×10^5	0.6±0.1	0.4±0.1	0.1±0.0	0.1±0.0
VF-36	11.2×10^5	2.4±0.2	1.9±0.2	0.8±0.1	0.4±0.0
L-3692	1.7×10^5	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0
Hagoromo	9.6×10^5	2.3±0.2	1.9±0.1	0.6±0.1	0.3±0.0

[a] The observations were conducted 10 days after protoplast plating. [b] L. C.=liquid culture. [c] D. L. C.=double-layer culture. [d] C. D.=rate of cell division of protoplasts. [e] C. F.=rate of colony formation of protoplasts.

rial with regard to several media and vice versa.

Ⅳ. Conclusion

Based on the above results, therefore, we could conclude that an effecient and reproducible culture method of tomato cotyledon protoplasts should be established as follows : when 'Kyoryokutoko' that geve a highest rate of cell division in this study, is used as plant material, young seedlings are grown on MS basal medium with 0.8% agar, at 20～25℃, 3,000～4,000 *lux*, 16 h photoperiod, for about 10 days. The pre-treatments, low temperature treatment (10～15℃, 10 h, in the dark), or the combination of the dark treatment (20℃, 24 h) and low temperature treatment (10～15℃, 10 h, in the dark) are conducted before protoplast isolation. After protoplast isolation is performed as described by CHEN, et al. (1987), 2 *ml* of TM-2 (3) (TM-2 basal medlum supplemented with 0.5% NAA and 0.5% BA) protoplast suspension (10^5 cells/*ml*) are transferred into 5 cm Petri dish and incubated at 25℃, 300～500 *lux* 16 h photoperiod for liquid culture.

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トマト栽培種(*L. esculentum*)の子葉の葉肉プロトプラストの細胞分裂とコロニー形成に及ぼす前処理の影響

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摘 要

トマト栽培種の葉肉プロトプラスト培養の効率化を図る目的で,栽培種の“強力東光”を主な供試材料として,その幼苗の子葉にプロトプラストを単離する前に行う処理(以下,前処理と称する)が子葉プロトプラストの収量,分裂率およびコロニー形成率に及ぼす影響について検討した.また,得られたプロトプラストの培養における培地組成や植物ホルモンの添加および培養方法の影響についても調査した.その結果,前処理の影響は処理液と培養培地によって異なることが分かった.すなわち,子葉の低温処理とその後のプロトプラスト培養に同じ培地を使用すると,プロトプラスト培養が効率化された.プロトプラストの収量,分裂率およびコロニー形成率においては,すべての処理区,つまり,暗黒処理,低温処理,暗黒処理+低温処理の組合せは対照区よりまさることが認められた.プロトプラストの液体培養法は重層培養法に対し,全般的によい結果を表した.0.5 mg/l NAA と 0.5 mg/l BA を含む TM-2 培養培地は液体培養法と重層培養法とのいずれにおいても最も高い分裂率をもたらした.一方,幼苗の生育とその後のプロトプラストの収量および分裂に MS 培地は TM-1 培地あるいは暗黒処理を行った TM-1 培地に比べてよい影響を与えることも分かった.