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Totipotency of tomato protoplasts

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Summary. An efficient and reliable protocol for tomato protoplast isolation, culture, and plant regeneration has been developed. Fourteen diverse cultivars were tested. Fertile plants were regenerated from all 14 cultivars without any modification in the protocol. Plating efficiency (percentage of the protoplasts that formed mini-calli) of up to 50% was achieved. Those mini-calli rapidly regenerated shoots at high frequencies. Regenerated shoots can be easily rooted on a basal medium with the appropriate auxin, and have been set to soil within two months after the isolation of the protoplasts.

Key words: Protoplast culture – *Lycopersicon esculentum* – Tomato – Plant regeneration

Introduction

The tomato is an important horticultural crop, and has proven to be one of the most versatile of cultivated plants. The horticultural value of the crop has been greatly enhanced through plant breeding. Many significant improvements in the culture of tomato have come about from the introgression of characters from the wild relatives of the cultivated tomato, e.g., changes in plant habit, disease and insect resistance, and high β-carotene content (Rick 1978). However, as useful as wild germplasm has been in the genetic improvement of the cultivated tomato, a vast reserve of additional genetic resources that exits in the wild species remains unexploited (Rick 1982). Sexual hybrids between *L. esculentum* and the more closely related species are easily obtained (Rick 1973). However, interspecific incompatibilities between some species limits the value of sexual hybridization for the introduction of important traits from those wild species. Somatic hybridization could be an alternate route to mixing of "wild" and cultivated tomato genomes. On the other hand, genetic engineering methods have been proposed (Fobes 1980) to facilitate gene transfer in tomato. These techniques, however, require efficient plant regeneration from protoplasts. Techniques for the regeneration of tomato plants from leaf sections and calli have already been reported (Behki and Lesley 1976). This paper describes an efficient technique for isolation, culture, and plant regeneration of protoplasts of different tomato varieties.

Materials and methods

Seeds of *Lycopersicon esculentum* Mill. were surface-sterilized by a 20 min immersion in a solution of 1.08% sodium hypochlorite. After being rinsed thoroughly with sterile distilled water, seeds were germinated on a pre-sterilized filter paper in glass petri-dishes. Three to five days after germination, the roots were cut off along with a portion of the hypocotyl, and the excised shoots then transferred to a "Plant Con" (Flow Laboratories) containing 100 ml of TM-1 medium (Table 1). The plants were kept in a growth chamber at 25 °C, 16 h light, 8 h dark, at 4,500 lux. After 3–4 weeks, the containers were removed from the incubator and placed in a dark chamber at 25 °C for two days. After the dark treatment, the leaves were cut into small fragments (0.5 cm) and placed into 50 ml of Pre-enzyme Treatment (PET) solution in 250 ml side-arm flasks for 12–14h at 10°C. PET solution is composed of 1/4XTM-2 major salts (Table 1), 0.3 M sucrose, vitamins as in TM-1 medium (Table 1), supplemented with 1.0 mg/l 2,4-D, and 0.5 mg/l BAP. The pH was adjusted to 5.80 before autoclaving the medium at 121 °C for 15 min. Tomato leaf protoplasts were isolated from leaf fragments by modifying the procedure used for tobacco (Shepard and Totten 1975). The filter-sterilized enzyme solution consisted of 0.1% (w/v) Macerozyme (Yakult Biochemicals Co. Ltd., Japan), 0.75% (w/v) Cellulysin (Calbiochem-Behring Corp., La Jolla, California), 0.3 M sucrose, TM-2 macronutrients and vitamins (Table 1), 1.0% polyvinylpyrolidine (PVP-10), 5 μM 2[N-Morpholino]-

Table 1. Constituents and concentrations of tomato culture media. Culture media were brought to pH 5.60–5.80 with 0.1 N KOH after addition of all ingredients, except agar, and then sterilized by autoclaving for 15 min at 115 °C. TM-2 medium was filter sterilized by using Nalgene filter

	mg/l except as noted				
	TM-1	TM-2	TM-3	TM-4	TM-5
Macronutrients					
KH_2PO_4	–	170	170	–	–
$CaCl_2 \cdot 2H_2O$	150	440	440	150	75
KNO_3	2,530	1,500	1,500	1,900	1,265
NH_5NO_3	320	–	–	320	160
$NH_4H_2PO_4$	230	–	–	230	115
$(NH_4)2SO_4$	134	–	–	134	67
$MgSO_4 \cdot 7H_2O$	250	370	370	247	125
Micronutrients					
KI	0.38	0.38	0.38	0.38	0.38
H_3BO_3	6.20	6.20	6.20	6.20	6.20
$MnSO_4 \cdot 4H_2O$	22.30	22.30	22.30	22.30	22.30
$ZnSO_4 \cdot 7H_2O$	8.60	8.60	8.60	8.60	8.60
$Na_2MoO4 \cdot 2H_2O$	0.25	0.25	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.025	0.025
$FeSO_4 \cdot 7H_2O$	13.90	13.90	13.90	13.90	13.90
$Na_2 \cdot EDTA$	18.50	18.50	18.50	18.50	18.50
Vitamins					
Nicotinic acid	2.50	2.50	5	5	5
Thiamine HCl	10	10	0.50	0.50	0.50
Pyridoxine HCl	1	1	0.50	0.50	0.50
Folic acid	0.50	0.50	0.50	0.50	0.50
Biotin	0.05	0.05	0.05	0.05	0.05
D-Ca-Pantothenate	0.50	0.50	–	–	–
Choline chloride	0.10	0.10	0.10	0.10	0.10
Glycine	0.50	0.50	2.50	2.50	2.50
Cacein Hycrolysate	50	150	100	–	–
L-Cysteine	1	1	–	–	–
Malic acid	10	10	–	–	–
Ascorbic acid	0.50	0.50	–	–	–
Adenine Sulfate	–	40	40	–	–
L-Glutamine	–	100	100	–	–
Myo-inositol	100	4,600	100	100	100
Riboflavin	0.25	0.25	–	–	–
Others					
Sucrose	30.0 g	68.40 g	50.0 g	30.0 g	30.0 g
Mannitol	–	4.56 g	–	–	–
Xylitol	–	3.80 g	–	–	–
Sorbitol	–	4.56 g	–	–	–
MES	–	97.60	97.60	97.60	–
Noble agar	6.0 g	–	7.0 g	7.0 g	9.0 g
Hormones					
NAA	–	1	–	–	–
Zeatin Riboside	–	0.5	–	1	–
2,4-D	–	–	0.20	–	–
BAP	–	–	0.50	–	–
GA_3[a]	–	–	–	0.20	–
IBA	–	–	–	–	0.1
pH	5.80	5.60	5.80	5.80	5.80

[a] GA_3 applied after autoclaving

ethane Sulfonic acid (MES) buffer, pH 5.6. Thirty ml of the enzyme solution was added to 1 g leaf material. Protoplasts were released after 4–6 h of rotation at 60 rpm in a 28 °C water bath shaker. The protoplasts were collected in babcock bottles (Kimble, series 1000), and washed twice with a washing solution (0.3 M sucrose, 1/2 X macronutrients of TM-2, vitamins of TM-2, 0.005 M MES buffer, and pH 5.8) by means of centrifugation at 50 × g for 10 min. Protoplasts were cultured in a thin layer (2 ml) of TM-2 medium in 60 × 15 mm plastic petri-dishes (COSTAR, Cambridge, Mass). The number of protoplasts ranged from 2×10^4 to 10^5/ml of culture medium. The petri-dishes were sealed with parafilm, and incubated at 25 °C in diffused light (500 lux), 16 h photoperiod, for one week. Multicellular colonies were transferred after 6–10 days to TM-3 medium (Table 1) supplemented with 0.2 mg/l 2,4-D, and 0.50 mg/l BAP to encourage their growth into mini-calli. At this stage, the cultures were kept under diffuse light (500 lux) of 16 h/day for one week. An automatic counter (BIO-TRAN III, New Brunswick Scientific Co., New Jersey) was utilized in counting the mini-calli. Two weeks after initial culture, the protoplast-derived mini-calli were transferred to TM-4 medium (Table 1). Ten mini-calli were placed on each plate and cultured under a 16 h photoperiod of 4,500 lux at 25 °C. Regenerated shoots were dissected from the callus and cultured on TM-5 medium (Table 1) for further shoot development and root initiation. Once the rooted plantlets were well developed (2–3 weeks after root initiation), they were transplanted to 4-inch pots containing a mixture of soil : sand (1 : 1). High humidity was maintained by using plastic containers to cover the plants for at least one week. Pots were placed in a growth chamber maintaining 16 h/day light of ≦ 10,000 lux at 25 °C constant temperature. The plants were fertilized weekly with a diluted solution of 20N : 20P : 20K fertilizer (1 g/l H_2O).

Results

Fourteen genetically-diverse cultivars of *L. esculentum* were used in our study. Protoplasts were readily obtained from leaves, stems and cotyledons of all cultivars tested, when the plants were preconditioned as described in Materials and methods. Consistent isolation of protoplasts was possible, with a yield of up to 6.6×10^6/g tissue (Table 2). Higher yield of protoplasts could be obtained, without damaging the protoplasts if the enzyme digestion process was extended to 12 h (unpublished data). Attempts to isolate and culture protoplasts from plants grown in the greenhouse and/or in the growth chambers failed due to the considerable amount of debris present, and variable quality of protoplasts obtained. Furthermore, bacterial and fungal contamination was a serious problem that could not be controlled without having an adverse effects on the growth and development of the protoplasts.

Tomato leaf protoplasts differed considerably in size, depending on the source cultivar, but were characteristically spherical with dense chloroplasts. Swelling of the protoplast was usually observed during the first 24 h after plating in TM-2 culture medium. Cell wall formation occurred within 48 h after plating, as confirmed by plasmolysis with 0.9 M mannitol solution. First division occurred within 2–4 days with further divisions noticed five days later. Colonies were macro-

Table 2. Protoplasts isolation, plating efficiency and organogenesis from *Lycopersicon peruvianum* and fourteen cultivars of *Lycopersicon esculentum*. Protoplasts were plated in the TM-2 medium at 4.0×10^4 per ml. All the data were taken from experiments conducted under same conditions as in Table 1

Tomato cultivar	Source of protoplasts	Protoplast yield no/g of tissue	Plating efficiency[a]	Degree of shoot response[b]
Lycopersicon peruvianum	Leaf	2.80×10^6	33%	+ + +
'Red Cherry'	Leaf	2.90×10^6	43%	+ + +
'Cocktail Cherry'	Leaf	4.40×10^6	50%	+ + +
'VFNT-Cherry'	Leaf	2.75×10^6	9%	+ +
'VF-36'	Leaf	4.20×10^6	21%	+ + +
'VF-36'	Stem	5.0×10^6	20%	+ + +
'Manapal'	Leaf	6.60×10^6	2%	+ + +
'Floradade'	Leaf	3.65×10^6	22%	+
'UC-82'	Leaf	2.65×10^6	2%	+ + +
'UC-82'	Cotyledon[c]	4.20×10^6	1.4%	+ + +
'Red Ace'-VF Type	Cotyledon	6.0×10^6	3%	+ +
'Roma'	Leaf	1.2×10^6	2%	+ + +
'Beefsteak'	Leaf	3.6×10^6	3%	+ + +
'San Marzano'	Leaf	2.1×10^6	9%	+ + +
'Improved Pearson'	Leaf	2.5×10^6	2%	+ +
'Heinz 733'	Cotyledon	4.2×10^6	7%	+ + +
'Heinz 2152'	Cotyledon	3.80×10^6	28%	+ + +

[a] Plating efficiency (PE) represents the percentage of protoplasts that produced mini-calli
[b] +: less than 25%; + +: from 25 – 50%; + + +: more than 50%
[c] Cotyledon were 12 days old

scopically observed 9–10 days after plating in the liquid medium. Soon after macroscopic observation, they were moved onto solid agar TM-3 medium to develope into mini-calli.

Browning of the cell colonies, followed by death, occurred in all tested cultivars when they were left in TM-2 liquid medium longer than 15 days. A similar phenomenon occurred at an earlier time (5–6 days after plating), when the protoplasts were plated at a high plating density > 100,000/ml. In this case, the protoplasts divided very rapidly (within 3 day) with high efficiency. However, the cells ceased to divide and grow after a few divisions, and turned brown afterward. To avoid such a problem, fresh TM-2 medium with lower osmolarity (0.15 M sucrose) was used to dilute the medium that supported the cell colonies. Or in some cases, the cell colonies were centrifuged at lower speed (50×g) and washed with fresh TM-2 medium, and then replated in the same medium.

By culturing the protoplasts from all 14 cultivars in the same medium and under the same conditions, it was possible to simply score their performance. Table 2 shows that the plating efficiency varied from 1.4% to 50%, depending on the genotype. Given the genetic diversity of those cultivars, it is not expected that an optimum plating efficiency can be attained for all of them via a universal medium. Nevertheless, substantial upgrading of the plating efficiency could be obtained by modifying the hormonal constituents of TM-2 medium as well as the plating density. In 'UC-82', for example, a 16% plating efficiency was obtained with protoplasts at a density of 5×10^4/ml, but only when the auxin (NAA) and the cytokinin (zeatin riboside) concentrations were reduced by half (data not shown).

It is important to note that the presence of both auxin and cytokinin in the TM-2 medium is critical for the continuous division of the tomato protoplasts. Tomato protoplasts plated in hormone-free TM-2 medium formed cell walls, but no division occurred. Tomato protoplast response to frequently-used growth regulators was studied in two tomato cultivars, 'Red Cherry' and 'Cocktail Cherry'. Protoplasts from the two cultivars were plated at a density of 30,000 protoplasts/ml in hormone-free TM-2 media, supplemented with a selection of auxins and cytokinins at various concentrations (Table 3). The results indicate that a great percentage of the protoplasts had divided after 12 days, irrespective of the supplied phytohormones in all experimental combinations.

Shoot regeneration in the protoplast-derived calli was noticed as early as 11 days after transfer to TM-4 medium. Greening of the callus seemed to be necessary prior to shoot emergence. Shoot organogenesis rarely occurred in calli that were not green, however, in a few cases green meristemoids were observed in white-friable calli. Shoot development in tomato protoplast calli was prolific; numerous green shoots appeared from each of several sites on the callus. Morphogenetic responses leading to shoot induction were found to vary depending upon the types of auxins used in conjunction with a given cytokinin (Table 4). The percentage of calli exhibiting shoot formation was always superior, in all tested cultivars, in the presence of both zeatin riboside and GA_3. Without GA_3, the use of zeatin riboside alone resulted in a very low frequency of organogenesis (< 25%), and no shoot induction in 'Cocktail Cherry', 'Floradade', and 'VFNT-Cherry'. High regeneration frequency (> 60%) occurred in 'VF-36' when GA_3 was used along with BAP and 2iP. Similar results were also obtained in 'UC-82' when both 2iP and GA_3 were present, however, no morphogenic response was observed in the other cultivars.

The majority of shoot buds regenerated from calli developed into vigorous plantlets when placed in TM-5 medium. However, many small, undeveloped shoot primordia degenerated when moved to TM-5 medium. To prevent such loss, those buds were transferred along with some portion of the callus onto modified TM-4

Table 3. Response of tomato protoplasts to various hormones, components and concentrations in the plating medium (TM-2). Protoplasts were plated at a density of 30,000/ml. Division rate in %: (2 and more divisions) was recorded 12 days after culture

Basal medium (TM-2) hormones (mg/l)						Division rate (%) cultivars	
NAA	BAP	Zeatin	2,4-D	Kinetin	p-CPA	'Cocktail Cherry'	'Red Cherry'
0.2	0.2	0.2	0.5	0.2	0.2	50	39
–	0.5	–	–	–	1.0	46	37
–	0.5	–	1.0	–	–	43	42
–	0.25	–	0.5	–	–	45	41
–	–	0.25	0.5	–	–	53	58
1.0	–	–	0.5	1.0	–	47	37

Table 4. Shoot initiation response of tomato protoplast-derived callus on basal TM-4 medium supplemented with various combinations of growth regulators. Results based on 10 replicates (10 calli/rep) at the end of the 3rd week. Protoplasts-derived calli were obtained as described in "Materials and methods"

Growth regulators (mg/l) in TM-4 basal medium						Shoot induction response[a]						
IAA	GA_3	ZnR	Kn	2iP	BAP	Red Cherry	Cocktail Cherry	VF-36	Floradade	Manapal	VFNT Cherry	UC-82
–	0.2	1.0	–	–	–	+++	+++	+++	++	+++	+++	+++
8.0	–	–	2.5	–	–	+	+	+	–	–	–	+
0.5	0.2	–	1.0	–	–	+	+	+	–	–	–	+
0.5	0.2	–	2.0	–	–	+	+	+	–	–	–	+
–	0.2	–	1.0	–	–	–	+	+	–	–	–	+
0.5	0.2	–	–	–	1.0	+	+	+++	–	+	+	+
0.5	0.5	1.5	–	–	–	+	+	+	–	+	–	–
–	0.2	–	–	1.0	–	–	+	+++	+	+	–	+
–	0.2	–	–	2.0	–	–	+	–	–	–	–	+++
–	–	1.0	–	–	–	+	–	+	–	+	–	+
0.175	–	–	–	4.0	–	+	+	–	+	–	–	+
1.0	–	–	3.25	–	–	+	+	–	–	–	–	+
2.0	–	–	3.25	–	–	+	+	–	–	–	–	+
4.0	–	–	2.5	–	–	+	+	–	–	–	–	+
–	0.2	–	2.0	–	–	–	–	–	–	+	–	+
–	–	–	0.5	–	–	+	–	–	–	–	+	–
–	–	–	1.0	–	–	+	–	+	–	–	+	+
–	–	–	1.5	–	–	+	–	–	–	+	–	+

[a] + < 25%; ++ between 25 – 50%; +++ > 60%

medium (contains 2.0% glucose instead of 3.0% sucrose). This second round of regeneration (12 days) enhanced more multiple shoot production and prevented shoot degeneration. Rooting could be induced within 10 days when the developed shoots were transferred to TM-5 medium. A few thousand plantlets with well developed roots were transplanted to pots and grown to mature plants. Fruits set on these plants, seeds were harvested, and the progenies of regenerants were planted in the field.

Discussion

Although tomato protoplasts have previously been isolated and cultured (Mühlbach 1980; Zapata et al. 1977, 1981; Morgan and Cocking 1982), no similar success in regeneration has been published. This failure could be due to the fact that proper preconditioning of the donor plants, prior to protoplasts isolation, was never attained. Researchers have spent most of their efforts in manipulating culture media and trying different genotypes, while neglecting the growth conditions of the donor plants. Factors such as plant and leaf age, as well as light intensity and photoperiod during growth of the donor plants, are critical to the viability and growth of protoplasts. The preconditioning of plant materials prior to protoplast isolation has resulted in increased protoplast viability and plant regeneration in petunia (Binding 1974) and eggplant (Bhatt and Fassuliotis 1981).

The finding of microscopic meristemic areas in three tomato cultivars ('Red Cherry', 'VF-36', and 'Manapal') as early as 12 days after protoplast culture, indicate that the cells were organized long before being subjected to the differentiation media (unpublished data). On the other hand, plants that were not preconditioned as described in the "Methods" section, yielded protoplasts that sustained few divisions, with no further proliferation, in spite of various media manipulations. It is suggested that tomato protoplasts are totipotent; i.e., they are in a stage, prior to isolation, of being able to divide, form cell colonies and organized tissue, and regenerate. Furthermore, preconditioning steps employed in this research may have triggered a shift in the behavior of cells in the intact plant that were incapable of division upon isolation, as well as maintained the status of those cells that are capable of division. We have no evidence as to what mechanism(s) might be involved in this switch.

Protoplasts isolated from wild tomato species, especially *L. peruvianum* (L.) Mill., have been reported to be highly totipotent (Mühlbach 1980). For this reason, and the previous difficulty in regenerating cultivated tomato protoplasts, some researchers (Thomas and Pratt 1981) have postulated the presence of regeneration-controlling genes in *L. peruvianum*, and have proposed interspecific hybridization as a means to transfer those "genes" into cultivated tomatoes. Bingham and coworkers (1975) demonstrated the presence of "regeneration genes" when they bred alfalfa strains

for high regenerability in vitro. In tomato, a similar approach would be very difficult due to the problems associated with interspecific hybridization: difficulty in effecting successful fertilizations, loss of fertility in subsequent generations, and the production of horticulturally undesirable progenies. Given the success of our protocol on such a wide range of genotypes, and the presumption that such genes exist in the cultivated tomato, we postulate that our defined procedure was favorable for the expression of "regeneration genes". Instead of breeding "regeneration ability" into the cultivated tomato, we recommend that the growth conditions of the source plant be optimized prior to isolation and culture of the protoplasts.

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