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Isolation of cells and protoplasts from leaves of in vitro propagated peach (*Prunus persica*) plants

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Abstract

Yields of 10^6–10^8 peach mesophyll cells and protoplasts · gfw^{-1} were obtained depending on factors such as digesting enzymes, and leaf size. Onozuka R-10 (2%) in combination with Macerase (0.5%) was found best for protoplast isolation and mediocre for cell isolation among several enzyme combinations tested. Viability was 90% for protoplasts and 60% for cells. Pectolyase Y23 was found to be ineffective in our investigation. Small leaves, 4–10 mm in length, were a superior source for protoplast isolation than medium or big expanded leaves, 22–30 mm in length. The high yields of protoplasts could be obtained only when keeping the ratio of leaf biomass to volume of digesting enzyme solution under 20 mg ml^{-1}. Purification of protoplasts on a sucrose gradient yielded about 10^7 protoplasts · gfw^{-1}, however, the preparation was still contaminated by intact cells. Protoplasts were cultured under different growth regulators and physical conditions. Limited growth and division of protoplasts embedded in agarose drops were observed.

Abbreviations: BA – 6-benzyladenine, IBA – indolebutyric acid, FDA – fluorescein diacetate, MES – 2-M-morpholinoethane sulphonic acid, MS – Murashige and Skoog, NAA – α-naphthaleneacetic acid, PVP – polyvinylpyrrolidone

Introduction

Protoplasts of temperate fruit crop species have been used to study sorbitol (Yamaki 1982) and ethylene (Mattoo & Lieberman 1977) metabolism and for breeding, mainly via somatic hybridization (Hidano & Niizeki 1988). Cells isolated from leaf tissue of deciduous fruit trees have been used for studying the effect of toxic compounds on membranes (Hammerschlag 1984; Mezzetti et al. 1992). The most frequently used deciduous fruit trees for isolating protoplasts are *Malus* x *domestica*, *Pyrus communis* and *Prunus spp.* (Ochatt et al. 1992). Only few studies have been reported on the isolation of protoplasts from peach [*Prunus persica* (L.) Batsch]. Salesses & Mouras (1977) isolated protoplasts from root tips for a cytological study. A brief report by Lee & Wetzstein (1986) described protoplast isolation from callus and leaf mesophyl cells. Matsuta et al. (1986) and Matsuta (1992) have succeeded in obtaining cell division and callus growth from protoplasts derived from cell suspensions.

Protocols for isolating apple cells of in vitro grown leaves (Mezzetti et al. 1992) and peach cells from field-grown trees (Hammerschlag 1984) have been previously published. Both

protocols were based on sucrose as the osmoticum component, one which is rarely used for protoplast isolation (Hidano & Niizeki 1988). In this paper we submit a protocol for the isolation of a mixture of peach cells and protoplasts from shoots of peach grown in vitro. This protocol will enable us to conduct further toxin/cell interaction studies using cells and protoplasts isolated and treated under identical conditions. We also extended the protocol toward the purification and culture of protoplasts, resulting in a procedure that should be useful for somatic hybridization studies.

Materials and methods

Plant material

Shoots of peach (*Prunus persica*) cv. Suncrest and Sunhigh were propagated in vitro on MS salts medium (Murashige & Skoog 1962) supplemented with 20 $g \cdot l^{-1}$ sucrose and in $mg \cdot l^{-1}$: 0.4 thiamine HCl, 100 myo-inositol, 0.05 nicotinic acid, 0.05 pyridoxine HCl, 0.01 *p*-aminobenzoic acid, 0.01 IBA, 2.0 BA and 6 $g \cdot l^{-1}$ agar (Hammerschlag et al. 1987). Shoots were subcultured onto fresh medium every three weeks. Axillary branches were excised and cultured separately. The pH was adjusted to 5.9 with NaOH prior to autoclaving for 15 min at 121°C and 1.1 $kg \cdot cm^{-2}$. Cultures were maintained at 25 ± 2°C under a 16-h photoperiod provided by cool white fluorescent lamps at a light intensity of 40–60 $\mu mol\ m^{-2} \cdot s^{-1}$.

Isolation of cells and protoplasts

Leaves of different sizes were excised from shoots approximately ten days after subculture. Mostly young leaves of 4–10 mm in length and 1 mm in width were used. These leaves were excised from the apex of 3–4 cm shoots or from a small side-shoot about 1 cm in length. In some experiments, medium and large fully expanded leaves were excised from the lower part of 3–4 cm shoots. The leaves were cut into sections smaller than 1 mm and incubated in a digestion enzyme solution. Enzymes (Table 1) added to digestion solutions included Onozuka R-10 (Yakult Pharmaceutical, Japan), Cellulysin (Calbiochem), Macerase (Calbiochem), Macerozyme R-10 (Yakult Pharmaceutical, Japan), Pectolyase Y23 (Seishin Pharmaceutical, Japan), Driselase and Hemicellulase (Sigma). The enzyme solutions were supplemented with CPW salts (Power et al. 1984) and either 0.71 M mannitol (CPW.71M), (Power et al. 1984; Ochatt et al. 1987) or 0.45 M sucrose (CPW.45S), usually 20 mg per ml. Both media were supplemented with 1% PVP (average molecular weight of 10,000) and 5 mM MES, pH 5.7. Incubation was

Table 1. Effect of digesting enzymes on yield of peach protoplasts and cells ($10^7 \cdot gfw^{-1}$) and viability (%) of protoplasts[a].

	Yield			Protoplast viability (%)
	Cells	Protoplasts	Protoplasts %	
ONO (2%), MAC (0.1%)[b]	8.3 ± 0.7[c]	1.38 ± 0.09	14	89
ONO (2%), MAC (0.5%)	6.0 ± 1.1	1.49 ± 0.12	20	94
ONO (2%), MAC (0.1%), DRI (0.1%)	6.9 ± 0.6	1.58 ± 0.13	19	94
ONO (2%), MAC (0.1%), DRI (0.5%)	4.1 ± 1.5	0.72 ± 0.12	15	51
ONO (2%), MAC (0.1%), PEC (0.1%)	13.5 ± 1.4	0.25 ± 0.05	2	0
ONO (2%), MACZ (0.1%)	7.8	0.40	5	83
ONO (2%), MACZ (0.5%)	14.4	0.49	3	70
ONO (2%), MAC (0.1%), HEM (1%)	1.9	0.87	5	90
CEL (2%), MAC (0.1%)	4.7	1.75	26	86

[a] Medium size leaves were digested in CPW.71M with a ratio of leaf wt/volume of enzyme solution of 21.5 ± 2.1 $mg\ l^{-1}$. Protoplasts were collected only in pellet I (centrifugation of 200 *xg* for 3 min).

[b] ONO – Onozuka R-10, CEL – Cellulysin, MAC – Macerase, MACZ – Macerozyme R-10, PEC – Pectolyase Y23, DRI – Driselase, HEM – Hemicellulase.

[c] Data are values from one experiment or the mean ± SE of 2–4 experiments, each consisting of 2–4 field counts in a hemocytometer.

carried out for 15–16h in the dark, at 25°C, with a 50 rpm agitation. After incubation, protoplasts were liberated from leaf sections with a Pasteur pipette and passed through 3 stainless steel sieves of 125, 94 and 43 μm. The filtrate was centrifuged for 3 min at 200 *xg* and then the supernatant was centrifuged for 8 min at 200 *xg*. Cells and protoplasts in the two pellets were resuspended in 1 ml of either CPW.71M or CPW.45S. When purer preparations of protoplasts were desired, the resuspended pellets were layered on top of 3 ml of CPW 21S medium (21% sucrose, Ochatt et al. 1987) and centrifuged (8 min, 200 *xg*). Protoplasts were collected from the interphase, diluted in CPW.71M, centrifuged (6 min, 200 *xg*) and resuspended in CPW9M (Power et al. 1984) or other culture media. Yield of cells and protoplasts was determined with a hemocytometer, viability by FDA (Widholm 1972) or Evans blue, and cell wall formation by Calcafluor white (Mattoo & Lieberman 1977).

Culture of protoplasts

Two culture media based on the shoot medium (see above) with different growth regulators were tested: a) 2.0 mg · l^{-1} NAA, 0.5 mg · l^{-1} BA, and 0.5 mg · l^{-1} zeatin (PrPr), and b) 1.0 mg · l^{-1} 2,4-D and 0.1 mg · l^{-1} BA (C5). Protoplast growth culture media also contained 9% mannitol as the osmoticum. Protoplasts were suspended in growth culture medium after being counted and density was adjusted to 1 or $2 \cdot 10^5$ ml^{-1}. Protoplasts were plated in 100 ul aliquots either as liquid or agarose (0.625% w/v, SeaPlaque) solidified cultures. Liquid aliquots were placed either in small (1.2 × 4.0 cm) vials or in droplets in 5-cm petri dishes. Droplets of agarose, 4–5 per 5-cm petri dish were allowed to settle for about 60 min prior to adding 1–3 ml of liquid growth medium. Cultures were maintained at 25°C in the dark either statically or on a shaker (50 rpm). After 14 days the medium in the solidified cultures was replaced by the same medium with 6 or 9% mannitol concentration according to the procedure described by Power et al. (1984). Protoplasts in liquid cultures were tested for viability with FDA from three up to 30 days after the beginning of culture.

Results and discussion

Our first efforts focused to isolate peach cells and protoplasts in a cell wall digesting medium containing sucrose as the osmoticum. In previous studies, cells of peach were isolated from leaf tissue of field-grown trees by Hammerschlag (1984) using 2% Cellulysin and 1% macerase in 0.45 M sucrose. Mezzetti et al. (1992) reported that 0.5% Cellulysin and 0.5% Macerase in 0.45 M sucrose resulted in the highest yield of viable apple cells which were also the most suitable for fluorescence emission studies (cells exhibited no change over time). Using sucrose in combination with CPW salts (Power et al. 1984), we found that the combination of 2% cellulase Onozoka R-10 with 0.5% Macerase yielded in a crude preparation $3.4 \pm 0.5 \times 10^7$ cells · gfw^{-1} with a viability of 60 ± 5%. Protoplast yield, however, was only $0.5 \pm 0.2 \times 10^7 \cdot gfw^{-1}$ (viability of 81 ± 3%). Since the proportion of 1:7 protoplasts to cells was not satisfactory for carrying out designated toxin/cell experiments with cells and protoplasts under the same experimental conditions, we looked for conditions to enrich the cell population with protoplasts.

Effect of digesting enzymes

The most effective cell wall digesting enzymes tested for production of viable peach protoplasts were the cellulases Cellulysin and Onozuka R-10 and the pectinase Macerase. Protoplast yields were between $1.38–1.75 \times 10^7 \cdot gfw^{-1}$ with viability of about 86 to 90% (Table 1). Driselase at a concentration of 0.1% did not improve yield, and at 0.5% caused reduction in viability. Lower yields were observed when hemicellulase was added or when Macerozyme replaced Macerase. Pectolyase Y23 proved to be significantly inferior to other pectinases, as all resulting protoplasts were non-viable. Interestingly, in contrast to the findings here, 0.1% Pectolyase Y23 was beneficial in isolating peach protoplasts from cell suspensions (Matsuta et al. 1986; Matsuta 1992) and was essential in isolation of apple protoplasts from callus (Niizeki et al. 1983).

More intact cells than protoplasts, ranging from $2–14 \times 10^7 \cdot gfw^{-1}$, were found in the crude preparations (Table 1). In the presence of

Macerozyme and Pectolyase Y23, more cells were released from leaf tissues. However, they exhibited a low viability of about 10%. Protoplasts of peach isolated under all conditions were quite small, 13 ± 0.3 μm in diameter and distinct from other protoplasts isolated from mesophyll cells by having only a few chloroplasts (Fig. 1A). This may be due to the origin of the leaves being from shoots grown in vitro for several years. In experiments described above and below, protoplasts and cells were isolated from both cultivars: Suncrest and Sunhigh. No differences were observed between the two cultivars.

Effect of shaking

In spite of cutting the leaf tissue into very small segments, overnight incubation in the enzyme solution under stationary conditions did not cause any tissue disintegration; very few protoplasts were released to the solution. Protoplast liberation was therefore enhanced by pumping the liquid with leaf sections up and down a Pasteur pipette. The same situation occurred when shaking under 30–50 rpm. Yields, however, were several times higher than under stationary conditions. Only when agitation at 125 rpm was applied, was tissue partially disintegrated and protoplasts released to the solution. The amount released was only a third of the total amount released by a Pasteur pipette-enforced breakage. Upon full tissue disintegration, shaking under either 50 or 125 rpm yielded the same amount of protoplasts. It should be mentioned that pumping the tissue with a Pasteur pipette caused some damage, judging by some vacuoles that were released along with the protoplasts; nevertheless, protoplast viability was usually about 90%.

Effect of leaf size

As with other deciduous species, the physiological conditions and the source of tissue play an important role in yield, viability and quality of isolated protoplasts (Hidano & Niizeki 1988). Young small leaves of peach resulted in the highest yield of protoplasts (Table 2). The proportion of cells to protoplasts was about 1:1. Medium and large leaves yielded more cells and less protoplasts. No difference in protoplast viability was found between the different leaf sizes.

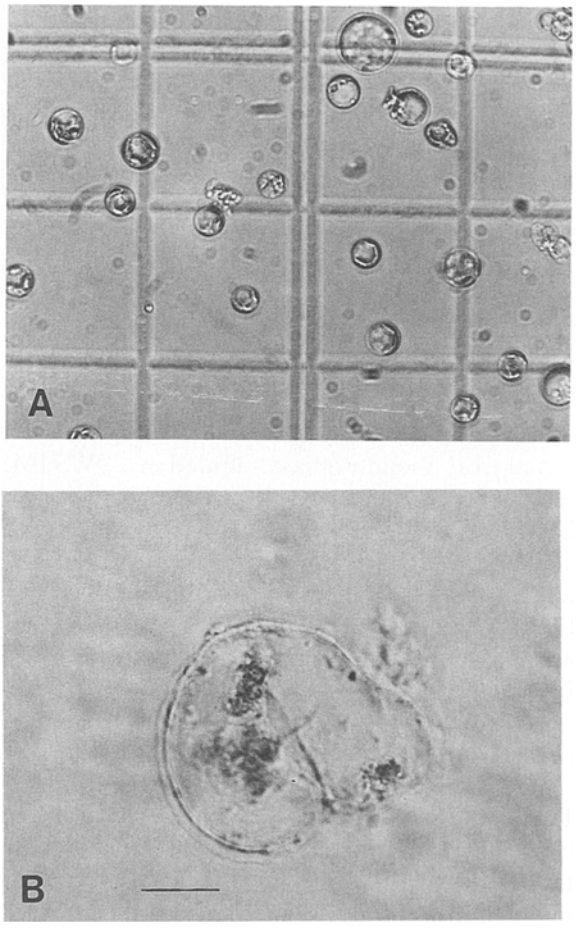

Fig. 1. Isolated protoplasts of peach from in vitro leaves. (*A*) fresh protoplasts after purification (squares are 50 × 50 μm); (*B*) first division after 7 days (bar represents 10 μm).

Small leaves of 3–4 mm were used by Wallin & Welander (1980) for isolation of apple protoplasts. In their study, expanded leaves did not yield any protoplasts. Jörgensen & Binding (1988), working with several woody species including *Prunus spp.* have reported that only protoplasts from shoot tips with small, not expanded leaves could be successfully cultured; no data, however was presented regarding the yield of different leaf sizes. In contrast to the above findings, Doughty & Power (1988) have reported that small leaves of *Malus x domestica* were found to be unsuitable for protoplasts isolation.

Table 2. Effect of leaf size on yield of peach protoplasts and cells, and viability of protoplasts[a].

Leaf size			Yield ($10^7 \cdot gfw^{-1}$)			Protoplast viability (%)
	Length (mm)	Width (mm)	Cells	Protoplasts	Protoplasts %	
Small	4–10	1	3.5 a	4.8 a	58 a	91 a
Medium	13–17	2	4.5 ab	1.0 ab	18 b	90 a
Big	22–30	3	9.4 b	0.3 b	3 b	90 a

[a] Leaves were digested with cellulase Onozuka R-10 (2%) and Macerase (0.5%) in CPW.71M. Leaf wt/volume of enzyme solution was 20.5 ± 2.1 mg ml^{-1}. Protoplasts and cells were collected only in pellet I (centrifugation of 200 *xg* for 3 min). Values in each column followed by the same letter do not differ significantly ($p = 0.05$) according to Fisher's test.

Ratio of leaf biomass to volume of digesting solution

Incubation of leaf tissue in a relatively small volume of the digesting solution, a ratio of 50–100 mg · ml^{-1}, resulted in a very ineffective protoplast release (Fig. 2). Much better yields were obtained when the ratio of leaf biomass to volume of digesting solution was 10–20 mg · ml^{-1}. No differences in viability were observed between the different treatments (data not shown). This ratio is rarely addressed and sometimes not even reported in the literature. Ratios of 10–20 mg · ml^{-1} have not been used for isolation of protoplasts from deciduous fruit trees. Ratios of 30 and 150 mg · ml^{-1} were applied by Wallin & Welander (1985) and Doughty & Power (1988), respectively, for *Malus* protoplasts and 100 mg · ml^{-1} by Ochatt et al. (1987) and Ochatt & Power (1988) for *Prunus* protoplasts. The negative effect of high ratios of leaf biomass to volume of solution on protoplast liberation may be attributed to the release of proteases from digested tissue that inhibit the digestion enzymes.

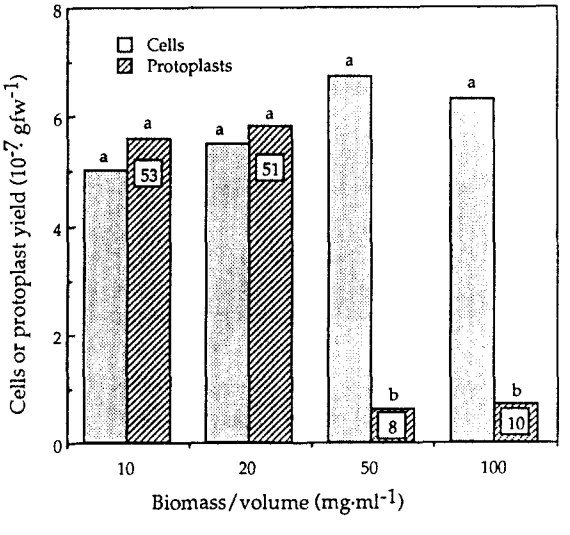

Fig. 2. Effect of tissue wt/digesting solution volume ratio on yield of peach protoplasts and cells. Small leaves were digested with cellulase Onozuka R-10 (2%) and Macerase (0.5%) in CPW.71M. Cells and protoplasts were centrifuged for 3 min (pellet I). Values in each column followed by the same letter do not differ significantly ($p = 0.05$) according to Fisher's test.

Protoplast purification

Extending the time of centrifugation from 3 to 10 min under a force of 200 *xg* resulted in about 2-fold increase in protoplast yield. However, this was accompanied by a decrease in protoplast viability from 97% to 79% (average of two experiments). Protoplasts were therefore harvested by splitting the long centrifugation into two centrifugations; after a 3 min run, the supernatant was pelleted for 8 additional min. Yield of the double centrifugation was similar to the one ten-min run and viability of protoplasts collected after the eight-min run was not impaired, 95% in comparison to 97% in the 3 min pellet. The yields of protoplasts harvested in the second eight-minutes runs were somewhat lower than the first pelleting, thus almost doubling the total yields (Table 3). Moreover, the second pellet was more enriched with protoplasts. In pellet II, protoplasts made up about 65% of total cells plus protoplasts in comparison to about 53% in the first pellet.

Further purification of protoplasts was obtained by layering the two pellets on top of a

Table 3. Yields of peach protoplasts and cells before and after purification on a sucrose gradient[a].

Fraction		Yield ($10^7 \cdot gfw^{-1}$)	Protoplasts %	Protoplast recovery (%)
Before sucrose gradient:				
3 min centrifugation	Cells	5.0 ± 0.6		
(Pellet I)	Protoplasts	5.7 ± 1.0		
	Total	10.7 ± 1.3	53 ± 4	
8 min centrifugation	Cells	2.2 ± 0.3		
(Pellet II)	Protoplasts	4.0 ± 0.2		
	Total	6.2 ± 0.2	65 ± 4	
After sucrose gradient:				
(Pellet I)	Cells	0.4 ± 0.1		
	Protoplasts	1.8 ± 0.3		
	Total	2.3 ± 0.4	82 ± 3	33 ± 2
(Pellet II)	Cells	0.1 ± 0.0		
	Protoplasts	0.8 ± 0.1		
	Total	0.9 ± 0.1	90 ± 0	21 ± 2

[a] Small leaves were digested with cellulase Onozuka R-10 (2%) and Macerase (0.5%) in CPW.71M. Cells and protoplasts were centrifuged for 3 min (Pellet I) and then the supernatant for 8 min (Pellet II). Recovery is expressed as the ratio (in %) between the yield after and before the sucrose gradient. Values are means (± SE) of 3 experiments.

21% sucrose gradient (Power et al. 1984). The percent protoplasts in the cell population increased from 53 to 65 for pellet I and from 65 to 90 for pellet II (Table 3). Purification on the sucrose gradient involved a loss of protoplasts to the lower sucrose layer. Protoplast recovery was 33% and 21% for pellet I and pellet II, respectively. The rate of impurity of the protoplast fraction by intact cells is rarely stated (Wichers et al. 1984); this suggests, therefore, that pure preparations contain approximately 100% protoplasts.

Culture of protoplasts

Trials to induce growth and division of peach protoplasts were attempted using different media and culture conditions. The two culture media used differed in their growth regulators. One designated PrPr was found to be the best combination for proliferation of callus from colt cherry protoplasts (Ochatt et al. 1987). Medium C5 was best for peach callus growth from embryos (Hammerschlag et al. 1985) and found in our lab (data not shown) to be very effective in obtaining highly proliferable peach cell suspensions. When kept static, protoplasts in liquid cultures of the two media were found non-viable after 3 days. Since dead cultured protoplasts exhibited some fluorescence in the absence of FDA, lack of viability was confirmed with Evans blue staining (Widholm 1972). Under shaking conditions, protoplasts were kept viable for somewhat longer periods: in PrPr medium up to 7–9 days and in C5 up to 14 days. Interestingly, protoplasts of pellet II were more viable than protoplasts of pellet I. Viability of protoplasts of pellet I was 1 and 2% after 7 days for media PrPr and C5, respectively, and 4 and 14% for protoplasts of pellet I. Absence of cell walls in protoplasts after isolation and the existence of a cell wall after 3 days of culture was confirmed with Calcafluor white. No division of protoplasts was detected in liquid cultures. In agarose, protoplast cell wall formation started and protoplasts changed shape after 3 days of culture, and division was detected after 7 days (Fig. 1B). The percentage of dividing protoplasts and extent of division were low, usually not exceeding 0.1% and a stage of 4–6 cells, respectively. After 2–3 weeks of culture, protoplasts turned brown. No growth of intact cells was observed in spite of careful observations.

Protoplasts embedded in agarose drops were also transferred to nurse cultures of peach cells originated in a suspension culture and embedded in C5 medium. Protoplasts under these conditions were alive for more than 6 weeks, ex-

panded to a size of 30–50 μm and had > 0·1% rate of division. This promising system has yet to be exploited.

Isolation of peach protoplasts from cell suspensions (Matsuta et al. 1986; Matsuta, 1992) represent the only full-length reports of peach protoplast isolation. Although actively growing suspension cells may be a better source of protoplasts amenable to culture (Ochatt et al. 1987), isolation directly from leaves of in vitro cultured plants has several advantages. These advantages include continuous accessibility to sterile plant material and the relatively limited risk of somaclonal variation. Because of increases in genetic variation in vitro over time (Skirvin & Janick 1976; Barbier & Dulieu 1980), mesophyll cell protoplasts, compared with suspension cell protoplasts, may more closely reflect cells of the intact plant and thus, be more appropriate for toxin/cell/protoplast studies. More work is needed to find conditions permitting further growth and development of in vitro leaf-derived protoplasts.

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