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Regeneration of Plants from Leaf Mesophyll Protoplasts of the Tetraploid Potato Cultivars Xenia and Bintje

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Abstract. An improved method for the isolation of leaf mesophyll protoplasts of *Solanum tuberosum* L. cvs. Xenia and Bintje, their culture and regeneration to plants is described. The improvements involve the culture of nodal stem explants on Schenk and Hildebrandt (SH) medium supplemented with $AgNO_3$ (7.5 mg l^{-1}) and Alar 85 (5 mg l^{-1}), and the pre-treatment of donor plants for 2–3 weeks at a short photoperiod (6 h), a low irradiance (17 μmol $m^{-2}s^{-1}$) and a low temperature (20 °C). Regeneration of cell wals was observed within 2–3 d and cell divisions within 7–12 d after resuspending freshly isolated protoplasts in Sidorov et al. (SW) medium. The resulting microcalli were cultured on solid media containing zeatin (1–2,5 mg l^{-1}) and/or NAA (0.1 and/or 0.01 mg l^{-1}) and subsequently on medium with BAR (0.25 mg l^{-1}) and GA_3 (0.1 mg l^{-1}) for shoot regeneration.

Protoplast culture techniques represent new approaches for crop improvement as well as for fundamental studies. For crop improvement by cell or gene manipulation techniques (fusion and transformation of protoplasts, microinjections) efficient regeneration techniques of plants from protoplasts is of essential significance. Concerning potato (*S. tuberosum* L.), regeneration of whole plants from mesophyll protoplasts has been described for several cultivars by a number of authors (*e.g.* Shepard and Toten 1977, Binding *et al.* 1978, Bokelmann and Roest 1983, Haberlach *et al.*. 1985, Foulger and Jones 1986, Carlberg *et al.* 1987, Grun *et al.* 1987, Taylor and Secor 1988). However, under our conditions none of the procedures reported in these papers appeared to be quite suitable for the cultivars Xenia and Bintje. Therefore, we have developed a procedure for conditioning of *in vitro* cultured donor plants as well as for isolation, culture and regeneration of protoplasts of these two potato cultivars. The cv. Xenia has been selected because of its very good regeneration ability in the stem internode system (Opatrný, personal communication), the cv. Bintje because of its high commercial importance.

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MATERIAL AND METHODS

Maintenance of Donor Plants

Virus free shoot cultures were obtained from the Research and Breeding Institute of Potato Growing, Havlíčkův Brod. They were propagated in vitro on Murashige and Skoog (1962) medium by transferring nodal stem explants. The explants were cultured in 200 ml jars (6–8 explants per 25 ml of medium) sealed with metal lids and incubated in a growth chamber at 25 °C and a daylength of 16 h at an irradiance of 40 μmol $m^{-2}s^{-1}$ (Tesla fluorescent tubes type "White"). In the reported experiments both culture medium and plant growth conditions before protoplast isolation were changed with respect to the leaf size of stem cuttings as well as to the yield of protoplasts capable of forming new cell walls and of starting cell divisions after isolation. Shoot cultures used for protoplast isolation were maintained on hormone-free SH medium (Schenk and Hildebrandt 1972, Table 1) supplemented with 7.5 mg l^{-1} $AgNO_3$ and 5 mg l^{-1} Alar 85. The medium was sterilized by autoclaving for 20 min at 114 °C. The jars were sealed with metal lids with an aperture (diameter 6 mm) closed by polyurethan foam to increase the exchange of air and were cultivated for 6–8 weeks in a growth chamber under the conditions described previously. For pre-treatment of donor plants the cultures were transfer ed for 2–3 weeks to another growth chamber at a daylength of 6 h, 20 °C and irradiance of 17 μmol $m^{-2}s^{-1}$.

Protoplast Isolation

The method is based on the procedure of Bokelmann and Roest (1983). Leaves from pre-treated shoots were used for protoplast isolation (Fig. 1A). About 0.5 to 1 g of leaves was cut into 1–2 mm pieces with a razor-blade and mixed in a Petri dish (diameter 6–7 cm) with 5–7 ml of an enzyme mixture (Table 1). The cellulase "Onozuka" R-10 and cellulase TC were commercial products of Serva, cellulase P-25 was obtained from Lachema Brno, Czechoslovakia. The dishes were incubated overnight (16 h) in the dark at 25 °C without shaking. At the end of the enzyme treatment, the mixture was gently shaken and filtered through a stainless steel mesh (75–100 μ, pore size). The filtrate was washed twice by density floatation using 0.5 M sucrose (centrifugation 5 min at 18 × *g*). The viable protoplasts in the upper sucrose layer were finally washed in W_5 medium (Menczel *et al.* 1981) and counted in a Bürker chamber. After 5 min centrifugation (14 × *g*)the protoplast pellet was resuspended in a liquid SW medium (Sidorov *et al.* 1987, Table 1) which was slightly modified: glucose was replaced by mannitol and sucrose, D-xylose was substituted by D-cellobiose and benzyladenine by zeatin. The medium was sterilized by autoclaving without hormones which were added later after being filter-sterilized.

Abbreviations used: IAA = indol-3-ylacetic acid; NAA = alpha-naphthyl-enacetic acid; 2,4-D = dichlorophenoxyacetic acid; BAR = benzyladenine riboside; KIN = kinetin; ZT = zeatin; GA_3 = gibberellic acid; MES = 2-(*N*-morpholino)ethanesulfonic acid.

Protoplast Culture and Plant Regeneration

The protoplasts (density 5.10^4 per ml) were plated in 10 cm Petri dishes sealed with Parafilm M and cultured at 25 °C, the first week in the dark and then under illumination of a sodium lamp at 2 μmol $m^{-2}s^{-1}$ and a 16 h daylength. After 18–21 d the growing colonies were diluted 1 : 1 with the liquid SH-M medium (Table 1) containing the macroelements according to Schenk and Hildebrandt (1972), and the microelements and vitamins in accordance with SH-1 medium of Sidorov *et al.* (1985). The formed microcalli were plated after three weeks in Petri dishes (Fig. 2A) on solidified (0.7 % agar Oxoid No. 3) SH-M medium and cultured under irradiance of a sodium lamp at 80 μmol $m^{-2}s^{-1}$ and a 16 h photoperiod. Subsequently 2–3 weeks later, the calli were transferred to agar SH-R_x medium (Table 1). For shoot regeneration, the calli were transferred three weeks later on to SH-R_{21} medium (Table 1). Regenerated shoots were periodically excised and transferred to SH medium containing 1 % sucrose, 0.7 % agar and 0.1 mgl^{-1} IAA for root formation (Fig. 2D).

RESULTS AND DISCUSSION

Stem cutting cultures grown on MS medium usually had very elongated internodes and small leaves. Replacement of MS medium by SH (Zubko, personal communication, Table 1) had a favourable effect on our donor plants as well as increasing the exchange of the air by ventilation aperture. However, a sufficient amount of larger leaves suitable for protoplast isolation (Fig. 1A) was obtained after an addition of $AgNO_3$ (for inhibition of ethylene action) and the morphoregulator Alar 85 to the culture medium (Table 1).

The yield of viable protoplasts isolated from donor plants cultured at a light intensity of 40 μmol $m^{-2}s^{-1}$ at 16 h daylength and 25 °C was very low and no cell wall formation was observed. Therefore, we examined the effect of light intensity and day length as well as temperature conditions on donor plants before protoplast isolation (see Shepard and Totten 1977, Tavazza and Ancora 1986). The highest protoplast yield (Fig. 1B) and the highest amount of protoplasts with synthesis of new cell walls (Fig. 1C) were achieved after a 2–3 week pre-treatment of donor plants at a short daylength (6 h), a low irradiance (17 μmol $m^{-2}s^{-1}$) and a low temperature (20 °C).

Three different types of cellulase (Table 1) were tested in the enzyme mixture for protoplast isolation from leaves of cv. Xenia. The highest yield of protoplast-derived calli (p-calli, Fig. 1D) was achieved with "Onozuka" R-10 (Table 2) and therefore this enzyme was used in combination with macerozyme in further experiments with both cultivars. Overnight (16 h) incubation of leaf pieces with enzyme mixture without vacuum infiltration and shaking turned out to be better than 2–6 h incubation with vacuum infiltration or shaking. The addition of auxin (NAA) and cytokinin (ZT and/or BAR and/or KIN) to the digestion medium resulted in a higher

TABLE 1

Media for preparation and cultivation of protoplasts and for plant regeneration of *Solanum tuberosum* L. cvs. Xenia and Bintje

	SH [mg l^{-1}]	Enzyme mixture [mg l^{-1}]	SW [mg l^{-1}]	SH-M [mg l^{-1}]	SH-R_x [mg l^{-1}]	SH-R_{21} [mg l^{-1}]
KNO_3	2500	–	950	2500	2500	2500
$CaCl_2.2\ H_2O$	200	735	220	200	200	200
$NH_4H_2PO_4$	300	–	–	300	300	300
$MgSO_4.7\ H_2O$	400	–	185	400	400	400
KH_2PO_4	–	–	85	–	–	–
NH_4Cl	–	–	133	–	–	–
H_3BO_3	5	–	3	6	6	6
$MnSO_4.4\ H_2O$	13.2	–	14	–	–	–
$ZnSO_4.7\ H_2O$	1	–	2	10	10	10
$CuSO_4.5\ H_2O$	0.2	–	0.025	0.025	0.025	0.025
$CoSO_4.7\ H_2O$	–	–	–	0.025	0.025	0.025
KI	1	–	0.75	0.83	0.83	0.83
$Na_2MoO_4.2\ H_2O$	0.1	–	0.25	0.25	0.25	0.25
$MnCl_2.4\ H_2O$	–	–	–	24	24	24
$CoCl_2.6\ H_2O$	0.1	–	0.025	–	–	–
$FeSO_4.7\ H_2O$	15	–	13.9	15	15	15
$Na_2EDTA.2\ H_2O$	20	–	37.3	20	20	20
Myo-inositol	1000	–	100	100	100	100
Adenine sulfate	–	–	–	40	80	80
Casein hydrolysate	–	–	500	300	100	100
Thiamine.HCl	5	–	10	0.5	0.5	0.5
Pyridoxine.HCl	0.5	–	1	0.5	0.5	0.5
Nicotinic acid	5	–	1	5	5	5
Glycine	–	–	–	2	2	2
Biotin	–	–	–	0.05	0.05	0.05

Folic acid	–	–	–	0.5	0.5	0.5
L-glutamine	–	–	100	–	–	–
Yeast extract	–	–	100	–	–	–
D-cellobiose	–	–	(0.5)	–	–	–
MES	–	1952	–	–	–	–
Cellulase R–10	–	1%	–	–	–	–
Macerozyme R–10	–	0.2%	–	–	–	–
Sucrose	10000	0.5M	0.01M	0.007M	0.007M	0.007M
D-mannitol	–	–	0.49M	0.3M	0.2M	0.2M
Agar	7000	–	–	(7000)	7000	7000
IAA	–	–	–	–	0.1[d]	–
NAA	–	5	2	0.1	0.01[d]	–
2.4–D	–	–	0.2	–	–	–
BAR	–	2[b]	0.5[c]	0.5	–	0.25
KIN	–	2[b]	–	–	–	–
ZT	–	2[b]	0.5[c]	–	1–2.5	–
GA_3	–	–	–	–	–	0.1
$AgNO_3$	7.5	–	–	–	–	–
Alar 85	5	–	–	–	–	–

[a]variants were: 2.5% cellulase TC and/or P 25; [b]BAR and/or KIN and/or ZT; [c]BAR and/or ZT: [d]IAA and/or NAA.

TABLE 2

Yield of protoplasts and *p*-calli as affected by 3 types of cellulase. Leaf fragments were incubated in the enzyme mixture for 16 h in the dark at 25 ψC without vacuum infiltration or shaking

Experiment No.	Cultivar[a]	Type of cellulase in enzyme mixture	Cytokinin in enzyme mixture	Protoplast cultivation medium[b]	Protoplast yield (per 1 g of leaf)	Yield of *p*-calli (per 100 plated protoplasts)
1	XE	R–10	ZT	SW_1	2.2×10^6	0.34
		TC	ZT	SW_1	2.5×10^6	0.15
		P–25	ZT	SW_1	0.9×10^6	0.06
2	XE	R–10	BAR	SW_1 + C	4.3×10^6	0.19
			ZT	SW_1 + C	6.3×10^6	0.19
3	XE	R–10	ZT	SW_1 + C	5.3×10^6	0.19
			ZT	SW_1	5.3×10^6	0.34
4	XE	R–10	ZT	SW_1 + C	3.9×10^6	0.22
			ZT	SW_2 + C	3.9×10^6	0.31
			ZT	SW_1	4.2×10^6	0.12
			ZT	SW_2	4.2×10^6	0.32
5	BI	R–10	KIN	SW_2	1.9×10^6	0.06
6	BI	R–10	KIN	SW_2	1.1×10^6	0.03
7	BI	R–10	KIN	SW_2	0.9×10^6	0.02

[a]XE = Xenia, BI = Bintje; [b]SW_1 = medium SW with 0.5 mg l^{-1} ZT, SW_2 = medium SW with 0.5 mg l^{-1} BAR, + C = supplement of 0.5% cellobiose.

and faster cell wall formation. After the addition of zeatin the synthesis of cell walls was observed 2–3 d after protoplast plating (Fig. 1C), whereas with BAR and/or KIN this stage was observed only after 4–5 d.

The composition of SW medium (Sidorov *et al.* 1987) for culture of freshly isolated protoplasts was modified (see Material and Methods) due to the reported favourable effect of D-cellobiose on cell wall regeneration and cell division of potato protoplasts (Kikuta *et al.* 1987). However, we have not confirmed this effect (Table 2). We have also shown that substitution of benzyladenine for zeatin in SW medium did not give a higher yield of *p*-calli (Table 2). Medium SW contained NH_4^+ ions reported as a component with a deleterious effect on the potato protoplast survival (Upadhya 1975, Shepard and Totten 1977, Bokelmann and Roest 1983). In spite of that SW medium gave much better survival of protoplasts compared to 1/2 MS where NH_4NO_3 was omitted (results are not presented).

Purified protoplasts (Fig. 1B) were plated in a density 5.10^4 per ml, which was chosen from the examined range 5.10^3–1.10^5 protoplasts per ml.

The first cell divisions appeared after 7–12 d of culture (Fig. 1C). The addition of SH-M medium in a ratio of 1 : 1 after 18–21 d was found to be crucial for the further growth of colonies (Fig. 1D).

In general, medium supplemented with zeatin is usually used for potato plant regeneration from *p*-calli (Shepard and Totten 1977, Shepard 1982, Haberlach *et al.* 1985, Tavazza and Ancora 1986). No morphogenesis was achieved in our experiments when *p*-calli were transferred to media containing ZT (1–2.5 mg l^{-1}) and low concentration of auxin (0.1 mg l^{-1} IAA and/or 0.01 mg l^{-1} NAA). Therefore, the

TABLE 3

Regeneration of shoots cvs. Xenia and Bintje from protoplast-derived calli as affected by different shoot regeneration media.

Cultivar	Sequence of media for shoot regeneration[a]	Number of calli	Calli producing shoots [%]	Number of shoots per callus
XE	$R_1 \rightarrow R_{21}$	150	58.0	1.6
XE	$R_2 \rightarrow R_{21}$	132	35.7	1.7
XE	$R_3 \rightarrow R_{21}$	151	42.0	1.9
XE	$R_1 - R_3 \rightarrow R_{21}$	66	75.5	2.7
XE	$R_1 - R_{31}$	73	34.0	1.7
BI	$R_1 \rightarrow R_{21}$	26	65.0	3.2

[a] R_1 = SH-R_x with 2.5 mg l^{-1} ZT + 0.1 mg l^{-1} IAA; R_2 = SH-R_x with 1 mg l^{-1} ZT + 0.01 mg l^{-1} NAA; R_3 = SH-R_x with 1 mg l^{-1} ZT + 0.1 mg l^{-1} IAA; R_{31} = SH-R_x with 1 mg l^{-1} BAR + 1 mg l^{-1} IAA + 10 mg l^{-1} GA_3; R_{21} = see Table 1.

calli were replaced after 2–3 week culture on the SH–R_x medium to plates containing SH-R_{21} medium with BAR and GA_3 according to Karp *et al.* (1982), which enabled the development of shoots (Fig. 2B and 2C). So we obtained a rapid and sufficient (up to 75 %) shoot formation in calli derived from protoplasts (Table 3). An even higher frequency of regeneration was achieved when ZT was combined in SH-R_x medium with IAA compared to NAA. We also tested a medium with a high level of GA_3 (10 mg l^{-1}) supplemented with IAA and benzyladenine (both 1 mg l^{-1}, Austin and Cassells 1983), but the frequency of *p*-calli with adventitious shoots was low in this case (Table 3). Plantlets were produced *via* rooting of regenerated shoots on SH medium supplemented with 0.1 mg l^{-1} IAA.

The regeneration of mature plants from mesophyll protoplasts of cv. Xenia has been reported by Schumann and Koblitz (1983), success in regenerating plants from mesophyll protoplasts of cv. Bintje was achieved by Bokelmann and Roest (1983). However, we had to modify the procedure described there for our conditions of culture of donor plants. Obviously, no uniform method exists for successful regeneration of plants from potato mesophyll protoplasts, not only for dihaploid and diploid species or clones but even for tetraploid genotypes (*e. g.*, commercial cultivars).

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